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Engineered immune cells in cancer immunotherapy (EICCI)

Edited by
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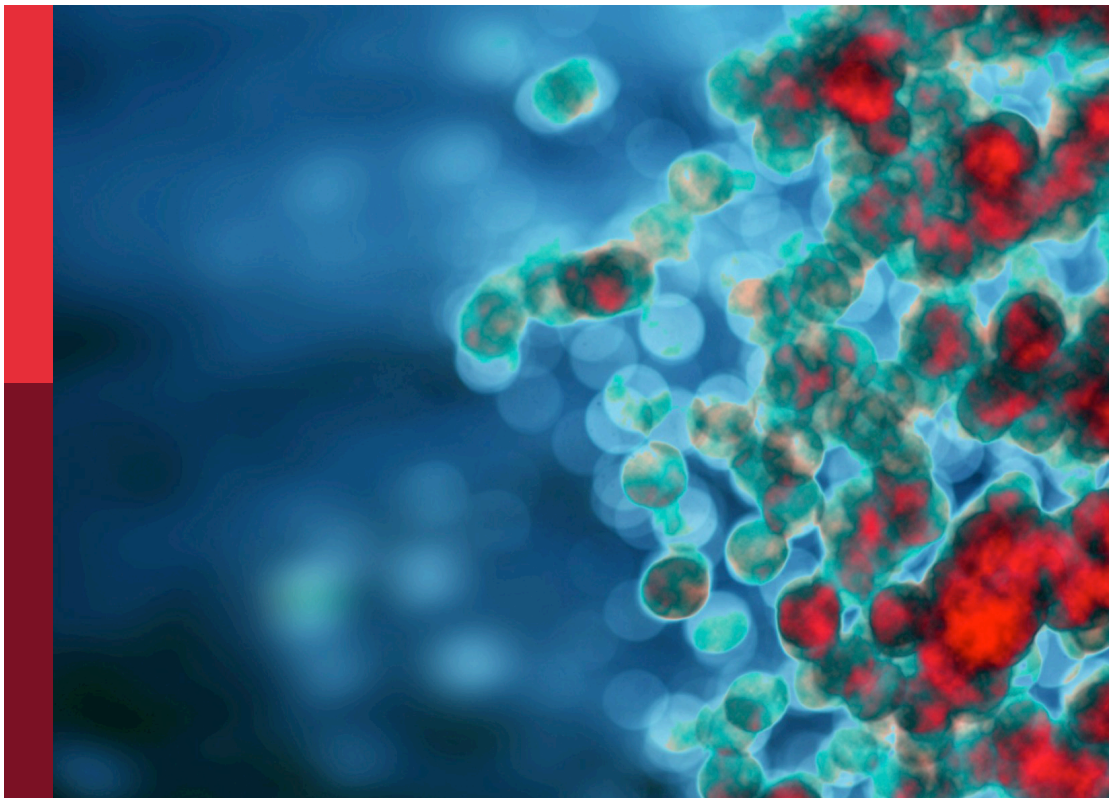

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Engineered immune cells in cancer immunotherapy (EICCI)

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Topic Editor Dr. Francisco Martin Molina is the founder of LentiStem Biotech, a start-up focused on the development of SAFER AND MORE EFFICIENT gene delivery systems for gene-cell therapy applications with special focus on lentiviral vectors and its applications to the genetic correction of rare disorders and cancer immunotherapy. Topic Editor Prof. Cameron Turtle receives financial support from Juno Therapeutics and Nektar Therapeutics. All other Topic Editors declare no competing interests with regards to the Research Topic subject.

Table of contents

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Editorial: Engineered immune cells in cancer immunotherapy (EICCI)

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Editorial on the Research Topic
Engineered immune cells in cancer immunotherapy (EICCI)

Adoptive Cell Therapy (ACT) to treat cancer represents a rapidly evolving field. New approaches for the genetic engineering of immune effector cells with either a T cell receptor (TCR) or a chimeric antigen receptor (CAR) (Figure 1 *Panels A and B, respectively*) have led to the increase of the clinical efficacy, the reduction or better control of toxicities and the expansion of the indications of these therapies. The Research Topic "Engineered Immune Cells in Cancer Immunotherapy (EICCI)" represents the venue for collecting studies, new evidence, advances in the technologies and the greatest knowledge for the translational application on the topic of cellular therapy for cancer. The great success of this Research Topic with the publication of total 46 articles, including 18 original articles, 20 reviews, 5 mini reviews, 2 case reports and 1 methodology manuscript, and the contributions of 360 authors, testify to the huge interest of the scientific community in this field and the numerous advancements.

The Research Topic is built on the outcomes of the 1st International Workshop on Engineered Immune Cells in Cancer Immunotherapy (EICCI), held at Sidra Medicine in Doha, Qatar, on 15th-16th February 2019. Worldwide experts in the field of cell therapy gathered in Doha to discuss progress and challenges in the field. The proceedings from the workshop by Guerrouahen et al. summarized the presentations from speakers from both academia and industry on pre-clinical and clinical development of genetic engineering ACT (GE-ACT), genetically modified immune cells, organizational needs and hurdles for the clinical grade manufacturing and the clinical application of these biological drugs.

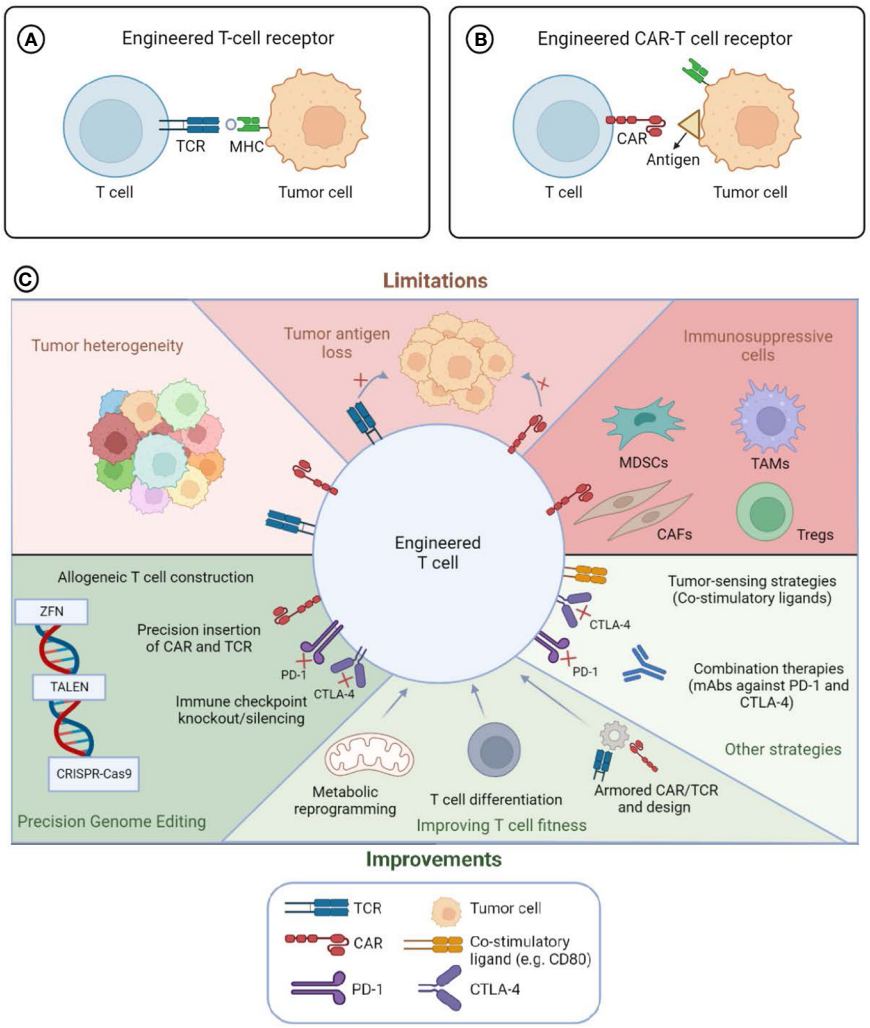

FIGURE 1
Engineered T cells to target tumor cells: limitations and advances. T lymphocytes engineered with T cell Receptor (TCR) (Panel **A**) or Chimeric Antigen Receptor (CAR) (Panel **B**) to redirect the immune cells toward tumor cells. The survival, proliferation and anti-tumor functions of engineered immune cells (Panel **C**) can be influenced by intrinsic factor of tumor cells, such as cell heterogeneity, antigen loss, or the immunosuppressive tumor microenvironment (TME) (Top part of Panel **C** entitled limitations). The administration of immune checkpoint blockade agents or co-stimulatory molecules in combination with engineered lymphocytes can modify the tumor milieu and implement the anti-tumor functions of the cellular products. Moreover, the modification of the fitness of the lymphocytes through actions that can change either the metabolomic profile, the differentiation status or the cytokine or immune checkpoint expression can lead to potentiate the functions of the immune cells (bottom part of Panel **C** entitled improvements). Precision genome editing (through the usage of either Zinc finger nuclease, ZFN, Transcription activator-like effector nuclease, TALEN or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (CAS9)) can increase the expression of the antigen-specific receptors, modify the fitness of the immune cells and/or implement the safety of the cell-therapy product. MDSCs, Myeloid-derived suppressor cells; TAMs, Tumor-associated macrophages; CAFs, Cancer-associated fibroblasts; Tregs, regulatory T cells. Created with BioRender.com.

11 years have passed since the first evidences that CAR-T cell therapy could revolutionize the clinical treatment of patients with hematological malignancies, such as acute lymphocytic leukemia (ALL) and non-Hodgkin lymphomas (NHL). Following these outstanding achievements, GE-ACT underwent an unprecedented growth, as testified by the 745 clinical trials, with 641 with CAR- and 102 with TCR-based therapies, that occurred for GE-ACT until the end of 2019 [Pinte et al.]. Interestingly, most clinical trials have been developed in China (47%) and the USA (44%), followed by the EU (6%) and other countries. Until 2013, the majority (91%) of GE-ACT clinical trials were spontaneously developed in academic settings, while from 2014 on, 54% of these studies were sponsored by biotech and pharma. Nevertheless, the majority of these clinical investigations are still in the early phase of development (Phase I/II) to demonstrate the safety and efficacy

of the novel product aimed at targeting a variety of tumor antigens and modified to implement their anti-tumor activities. A bibliometric analysis performed by Ou et al. confirmed the rapid progress of the field of CAR-T cells over the 2001-2021 timeline, with a total of 5981 articles reviewed and with the US being the leading country in terms of the number of publications, followed by China and Germany. Similar results were reported by Miao et al., with hot topics represented by cytokine release syndrome (CRS) associated with CAR-T cells, CD19-CAR-T cells, emerging tumor antigens to redirect the immune cells, GE-ACT for solid tumors and universal CAR-T cells.

Regulatory/manufacturing

A comprehensive overview of the regulatory and organizational requirements of clinical centers dedicated to the administration of cell therapies to cancer patients have been provided, also introducing the novel professional profile of the CAR-T specialist deputed to the overall coordination of GE-ACT clinical unit [Gotti et al.].

An important consideration is represented by the clinical-grade manufacturing of CAR-T cells that might occur in centralized facilities vs. academic/hospital environments with associated advantages or hurdles and that might affect the accessibility as well to these peculiar therapies [Landazuri et al.]. This review also provided the difficulties posed by the COVID-19 pandemic in the preparation and administration of engineered T cells to patients.

Clinical trials/results/reviews

Recently, two clinical grade CAR-T cell drugs have been approved for the treatment of multiple myeloma (MM), which generally relapses or is refractory to the available therapies, representing a salvage intervention for these patients. García-Guerreo et al. and Jasiński et al. provide an overview of the knowledge of CAR-T cells for MM, describing candidate antigens and multiple designs of CARs to combine costimulatory domains and/or growth factors and cytokines to modulate the strength of antigen binding of the CARs, increase the T cell survival and prevent their exhaustion. This review also suggested that the combination of CAR-T cells with other drugs currently utilized for MM, such as immunomodulatory agents, proteasome inhibitors, or antibodies, might represent the future in the therapeutic field for this type of malignancy. CD19-CAR-T cells introduced a major advance for pediatric hematological malignancies (B-acute lymphocytic leukemia; CLL) demonstrating 65-90% of complete responses across clinical trials. However, one of the major challenges following this cell therapy is the durability of the responses and the frequency of relapse. Schultz et al. addressed the limitations of the clinical outcomes associated with the CD19-CAR-T cell therapy in pediatric cancer patients highlighting the needs of identifying the predictors of responsiveness to help the stratifications of patients between long term responder and high risk of remission who might need further infusion of the engineered cells or stem cell transplantation.

Improving CAR-T cell products: New CAR designs

The long-term clinical outcomes of these live drugs correlate with the *in vivo* persistence of T cells. Therefore, the optimization of CAR design and/or cell engineering and T cell subset selection are important considerations for the translation of cell products to clinical application.

The cytoplasmic signaling of CARs

The cytoplasmic signaling domains of the receptor determine the persistence and survival, as well as the cytotoxic functions of T cells. The introduction of modules encoding for co-stimulatory molecules (e.g., CD28, ICOS, 4-1BB, OX40, etc.) into the design of CARs has overcome initial limitations, empowering the engineered T cells with activation, proliferative and cytotoxic properties. In this direction, Meng et al. reviewed the studies aimed at improving CAR-T cell functions by the modification of the immunoreceptor tyrosine-based activation motif (ITAM) sequence in both CD3ζand CD28ζ. The augmentation of CAR-T cell survival and persistence could also be achieved through the insertion in the modular structure of the chimeric receptor of anti-apoptotic genes (Bcl-xL), as described by Fan et al. in a model of adoptive cell therapy for solid tumor.

Hinge domain and the transmembrane domain

Several articles in this topic analyzed how to improve CARs through the modifications of the hinge domain (HD)/transmembrane domain (TMD). Muller et al. reported that the choice of the modular structure of the intracellular signaling can affect the functions of the engineered T cells. Biochemical differences can occur between CD28-TMD and CD8-TMD in the CAR. While CD28-TDM can have superior sensitivity to low abundant antigens on tumor cells as compared to CD8-TDM, the first TMD can augment the sensitivity to antigens expressed at low levels, leading to toxicities. CD28-TMD can form heterodimers leading to reduced CD28 expression that could lead to lower persistence and affect the differentiation of CAR-T

cells. These authors showed that the extracellular spacer domain, usually the CH2-CH3 components of IgG1, of CARs is critical for redirecting T lymphocytes towards tumor cells, since this regulates the distance between effectors and target cells. CARs containing long spacer can recognize membrane proximal antigens efficiently, while membrane distal epitopes require CARs containing short spacers for the receptor engagement. Along the same line, Schafer et al. developed a novel spacer derived from Sialic acid-binding immunoglobulin-type lectins (Siglecs). The advantage of this spacer is the efficiency in binding membrane proximal antigens expressed by either liquid or solid tumor cells, such as CD20 and TSPAN8, combined with central memory CAR-T cell phenotype, high cytotoxic and low inflammatory profile. This spacer also reduces the unspecific off-target binding of the CARs.

Binding domain

As expected, other groups have centered their attention on the CAR binding domain, as a first domain that interacts with the targeted tumor. In this direction, McComb et al. showed that CAR-T cells generated with anti-EGFR nanobodies linked with truncated CD8 hinge target selectively tumor cells over-expressing EGFR and not normal cells expressing low levels. They also showed that epitope location was critical for determining hinge-domain requirements for CARs and therefore, hinge length tuning can be used for controlling antigenic sensitivity in CARs-T cells.

Improving CAR-T cell products: Improving manufacturing

The understanding of the mechanisms underlying the development of toxicities helped to overcome or prevent some of these effects. Few combinatorial strategies with either antagonistic antibodies, immune checkpoint blockade, Jak inhibitors, or conditional regimens have been shown either at the pre-clinical or clinical levels to overcome the inflammatory reactions associated with CAR-T cells [Safarzadeh Kozani et al.; Miao et al.. Further studies aimed at dissecting the mechanisms leading to severe side effects and to the design and manufacturing of CAR-T cells are warranted to improve the safety of these live drugs. The variability of procedures for the generation of CAR-T cells at different manufacturing sites can affect the final characteristics of the product, including the differentiation status and the survival and cytotoxic features. This field requires optimization and standardization. In this context the investigations of Arcangeli et al. and Jackson et al. analyzed the automated manufacture of CAR-T in autologous setting and could reveal important technical approaches also demonstrating that patient's intrinsic characteristics can affect the final QC of the therapeutic cell products. Moreover, reliable tools to perform the quality controls of the cell-based product and to monitor their changes along with the manufacturing or even upon the administration into patients are desirable. One example is represented by the design and validation of immunofluorescence panels that, in a relatively simple manner, can be exploited to evaluate at the same time the expression of the CARs, the differentiation status and the cytotoxic functions of engineered T cells [Blache et al.]. These tools need to be accessible and exploitable in a large scale by laboratories at the manufacturing and/or clinical sites, without the need of complex platforms.

CAR-T cells are a gene therapy-based advanced therapy medicinal products (ATMP), and therefore, different tools and protocols to achieve these genetic modifications will render different CAR-T cell products. In this direction, several articles focus on how different gene therapy protocols can generate better products using ɣ-retroviral or lentiviral vectors. Jin et al. demonstrated that the temperature of transduction of T lymphocytes with lentiviral vectors (LVs) encoding for CARs is an important factor that can affect the differentiation and cytotoxic features of CAR-T cells. Brandt et al., Kozani et al. and Tristan-Manzano et al. [22] described the importance of using the appropriate promoters to express the CAR. Generally, strong CAR expression has been envisioned as an advantage for tumors with low antigen expression. However, if that is not the case, this strong CAR expression can lead to tonic signaling and premature exhaustion of CAR-T cells, lowering the overall anti-tumor efficacy. Another important factor is to be able to expand the CAR-T cell product without losing their most wanted properties (high % of early stage of differatiated cells). In this line, Arcangeli et al. and Jackson et al. presented optimized protocol for CAR T cell production using methods that are compatible with automated manufacturing and can generate CAR-T cells products highly enriched of stem cell memory T cells (TSCM) from patients. Although retroviral vectors are rendering impressive results so far, their production is time-consuming and costly, and other gene transfer system alternatives to achieve stable expression of CAR in T cells are being investigated. In this topic, Li et al. reported a case report of triple-hit relapse/refractory (RR) DLBCL with TP53 mutation treated with CAR-T cells generated with piggyBac transposons. This emerging non-viral methodology possesses a large cargo capacity and, more importantly, the manufacturing is simple and cost-effective. This team reported complete responses in patients, in which durability could be achieved by the combination with lenalidomide. Although further investigations are required to confirm the efficacy and safety of the combination therapy, this evidence proves the feasibility for clinical application of a different modality of gene transfer. However, this vector has been implicated in the generation of lymphoma in 2 patients from a clinical trial performed by Micklethwaite et al. (1)

Alternatively, transposons based on sleeping beauty are also being investigated.

Controlling CAR-T cell activity

Besides the excellent clinical outcome of CAR-T cells for several blood cancers, several toxicities have been observed in patients treated with these "living drugs"; some of them are life threatening if not recognized and treated at the onset of the development as discussed by Miao et al. The most common side effects observed in patients are the CRS, the immune effector cell-associated neurotoxicity syndrome (ICANs), off-target effect, anaphylaxis, B cell aplasia tumor lysis syndrome and infections. Also, to improve the efficacy of CAR-T cells against solid tumors, the secretion of potent active molecules such as IL-12 or interferons (IFNs) is required and can therefore generate even stronger adverse side effects. This toxicity also limits the efficacy, due to the impossibility to reach the appropriate concentrations in target organs. There is therefore a clear necessity to develop strategies that can control CAR-T activity in a time and spacial manner. In this direction, Brandt et al. and Tristán-Manzano et al. described the different strategies developed by different groups to control the potency and duration of the CAR-T activity.

Efforts have been devoted to regulating the expansion and the activity *in vivo* of CAR-T cells through different approaches that can be divided into those that allow the CAR-T cells to "take" decisions based on the environment (endogenous regulation) or based on the external addition of an inductor (exogenous regulation).

The idea of generating CAR-T cells that can be controlled by themselves and be active only in the presence of the tumor antigen is in the CAR-T cell DNA. However, these cells can be activated by different factors other than the targeted tumor, generating undesired side effects. This is even more problematic when designing CAR-T cells for solid tumors that require the secretion of potent immune regulators. There are multitude of approaches under study to achieve this autonomous control, which is reviewd by Brandt et al., such as the Split-CARs (the CAR binding domain and signaling domains are separated), logic gates, such as the requirement of several antigens for the activation of CARs (AND GATE) or the generation of inhibitory CARs (iCAR) targeting specifically normal cells, thus the recognition of antigen expressed by healthy cells leads to inhibitory signaling (NOT GATE). Another interesting approach uses hypoxia-regulated CAR-T cells that only express other CARs or molecules under hypoxic conditions.

Alternatively, other groups have aimed at generating smart CAR-T cells which can be controlled by the addition of an inductor, allowing the clinicians to manage the intensity and durability of the therapy reviewed by Tristan-Manzano et al. There are various systems that have been used to kill CAR-T cells if necessary, such as inducible suicide herpes simplex virus tyrosine kinase (HSV-TK) or the iCaspase systems that trigger cell death upon a small molecule administration. In addition, several other systems can control CAR-T activity by controlling the binding of Split-CARs (the CAR binding domain and signaling domains are separated and get together only upon the addition of the inductor) or by controlling the expression of the CAR itself or other molecules (IL12, IFN-β). Several systems have been investigated with this aim, including several TetOn system that uses doxycycline as inductor, the RheoSwich that uses veledimex and the Gene Switch system that use synthetic steroid mifepristone (MFP).

CAR-T cells for solid tumors

Efforts are ongoing to utilize the CAR-T cell strategy for the treatment of solid tumors, however, the clinical efficacy for these types of tumors is limited as compared to hematological malignancies, due to intrinsic mechanisms of cancer cells leading to their escape from immune responses. The principal factors limiting the clinical development of CAR-T cells are the immunosuppressive tumor microenvironment and the inability of T cells to penetrate the tumor-associated vasculature and the extracellular matrix. Rodriguez-Garcia et al. and Kozani et al., have reviewed the possible strategies to release the brakes of CAR-T cells and implement their efficacy in the targeting of solid tumors.

Examples of these approaches are the manipulation of the TME by either the combination of CAR-T cell infusion with monoclonal antibodies targeting regulatory immune cells or immune checkpoint molecules, the expression of cytokines or chemokine ligands by the CAR-T cells or by modifying the T lymphocytes to be resistant to immune suppression. CAR-T cells targeting stromal cells can also be employed to overcome the inhibitory effect of TME [25]. This knowledge can also be applied to improve CAR-T cell potency. Pavlovic et al. reviewed how CAR-T cells can be re-engineered using genome editing tools to make them resistant to the TME environment or to become more resistant to exhaustion.

The combination of CAR-based cell therapy with chemotherapy or antiangiogenic agents can facilitate the migration of T cells to the tumor milieu. Additionally, the addition of either cancer vaccines or photothermal therapy with cell therapy have been explored to engage the antigen presenting cells and augment the CAR-T cell proliferation, multifunctionality and anti-tumor activity or to recruit bystander immune cells through antigen spreading Rodriguez-Garcia et al., Kozani et al.]. Along this line, the lack or a low number of tumor-specific antigens in solid tumors that are not expressed by normal tissues to prevent the risk of "off-target' reactivity and toxicities has limited the number of clinical trials of CAR-T cells in solid tumors. The high grade of tumor

heterogeneity and the loss of antigen along with tumor progression can also limit the clinical efficacy of CAR-T cells targeting a single antigen. In the search for novel tumor-associated antigens, Ponterio et al. investigated the role of glycosylated antigens in redirecting CAR-T cells toward glioblastoma (GBM) and colorectal cancer. Few other reports explored the development of CAR-T cells targeting PIK7, and αvβ6 integrin in lung cancer and cholangiocarcinoma, respectively [Jie et al., Phanthaphol et al.]. Promising results of the ability of CAR-T cells to provide clinical benefit in recurrent solid tumors have been reported for the targeting of EGFR vIII in a patient with aggressive GBM [Durgin et al.]. This case reports the persistence of the infused CAR-T cells in the circulation for 29 months of follow-up with improvement of patient's survival (36 months) after tumor recurrence. The analysis of the tumor at the post-infusion timeline showed anti-inflammatory adaptations with decreased levels of EGFRvIII and a moderate expression of the programmed cell death protein 1 (PD-1) by the infiltrating T lymphocytes. This evidence suggests that EGFRvIII CAR-T cells might represent a valuable therapeutic therapy for GBM patients with advanced disease upon its optimization, for instance, through the combination with anti-PD-1 antagonistic antibody. The combination of CAR-T cells with PD-1 blockade indeed revealed its efficacy in a mouse model of Her-2^+ breast cancer [Li et al.].

An additional approach to implement the targeting of tumor cells by CAR-T cells is the combination with bispecific antibodies that can facilitate the redirection and interaction between effectors cells with target cells of both the engineered T cells and possible bystander lymphocytes [Blanco et al.].

Universal GT-ACTs

Most CAR-T cell approaches use autologous T cells from the same patient to generate the ATMP. However, there are important limitations of this personalized strategy such as the availability, inclusion criteria and cost. The approach of developing allogeneic/"off-the-shelf" CAR-T cells has been pursued by several groups in order to render the manufacturing of these "living drugs" independent of the availability of patients' derived peripheral blood lymphocytes (PBLs) and shortening the delay that elapses from the patient's enrollment and the time of infusion of the drug. Morgan et al. and Pavlovic et al. describe how genome editing (GE) can be used to generate "of-the-self" CAR-T cells that can be used for the treatment of patients with type B malignances. They illustrate how GE can be used to eliminate the TCR and the MHC class I and/or MHC class II molecules to prevent GVHD and allograft rejection. However, the knock-out of MHC can result in the recognition and destruction of the modified CAR-T cells by NK cells. Expression of ligands that inhibit NK cell cytotoxicity (HLA E or HLA G) can be implemented to avoid these situations.

Other cell types rather than T cells to generate "off-the-self" GT-ACT products have also been suggested. An overview of this approach has been provided by Guerrouahen et al. The source of allogeneic immune cells, e.g., Natural Killer (NK cells), PBLs, Cord blood, induced pluripotent stem cells (iPSCs), is a critical point in order to lower the risk of allogeneic toxicities and to allow availability of large numbers of cells for the optimal manufacturing CAR-engineered immune cells [34].

NK-CAR cells provide the advantage of rare induction *in vivo* of toxicities, such as cytokine release syndromes or graft-versus-host disease (GvHD). However, challenges in the manipulation of these cells with difficulties in the engineering with exogenous receptors and the sensitivity to apoptosis, warrant the investigations to optimize the manufacturing of clinical-grade CAR-NK cells [Scmidt et al.]. Efforts are undergoing to optimize the isolation, engineering and *ex vivo* expansions of NK cells [Mantesso et al.]. The dual engineering of primary NK cells with CD19 CAR and chemokine receptor (CXCR4) equipped the engineered immune cells with high efficiency in migrating to the bone marrow as compared to conventional CD19-CAR-NK cells, providing a further tool to implement the efficacy of NK cells for cell therapy [Jamali et al.]. Along this line, Huang et al. have identified a robust CRISPR-based genome engineering platform for NK-92 cells leading to the isolation of immune cells endowed with high cell-mediated and antibody-dependent cellular cytotoxic functions.

Transgeneic TCR-T cells

Endogenous tumor antigens can be targeted by T cells engineered with a TCR that has been isolated from antigen-specific lymphocytes. This approach has shown clinical efficacy in the context of clinical trials for patients with solid tumors. However, the improvement of the generation of anti-tumor T cells is required to endow these cells with potent cytotoxic activity, the ability to migrate to the tumor site, to penetrate the TME and long-term survival and proliferation. T lymphocytes are engineered with antigen-specific TCRs that could be encoded by viral vectors but also by no-viral gene transfer vectors, such as transposons or messenger RNA incorporated into lipid nanoparticles. The advent of gene editing allowed to make a precise insertion of the exogenous TCR gene transfer and the disruption of endogenous genes encoding for α and β chains of the receptors to prevent the TCR mispairing and to augment the expression on the cells of the novel antigen-specific receptors. Gene editing can also be utilized to control the differentiation of T lymphocytes and maintain their early-stage maturation, similar to what has been described above for CAR-T cells.

Moreover, the selection of the target antigen and of tumor-specific TCR is critical for the downstream manufacturing of efficient anti-tumor cell therapy. Neoantigens generated by somatic mutation in tumor cells represent promising tools for

the isolation of highly specific" living drug" bearing exogenous TCRs, as they overcome the risk of inducing anergic responses as in the case of self/endogenous antigens. Moreover, the targeting of this type of antigen can prevent the "off-target" toxicities since they are not expressed by normal tissues. Nevertheless, continuous efforts by several groups are ongoing in the search for the "optimal" candidate tumor antigen(s) and to decrease the complexity and lengthy process of the selection of TCR. Of note, soluble T cell redirecting biologics, either TCR or immunoglobulin-based molecules, represent a novel approach to redirect and facilitate T-cell mediated responses toward cells, also providing the advantage of being independent of the availability of autologous patient's cells. An overview of the topics listed above was provided in this issue by Manfredi et al., Cai et al. and Jones et al. The selection process of TCR for clinical application and targeting preferentially the alpha-fetoprotein on hepatocellular carcinoma cells and not cross-reacting with proteins expressed by normal cells is described by Luo et al. In addition, the TCR proximal signaling and the molecules involved in this process constitute a complex cascade of molecules, in which genomic variations can lead to the development of different diseases including malignancies. The knowledge of the molecular structure, the functions and the relationship of these molecules is relevant to implement the development of TCR gene transfer and engineering for cell therapy [Kent et al.]. The novel strategy to genetically modify tumor-infiltrating lymphocytes (TILs) with retroviral vectors has shown its feasibility to improve the survival machinery of cells that are physiological endowed with anti-tumor properties [Weinstein-Marom et al.].

Novel approaches

Among novel perspectives of therapeutic approaches for anti-tumor therapies are the reports of *i.* Fereydouni et al. and Fereydouni et al. suggesting the usage of mast cells (MC), sensitized with Her-2/neu IgE in order to polarize their anti-tumor activities and to be utilized for cell-based therapies. *ii.* The engineering of extracellular vesicles, which are secreted by tumor cells, to be used as vaccines or to block the immunosuppressive functions to elicit or implement the anti-tumor immune responses in solid tumors [Yin et al.].

Overall, the Research Topic documents and collects the huge effort ongoing worldwide in the field of GT-ACT, highlighting the progress and the limitations and how the advent of innovative technologies can accelerate the progress toward the development of more precise manufacturing and superior safety for cell-based therapies (Figure 1 *Panel C summarizes these aspects*).

Author contributions

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Conflict of interest

FM is founder of LentiStem Biotech involved in generating improved gene transfer systems to improve CAR-T cells.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Emerging Approaches for Regulation and Control of CAR T Cells: A Mini Review

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Chimeric antigen receptor (CAR) T cells have emerged as a promising treatment for patients with advanced B-cell cancers. However, widespread application of the therapy is currently limited by potentially life-threatening toxicities due to a lack of control of the highly potent transfused cells. Researchers have therefore developed several regulatory mechanisms in order to control CAR T cells *in vivo*. Clinical adoption of these control systems will depend on several factors, including the need for temporal and spatial control, the immunogenicity of the requisite components as well as whether the system allows reversible control or induces permanent elimination. Here we describe currently available and emerging control methods and review their function, advantages, and limitations.

Keywords: chimeric antigen receptor, cancer, immunotherapy, T cell, synthetic, regulation, cell therapy

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INTRODUCTION

Chimeric antigen receptor (CAR) T cells have emerged as a promising treatment for patients with advanced B-cell cancers (1–3) but more effective control of the therapy is needed to combat associated toxicity and to expand CAR therapy toward other cancer types. CAR T cells are a personalized immunotherapy, in which allogeneic or autologous T cells are genetically modified to express a synthetic construct, combining an extracellular binding domain, often an antibody-derived single chain variable fragment (scFv), with activating signaling domains from the T-cell-receptor complex, such as CD3ζ, CD28, and 4-1BB. Recognition of cell-surface proteins through the extracellular domain allows CAR T cells to target cancer cells for cytotoxic killing (4).

As a living drug, CAR T cells bear the potential for rapid and massive activation and proliferation, which contributes to their therapeutic efficacy but simultaneously underlies the side effects associated with CAR T-cell therapy. The most well-known toxicity is called cytokine release syndrome (CRS) which is a systemic inflammatory response characterized by fever, hypotension and hypoxia (5–7). CRS is triggered by the activation of CAR T cells and their subsequent production of pro-inflammatory cytokines including IFNγ, IL-6 and IL-2 (8). This is thought to result in additional activation of bystander immune and non-immune cells which further produce cytokines, including IL-10, IL-6, and IL-1 (9). The severity of CRS is associated with tumor burden, and ranges from a mild fever to life-threatening organ failure (10, 11). Neurologic toxicity is another serious adverse event which can occur alongside CRS (12). Although the pathomechanism is unknown, it is believed to be the result of cerebral endothelial dysfunction (13). Finally, since few antigens are truly tumor specific, toxicities can arise if CAR T cells target healthy cells expressing the

recognized antigen i.e., on-target, off-tumor activity. Unfortunately, this has led to severe and fatal outcomes, especially when targeting antigens in solid tumors, hampering CAR T-cell application in these patients (14–17).

Current clinically approved CAR designs do not enable control over CAR T cells following infusion, and so management of toxicities depends on immuno-suppression using systemic corticosteroids as well as an IL-6 receptor antibody, tocilizumab. Unfortunately, the use of immunosuppressive drugs severely limits the time span CAR T cells are functional (11). Given the severity of the toxicities, as well as the manufacturing costs, there is a clinical need to regulate CAR T-cell numbers and activity once deployed in patients. In this mini review, we describe existing and emerging approaches to regulation and control of CAR T cells, and discuss each method's advantages and disadvantages.

PASSIVE CONTROL

Passive control methods provide straightforward opportunities to limit CAR T-cell mediated cytotoxicity, but offer no downstream control over engrafted cells following transfusion (**Figure 1**, left panel).

Transient Transfection

A simple but effective way of regulating CAR T cells consists of transiently transfecting T cells with CAR-encoding mRNA (18–23). Due to the lack of genomic integration, CAR expression is limited by the degradation of the CAR-encoding mRNA and dilution following each T-cell division (18). The result is a steady decrease in CAR-expressing T-cell numbers, unless new cells are infused. Repeated infusions are however associated with a higher risk of an anaphylactic reaction due to the CAR T cells (24). While the inherently limited persistence of these CAR T cells might compromise continued anti-leukemic effect (25), it also limits long-term hematologic toxicities and off-target effects.

Affinity Tuning

Lowering the binding domain's affinity toward the targeted antigen aims to prevent on-target, off-tumor toxicities from arising in the first place (26, 27). While affinity-tuned CARs retain the ability to bind to cancer cells with a high antigen expression, healthy tissues with lower expression are spared (28). The use of low-affinity CARs is therefore especially interesting when targeting antigens known to be expressed on healthy tissue in low amounts, e.g., HER2 or EGFR (26, 27). This, however, might also lead to cancer cell escape variants with low antigen expression (29). Additionally, both promoter usage and transduction level of T cells might result in heterogeneous expression of the CAR protein, making it hard to ensure consistent behavior among individual CAR T cells as their avidity toward the antigen can vary. One promising strategy to overcome heterogeneous CAR expression is to instead integrate the CAR construct into the endogenous TCR alpha chain (TRAC) locus using the CRISPR/Cas9 system (30).

INDUCIBLE CONTROL

Recognizing that CAR T-cell toxicities arise rapidly, researchers have developed several exogenous methods to quickly regulate

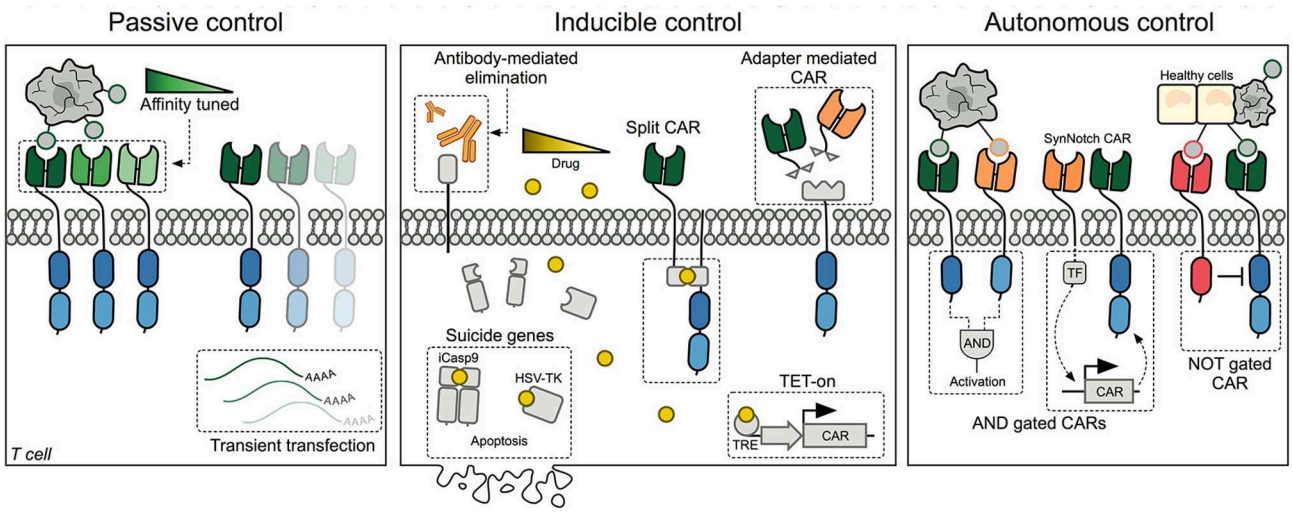

FIGURE 1 | Schematic representation of the three major methods designed for controlling CAR T cells today. **Left panel:** Passive control methods include affinity tuned CARs and transient transfection of T cells. **Middle panel:** Inducible control includes methods to eliminate CAR T cells using antibodies or inducible suicide systems. Additionally, different drugs have been utilized to either control CAR expression at the transcriptional level or assembling of a split-CAR, where the extra- and intracellular domains have been separated. Another approach has been to decouple the binding domain from the intracellular signaling domain, such that binding adapters can be supplied and titrated. **Right panel:** Autonomous CAR T cells are self-regulated and can decide whether to initiate or withhold cytotoxic killing of target cells based on surface proteins expressed by healthy and cancerous cells. CAR, Chimeric Antigen Receptor; TRE, Tetracycline Response Element; TF, Transcription Factor; SynNotch, Synthetic Notch receptor.

the activity of the CAR T cells or to eliminate them completely. These methods rely on co-administration of a drug, thereby making their use dependent on pharmacokinetics, tissue availability, and potential adverse effects of the chosen drug (**Figure 1**, middle panel).

Suicide Genes

Depletion of CAR T cells can be achieved by designing CAR constructs that also express a suicide gene, such as inducible Caspase 9 (iCasp9) (31–39), herpes simplex virus tyrosine kinase (HSV-TK) (40, 41) or human thymidylate kinase (TMPK) (42). In cells expressing iCasp9 and TMPK, elimination is achieved through activation of the caspase 3 apoptotic pathway when a small molecule is administered. The iCasp9 system has successfully been validated in patients receiving haploidentical stem-cell transplants (HSCT) in which iCasp9-expressing T cells were rapidly removed at onset of graft-versus-host disease (GvHD) (31). Likewise, administration of ganciclovir to T cells co-expressing HSV-TK causes formation of a toxic metabolite but cell death may take up to several days as it depends on cell proliferation (40, 41). The use of HSV-TK is severely limited by the high immunogenicity of the virally-derived protein (43). Furthermore, the HSV-TK suicide system is complicated by the fact that ganciclovir is used as a first-line treatment against cytomegalovirus (CMV) infection, a virus which is often reactivated in HSCT and other immunocompromised patients (44). As TMPK and iCasp9 are of human origin, the risk of immunogenicity is low. Indeed long-term engraftment up to several years in patients infused with iCasp9 expressing cells has been reported (45).

Elimination Markers

Co-expression of a cell-surface elimination marker, not normally present on T cells, allows for antibody-mediated degradation and control of the CAR T cells (22, 46–52). By utilizing clinically approved antibodies, e.g., rituximab targeting CD20 (48–50) or cetuximab targeting EGFR (51, 52), complement- or antibody-dependent cytotoxicity (CDC/ADCC) can be achieved toward the CAR T cells (51). Choosing a marker that is co-expressed on cancer cells, allows this method to create additional tumor killing, with the caveat that further collateral toxicity might arise. Cell-surface markers also allow for positive selection of transduced T cells in the manufacturing process, and subsequent monitoring of CAR T-cell levels *in vivo*. However, the efficacy of the strategy can be compromised by the fact that CDC/ADCC capacity is limited in patients treated with chemotherapy prior to CAR T infusion (53). In addition, antibodies can have limited biodistribution and tissue penetration, especially in poorly vascularized tumors (54). In order to address these problems, researchers have instead created anti-idiotype CARs recognizing murine CD19-specific CARs (55) or incorporated a short peptide epitope, called an E-tag, into the extracellular domain of the CAR, and created anti-E-tag CARs which could then be used to eliminate the anti-tumor CARs (56).

Since the use of suicide genes and elimination markers result in irreversible depletion of this complex treatment, researchers have developed a number of reversible methods to control CAR T cells as well.

Systemic T-Cell Inhibition

Current methods for controlling CAR T cells include systemic immunosuppressive agents, e.g., corticosteroids (57). The lymphocytotoxic anti-CD52 antibody alemtuzumab, has also been proposed as a method of depleting CD4- and CD123-specific CAR T cells (47, 58), as targeting these proteins might cause hematological aplasia and toxicity. More recently, it was shown that CAR signaling could be inhibited using the tyrosine kinase inhibitor dasatinib. Dasatinib inhibits phosphorylation of lymphocyte-specific protein tyrosine kinase (LCK), a critical component in the T-cell signaling pathway. Preclinical studies suggest that treatment with dasatinib can reversibly inhibit CAR T-cell proliferation and cytokine production without negatively affecting viability (59, 60). Although dasatinib cannot adequately inhibit already activated CAR T cells, limiting its usage against acutely arising CRS or neurotoxicity, the drug was shown to be superior to dexamethasone in inhibiting further activation in a preclinical study (59). Finally, while corticosteroids and alemtuzumab cause widespread inhibition or complete elimination of both CAR T cells and healthy lymphocytes, dasatinib has the advantage of acting as a faster on/off switch, due to its short half-life of 4 h (61).

Adapter Mediated CARs

Aiming to specifically control CAR T-cell activity toward the antigen, several models of adapter-mediated CARs, also known as universal CARs, have been developed (62–71). A shared feature is their method of tumor recognition, which is achieved by linking an adaptor, a molecule recognized by the CAR, to an antibody or ligand that recognizes the tumor antigen. While current clinically approved CARs are designed to be constitutively active, adapter-dependent CAR T cells can only recognize and kill when the adapter is administered, allowing for titratable and reversible control of the CAR T cells. A major advantage of this approach is the ability to target different antigens without the need to re-engineer and re-transfuse T cells. Adapters have also been designed to redirect anti-CD19-specific CARs to another target, using CD19-fusion proteins, suggesting that adapter proteins might be used with current clinically approved CAR T cells (72). However, large differences in adapter kinetics and subsequent effects on CAR T cells have been reported, probably reflecting differences in models used, affinity differences between the adapter and both target and CAR T cells, and biodistribution of the adapter molecules.

Split-CARs

Instead of directly regulating CAR binding to antigen, pharmacological inducers can also be used to control the activity of CAR T cells themselves, by splitting the CAR's extracellular antigen-binding domain from its intracellular signaling domains (73, 74). Assembling of the fully functional CAR is therefore dependent on administration of a dimerizing drug, limiting the CAR activity by the half-life of the drug.

Consequently, CAR T-cell activation requires two inputs: the tumor antigen and the dimerizing drug. The split-CAR can thus also be considered an AND gate CAR (see below). The split-CAR allows temporal and reversible control over the number of functional CARs, but the design does not prevent on-target, off-tumor toxicities as no spatial control is achieved due to a lack of control over the distribution of the drug.

Protease Inhibitors

Strategic incorporation of the autocleaving hepatitis-C-derived NS3 protease in the CAR construct has also been suggested as a way to control CAR T-cell activity. Juillerat et al. incorporated the NS3 protease between the CAR and a degradation moiety, thereby tagging the CAR construct for degradation when a NS3 protease inhibitor was administered (75). This construct showed reversible as well as tunable control over CAR T-cell cytotoxicity *in vitro*. As the NS3 protease is virally derived, it holds immunogenic potential which could potentially limit CAR T-cell persistence.

TET-On Regulation

It has also been suggested to regulate CARs at the transcriptional level. Several groups have shown that a drug-inducible CAR system can be generated by controlling CAR transcription using the TET-on system, allowing reversible control of CAR T cells (76–78). CAR mRNA is thus only produced in the presence of doxycycline, although some background CAR expression was observed (76). As the TET-on system is derived from both bacteria and virus, it holds significant immunogenic potential with the risk of host mediated elimination of the CAR T cells. Another drawback is the lack of rapid control, should life-threatening side effects occur, due to the control occurring on a transcriptional level. However, for highly proliferative CAR T cells that are constantly diluting the CAR protein amongst daughter cells, transcriptional regulation may be sufficient to limit the quantity of functional CAR complexes.

LOGIC GATES AND AUTONOMOUS CONTROL

As CAR T-cell therapy is applied against solid tumors, the distinction between healthy and malignant tissue becomes increasingly important (79). The use of so-called boolean logic gates and tumor selectivity mechanisms is envisioned to generate autonomous CARs with a higher target specificity, capable of better distinguishing tumor cells from healthy cells (**Figure 1**, right panel).

AND Gates

One approach to enable better decision-making in CARs is the incorporation of logic AND gates, such that a combination of antigens are required for activation. Often this dual CAR design consists of two extracellular domains, with specificities toward different antigens, each coupled to separate components of the intracellular stimulatory apparatus, e.g., CD3ζ and CD28 or 4-1BB (80–83). Such approaches have been tested in preclinical prostate and breast cancer models and might allow for targeting of proteins that are also present in healthy tissue (81, 82). A worry, however, is that even partial signaling through one receptor may generate sufficient T-cell activity to cause off-target damage (81, 83). Another approach has therefore been to use one receptor exclusively as a priming signal, with no activating signaling capacity itself. This was achieved using so-called SynNotch receptors, which were coupled to orthogonal transcription factors that were released upon binding. This "priming" leads to expression of a fully functional CAR targeting another cancer-associated antigen, ensuring localized activity of the CAR T cell (84, 85). Importantly, in mouse models the SynNotch CARs did not migrate, but retained their function only in dual positive tumors, indicating that this approach ensured good spatial control of the CAR (84, 85).

NOT Gates

Better discrimination between malignant and healthy cells can also be achieved by designing an inhibitory CAR (iCAR). The iCAR contains a binding domain specific for an antigen expressed on healthy cells fused to the signaling domains of CTLA-4 or PD-1, such that recognition of the healthy antigen leads to an inhibitory signaling cascade that overrides activating signals by dephosphorylating the receptor complex (86). The iCAR should restrict CAR T-cell activity to tumor tissue lacking the healthy antigen, limiting on-target, off tumor activity. Such a system might allow for CAR T cells previously shown to cause lethal off-tumor activity, such as ERBB2-specific designs, to be re-introduced.

While the AND gate and iCARs restrict CAR T-cells' activity spatially, they cannot control the intensity of the CAR T-cell activity nor control them in a temporal manner.

Tumor Localizing Mechanisms: Hypoxia Sensitivity and Masked CARs

In order to gain better temporal control and limit CAR T-cell activity to the tumor microenvironment (TME), CAR expression can be controlled by incorporating a hypoxia inducible factor (HIF) in the CAR construct (87). The CAR-HIF construct is continuously targeted for degradation when the CAR T cell is present in a normoxic environment, i.e., most healthy tissues, ensuring that CAR expression only occurs under hypoxic conditions, as seen within parts of the TME. Because degradation of the CAR is regulated at the protein level, control is thought to occur quickly, which is favorable to avoid CAR activity outside of the tumor. This method has only been tested *in vitro*, and may fail to eradicate tumor cells residing in normoxic tissues, e.g., the peripheral parts of the tumor. Moreover, these CAR T cells may show off-target effects in healthy, hypoxic tissues like the bone marrow.

Another method to constrict CAR activity to the TME is by designing a masked CAR, as proposed by Han et al. (88). Here the CARs antigen-binding site is hidden by a masking peptide with a linker sensitive to proteolytic cleavage. Tumor associated proteases present in the TME

TABLE 1 | Current approaches to regulation and control of CAR T cells.

	CAR specific?	Default state (On or Off)	On/Off kinetics	Origin of system	State of control (Permanent or reversible)	Tested in clinical studies	References
Transient transfection	Yes	ON	11–13 days		Permanent	Phase I NCT03060356 NCT02277522 NCT02624258 NCT02623582	(18–23, 25)
Affinity tuning	Yes	ON			Permanent	Phase I NCT02443831	(26–28)
Suicide genes	Yes	ON	<24 h	Modified human and Viral	Permanent	Phase I/II (iCasp9) NCT03579927 NCT02414269 NCT02107963 NCT01822652	(31–42)
Elimination markers	Yes (not CD52)	ON	<1 h–1 week	Modified human	Permanent	Phase I (RQR8, tEGFR) NCT03590574 NCT02746952 NCT03085173 NCT03618381 NCT02051257 NCT03070327 NCT02028455 NCT02146924 NCT01865617 NCT02937844 NCT03638167 NCT02311621 NCT03114670 NCT02159495	(22, 46–52)
Anti-E-tag CARs	Yes	ON/OFF	>48 h	Murine	Permanent	No	(56)
Dasatinib	No (all T cells)	ON	OFF: 1–2 h ON: 7 h		Reversible	No	(59, 60)
Adapter mediated CARs	Yes	OFF	1 h–11 days	Human or murine	Reversible	No	(62–72)
Split-CARs	Yes	ON	<36 h	Human	Reversible	No	(73, 74)
Protease inhibitors	Yes	ON	<48 h	Viral	Reversible	No	(75)
TET-on regulation	Yes	OFF	12–24 h	Viral and bacterial	Reversible	No	(76–78)
AND gates	Yes	OFF		Synthetic	Reversible	No	(80–83)
SynNotch	Yes	OFF	OFF: 8 h ON: 13 h	Murine	Reversible	No	(84, 85)
NOT gates	Yes	ON (CAR) OFF (iCAR)	<24 h	Human	Reversible	Phase I NCT03824951 NCT02442297	(86)
Hypoxia sensitive CARs	Yes	OFF (normoxia)	6 h	Human	Reversible	No	(87)
Masked CARs	Yes	OFF	<6 h	Bacterial	Reversible	No	(88)
IL-1Ra producing CARs	Yes	ON		Murine		No	(9)

can then cleave the linker, removing the masking peptide and allowing CAR T cells to target antigen presenting cells. However, a possibility remains that endogenous proteases cleave the masking peptide, paving the way for on-target, off-tumor toxicities.

CAR T Cells Producing IL-1 Receptor Antagonist (IL-1Ra)

One of the central cytokines involved in CRS is IL-1. Giavridis et al. recently designed a CAR T cell constitutively producing IL-1 receptor antagonist, protecting mice against CRS associated

mortality without affecting the anti-tumor efficacy (9). One major advantage of using IL-1 receptor antagonist is its ability to cross the blood-brain barrier, thereby potentially reducing CAR T-cell-related neurotoxicity (89).

While the above mentioned logic gates and hypoxia sensitive CARs seek to enhance tumor specificity, they do not allow the clinicians control over the CAR T cells and as such offer no solution should life-threatening toxicities arise.

DISCUSSION

The recent years' success of CAR T cells in the clinic has revealed the serious and potentially lethal side effects associated with the potent treatment, including off-tumor, on-target effects, systemic inflammatory conditions such as CRS and acute neurotoxicity (90, 91). More recently it has become clear that cardiovascular and gastrointestinal events can also occur post-CAR-T (92, 93). As the range of specificities and tumor-types targeted using CAR T cells increase, new side-effects will likely come to light. It is therefore important to consider how to best regulate and control engineered T cells.

Because CAR-related toxicities often arise acutely, control mechanisms should ideally grant the clinician swift control over CAR T-cell activity. A direct comparison of the on/off kinetics for each method is made difficult by differences in study design, but regulation on the protein level as well as the use of suicide genes or elimination markers are expected to act faster than regulation at the transcriptional level (**Table 1**). Permanent elimination of CAR T cells however abrogates the long term anti-leukemic effect and many methods therefore aim at reversible control, allowing the clinician to turn off the CAR T cells when toxicities occur. In the future, an appropriately designed recombinase-mediated switch would allow CAR activity to be stably switched into an OFF state with one small molecule, and subsequently flipped back into an ON state using a second molecule (94). This would give clinicians the power to halt CAR T activity without permanently destroying a costly and life-saving therapeutic product, while avoiding the need to constantly administer the suppressive molecule to maintain an OFF state. Ideally, small molecules that have already gained regulatory approval and show minimal side effects can be co-opted to rapidly and reversibly modulate CAR T-cell activity. The choice of drug however must also be guided by the tumor-type targeted, as endothelial barriers, such as the blood-brain-barrier, and poor vascularization can prevent proper biodistribution and concentration in the effected organs.

Another approach is to avoid unwanted immune responses from arising at all. This is the rationale behind many of the logic-gated CAR designs developed, including iCARs and tumor-localized CARs (84, 87). Passive and sustained fine-tuning of CAR expression levels could also be achieved by targeted genomic integration (30) or by using degrons (95) or synthetic miRNA regulation (96). Not only does this have the potential to mitigate dangerous cytokine release, reducing CAR expression has also been shown to combat T-cell exhaustion (30).

The adaptation of CARs to recognize and respond to soluble ligands, such as secreted cytokines, were recently reported by Cheng et al. and creates exciting new possibilities in CAR engineering (97). CARs targeting immunosuppressive soluble ligands, such as TGF-beta, could possibly contribute in overcoming the hostile TME, which has proven a major obstacle especially in solid tumors. Likewise, a possible combination of iCARs or SynNotch receptors with the ability to sense inflammatory cytokines could be used to achieve autonomous dynamic feedback control of CAR activity (98). Consequently, this could lead to the creation of CAR T cells capable of responding to heightened levels of inflammatory cytokines, preventing accompanying toxicities. Simultaneously leveraging inducible and autonomous CAR T-cell control methods could substantially improve the safety of CAR therapy. This might be especially useful when targeting solid tumors, where targeted antigens can often be found in other healthy tissues. While autonomous CAR control designs can restrict cytotoxic activity to the time, location and target cells of interest, also including an inducible kill switch will provide an additional fail-safe in the event the engrafted T cells behave unexpectedly or undergo oncogenic transformation.

Early clinical success and challenges have led to an explosion in new technologies for inducibly, autonomously and passively controlling CAR T cell function, providing the community with a growing menu of solutions for safe and effective anti-cancer therapy. Ultimately the desired regulation of CAR T cells will depend on the location, aggressiveness and targetability of the tumor.

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LB, MB, and YM wrote the manuscript. JH and TB reviewed and edited the manuscript.

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Conflict of Interest: YM and MB have filed a patent relating to a technology presented in this manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Selection of a Clinical Lead TCR Targeting Alpha-Fetoprotein-Positive Liver Cancer Based on a Balance of Risk and Benefit

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Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer with a poor prognosis and limited therapeutic options. Alpha-fetoprotein (AFP), an established clinical biomarker of HCC, has been employed as an attractive target for T cell-based immunotherapy against this disease given its high expression in the tumor and restricted expression in normal tissues. We have identified a number of T cell receptors (TCRs) recognizing the HLA-A*02:01 restricted $AFP_{158-166}$ peptide FMNKFIYEI, providing a TCR candidate pool for identifying TCRs with optimal clinical benefit. To select the ideal AFP TCR for clinical use, we evaluated the efficacy and safety profile of 7 TCRs by testing their potency toward AFP-expressing HCC cells and their specificity based upon reactivity to normal and transformed cells covering a wide variety of primary cell types and HLA serotypes. Furthermore, we assessed their cross-reactivity to potential protein candidates in the human genome by an extensive alanine scan (X-scan). We first selected three TCR candidates based on the *in vitro* anti-tumor activity. Next we eliminated two potential cross-reactive TCRs based on their reactivity against normal and transformed cells covering a variety of primary cell types and HLA serotypes, respectively. We then excluded the potential cross-reactivity of the selected TCR with a protein candidate identified by X-scan. At present we have selected an AFP TCR with the optimal affinity, function, and safety profile, bearing properties that are expected to allow AFP TCR redirected T cells to specifically differentiate between AFP levels on tumor and normal tissues. An early phase clinical trial using T cells transduced with this TCR to treat HCC patients (NCT03971747) has been initiated.

Keywords: T cell receptor (TCR), hepatocellular carcinoma (HCC), alpha-fetoprotein (AFP), cross-reactivity, immunotherapy, alloreactivity, X-scan

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for 75–85% of all liver cancers (1). There are about 841,000 new cases and 782,000 deaths annually caused by liver cancer (1). Liver cancer ranks fifth in terms of global cancer cases and second in terms of cancer death in males (1). In China, there are ∼466,000 cases of liver cancer diagnosed

annually, with the mortality reaching 422,000 each year (2), more than half of the world rate. HCC in adults is often diagnosed in later stages. Current treatments of HCC are limited to surgery, local ablation, liver transplantation, and targeted therapies, which show restricted efficacy and minimal survival benefits for the majority patients (3). New therapeutic means are urgently needed to effectively treat this malignancy. Among them, T cell-based immunotherapy appears to be a promising clinical intervention for HCC patients due to the following observations. First, it is found that there is a correlation of improved overall survival rate with the number of $CD8^+$ T cells infiltrated into HCC (4, 5). Second, the down-regulation of HLA-class I expression in most solid tumors generally does not occur in HCC (6). Instead, HLA-A is upregulated in more than 50% of the patients (6).

T cell-based immunotherapies have proven to be one of the most potent ways to treat late-stage malignancies, especially B-lineage hematologic malignancies (7–9). Efficacy of T cell-based immunotherapies against solid tumors, however, are still limited due to lack of optimal tumor specific targets, heterogeneity of tumor antigen expression, poor persistence of transferred cells, and immunosuppressive tumor microenvironment (7–10). Among these factors, lack of tumor-specific targets is a major obstacle, limiting the effectiveness and safety of T cell-based immunotherapies (9, 11, 12). Therefore, new immunotherapies targeting a HCC specific antigen holds promise to improve the treatment of this devastating disease.

Alpha-fetoprotein (AFP), a secreted 70 kD glycoprotein, has been used as a biomarker for HCC as elevated expression of AFP in tumors and serum is found in 60–80% of HCC patients and correlates with poor prognosis (13). AFP is commonly expressed in the fetal liver and yolk sac during the first trimester of pregnancy, but declines sharply after birth and remains low in adulthood (13). While vital for the developing fetus, the role of AFP in adult tissues is less well-understood but appears dispensable, thus making it a promising tumor antigen for T cell-based immunotherapies (13). A human TCR specific for $AFP_{158-166}$ was recently identified from healthy donors, but the antitumor effect is restricted, probably due to its low affinity (14). Recently, TCR (15) (NCT03132792) and TCR-mimic CAR (16) (NCT03349255) developed to target AFP expressing HCC have been employed in clinical trials. To date, the safety profile remains good, and promising signs of clinical efficacy have been observed (17, 18), suggesting that AFP is a good target for T cell-based immunotherapies.

We have identified a number of TCRs against $AFP_{158-166}$ from HLA-A2 transgenic AAD mice with varied affinity using a lentivector-prime and peptide-boost approach (19). To select the most optimal AFP TCR for clinical use, potent anti-tumor activity would need to be achieved while avoiding severe off-tumor toxicity previously observed in a few clinical trials (12, 20–23). To accomplish this, we first performed serials of *in vitro* assays to select TCRs with potent activity against AFP-expressing tumor cells. Next we evaluated the safety profile of the three selected TCRs by testing the TCR expressing cells against normal and transformed cells, which include a variety of primary cell types and HLA serotypes, respectively. In addition, our colleagues [accompanied study, (24)] performed an X-scan screening to exclude the potential cross-reactivity of TCR 1-3 with other protein candidates in the human genome. We further confirmed that the selected TCR did not cross-react with the potential candidate with serials of validation assays. Based on these analyses, we have selected a TCR based on the balance of its activity and safety profile. This AFP TCR bears properties that are expected to allow T cells, redirected with this TCR, to specifically differentiate between AFP levels on tumor and normal tissues. An early phase clinical trial using T cells transduced with this TCR to treat HCC patients (NCT03971747) has been initiated.

Abbreviations: HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; UT, untransduced; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; PBMC, peripheral blood mononuclear cells; TCR, T cell receptor; BLAST, Basic Local Alignment Search Tool.

MATERIALS AND METHODS

TCR Cloning

For each TCR, the coding sequences of its α and β chain were codon-optimized, joined with a P2A linker, and cloned into a lentiviral backbone under the EF1α promoter.

Lentivirus Production

For packaging, 293T cells (ATCC) were seeded in poly-L-Lysine coated plates (Corning) and transfected the next day with the mix of AFP TCR transfer plasmid and 3 packaging/envelope plasmids, using lipofectamine 3000 (Thermo Fisher). Forty-eight hours after transfection, the virus-containing media were harvested and centrifuged to remove cell debris. The virus supernatant was then directly used for transduction or immediately stored at −80°C.

Generation of AFP TCR-T Cells

Peripheral blood mononuclear cells from healthy donors were obtained from Precision for Medicine (Fredrick, MD). Total or $CD8^+$ T cells were isolated using either EasySep™ Human T Cell Isolation Kit or EasySep™ Human $CD8^+$ T Cell Isolation Kit (both from StemCell Technologies), respectively, following the manufacturer's protocol. The isolated cells were then cultured in AIM V medium (Thermo Fisher) supplemented with 10% fetal bovine serum (FBS; VWR) and 200 IU/mL IL-2 (Peprotech), along with Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher; cell to bead ratio 1:1). After 24 h of activation, cells were transduced with AFP TCR lentivirus in the presence of 10 μg/mL Protamine Sulfate (Sigma). The transduced cells were expanded for 9–11 days and then used for downstream analysis or cryopreserved with Cryostor D10 media (Biolife Solutions).

Cell Lines, Primary Cells, and iCells

HepG2 and Huh7 cells were obtained from ATCC. MDA-MB231 cells were obtained from Dr. Hasan Korkaya who originally purchased from ATCC. All cell lines were maintained in DMEM medium supplemented with 10% FBS (VWR). The Epstein-Barr virus (EBV)–transformed B-lymphoblastoid cell

lines (B-LCL) used for alloreactivity test were obtained from either Sigma or Fred Hutchinson Cancer Research Center, and maintained in RPMI 1640 medium supplemented with 15% FBS (VWR). Primary adult human hepatocytes were obtained from Lonza. Primary human lung and kidney epithelial cells were purchased from Novabiosis and Lifeline, respectively. Induced pluripotent stem cell-derived iCell® Neurons, Astrocytes, Cardiomyocytes and Endothelial Cells were all from FUJIFILM Cellular Dynamics, Inc. The culture medium, supplements and plate coating reagents for each cell type were purchased from vendors as designated by the cell suppliers. Primary hepatocytes and the four iCells were originally from HLA-A*02:01$^+$ donors, according to vendor-provided information. Primary lung and kidney epithelial cells were originally from HLA-A2$^+$ donors, in-house PCR (25) and sequencing further confirmed they also carry HLA-A*02:01 allele.

Co-culture

HepG2, Huh7, and B-LCL cells were harvested and resuspended in IL-2 free T cell medium. Total 50,000 cells were then seeded in each well of a 96-well plate, followed by addition of equal number of AFP TCR T or untransduced control T cells. After overnight co-culture, the supernatant was saved for ELISA, and cells from replicate wells were combined for FACS analysis of 4-1BB activation wherever indicated. Primary lung and kidney epithelial cells and iCell Endothelial Cells were expanded after thawing, and the 2nd passage cells were harvested for co-culture with T cells as stated above. Co-culture with other primary cells or iCells was done similarly except that primary hepatocytes, iCell Neurons, Astrocytes or Cardiomyocytes were seeded in 96-well plates immediately after thawing, and T cells were added the next day for overnight incubation.

IFN-γ ELISA

The ELISA plates were coated with a human IFN-γ monoclonal capture antibody (Thermo Fisher, cat. M700A) overnight at 4°C. After washing and blocking with assay buffer, cell culture supernatants or diluted IFN-γ standards (Biolegend, cat. 570209) were added together with the biotin-labeled IFN-γ antibody (Thermo Fisher, cat. M701B) and incubated at room temperature for 1.5 h. HRP-conjugated Streptavidin (Thermo Fisher, cat. N100) and TMB Substrate (Thermo Fisher, cat. 34021) was then sequentially added for detection. After stopping the reaction, the absorbance was measured at 450 nm by a SpectraMaX iD3 plate reader (Molecular Device).

FACS

Cells were first stained with LIVE/DEAD™ Fixable Aqua (Thermo Fisher, cat. L34957), followed by staining with antibodies against various surface markers. Samples were then acquired by a FACS instrument (CytoFLEX LX, Beckman Coulter). For intracellular staining, GolgiPlug (BD) was added upon at the beginning of T cell and target cell co-culture and cells were cultured for 6 h. Samples were then processed with the Cytofix/cytoperm kit (BD, cat. 555028) following the manufacture's protocol. The following antibodies were used: APC-eFluor 780 anti-human CD3 (Thermo Fisher, cat. 47003642), FITC anti-human CD8a (Biolegend, cat. 301006), BV605 anti-mouse TCRβ (Biolegend, cat. 109241), APC anti-human CD137 (Biolegend, cat. 309810), PECy7 anti-human IFN-γ (Biolegend, cat. 502528), Alx700 anti-human TNF-α (Biolegend, cat. 502928), PE anti-human IL2 (Biolegend, cat. 500307), PerCPCy5.5 anti-human CD107a (Biolegend, cat. 328616). HLA-A2/AFP158 tetramer was kindly provided by NIH Tetramer core facility at Emory University. All data were analyzed with FlowJo V10 software (FlowJo).

Cytolytic Assay

HepG2 cells were seeded in the 96-well E-plate (30,000 cells/well) and placed in the Real-time Cell Analyzer (RTCA MP, ACEA Biosciences) to record the cell index. The next day T cells were added at the indicated effector to target ratio, and cell index was recorded continuously for another 4 days. The cell index curve was then normalized to the time point just before adding T cells, and transformed as percentage cytolysis by comparing the average of triplicate in each T cell group with that of target cell only group (i.e., only T cell medium was added).

Western Blot

Cell lysates were extracted with RIPA buffer (Thermo Fisher), separated on a 4–12% SDS-page gel (Thermo Fisher), and transferred onto nitrocellulose membrane using iBlot 2 gel transfer device (Thermo Fisher). The membrane was blotted with indicated primary antibodies. After incubating with corresponding secondary antibody, signals were detected on an Odyssey Fc imaging system (Licor). ENPP1 antibody was purchased from Abcam (cat. ab223268). AFP and GAPDH antibodies were from Santa Cruz Biotechnology (cat. sc-8399 and sc-47724, respectively). IRDye® 800CW Goat anti-Rabbit IgG and IRDye® 680RD Goat anti-Mouse IgG secondary antibodies were from Licor.

Bead-Based Immunoassay

The LEGENDplex™ Human CD8/NK panel (BioLegend, cat. 740267) was used for simultaneous quantification of 13 soluble analytes in cell culture supernatants, following the manufacture's protocol. The assay was read on a CytoFLEX LX FACS instrument (Beckman Coulter).

Statistical Analysis

Data were analyzed using the GraphPad Prism software (Version 5). Unpaired Student *t*-test was conducted for comparison and two-tailed $P < 0.05$ was considered statistical significant.

RESULTS

Screening AFP TCRs for Their Anti-tumor Activities *in vitro*

We have previously reported the identification of 3 TCRs from HLA-A2 transgenic AAD mice after immunization with human $AFP_{158-166}$ peptide (19). Four additional TCRs were identified from newly sequenced hybridomas, following the same method described previously (19). In order to identify the optimal AFP TCRs based on potency toward their targets,

we first codon-optimized and cloned the 7 AFP TCRs (19) into a lenti-viral backbone and enforced their expression in human T cells. Eight days after transduction, the relative T cell expansion and phenotype of 7 AFP TCR-transduced cells are comparable (**Figure S1**). Next we evaluated the expression of TCR β chain as well as the pairing of TCR α and β chains by measuring the surface expression levels of TCR β chain and AFP tetramer binding capacities. The expression level of TCR β chains is comparable, ranging from 80 to 90% for these 7 TCRs. Interestingly, the tetramer binding capacity of these TCRs is highly variable, although the CDR3 regions of these TCRs are quite similar at the amino acid level especially when compared in pairs (**Figure 1A**). TCR 3 demonstrated the highest tetramer binding capacity reaching almost 60%, followed by TCR 1, 2, and 6, reaching 40%, while TCR 8, 10, and 11 were much lower in the range of 7–26% (**Figure 1B**). We reasoned that the different tetramer binding activity is very likely due to the pairing difference between TCR α and β chain given the comparable expression level of 7 TCR β chains and the assumption that the expression of TCR α chain is similar since the codon-optimized α chain is cloned upstream of TCR β chain.

To evaluate the cytokine secretion capacity of these AFP TCRs, we co-cultured the TCR expressing cells with HCC cell line HepG2, which displays high AFP expression in a HLA-A2 setting (26). A significant fraction of T cells redirected with TCR 1, 2, and 3 were capable of producing multiple cytokines including IL-2, IFN-γ, TNF-α, as well as degranulation factor CD107a. Nevertheless, TCR 8 and 11 expressing T cells produced very few cytokines and TCR 6 and 10 expressing T cells displayed intermediate cytokine producing capacity (**Figure 1C**). This observation is consistent with their respective tetramer binding capacities, except for TCR 6 expressing T cells, which produced less cytokines compared to TCR 1 and 2 redirected T cells, even though their tetramer binding capacities were close, suggesting that the cytokine production capacity is not solely dependent on the ability to bind target. Consistently, the IFN-γ secretion capacity of different TCRs aligned well with their intracellular cytokine production capacities, i.e., T cells expressing TCR 1, 2, and 3 secreted the highest amount of IFN-γ (**Figure 1D**).

We next examined the cytolytic activity of these TCR expressing cells upon encountering their targets. Again, T cells expressing TCR 1, 2, and 3 displayed the highest cytotoxicity, killing almost 95% target HepG2 cells after co-culturing for 48 h (**Figure 1E**) and taking virtually the same time to kill 50% of the targets at the effector to target ratio of 0.25:1 and 0.5:1, surpassing the other 4 TCRs (**Figure 1F**). Taken together, TCR 1, 2, and 3 demonstrated the best TCR expression and pairing profile, strong cytokine production capacity, and most potent target killing efficacy. These three TCRs were therefore selected for the next step of safety profile screening.

Selection of AFP TCR 2 Based on Its Balance of Potency and Specificity

AFP protein shows a specific expression pattern in HCC that rarely appears in human adult tissue (13, 15). However, to more rigorously evaluate potential "on-target, off-tumor" toxicity for AFP TCRs, we examined the ability of AFP TCR engineered T cells to react with a range of primary cells, especially those from essential human organs. First we tested the reactivity of AFP TCR transduced cells to HLA-A*02:01$^+$ iCell derived neurons. T cells transduced with TCR 1 and 2 showed minimal reactivity against iCell neurons but the reactivity of TCR 3 transduced cells was relatively higher in one of the two donor cells tested (**Figure 2A** and the data of the other donor is not shown), suggesting that TCR 3 is less specific compared to TCR 1 and 2.

Simultaneously, we tested the reactivity of TCR 1, 2, and 3 against a panel of EBV–transformed B cell lines designed to evaluate alloreactivity (15, 22). We did observe low level of IFN-γ release from untransduced (UT) control T cells after co-culture with these B cell lines, most likely due to the recognition of EBV antigen by the endogenous TCRs. Therefore, only increases in IFN-γ release above such background would be considered as alloreactive. To this end, TCR 1 engineered T cells did show higher activity against cell line 1332-8265, FH43, and KT14 compared to other T cell products including UT, TCR 2 and 3 engineered T cells (**Figure 2B**), suggesting that the relative alloreactivity of TCR 1 is higher than the other two TCRs.

Taken together, we decided to focus on TCR 2 to further test the toxicity of this TCR as our leading clinical candidate based on its optimal balance of activity and specificity relative to TCR 1 and 3. Furthermore, TCR 2 also displayed strong anti-tumor activity in an NSG mouse model (19), consolidating the choice of this TCR for further safety profile testing.

No Off-Target Recognition Was Detected for TCR 2 Engineered Cells Toward a Panel of HLA-A*02:01$^+$ Primary Cells

To exclude any potential cross-reactivity of AFP TCR 2 engineered T cells against essential human tissues, we further tested the reactivity of TCR 2 expressing T cells toward a panel of HLA-A*02:01$^+$ primary cells derived from different human tissues. First we co-cultured AFP TCR 2 engineered T cells with primary hepatocytes from various donors and resources and measured the IFN-γ release as an indicator of reactivity. Again, TCR 2 engineered T cells released high levels of IFN-γ toward their target HepG2 cells, but only background levels of IFN-γ were detected when co-culturing with primary hepatocytes (**Figure 3A**). Similarly, no significant amount of cytokines or effector molecules were detected from the co-culture supernatant including IL-2, IL-4, IL-6, IL-10, IL-17a, TNF-α, sFas, sFasL, Granzyme A, Granzyme B, Perforin, and Granulysin (**Figure S2**). This suggests that there is no cross-reactivity of TCR2 engineered T cells with primary hepatocytes, further reassuring that AFP is a safe target for HCC. To further test the potential reactivity on a per cell basis, we checked the 4-1BB level in TCR 2 engineered T cells co-cultured with primary hepatocytes. No up-regulation of 4-1BB was detected in TCR 2 engineered cells co-cultured with hepatocytes while a significant fraction of 4-1BB was upregulated when co-cultured with HepG2 cells, demonstrating that TCR 2 engineered T cells do not recognize primary HLA-A*02:01$^+$ hepatocytes (**Figure 3B**).

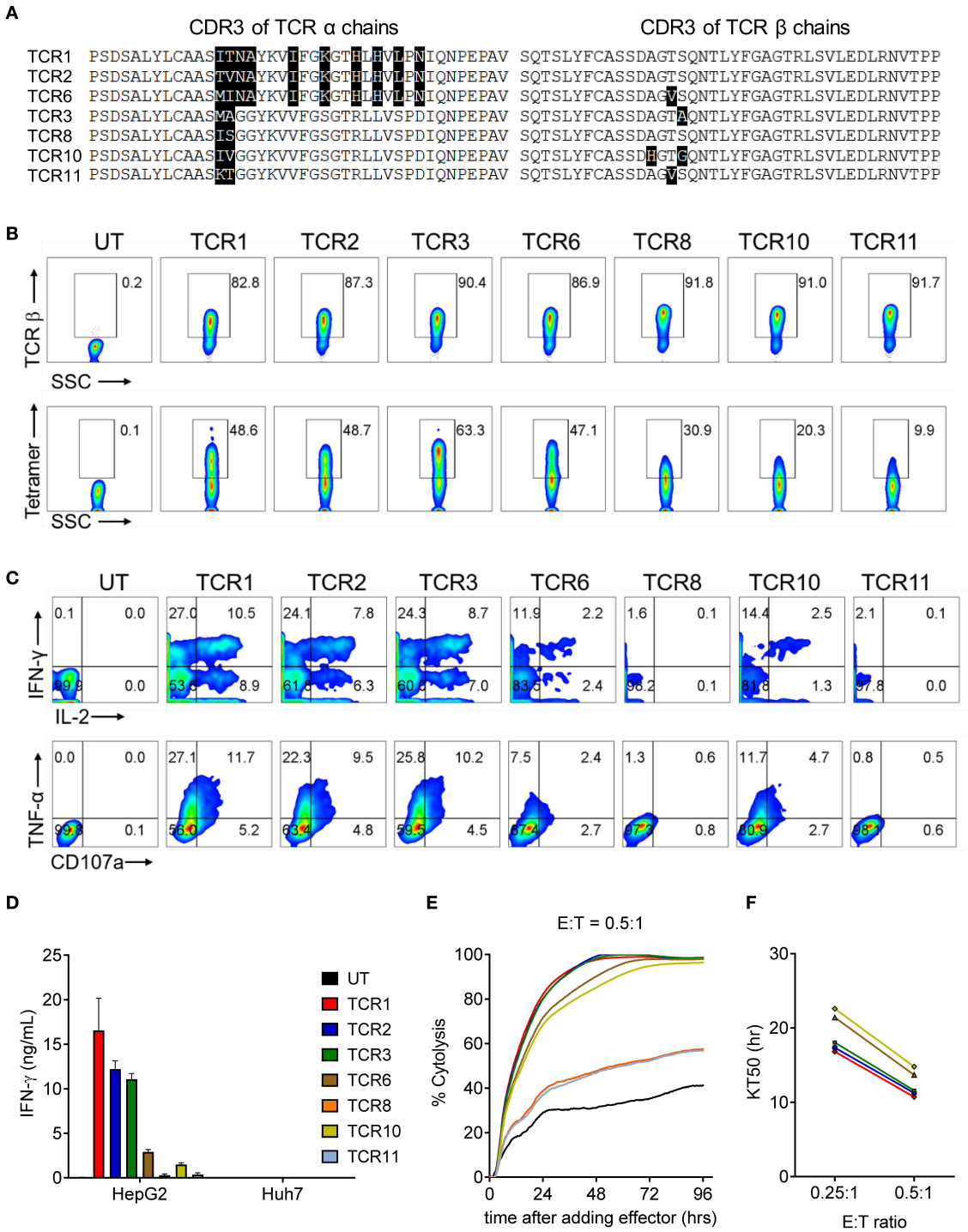


FIGURE 1 | AFP TCR1, 2, and 3 demonstrate comparable potent reactivity toward the target cells *in vitro*. **(A)** Alignment of amino acid sequence of CDR3 regions of the α (left) and β (right) chains of the 7 AFP TCRs. **(B)** FACS analysis of the surface expression of TCR β chain and AFP tetramer staining of T cells 8 days after transducing of indicated AFP TCRs. The percentage of TCR β (top) or AFP tetramer (bottom) positive cells as indicated. **(C)** Cytokine production and degranulation of AFP TCR T cells upon encountering HepG2 target cells as revealed by intracellular staining of IL-2, IFN-γ (top), TNF-α and CD107a (bottom). **(D)** IFN-γ concentration in the supernatant of overnight, 1:1 ratio co-culture of AFP TCR T cells with HepG2 target cells or control Huh7 cells measured by ELISA. Data represents the mean + s.d. of quadruplicate co-culture samples. **(E)** Cytolytic capacity of AFP TCR T cells toward HepG2 target cells over a 4-day co-culture. Data represents the mean of triplicate samples derived from RTCA instrument, as compared to target only wells. (E:T): effect to target ratio. Colors are represented as in **(D)**. **(F)** Time to eradicate 50% of target cells (KT50) of different AFP TCR T cells at the indicated effect to target (E:T) ratio. Colors are represented as in **(D)**. T cells transduced with TCR 8 and TCR 11 did not eradicate 50% of the target by the end of the analysis at E:T = 0.25:1, thus were not plotted. Data shown in b-f is representative of at least 3 independent experiments on T cells isolated from 3 healthy donors. UT, untransduced T cells.

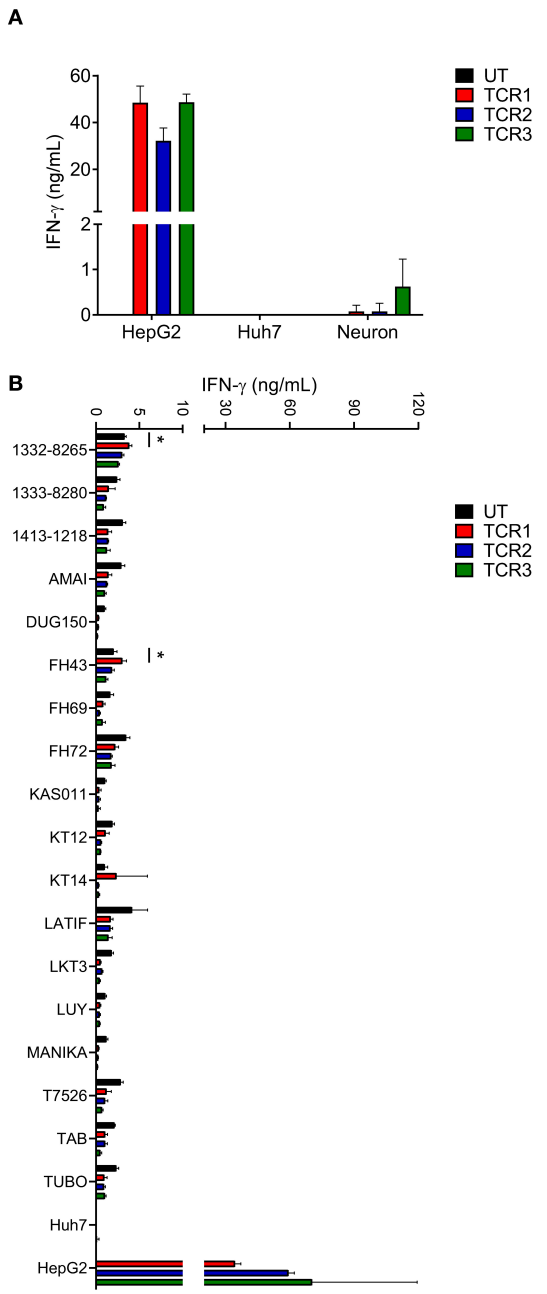
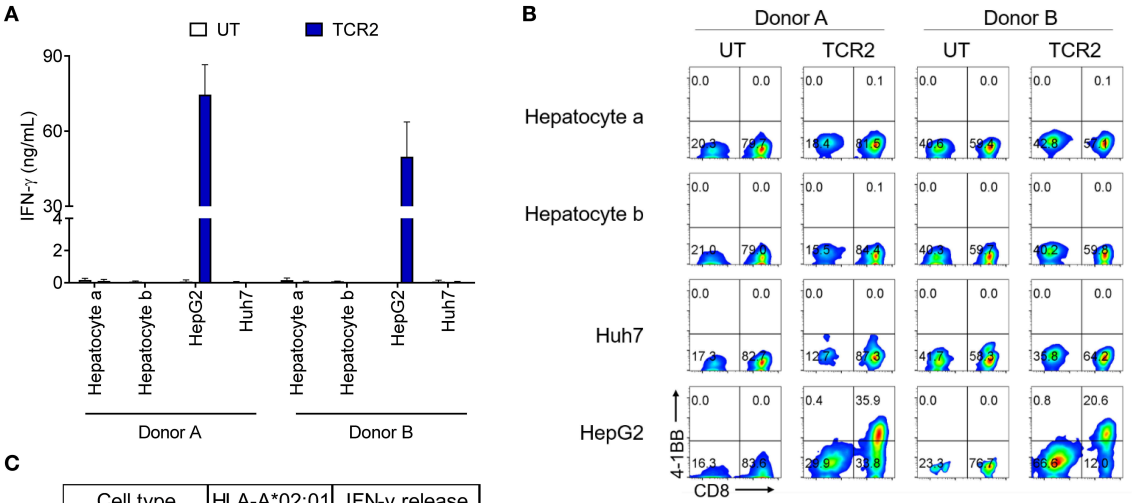


FIGURE 2 | AFP TCR 2 shows lower than background activity toward primary neurons and no alloreactivity compared to TCR 1 and 3. **(A)** IFN-γ concentration in the supernatant of overnight, 1:1 ratio co-culture of the indicated AFP TCR T cells with iCell derived neurons measured by ELISA. Data represents the mean + s.d. of the triplicate co-cultures and is representative of T cells prepared from 2 healthy donors. **(B)** IFN-γ concentration in the supernatant of overnight, 1:1 ratio co-culture of the indicated AFP TCR T cells with a panel of Epstein-Barr virus transformed B cell lines expressing various HLA alleles measured by ELISA. Data represents the mean + s.d. of quadruplicate co-cultures and is representative of T cells prepared from 2 healthy donors. HepG2 and Huh7 cells were included as positive and negative controls, respectively, in both **(A,B)**. *$P < 0.05$ (unpaired two-tailed Student's *t*-test).

We therefore performed serials of assays including IFN-γ release and 4-1BB upregulation by TCR 2 engineered T cells co-cultured with a number of HLA-A*02:01$^+$ primary or iCell derived cells including astrocytes, cardiomyocytes, lung, kidney, and endothelial cells. Thus far, there was no above background cross-reactivity detected (**Figure 3C** and **Figure S3**). Taken together, we conclude that TCR 2 engineered T cells demonstrate no above-background cross-reactivity toward the HLA-A*02:01$^+$ primary cells tested.

TCR 2 Engineered T Cells Showed Minimal Alloreactivity

Next we performed a comprehensive screening of the alloreactivity of TCR 2 engineered T cells using a large panel of EBV-transformed B cell lines, which has been widely used as a surrogate for the antigen presenting cells with different HLA types (15, 22). Due to the large number of HCC cases and unmet medical need in the Chinese population, we first chose EBV-transformed B cell lines covering a significant percentage of HLA serotypes in Chinese population (27). In addition to the previous 14 EBV-transformed lines tested (**Figure 2B**), we examined another set of EBV-transformed B cell lines (**Figure 4A**) and later expanded to a total of 38 lines (**Table S1**), covering more than 98% of the HLA class I serotype of Chinese population (**Figures 4B,C**). There was generally no above-background level of IFN-γ detected in the allo-lines tested (**Figure 4A** and the data of other 10 cell lines is not shown). However, in the co-culture supernatant from cell line 1332-8265 and FH43, we found that the IFN-γ release was slightly higher than UT, although this observation was donor dependent (**Figure S4A**). All the HLA class I alleles expressed by these two cell lines are also found in other non-reactive cell lines. However, they share a common HLA class II allele DPB1*11:01, which is unique to these two B cell lines and absent from the rest in the same experiment. To test whether there is alloreactivity of TCR 2 against DPB1*11:01$^+$ cells, in an independent experiment we measured the 4-1BB upregulation in TCR 2 engineered T cells against 1332-8265 and FH43, together with an additional DPB1*11:01$^+$ line FH5, and other DPB1*11:01 negative controls. Again, 4-1BB was highly upregulated when TCR 2 engineered CD8$^+$ T cells encountered the target cell HepG2. We also detected marginal upregulation of 4-1BB in the CD4$^+$ fraction of TCR 2 engineered T cells, when they were co-cultured with all three DPB1*11:01$^+$ cell lines from our collection of 38 lines. We considered it marginal because the highest percentage of upregulation was only about 1.8%, compared to the background 0.4% in its UT counterpart. Interestingly, we did not observe upregulation in TCR 2 engineered CD8$^+$ T cells (**Figure S4B**), suggesting that the reactivity to DPB1*11:01 is restricted to CD4$^+$ T cells transduced with TCR 2. To further examine whether CD4$^+$ T cells are the only resource inducing the alloreactivity, we tested the alloreactivity to DPB1*11:01 cell lines with AFP TCR 2 engineered pan T cells including both CD4$^+$ and CD8$^+$ T subsets and AFP TCR 2 engineered CD8$^+$ only T cells from the same donor. Consistent to our hypothesis, the increased



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Cell type	HLA-A*02:01	IFN-γ release
Hepatocyte	+	No
Neuron	+	No
Astrocyte	+	No
Cardiomyocyte	+	No
Lung	+	No
Kidney	+	No
Endothelial	+	No

FIGURE 3 | AFP TCR 2 displays no reactivity toward a variety of HLA-A*02:01$^+$ primary cells. **(A)** IFN-γ concentration in the supernatant of overnight, 1:1 ratio co-culture of UT or T cells expressing AFP TCR 2 with primary hepatocytes measured by ELISA. T cells were prepared from 2 healthy donors and human primary hepatocytes were obtained from 2 adult HLA-A*02:01$^+$ donors. Data represents the mean + s.d. of quadruplicate co-cultures. **(B)** 4-1BB upregulation by T cells after co-culture as described in a. Cells were further analyzed by FACS for surface expression of 4-1BB on total CD3$^+$ untransduced T cells or AFP TCR 2 transduced T cells. HepG2 and Huh7 cells were included as positive and negative controls, respectively, in both **(A,B)**. **(C)** Summary of reactivity from co-culture experiment of T cells expressing AFP TCR 2 with primary hepatocytes, primary lung and kidney epithelial cells, and iCell derived primary cells including neurons, astrocytes, cardiomyocyte, and endothelial cells.

IFN-γ release compared to UT from pan T cells diminished in CD8$^+$ T cell only population (**Figure S4C**), suggesting that the minimal reactivity to DPB1*11:01 allele is associated with TCR 2 transduced CD4$^+$ T cells. In other words, TCR 2 may show minimal alloreactivity to DPB1*11:01 allele.

In total we have tested 38 EBV-transformed B cell lines covering 52 HLA serotypes, which represent more than 98% of HLA-A, B, and C in the Chinese population, accounting for almost half of the HCC patients worldwide (**Figures 4B,C**). These selected B cell lines also cover a significant percent of other ethnic groups including European Caucasian, African American, Hispanic, Mexican/Chicano, South Asian Indian based on data from the Allele Frequency Net Database (28) (**Table S2**). This shows the eligibility of a significantly greater patient population for this AFP TCR worldwide.

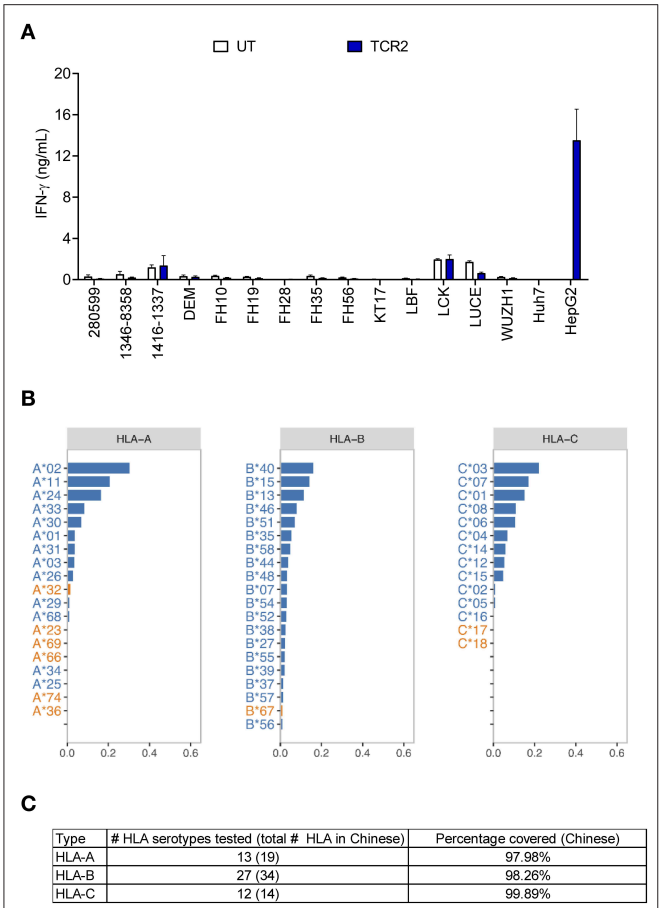
AFP TCR 2 Is Unlikely to Cross-React With Other Proteins in the Human Genome

In order to detect whether there is any cross-reactivity of AFP TCRs to any other proteins in the human genome, an X-scan screen was performed to exclude the possibilities [accompanied study, (24)]. Basically we identified the key irreplaceable amino acids of AFP$_{158-166}$ peptide for TCR 2 recognition (**Figure 5A**). In other words, any amino acid replacement that induces more than 90% activity loss is considered as irreplaceable. Using this criteria, we identified an AFP TCR 2 binding motif defining the essential positions critical for AFP TCR 2 activity (**Figure 5A**).

We next performed a BLAST search of the UniProtKB protein database with the identified key motif for AFP TCR 2. Further analysis and testing identified peptide YLNKYLGDV as the only candidate that is able to activate TCR 2 transduced T cells *in vitro* [for details see the accompanied study, (24)].

YLNKYLGDV could potentially be processed from transmembrane glycoprotein ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) protein, a key player involved in inhibiting insulin receptor signaling and the development of insulin resistance (29). We next set to test whether ENPP1 expressing cells can activate TCR 2 transduced T cells. It is found that ENPP1 is highly expressed in MDA-MB-231, a HLA-A*02:01$^+$ cell line, according to the Broad Institute Cancer Cell Line Encyclopedia[1]. We therefore evaluated the ENPP1 level in MDA-MB-231 cells by Western blot. Consistent with the reported mRNA expression in the database, ENPP1 protein was expressed in MDA-MB-231 cells, similar to the level in HepG2

[1]https://portals.broadinstitute.org/ccle



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Type	# HLA serotypes tested (total # HLA in Chinese)	Percentage covered (Chinese)
HLA-A	13 (19)	97.98%
HLA-B	27 (34)	98.26%
HLA-C	12 (14)	99.89%

FIGURE 4 | AFP TCR 2 shows no alloreactivity. **(A)** IFN-γ concentration in the supernatant of overnight, 1:1 ratio co-culture of UT or AFP TCR 2 transduced T cells with a panel of 14 Epstein-Barr virus transformed B cell lines expressing various HLA alleles measured by ELISA. Data represents the mean + s.d. of quadruplicate co-cultures and is representative of 3 healthy donors. A total of 38 transformed B cell lines were tested. **(B)** Frequencies of class I HLA serotypes in Chinese Han population. Serotypes were sorted by frequency for each subclass from high to low; those covered by the 38 transformed B cell lines in our test are shown in blue while those not covered are shown in orange. For HLA-B, only the top 20 serotypes are displayed. **(C)** Summary of the number of class I HLA serotypes covered in this study and corresponding cumulative percentage in Chinese Han population.

cells (**Figure 5B**). Interestingly, ENPP1 protein was also detected in primary hepatocytes [accompanied study, (24)], however AFP was only detected in HepG2 cells (**Figure 5B**). We therefore performed a co-culture assay to test the reactivity of TCR 2 transduced T cells toward different cell lines. Again, high IFN-γ release was detected from the co-culture supernatant of HepG2 cells, but there was virtually no IFN-γ release from the co-culture supernatant of MDA-MB-231 (**Figure 5C**). Previously, we also showed that there is no IFN-γ or other cytokine release as well as T cell activation when TCR 2 expressing cells were co-cultured with primary hepatocytes (**Figures 3A,B** and **Figure S2**). Taken together, it is clear that TCR 2 transduced T cells do not recognize HLA-A*02:01 restricted, ENPP1 expressing cells but only recognize AFP expressing targets. Because ENPP1 is the only candidate that may be recognized by AFP TCR 2 in X-scan screening, we can conclude that there is virtually no cross-reactivity of TCR 2 transduced T cells to other antigens in the human genome under physiological condition.

DISCUSSION

In this article, we described a pipeline for systematically selecting a clinical lead AFP TCR for translational purposes. We first screened our TCR candidates based on their reactivity toward the target *in vitro*. Once we shrunk the pool of candidates by selecting the most potent ones, we further tested their cross-reactivity toward primary cells, EBV-transformed B cell lines covering a wide range of HLA class I, and also reactivity toward other potential targets in the human genome. Based on the balance of all the above factors, we selected TCR 2 to start our clinical trial (NCT03971747).

Our TCR pool is generated from natural occurring TCRs after immunizing AAD mice with $AFP_{158-166}$ peptide (19). Interestingly, these TCRs displayed different TCR α and β chain pairing capability and also varied activities with a few amino acid differences in the CDR3 region. We found that the CDR3 region of our TCR β chains is quite conserved, with only 2–3 amino acid differences (**Figure 1A**), which is consistent with the findings that different TCR clonotypes specific for the same antigen often utilize the same Vβ gene segment (30, 31). However, we observed significant differences among the activity of these TCRs. For example, TCR 3 and TCR 8 only vary by 3 amino acids in the CDR3 region: 1 in the β chain and 2 in the α chain. However, the pairing capacity of their α and β chains is quite different and the reactivity against targets is even more different in the *in vitro* assays (**Figures 1B–F**), suggesting that only a few amino acid changes in CDR3 region can cause substantial difference in TCR activities. Because all of these TCRs are naturally occurring, the information embedded in their CDR3 region together with their corresponding activity can provide a reference for gene engineering strategies aimed to improve TCR affinity and potency.

For effective adoptive cell therapy, one would like to choose a TCR with optimal affinity to allow the TCR engineered T cells to efficiently eradicate their targets. However, TCRs with higher activity are not always the best clinical option as they may pose a safety issue. It is established that TCRs of high affinity within the natural range are more efficacious but also prone to be associated with less specificity (32). In our case, TCR 1 and 3 most times outperformed TCR 2 (we repeated the same result many times besides the data shown in **Figures 1D–F** and **Figure 2**) in IFN-γ release. But the increase of activity, again, is associated with a higher level of reactivity toward primary cells and also alloreactivity. Furthermore, TCR 2 also displayed potent anti-tumor activity in an NSG mouse model (19). Taken together, we chose TCR 2 as our clinical lead candidate even though the anti-tumor activity is comparable or even less potent compared to TCR 1 and 3 *in vitro*.

All of our TCRs are not gene engineered so the chance of non-specificity induced by gene engineering is relatively

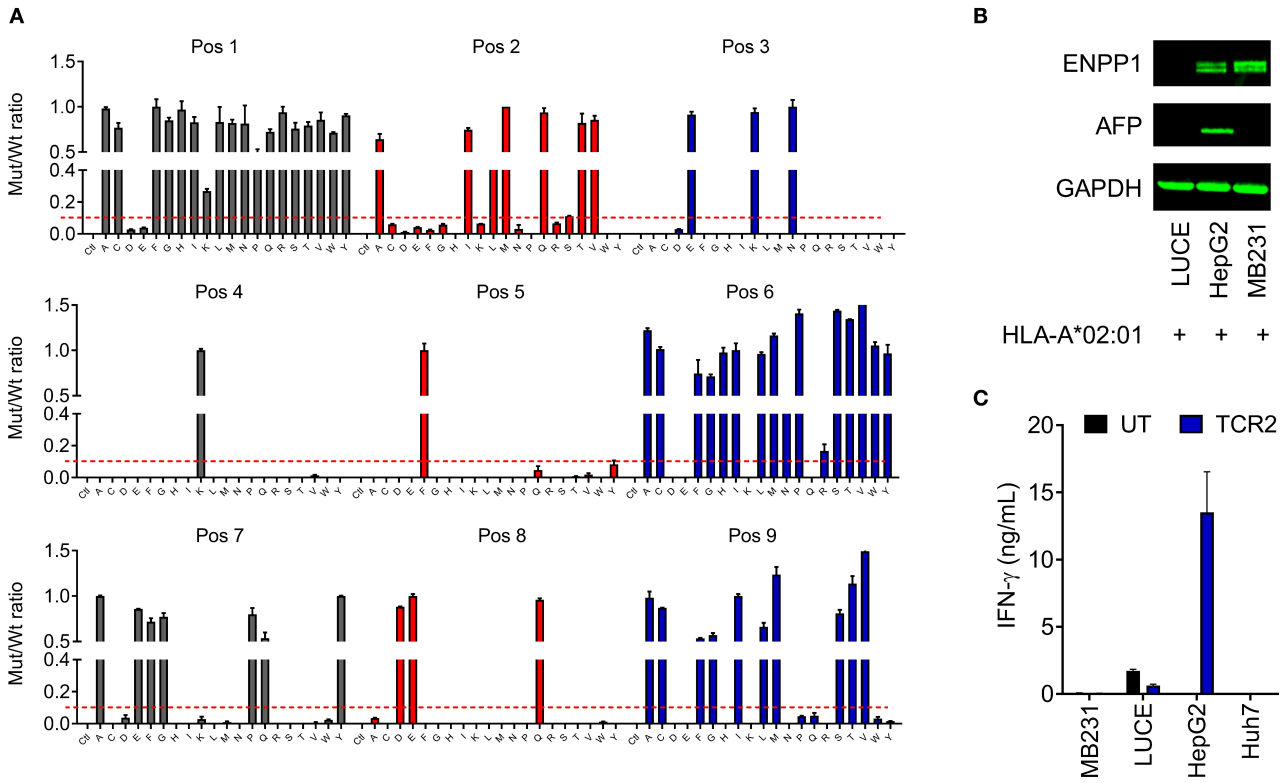

FIGURE 5 | AFP TCR 2 is unlikely to cross-react with other proteins in the human genome. **(A)** Graphic presentation of X-scan results showing the replaceability of each amino acid of $AFP_{158-166}$ epitope. Peptides with replacement of each amino acid residue with every other 19 amino acids were used to activate the TCR 2 transduced T cells. The amount of IFN-γ produced by AFP TCR 2 transduced T cells after stimulation with mutated epitope (mut) was compared to that from wild type $AFP_{158-166}$ peptide (wt) and the ratios are presented. Dashed line indicates the cut-off for tolerance. Data represents the mean + s.d. of duplicate co-cultures and is representative of 3 healthy donors. **(B)** ENPP1 and AFP expression in the indicated cell lines tested by Western blot. GAPDH was used as loading control. **(C)** IFN-γ concentrations in the supernatant of overnight, 1:1 ratio co-culture of UT or AFP TCR 2 transduced T cells with the indicated cell lines measured by ELISA. Data represents the mean + s.d. of quadruplicate co-cultures and is representative of 3 healthy donors.

low. However, we think it is still critical and necessary to perform safety studies to select the best candidate for clinical use. Unfortunately, there are no optimal animal models to test the cross-reactivity of TCRs to human tissues due to the absence of human antigen and HLA expression and processing in these models. Therefore, carefully designed *in vitro* assays become critical. We, again, confirmed that the combination of using primary cells, EBV-transformed cell lines with different HLA alleles, and X-scan is a valid platform for evaluating TCR safety profile, which is supported by numerous retrospective and prospective studies (15, 22, 33, 34). Although the actual safety of a TCR needs to be eventually evaluated by carefully designed phase I dose-escalation safety trials, which are now performed by almost all T cell-based therapy trials, it is worth the effort to perform an *in vitro* preclinical safety analysis using the above methodologies considering the low cost and the quick turnaround of these *in vitro* assays compared to clinical trials.

Considering the urgent unmet medical need for treating HCC in China and worldwide, adoptive cell transfer appears to be a promising method to effectively treat this malignancy. Our study provides a basis for advancing a TCR targeting HCC into the clinic based on the balance of activity and safety. The extensive panel of selected EBV-transformed cell lines in our assay covers majority of HLA serotypes, offering a selection criterion for patient recruitment across a wide range of ethnic groups worldwide. Furthermore, our study also provides a reference for generating different recruitment criteria for patients from different ethnic backgrounds. For example, we did not exclude DPB1*11:01 in our first-in-human trial in Han Chinese due to the following reasons: First, the percentage of DPB1*11:01 in Han Chinese, which accounts for 92% of Chinese population, is just 0.03%. Considering that all the patients we plan to recruit must be $A*02:01^+$, which is around 14%, the chance of encountering an $A*02:01^+$ $DPB1*11:01^+$ patient is extremely low. Second, the alloreactivity of TCR 2 toward DPB1*11:01 positive cells is relatively marginal, only around 2% of the amount from positive control HepG2 cells, and is donor dependent. Therefore, we believe it is relatively safe to move TCR 2 further into clinical trial without excluding $DPB1*11:01^+$ patients in Han Chinese. However, it will be necessary to exclude DPB1*11:01 if the patient pool includes a higher percentage of this allele. Currently an early phase clinical trial using T cells transduced with TCR 2 to treat HCC patients (NCT03971747) has been initiated in China. We hope the safety and efficacy data

obtained from this trial will provide a solid foundation for its application worldwide.

In conclusion, we have selected a TCR with a balance of affinity, function, and safety profile, bearing properties that are expected to allow AFP TCR redirected T cells to specifically differentiate between AFP levels on tumor and normal tissues. The safety and efficacy signals obtained from the clinical trial (NCT03971747) with this TCR will provide a solid foundation for its application worldwide.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

YJ, XL, LC, and YH designed and interpreted the study. XL, HC, LC, W-CY, and MP performed the experiments. XL, YJ, HC, SZ, JH, XY, and YY analyzed the data. WZ performed bioinformatics analysis. YJ and XL wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00623/full#supplementary-material

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ORIGINAL RESEARCH
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Preclinical Studies of the Off-Target Reactivity of AFP_{158}-Specific TCR Engineered T Cells

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Autologous T cells engineered with T receptor genes (TCR) are being studied to treat cancers. We have recently identified a panel of mouse TCRs specific for the HLA-A0201/alpha fetoprotein epitope (AFP_{158}) complex and have shown that human T cells engineered with these TCR genes (TCR-Ts) can eradicate hepatocellular carcinoma (HCC) xenografts in NSG mice. However, due to TCR's promiscuity, their off-target cross-reactivity must be studied prior to conducting clinical trials. In this study, we conducted *in vitro* X-scan assay and *in silico* analysis to determine the off-target cross-reactivity of 3 AFP_{158}-specific TCR-Ts. We found that the 3 AFP_{158}-specific TCR-Ts could be cross-activated by $ENPP1_{436}$ peptide and that the TCR3-Ts could also be activated by another off-target peptide, $RCL1_{215}$. However, compared to AFP_{158}, it requires 250 times more $ENPP1_{436}$ and 10,000 times more $RCL1_{215}$ peptides to achieve the same level of activation. The EC_{50} of $ENPP1_{436}$ peptide for activating TCR-Ts is approximately 17–33 times higher than AFP_{158}. Importantly, the ENPP1+ tumor cells did not activate TCR1-Ts and TCR2-Ts, and only weakly activated TCR3-Ts. The IFNγ produced by TCR3-Ts after ENPP1+ cell stimulation was >22x lower than that after HepG2 cells. And, all TCR-Ts did not kill ENPP1 + tumor cells. Furthermore, ectopic over-expression of ENPP1 protein in HLA-A2+ tumor cells did not activate TCR-Ts. *In silico* analysis showed that the $ENPP1_{436}$ peptide affinity for HLA-A0201 was ranked 40 times lower than AFP_{158} and the chance of $ENPP1_{436}$ peptide being processed and presented by HLA-A0201 was 100 times less likely than AFP_{158}. In contrast, the two off-targets (Titin and MAGE-A3) that did cause severe toxicity in previous trials have the same or higher MHC-binding affinity and the same or higher chance of being processed and presented. In conclusion, our data shows that TCR-Ts can be activated by off-target $ENPP1_{436}$ peptide. But, compared to target AFP_{158}, it requires at least 250 times more $ENPP1_{436}$ to achieve the same level of activation. Importantly, $ENPP1_{436}$ peptide in human cells is not processed and presented to a sufficient level to activate the AFP_{158}-specific TCR-Ts. Thus, these TCR-Ts, especially the TCR1-Ts and TCR2-Ts, will unlikely cause significant off-target toxicity.

Keywords: T cell receptors, T cell engineering, alpha fetoprotein, hepatocellular carcinoma, TCR cross-reactivity, off-target toxicity, immunotherapy

INTRODUCTION

With 840,000 new diagnoses and 781,000 deaths annually, liver cancer is the 6th most common cancer, and the 3rd most common cause of cancer deaths due to the lack of effective treatment (1). The majority of liver cancer is hepatocellular carcinoma (HCC). Recently, several immunotherapies are being developed for HCC (2). The PD1 blockade has significantly increased the overall response rate (3). But its effect may depend on the presence of tumor reactive T cells (4), which are not always present in most HCC lesions. Redirecting autologous T cells with tumor antigen-specific T cell receptor (TCR) genes will provide the tumor-specific T cells, and thus has a great potential for cancer immunotherapy (5). The feasibility of TCR gene transfer to render T cell specificity was published in 1986 (6). And the first evidence that TCR gene engineered T cells (TCR-Ts) generated antitumor effect in treating human cancers was reported 20 years later in 2006 (7). Since then, a number of human tumor antigen specific TCRs derived from both mouse and human sources, including the TCRs specific for MART1 (human TCR) (8), GP100 (mouse TCR) (8), CEA (mouse TCR) (9), NY-ESO1 (human TCR) (10, 11), and MAGE-A3 (mouse and human TCR) (12, 13), have been tested in clinical trials. Adoptive transfer of TCR-Ts has generated significant antitumor effect in several cancers (14). The clinical trial data from NY-ESO1 specific TCR-Ts in treating melanoma, multiple myeloma, and synovial carcinoma is very promising with great safety profile (10, 11). Recently, we (15) and others (16) identified the human alpha fetoprotein (AFP)- specific TCR genes from mouse and human and showed that human T cells genetically modified with the AFP-specific TCRs could effectively kill HCC tumor cells and eliminated HCC xenografts in immune compromised NSG mice (15), demonstrating the potential of the TCR-Ts for HCC immunotherapy.

While the antitumor potency of TCR-Ts is evident, many of the TCR-Ts trials, such as those against CEA, GP100, MART, and MAGE-A3 antigens, also showed significant toxicity including patient death. The toxicity of TCR-Ts can come from three aspects: (1) the on-target/off tumor toxicity due to the low level expression of shared-tumor antigen in normal tissues (8, 9); (2) the off-target toxicity due to the TCR's promiscuous recognition of unrelated epitopes derived from normal proteins (12, 13, 17); and (3) the alloreactivity of TCR-Ts recognizing different HLA presented random peptides. All three aspects of the TCR-T's toxicity must be properly evaluated prior to conducting clinical trials. As investigations of TCR-Ts in animal models offers little value in evaluating their toxicity in human, an *in vitro* preclinical toxicity study strategy was proposed to assess the TCR-T's risk (18). Ideally, tumor-specific or relatively tumor-specific antigens should be selected as the TCR-T's target to reduce on-target/off-tumor reactivity. However, even with highly tumor-specific targets, the off-target cross-reactivity of TCR-Ts in recognition of MHC-peptide complex may still cause severe toxicity.

Abbreviations: AFP, alpha fetoprotein; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; HCC, hepatocellular carcinoma; RCL1, RNA 3'-terminal phosphate cyclase-like protein; TCR, T cell receptors.

In this report and the accompanying study, we determined the optimal TCRs out of the 7 AFP_{158}-specific TCRs based on their preclinical antitumor efficacy and toxicities. The selection of optimal TCR-Ts for HCC immunotherapy and the on-target/off-tumor toxicity and alloreactivity of the AFP_{148}-specific TCR-T's were reported in the accompanying paper (Luo et al). In this study, we investigated the off-target cross reactivity of 3 potent TCR-Ts by using X-scan. We found that TCR3-Ts could be cross-activated by 2 synthetic peptides, the $ENPP1_{436}$ and $RCL1_{215}$, while the TCR1-Ts and TCR2-Ts were activated by only $ENPP1_{436}$. The EC_{50} of $ENPP1_{436}$ peptide for activating AFP_{148}-specific TCR-Ts was 17–33 times higher than the EC_{50} of AFP_{158}. And it required 250–400 times more of $ENPP1_{436}$ and 1000 times of $RCL1_{215}$ peptide to achieve the same level of TCR-T activation as AFP_{158} peptide. Importantly, the HLA-A020 + ENPP1 + human cells do not activate TCR1-Ts and TCR2-Ts. In addition, *in silico* analysis showed that the $ENPP1_{436}$ peptide's MHC binding affinity and its chance of being processed and presented by HLA-A0201 were significantly lower than that of AFP_{158}. In contrast, the two off-targets (Titin and MAGE-A3) that indeed caused severe toxicity in previous trials had the same MHC binding affinity and the same or higher chance of being processed and presented by host cells. Altogether, we conclude that the AFP_{158}-specific TCR-Ts, especially TCR1-Ts and TCR2-Ts, will not cause significant off-target toxicity.

MATERIALS AND METHODS

Cells

The cell lines of $TAP^{-/-}$ T2 (19), HepG2, and HEK293 were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States). Breast cancer cell lines of MCF7, MDA-MB231, and brain tumor U87MG cells were purchased from ATCC. MDA-MB231-Luc cells were derived from MDA-MB231 by transfecting with luciferase gene and kindly provided by Dr. Hasan Korkaya of Georgia Cancer Center. The cells were cultured in standard DMEM or RPMI1640 media for no more than 8–10 passages to maintain their authenticity. Mycoplasma test was conducted according to manufacturer instructions (Thermo Fisher, MA, United States). Primary normal adult human hepatocytes were purchased from Lonza (Walkersville, MD, United States) and Novabiosis (Research Triangle Park, NC, United States).

Peptides

Peptides were synthesized by GenScript (Piscataway, NJ, United States) and Chinapeptides (Shanghai, China) to a purity of >95%. The stock peptides are dissolved in DMSO at 5 mg/ml and aliquoted and stored at −20°C.

T Cell Isolation and TCR Transduction

Buffy coat was obtained from local Shepard blood center. PBMCs were harvested by centrifugation on a Lymphoprep Ficoll gradient, diluted to 1×10^7 cells/ml, aliquoted, and frozen. Lentiviral vectors expressing TCRs were prepared by 4 a plasmid co-transfection as previously described (20–22). Total T cells

were isolated from PBMC by negative isolation kit (STEMCELL Technologies, Vancouver, BC, Canada) and then transduced by lentiviral vectors at MOI of 20–40 as described (15). Between 12–15 days after transduction, tetramer (NIH Tetramer Core Facility) staining was conducted to measure the percentage of TCR+ T cells. The TCR-Ts were then aliquoted and frozen.

X-Scan Assay

X-Scan assay was performed as described (23). Briefly, one vial of TCR-Ts were thawed and recovered 2–3 days before use. For each well of the 96 well plate, 15,000 T cells (∼5,000 TCR-Ts) were cultured with 20,000 T2 cells in the presence of 10 ng/ml of X-peptides, which is equal to the EC_{90} of the index AFP_{158} peptide. After co-culture overnight, the media was collected and assayed for IFNγ by ELISA. The absolute amount of IFNγ after X-peptides was then compared to the IFNγ level after AFP_{158} peptide stimulation, and the ratios were calculated and presented.

Immune Analysis

IFN-γ ELISA kits and antibodies were from Biolegend (San Diego, CA, United States). HLA-A0201/AFP_{158} Tetramer was synthesized by NIH Tetramer Core facility. Antibody staining was done according to each antibody's instructions. Flow cytometry was done on BD LSRII (San Jose, CA, United States). Data was analyzed using the FCS express software (*De Novo* Software, Pasadena, CA, United States).

Ectopic Over-Expression of Ectonucleotide Pyrophosphatase/Phosphodiesterase 1 (ENPP1) Protein

MCF7 cells were transduced by ENPP1 gene (Addgene, Watertown, MA, United States) by utilizing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States). The expression of ENPP1 was detected by Western blot (WB) analysis (**Figure 5A**).

WB Analysis

Protein expression in cell lines was examined by WB analysis. Briefly, cells was homogenized in RIPA lysis buffer containing 25 mmol/L Tris–HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, United States). Protein samples were resolved on 8% SDS polyacrylamide gel. The protein was transferred onto nitrocellulose membrane, which was blocked by using 5% (w/v) non-fat dried milk in Tris–buffered saline containing 25 mM Tris–HCl (pH 7. 4), 130 mM NaCl, 2.7 mM KCl and 0.1% Tween 20. The blot membranes were probed with anti-ENPP1 antibody (Genscript, Piscataway, NJ, United States) and the horseradish peroxidase conjugated anti-IgG secondary antibody (Cell Signaling, Danvers, MA, United States), followed by lightning ECL (PerkinElmer, Waltham, MA, United States).

In silico Analysis

The online software of NetMHC4.0 (24, 25), IEDB (26), and NetCTLpan (27) were used to analyze the ranks of peptide's MHC binding affinity and peptide's chance of being processed and presented. The rank of MHC binding affinity by NetMHC4.0 is based on a total of 400,000 random natural peptides in its databank and the rank of peptide's chance of being processed and presented by HLA-A0201 is based on 200,000 random natural peptides in the NetCTLpan databank.

Statistics

Statistical analysis was done with Prism software using either ANOVA or student *t*-test.

RESULTS

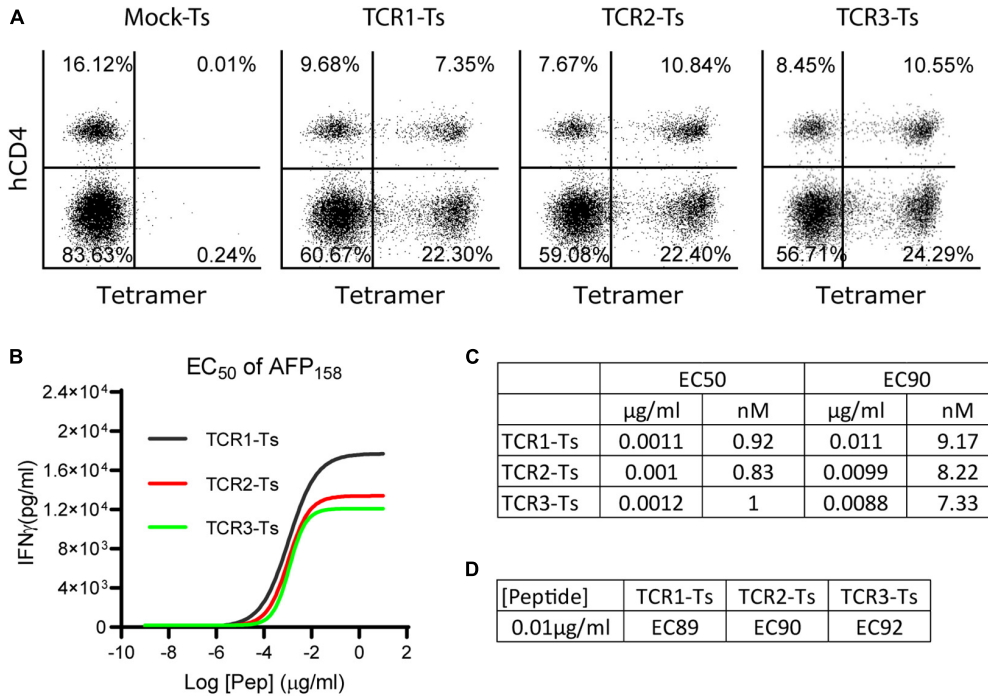
We have identified 7 unique AFP_{158}-specific TCR sequences. A functional screening (Refer to **Figure 1** of the accompanying paper, Luo et al) identified that human T cells engineered with TCR1, 2, and 3 genes (TCR1- TCR2-, and TCR3-Ts) generated strong cytotoxicity and produced high level of cytokines, which were consistent with our previous report (15). Thus, in the study of off-target cross-reactivity, only the TCR1-, 2-, and 3-Ts were used.

The EC_{50} and EC_{90} of Target AFP_{158} and the Peptide Concentration for X-Scan

To comprehensively study the TCR-T's off-target reactivity, X-scan assay was recently developed (23). To properly conduct an X-scan, we first need to decide the right peptide concentration for the assay. To this end, the EC_{50} of index AFP_{158} peptide for activating AFP_{158}-specific TCR-Ts was determined. TCR-Ts were prepared by transducing primary human T cells with lentiviral vectors. Approximately 30–35% of T cells were stained positive by HLA-A0201/AFP_{158} tetramer, and 2/3 of the TCR + T cells were CD8 (**Figure 1A**). TCR-Ts were co-cultured with T2 cells in the presence of different concentrations of AFP_{158} peptide. The IFNγ in the media was measured 20 h later. Using this approach, we determined the EC_{50} of AFP_{158} peptide for activating the TCR1-Ts, TCR2-Ts, and TCR3-Ts were 1.1 ng/ml (0.92 nM), 1 ng/ml (0.83 nM), and 1.2 ng/ml (1.0 nM), respectively (**Figures 1B,C**). This AFP_{158} EC_{50} value for activating our TCR-Ts is similar to the EC_{50} of AFP_{158} peptide for activating human TCR-Ts recently reported by Docta et al. (16), but is slightly higher than several EC_{50} previously reported for other affinity-enhanced TCR-Ts. For example, the EC_{50} of cognate peptide for activating the MAGE-A10 specific TCR-Ts was 0.3–0.5 nM (23), and the EC_{50} of NY-$ESO1_{157}$ peptide for activating cognate TCR-Ts was around 0.2 nM (28). In contrast, the EC_{50} of cognate peptide for activating the MAGE-C2 specific TCR6-T cells was 3.3 nM (18). Next, based on the EC_{50}, the EC_{90} of AFP_{158} peptide was calculated as 10 ng/ml (**Figures 1C,D**). As recently reported by Border et al. (23), the EC_{90} of AFP_{158} was selected as the peptide concentration for conducting X-scan assay.

X-Scan Assay Determines the Peptide Motifs That Are Potentially Recognized by TCR-Ts

To conduct X-scan, each amino acid residue of the index AFP_{158} peptide was replaced with every other possible amino acids



C

	EC50		EC90	
	μg/ml	nM	μg/ml	nM
TCR1-Ts	0.0011	0.92	0.011	9.17
TCR2-Ts	0.001	0.83	0.0099	8.22
TCR3-Ts	0.0012	1	0.0088	7.33

D

[Peptide]	TCR1-Ts	TCR2-Ts	TCR3-Ts
0.01μg/ml	EC89	EC90	EC92

FIGURE 1 | The EC_{50} and EC_{90} of AFP_{158} peptide for activating TCR-Ts. **(A)** TCR-Ts were generated by transducing primary human T cells with TCR genes. Representative dot plots showed the% of TCR + T cells 12–15 days after transduction. **(B,C)** The EC_{50} of AFP158 peptide for activating TCR-Ts were presented. The EC_{90} was calculated using Prism software. **(D)** The EC_{XX} value corresponding to the 10 ng/ml of AFP_{158} peptide for activating different TCR-Ts was calculated using Prism software. The experiments were repeated three times with similar data.

to create a library of X-peptide (**Figure 2A**). A total of 171 X-peptides and the original index AFP_{158} peptide were used to stimulate TCR-Ts. An amino acid substitution in the X-peptide was defined as tolerant if the TCR-T response stimulated by this peptide was >10% of original AFP_{158} peptide. X-scan allowed us to identify all tolerant amino acid replacements in each position (**Figure 2B**). The complete X-scan data was shown in **Supplementary Figure S1**. Based on the X-scan data, a peptide motif for each TCR-T was generated (**Figure 2C**). We also conducted the Alanine (A)-scan and Glycine (G)-scan according to previous report (17) (replacing each residue in the target epitope with Alanine or Glycine) (**Supplementary Figure S2**). While the data from A- or G- Scan can only indicate either 1 (intolerant) or 20 (tolerant), the motif obtained from the X-scan reveals more precise amino acid replacements (**Figures 2C,D**).

AFP_{158}-Specific TCR-Ts Can Be Cross-Activated by the $ENPP1_{436}$ Peptide

The peptide recognition motifs of TCR1, 2 and 3 are slightly different (**Figure 2C**). To make sure that we would not miss any potential reactive peptides, we used a common peptide motif (**Figure 2C**) that cover all 3 TCRs to search the SwissProtein databank using the ScanProsite program (29). A total of 93 peptides with the potential capability of activating the TCR-Ts were identified and synthesized (**Supplementary Table S1**). In the 1st experiment, we used high peptide concentration (1 μg/ml) in the stimulation assay to catch all potential peptides that may activate TCR-Ts. By using 10% of the AFP_{158} response as cut-off, we found that TCR1-Ts and TCR2-Ts could be cross-activated by $ENPP1_{436}$ and $FL2D_{189}$ peptide. TCR3-Ts could be cross-activated by four peptides, the $ENPP1_{436}$, $FL2D_{189}$, $EPG5_{1033}$, and $RCL1_{215}$ peptides (**Figure 3A** and **Supplementary Figure S3**). But, at a lower peptide concentration (10 ng/ml), TCR1-T and TCR2-T could be cross-activated by $ENPP1_{436}$ only, while TCR3-Ts were cross-activated by both $ENPP1_{436}$ and $RCL1_{215}$ peptides. A peptide titration study showed that it required high concentration (1 μg/ml) of $FL2D_{189}$, $EPG5_{1033}$, and $RCL1_{215}$ to activate TCR-Ts. At 10 ng/ml, the $RCL1_{215}$ could weakly cross-activate TCR3-Ts. The data also showed that it required 10,000 times more $RCL1_{215}$ peptide to achieve the same level of activation as AFP_{158}. Even for the more reactive $ENPP1_{436}$, it would require 2 log more (250–400 times) of peptide to generate the same level of response as AFP_{158} (**Figure 3B**). Furthermore, the EC_{50} of $ENPP1_{436}$ for cross-activating the AFP_{158}-specific TCR-Ts were 17–33 times higher than AFP_{158} (**Figures 3C,D**).

ENPP1 Expressing Cells Do Not Activate TCR-Ts

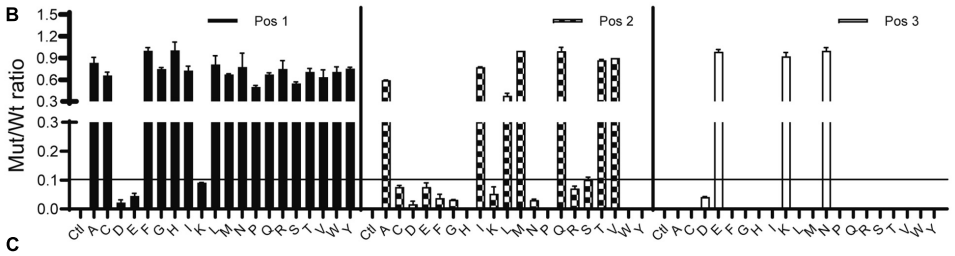
A number of human tissues express ENPP1[1]. Thus, we studied whether the ENPP1 expressing cells could cross-activate

[1]www.proteinatlas.org

A

AFP158 →	F1	M2	N3	K4	F5	I6	Y7	E8	I9	1 peptide
Replacement of each residue with other 19 AA	X	M	N	K	F	I	Y	E	I	19 peptides
	F	X	N	K	F	I	Y	E	I	19 peptides
	F	M	X	K	F	I	Y	E	I	19 peptides
	F	M	N	X	F	I	Y	E	I	19 peptides
	F	M	N	K	X	I	Y	E	I	19 peptides
	F	M	N	K	F	X	Y	E	I	19 peptides
	F	M	N	K	F	I	X	E	I	19 peptides
	F	M	N	K	F	I	Y	X	I	19 peptides
	F	M	N	K	F	I	Y	E	X	19 peptides

B



C

	F1	M2	N3	K4	F5	I6	Y7	E8	I9
TCR1	ACFGHILMN PQRSTVWY	AILM QSTV	EKN	K	FY	ACFGHILM PRSTVWY	AEFG PQY	DEQ	ACFGI LMSTV
TCR2	ACFGHIKLMN PQRSTVWY	AILM QSTV	EKN	K	FY	ACFGHILM PRSTVWY	AEFG PQY	DEQ	ACFGI LMSTV
TCR3	ACEFGHIKLM NPQRSTVWY	AILM QSTV	EKN	K	F	ACFGHILM PSTVWY	AEFG PQY	DEQ	ACFGI LMSTV
Common motif	ACEFGHIKLM NPQRSTVWY	AILM QSTV	EKN	K	FY	ACFGHILM PRSTVWY	AEFG PQY	DEQ	ACFGI LMSTV

D

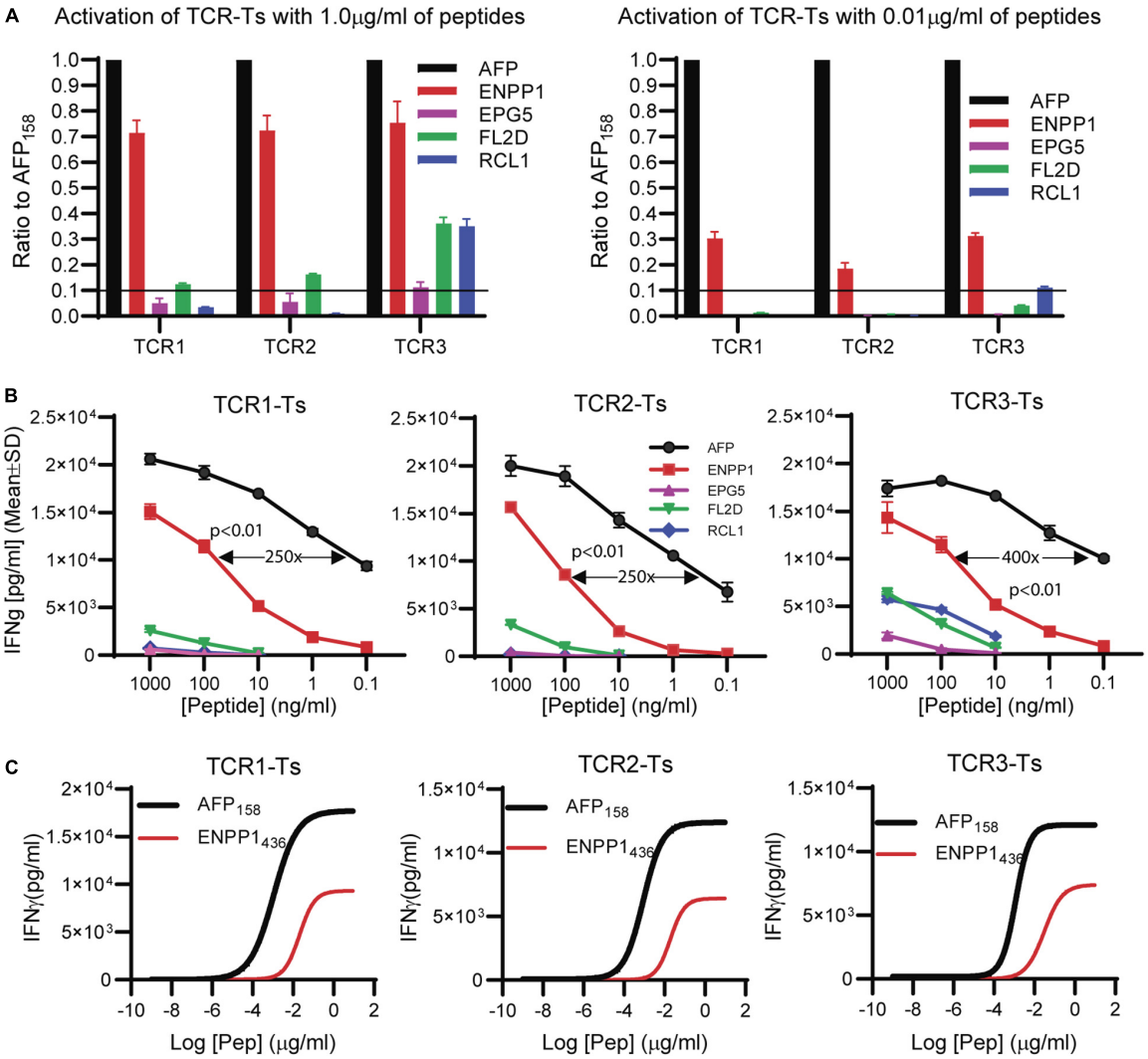
AFP158	F1			M2			N3			K4			F5			I6			Y7			E8			I9		
Scan	A	G	X	A	G	X	A	G	X	A	G	X	A	G	X	A	G	X	A	G	X	A	G	X	A	G	X
TCR1	20	20	17	20	1	8	1	1	3	1	1	1	1	1	2	20	20	15	20	20	7	1	1	3	20	20	10
TCR2	20	20	18	20	1	8	1	1	3	1	1	1	1	1	2	20	20	15	20	20	7	1	1	3	20	20	10
TCR3	20	20	19	20	1	8	1	1	3	1	1	1	1	1	1	20	20	14	20	20	7	1	1	3	20	20	10

FIGURE 2 | Identification of peptide motifs that can be recognized by TCR-Ts. **(A)** The scheme of X-Peptides. "X" in each position represents any of the other 19 amino acids. **(B)** A representative data (position 1–3 of TCR1-T) of X-Scan assay showed the response ratio of X-peptide vs. AFP_{158} peptide in stimulating TCR-Ts to produce IFNγ. The Mean ± SD from 3 wells was shown. The X-scan assay for each peptide was repeated 3–5 times and consistent observation was obtained. **(C)** X-Scan identified the peptide motifs potentially recognized by TCR-Ts. The colored letters indicate the difference among 3 TCR-Ts. A common motif that cover all 3 TCR-Ts is also shown. **(D)** Comparison of A-, G-, and X-Scan to reveal the number of the tolerant amino acids at each position AFP_{158} epitope.

AFP_{158}-specific TCR-Ts. To this end, we detected ENPP1 in several cell lines and found out that MB231 and MCF7 are ENPP1 + (**Figure 4A**) and HLA-A2 +. The MDA-MB231 cells and MB231-luc (derived from MB231 and expressing luciferase) have a very high level of HLA-A2 (10 times more A2 than 293T cells) (**Figure 4B**). Consistent with the highest level of HLA-A2, MB231 pulsed with ENPP1 peptide stimulated the highest activation of TCR-Ts (**Figure 4C**). However, even though the MB231 and MB231-luc express high level of ENPP1 and HLA-A2, they did not activate TCR-Ts (**Figure 4D**). A detail analysis revealed that, while TCR1-Ts and TCR2-Ts did not produce more IFNγ than Mock-T cells after MB231 stimulation, the TCR3-Ts produced slightly more IFNγ (∼300 pg/ml by TCR3-Ts vs. 100 pg/ml by Mock-Ts) (**Figure 4E**). But the IFNγ level produced by TCR3-Ts after MB231 stimulation was 22x lower than that after HepG2 stimulation (**Figures 4D,E**). The *in vitro* CTL assay showed that all 3 TCR-Ts were unable to kill MB231 tumor cells (**Figure 4F**). To further test whether TCR-Ts could be activated by ENPP1 expressing cells, we then used U87MG brain tumor cells, which express higher level of ENPP1 than MB231 and similar level of HLA-A2. Again, all TCR-Ts were not cross-activated by and did not kill the U87MG tumor cells (**Supplementary Figure S4**). In addition, normal primary hepatocytes also express ENPP1 (**Supplementary Figure S5A**). The hepatocytes from 2 donors were also HLA-A2+ (refer to **Figure 3C** of the accompanied paper, Luo et al). The data showed that hepatocytes did not activate TCR-Ts (**Supplementary Figure S5B**), consistent with our previous report (15).

Ectopic Over-Expression of ENPP1 in MCF7 Did Not Activate TCR-Ts

In this experiment, we studied whether ectopic over-expression of ENPP1 in MCF7 tumor cells could cross-reactivate TCR-Ts. We found that while all 3 TCR-Ts could be activated by and kill the MCF7 pulsed with $ENPP1_{436}$ peptide, they were not



	EC50		
	AFP_{158}	$ENPP1_{436}$	$ENPP1_{436}/AFP_{158}$
TCR1-Ts	0.92nM	19.44nM	21.1
TCR2-Ts	0.83nM	27.78nM	33.4
TCR3-Ts	1.0nM	17.59nM	17.6

FIGURE 3 | Synthetic off-target peptides cross-activate TCR-Ts. **(A)** The activation of TCR-Ts to produce IFNγ by 5 peptides at high (1 μg/ml) and low (0.01 μg/ml) concentrations is presented. The data shown was the response ratios of off-target peptides vs. AFP_{158}. **(B)** Shown is the dose-dependent production of IFNγ by TCR-Ts after stimulation with different peptides. ANOVA was used for statistical analysis. **(C,D)** The EC_{50} of AFP_{158} and $ENPP1_{436}$ was measured and compared.

activated by and did not kill the ENPP1 overexpressing MCF7 tumor cells (**Figures 5A–C**). In conclusion, all the data indicates that it is unlikely that the ENPP1 expressing cells in the patients will cross-activate AFP_{158}-specific TCR-Ts.

In silico Analyses May Predict TCR-T's Off-Target Reactivity

Here we studied whether *in silico* analysis would help predict TCR-T's off-target reactivity. First, we used the NetMHC4.0 program to rank the 93 peptide's MHC binding affinity based on databank of 400,000 random natural peptides. The results were then correlated to the experimental data of peptide's TCR-T activation. The data was summarized in **Table 1** and **Supplementary Table S1**. According to the default criteria of 0.5% set by the software, only AFP_{158} (rank: 0.01%) and $ENPP1_{436}$ (rank: 0.4%) peptides are strong binders of HLA-A0201. Consistent with *in silico* analysis, the experimental data showed that these two peptides could activate all 3 TCR-Ts at physiological concentration (10 ng/ml). In contrast, $RCL1_{215}$

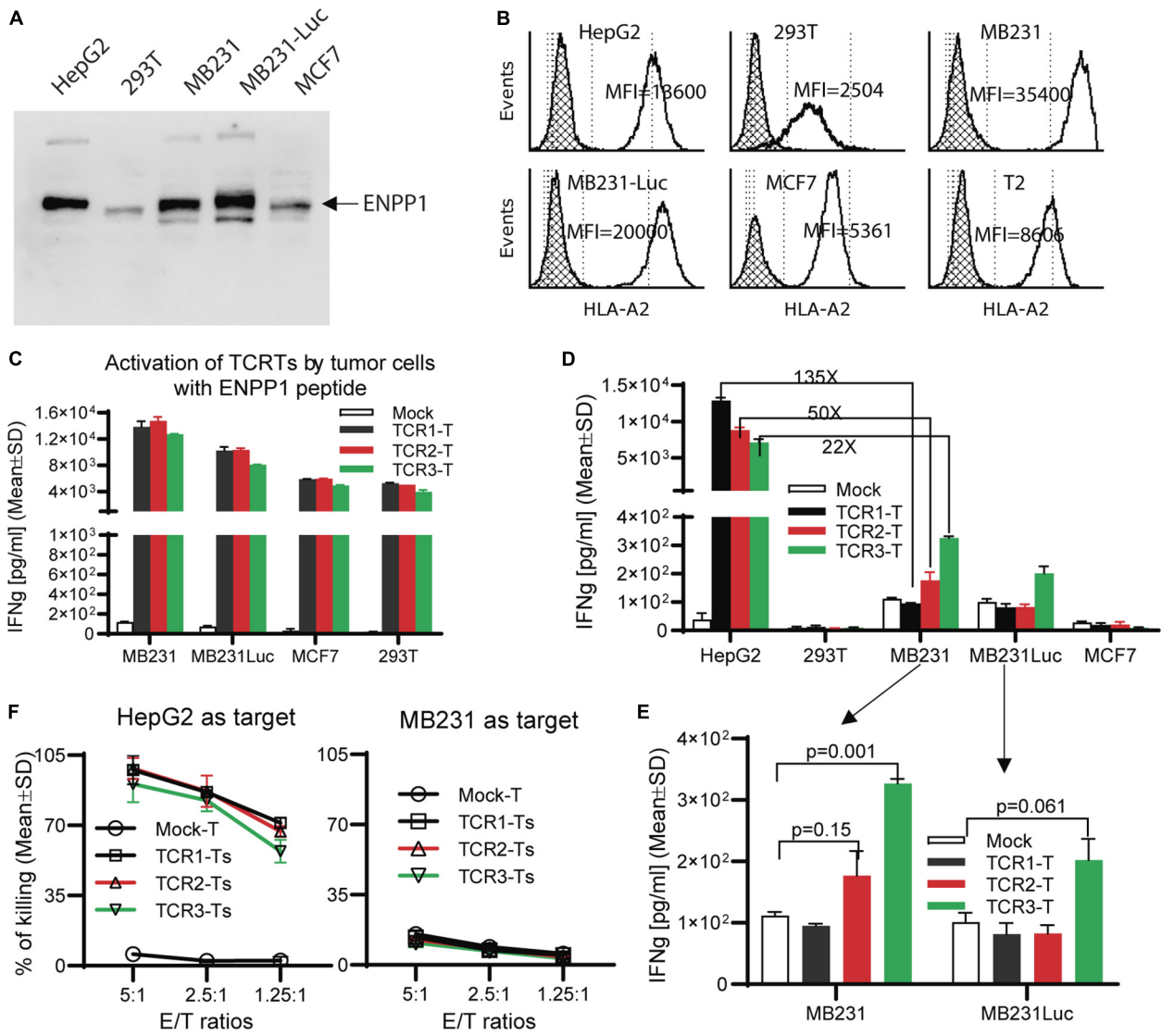


FIGURE 4 | TCR-Ts do not recognize and kill ENPP1 + tumor cells. **(A)** Western Blot analysis showed ENPP1 expression in different cell lines. MB231-Luc was derived from MDA231 by transfecting Luciferase gene. **(B)** Flow cytometry data demonstrated the HLA-A2 expression level on the surface of different cell lines by anti-HLA-A2 antibody staining. The hatched lines indicate the isotype staining controls. **(C)** ELISA assay showed the level of IFNγ production by TCR-Ts after stimulation with indicated cell lines in the presence of 1 μg/ml ENPP1 peptide. **(D,E)**. The activation of TCR-Ts by different cell lines in the absence of ENPP1 peptides was presented. Only the TCR3-Ts were weakly activated, while TCR1-Ts and TCR2-Ts could not be activated by ENPP1 + MB231 and MCF7 cells. **(F)** LDH assay showed that TCR-Ts do not kill the ENPP1 + MB231 cells. HepG2 cells were used as positive control. Student *t*-test was used for statistical analysis. This experiment was repeated 4 times with similar data.

(rank: 0.8%) is a weak binder. The experimental data showed that $RCL1_{215}$ cross-activated only TCR3-Ts (**Figure 3A**). $FL2D_{189}$ (rank: 17%) is a non-binder. It weakly cross-activated TCR-Ts only at high concentration. The EPG_{1033} peptide (rank: 29%) is also a non-binder. It activated only TCR3-Ts at high concentration. The MHC binding affinity of peptides was also analyzed by IEDB software and its rank was similar to NetMHC4.0 (**Table 1**). Secondly, we used the NetCTLpan program to rank a peptide's chance of being processed and presented by MHC molecule. Based on the data of 200,000 random natural peptides, $RCL1_{215}$ and $ENPP1_{436}$ peptides are ranked at 0.8% and 1% (**Table 1**), which means they are the top 1600 and 2000 peptide of being processed and presented, respectively. In contrast, the AFP_{158} is ranked at 0.01% (**Table 1**), which means it is the top 20 out of 200,000 peptides that are processed and presented. Thus, AFP_{158} has much higher probability of being processed and presented. In conclusion, the *in silico* analysis data of peptide's MHC binding affinity and their chance of being processed and presented correlate well with the experimental data of cross-reactivity.

In addition, we did retrospective *in silico* analysis of the peptides that were known to cause severe toxicity in previous

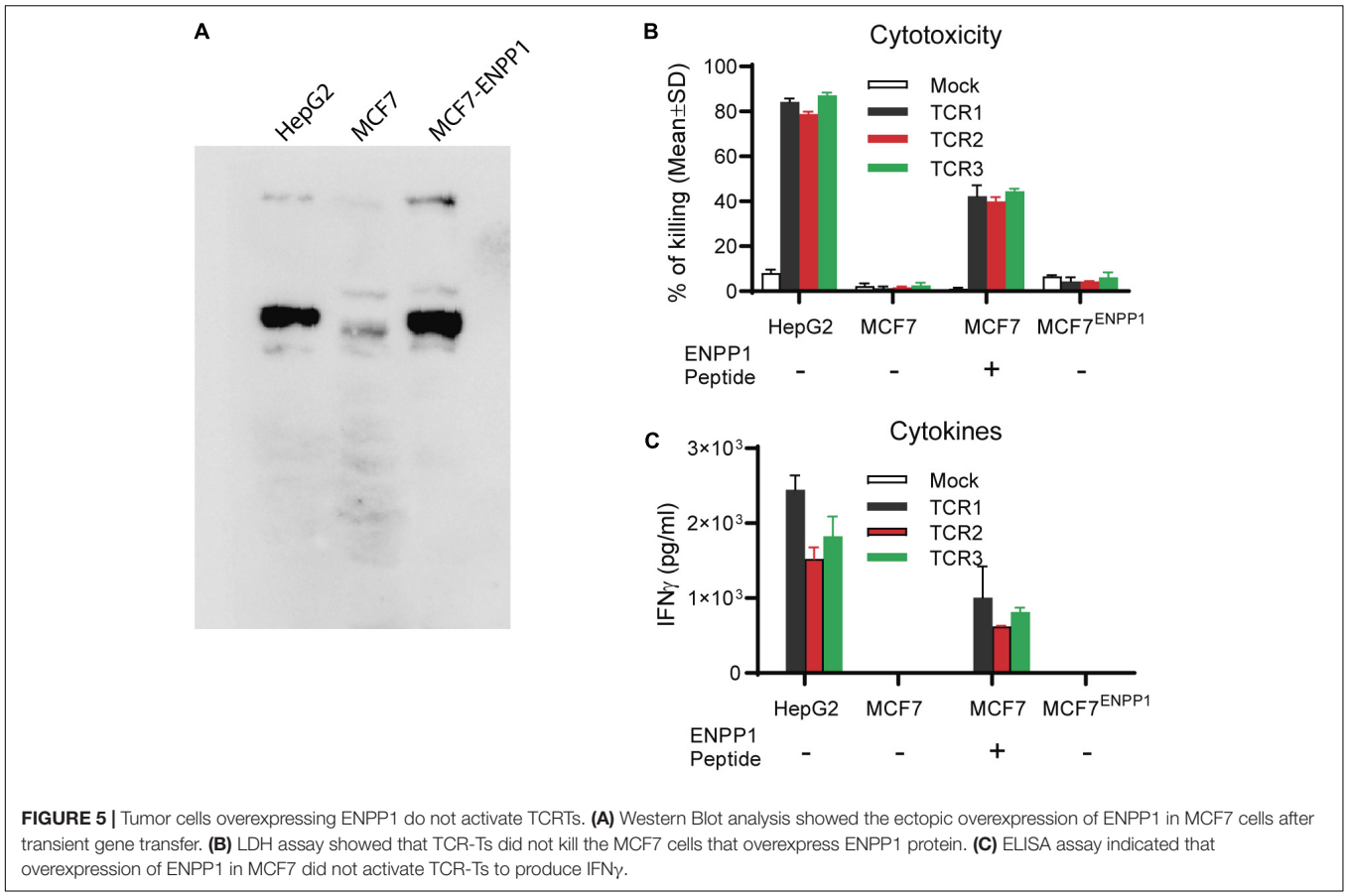


FIGURE 5 | Tumor cells overexpressing ENPP1 do not activate TCRTs. **(A)** Western Blot analysis showed the ectopic overexpression of ENPP1 in MCF7 cells after transient gene transfer. **(B)** LDH assay showed that TCR-Ts did not kill the MCF7 cells that overexpress ENPP1 protein. **(C)** ELISA assay indicated that overexpression of ENPP1 in MCF7 did not activate TCR-Ts to produce IFNγ.

TABLE 1 | Correlation of the *in silico* analysis data with experimental data of cross-reactivity.

Epitope	Peptide Sq	MHC binding affinity			Ranks (%) of being presented	Experimental data	
		NetMHC 4.0		IEDB	NetCTLpan	[Peptide] (μg/ml)	
		Affinity (nM)	Rank (%)	Rank (%)		1.0	0.01
AFP_{158}	FMNKFIYEI	3.2	0.01	0.2	0.01	++++	++++
$ENPP1_{436}$	YLNKYLGDV	28.67	0.4	0.6	1	+++	+
$RCL1_{215}$	ILNKFIPDI	64.06	0.8	2	0.8	–/–/++	–/–/+
$FL2D1_{89}$	LQKKYSEEL	11170.64	17	22	6	+/+/++	–/–/–
$EPG5_{1033}$	SIEKFCAEG	20847.93	29	37	50	–/–/+	–/–/–

The peptide's HLA-A2 binding affinity is analyzed by NetMHC4.0 and IEDB software. The likelihood (rank) of the peptides being processed and presented by MHC molecules is analyzed by NetCTLpan program. The lower the number, the higher the chance of that peptide being processed and presented. The rank of NetMHC4.0 binding affinity is based on 400,000 random natural peptides. The ranks of the peptide being processed and presented is based on 200,000 random natural peptides. The wet laboratory experimental data of cross reactivity is shown in the last column. In the last column where the reactivity is different among TCR1, 2, and 3, their reactivity is indicated as TCR1/TCR2/TCR3.

trials. It was reported that the HLA-A01/MAGE-A3 specific TCR-Ts (17) and HLA-A0201/MAGE-A3 specific TCR-Ts (30, 31) caused severe cardiac and neurological toxicity by cross-reacting with off-target peptides of Titin in the heart and MAGE-A12 in the brain, respectively. We compared the peptides of MAGE-A3 vs. Titin and MAGE-A3 vs. MAGE-A12 regarding to their MHC binding affinity and chance of being processed and presented by *in silico* analysis. The MAGE-A3 and Titin peptides have the same ranking of binding to HLA-A01 (0.01% vs. 0.01% by NetMHC4.0; 0.12% vs. 0.17% by IEDB software) (**Table 2**). Both epitopes also have the same chance (0.05%) of being processed and presented (**Table 2**). In the second example, MAGE-A12 epitope (rank 0.01%) has an even higher affinity than index MAGE-A3 peptide (rank 0.03%) for binding HLA-A0201. The MAGE-A12 epitope also has higher chance of being processed and presented by HLA-A0201 than the index MAGE-A3 peptide (0.2% vs. 0.01%) (**Table 2**). Thus, in both cases that TCR-Ts caused severe toxicity, *in silico* analysis showed that the off-target

TABLE 2 | *In silico* analysis of two previous TCR-Ts that showed severe toxicity in clinical trials.

	Antigen	Epitope	NetMHC4.0		IEDB Rank (%)	NetCTL pan	Cross-Reactivity
			Affinity (nM)	Ranks (%)			
A0101/MAGE-A3	MAGE-A3	EVDPIGHLY	11.43	0.01	0.12	0.05	+++
	Titin	ESDPIVAQY	8.07	0.01	0.17	0.05	++
A0201/MAGE-A3	MAGE-A3	KVAELVHFL	16.05	0.25	0.9	0.2	+++
	MAGE-A12	KMAELVHFL	3.28	0.01	0.2	0.01	++++
	MAGE-A2	KMVELVHFL	4.61	0.03	0.3	0.05	+
	MAGE-A4	KVDELAHFL	60.75	0.70	1.9	0.8	–
	MAGE-A6	KVAKLVHFL	109.34	1.10	2.2	1.5	+
	MAGE-A1	KVADLVGFL	165.35	1.50	2.8	1.5	–
	MAGE-A8	KAVELVRFL	2082.94	6.00	7.6	3	–

The cross-reactivity in vitro experimental data are taken from the references of 17 and 31. The shade data indicate the cross reactivity that cause severe in vivo toxicity. The rank of NetMHC4.0 binding affinity is based on 400,000 random natural peptides. The ranks of the peptide being processed and presented by NetCTL program is based on 200,000 random natural peptides.

peptide's MHC binding affinity and chance of being processed and presented were at least at the same level as the intended index epitopes.

DISCUSSION

The Reliability of X-Scan

T cell activation requires their TCR to specifically bind to cognate MHC/peptide complex. However, the TCR's recognition of MHC/peptide complex is degenerate (32), which may cause lethal off-target cross-reactivity (12, 30). X-scan is a comprehensive approach to study TCR-T's off-target reactivity (16, 23). But its reliability of X-scan may depend on the X-peptide concentration used for the assay. Conducting the assay with too high concentration of X-peptide will unnecessarily find many peptides that cross-activate TCR-Ts, but in reality will never do so because of their lower physiological level. On the other hand, too low of concentration will miss out the peptides that may do cause cross reactivity. In our study, we found that the EC_{50} of AFP_{158} peptide for activating AFP_{158}-specific TCR-Ts was ∼1 nM. According to previous study (33), T2 cells pulsed with 1 nM of gp100 peptide and WT-1 peptides yielded 12–47 copies of MHC/peptide complex on cell surface. This number of MHC/peptide complex is similar to the naturally presented gp100 epitopes on the melanoma Mel526 and Mel624 cells (9–68 copies) (33) and the naturally processed MHC/NY-ESO1 peptide complex on tumor cells (34). Therefore, 1 nM is the relevant concentration for X-scan that generates physiological level of MHC/peptide complex on cell surface. Thus, we agree that the EC_{90} of index peptide used in a recent study (23) is the right concentration for X-scan assay in order to make sure that no potential peptides will be missed out.

The reliability of X-scan may also be affected by the fact that the X-peptide contains only one amino acid residue replacement while all other amino acid residues remain original. It does not consider the effect of other amino acid residues in the epitope that may also affect its capability of activating TCR-Ts. Thus, it is possible that peptides with more than one amino acids being simultaneously changed may not follow the rule concluded from X-scan assay. For example, the Y replacement at position 5 of AFP_{158} was intolerant for TCR3-T activation (even though it is tolerant for TCR1-T and TCR2-T) (**Supplementary Figure S1**). However, contradictory to this rule, the $ENPP1_{436}$ peptide is capable of activating TCR3-Ts (tolerant) even it has Y at position 5. Compared to AFP_{158} (FMNKFIYEI), the $ENPP1_{436}$ peptide (YLNKYLGDV) has Y at position 5, but also has other 7 amino acid replaced. Those additional amino acid changes may work together to enhance the peptide's activation of TCR-Ts. To minimize the chance of missing any potential reactive peptides, for the three positions (E and K at Position 1; Y at Position 5; R at Position 6) in the peptide motifs that are different among the 3 TCR-Ts, we used a common peptide motif to cover all potential amino acids to search the protein databank. In this case, as long as one TCR-T is tolerant for one particular amino acid replacement, it will be included in the common motif.

In silico Analysis of Peptide's MHC Binding Affinity Helps Predict TCR-T's Off-Target Toxicity

The X-scan strategy may generate a long list of potential off-target peptides. In this study, 93 peptides were identified by X-scan that may potentially cross-activate AFP_{158}-specific TCR-Ts. But, the experimental data showed that the peptides indeed cross-activated TCR-Ts at physiological concentration were within the top 1% of their MHC binding affinity ranked by NetMHC4.0 analysis (**Supplementary Table S1**). The default cut-offs of strong and weaker binder are 0.5 and 2%, respectively. Although more experimental data will be needed, it is reasonable to assume that, at physiological concentration, only the top 2% of peptides ranked by NetMHC4.0 have the potential to truly cross-activate TCR-Ts. Thus, the programs of calculating peptide's MHC binding affinity, such as NetMHC4.0 and IEDB, may help

shorten the peptide list so that wet laboratory experiments will not be too costly.

Secondly, the computer program of NetCTLpan (27), which integrates peptide's proteasomal C terminal cleavage, TAP transport efficiency, and MHC binding affinity, may help predict the probability of a particular peptide being processed and presented by MHC. The NetCTLpan analysis showed that the two off-target peptides (Titin and MAGE-A12) that indeed cause severe toxicity in human trials had similar or higher chances of being processed and presented as the intended target of MAGE-A3 (**Table 2**). In contrast, the NetCTLpan ranks that the chance of $ENPP1_{436}$ peptide being processed and presented by HLA-A0201 is 100 times lower than the target epitope AFP_{158} (top 1% vs. top 0.01% in **Table 1**). Thus, the data of *in silico* analysis is in agreement that the $ENPP1_{436}$ peptide is not efficiently processed and presented.

Thirdly, will TCR bind the index and cross-reactive peptides with similar strength? The recent software to predict TCR and MHC/peptide interaction is highly immature (35). However, this may not be necessary as the ultimate test is the wet experiments. According to previous study (33), as low as 0.1 nM of peptides can stimulate TCR-T to produce IFNγ that can be detected, but it requires 1 nM peptides to generate sufficient level of MHC/Peptide complex on cell surface to be detected by microscope. And, it needs 10 nM of peptides to generate measurable MHC-peptide complex by flow cytometry. Thus, the TCR-T functional activation by measuring the IFNγ level is the most sensitive way of finding potential off-target peptides.

TCR-T's Cross-Reactivity to Synthetic $ENPP1_{436}$ Peptide Unlikely Causes Severe Off-Target Toxicity

The cross-activation of AFP_{158}-specific TCR-Ts by synthetic $ENPP1_{436}$ peptide raises the concern that AFP_{158}-specific TCR-Ts may cause off-target toxicity. However, our experimental data indicates such off-target toxicity is highly unlikely. 1st, the cross-activation of AFP_{158}-specific TCR-Ts by $ENPP1_{436}$ peptide is significantly weaker than AFP_{158} peptide. The EC_{50} of $ENPP1_{436}$ peptide for activating the TCR-Ts is 17–33 time higher than AFP_{158} peptide. It requires 250–400 times more of $ENPP1_{436}$ peptide to achieve the same level of activation as AFP_{158} peptide. The significant weak activation of TCR-Ts by $ENPP1_{436}$ peptide is further supported by *in silico* analysis showing that the MHC binding affinity of $ENPP1_{436}$ peptide is ranked 40 times lower than AFP_{158} peptide. Thus, there is a wide concentration window to distinct the intended target from off-target activation. 2nd, the chance of $ENPP1_{436}$ peptide being processed and presented is low. The activation assay of using peptide pulsed T2 cells to stimulate TCR-Ts omits the process of antigen processing which including protein degradation and TAP transport and MHC loading (36). Not all so-called epitopes identified by synthetic peptides can be truly processed and presented by cells (37, 38). In fact, the process of antigen processing and presentation is rather very inefficient, estimating only 1/10,000 peptides being processed and presented (39). The incapability of ENPP1+ MB231, MCF7, U87MG, and normal hepatocytes to activate TCR-Ts is in agreement with the computer prediction that $ENPP1_{436}$ epitope is unlikely being processed and presented by HLA-A0201 to the level that is sufficient to activate TCR-Ts. Thus, we have strong evidence that the AFP_{158}-specific TCR-Ts will unlikely cause severe off-target toxicity. That being said, the concern of causing off-target toxicity cannot be completely excluded. Further safety switches such as incorporation of suicide gene (40) and truncated EGFR (41) tag may be added in case of the TCRTs need to be removed.

CONCLUSION

In conclusion, our off-target toxicity study showed: (1) The TCR3-T has a broader cross-reactivity than TCR1-T and TCR2-T, highlighting the need of multiple TCRs to find a proper one; (2) The $ENPP1_{436}$ peptide can weakly activate AFP_{158}-specific TCR-Ts, but, it requires 250–400 times more of peptide to achieve the same level of activation as AFP_{158} peptide; (3) Importantly, the ENPP1 expressing cells do not activate TCR1-Ts and TCR2-Ts even though they may slightly activate TCR3-Ts; (4) *In silico* analysis show that $ENPP1_{436}$ peptide has a much lower HLA-A0201 binding affinity than AFP_{158} and is less likely of being processed and presented. In contrast, the off-target peptides that indeed cause severe toxicity in previous studies have a similar HLA binding affinity and a similar or even higher chance of being processed and presented than the intended target peptides. Thus, we conclude that it is unlikely that the AFP_{158}-specific TCRs will cause significant off-target toxicity.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

LC and YH designed the study, conducted majority of the experiments, summarized the data, and wrote the manuscript. LDC and YP prepared the recombinant DNA, lentiviral vectors, and the TCR-Ts. WZ did the A- and G- Scan experiments. XL, YY, and YJ conducted the Western Blot of ENPP1 in primary hepatocytes and the TCR-T activation by primary normal hepatocytes. All authors agreed the manuscript for publication.

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developing TCR-T based immunotherapy for liver cancers. The data and materials are open for anyone who is interested.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00607/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Enhanced NK-92 Cytotoxicity by CRISPR Genome Engineering Using Cas9 Ribonucleoproteins

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Natural killer (NK) cells are an attractive cell-type for adoptive immunotherapy, but challenges in preparation of therapeutic primary NK cells restrict patient accessibility to NK cell immunotherapy. NK-92 is a well-characterized human NK cell line that has demonstrated promising anti-cancer activities in clinical trials. Unlimited proliferation of NK-92 cells provides a consistent supply of cells for the administration and development of NK cell immunotherapy. However, the clinical efficacy of NK-92 cells has not reached its full potential due to reduced immune functions as compared to primary NK cells. Improvements of NK-92 functions currently rely on conventional transgene delivery by mRNA, plasmid and viral vector with limited efficiencies. To enable precise genetic modifications, we have established a robust CRISPR genome engineering platform for NK-92 based on the nucleofection of Cas9 ribonucleoprotein. To demonstrate the versatility of the platform, we have performed cell-based screening of Cas9 guide RNA, multiplex gene knockout of activating and inhibitory receptors, knock-in of a fluorescent gene, and promoter insertion to reactivate endogenous CD16 and DNAM-1. The CRISPR-engineered NK-92 demonstrated markedly enhanced cytotoxicity and could mediate antibody-dependent cellular cytotoxicity against hard to kill cancer cell lines. Our genome editing platform is straightforward and robust for both functional studies and therapeutic engineering of NK-92 cells.

Keywords: CRISPR, Cas9, RNP, nucleofection, NK-92, immunotherapy, ADCC

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INTRODUCTION

Natural killer (NK) cells are potent innate effectors capable of targeting and killing virally infected and malignant cells (1). Unlike T cells, NK cells do not require a matching human leukocyte antigen for activation or functions. Instead, NK cells rely on an array of germline-encoded activating and inhibitory receptors that engage their cognate ligands on the target cells and initiate cytotoxicity (2). NK cells not only secrete granzyme B and perforin that lyse the target cells, but also cytokines and chemokines to orchestrate the subsequent immune responses (2). These unique attributes make NK cells an attractive cell type for adoptive immunotherapy.

Evidence from clinical studies demonstrates that NK cell immunotherapy is effective and safe (3–8), but there are still challenges, particularly in the manufacture of therapeutic NK cells. Because NK cells represent only 10% of peripheral lymphocytes, the supply of NK cells from leukapheresis is limited (9). *Ex vivo* expansion is necessary to generate clinically relevant levels of primary NK cells for infusion; however, this process is complicated by telomere shortening and reduced cytotoxicity

of the resulting cells (9). Although allogeneic transfer of NK cells is safe, depletion of contaminating allogeneic T cells is crucial to prevent graft-vs.-host reaction. The logistics and costs associated with the preparation of primary NK cells have restricted NK cell immunotherapy to highly selected patients (9).

To overcome the limitations of primary NK cells, several clonal NK cell lines were established from patients with NK-cell lymphoma (10). Among them, NK-92 cell line has shown consistent anti-cancer activities in several clinical studies (10). NK-92 cells possess many hallmark activating receptors (for example, NKG2D, NKp30, NKp44, and NKp46), and yet lack several inhibitory receptors (for example, TIGIT and PD-1) (11). Infusion of gamma-irradiated NK-92 cells has also been proven safe to patients (9, 12). Furthermore, unlimited proliferation of NK-92 generates a consistent supply of homogeneous NK cells to allow multiple infusions, improve the logistics of treatment and reduce the cost for therapeutic development (9). However, NK-92 has reduced anti-cancer activities when compared to primary NK cells due to compromised immune functions. A better understanding of NK-92 immunobiology is vital to increase the efficacy of NK-92 adoptive immunotherapy, and efficient genetic toolkits are required to achieve that goal.

Recent advances in genome editing technologies such as CRISPR has reinvigorated interests in NK cell genetic engineering. However, genetic modifications of NK-92 still rely largely on transgene delivery by mRNA, plasmid DNA and viral vectors (13–17). These conventional methods are confined by transient expression, low transfection efficiency, inconsistent transduction, and random genomic integration of vector DNA. A more robust and precise method is needed for next generation NK-92 engineering. Recently, nucleofection of pre-assembled CRISPR-Cas9 ribonucleoprotein (Cas9 RNP) has shown promising genome editing efficiencies in primary NK cells (18–20). We sought to explore this approach for NK-92 genome engineering, only to discover that the conditions for primary NK cells were not transferable to NK-92.

Here we describe a powerful and versatile Cas9 RNP-based genome editing platform for NK-92 cells. We have used this platform for cell-based screening of Cas9 single guide RNA (sgRNA). We also demonstrated that multiplex KO of activating and inhibitory receptors was effective and viable, as opposed to plasmid-based CRISPR editing. Finally, we performed Cas9-mediated homology-directed repair (HDR) to insert restriction sites, a fluorescent gene, and also a synthetic promoter to reactivate silenced endogenous genes. The CRISPR-engineered NK-92 cells were enriched and expanded to demonstrate significantly enhanced cytotoxicity against cancer cell lines. This work represents the first reliable gene editing method for this clinically important NK cell line.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CRISPR, clustered regularly interspaced short palindromic repeats; FACS, fluorescence-activated cell sorting; dsDNA, double-stranded DNA; HDR, homology-directed repair; indel, insertion-deletion; IVT, *in vitro* transcription; KI, knock-in; KO, knockout; NGS, next generation sequencing; NHEJ, non-homologous end joining; NK, natural killer; RNP, ribonucleoprotein; NT, untreated; SFFV, spleen focus-forming virus; sgRNA, single guide RNA; Sol2, solution2+mannitol; ssDNA, single-stranded DNA; 7-AAD, 7-aminoactinomycin D.

MATERIALS AND METHODS

Cell Culture

All cell culture reagents and media were purchased from Gibco (Thermo Fisher Scientific) unless stated otherwise. All cell lines were of human origin, maintained in 37°C incubator with 5% CO_2 in specific media, and routinely tested for mycoplasma contamination by EZ-PCR detection assay kit (Biological Industries). The malignant non-Hodgkin's lymphoma cell line NK-92 (ATCC) were maintained in RPMI 1640 Medium (ATCC modification) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, 1X GlutaMAX, 1X Antibiotic-Antimycotic, and 100 U/ml IL-2 (PeproTech). NK-92 cells were passaged every 2–3 days to maintain the cell density at 2×10^5 – 8×10^5 cells/ml. The Burkitt's lymphoma cell line Raji was maintained in RPMI 1640 Medium (ATCC modification) supplemented with 15% heat-inactivated FBS, 25 mM HEPES, 1X GlutaMAX and 1X Antibiotic-Antimycotic. The adenocarcinoma cell line HeLa (ATCC), the embryonic kidney cell line HEK293T (ATCC), the adenocarcinoma cell line MDA-MB-231 (gift from Dr. Ruey-Hwa Chen in Academia Sinica), and the ductal carcinoma cell line BT-474 (BCRC, Taiwan) were maintained in DMEM with high glucose (HyClone) supplemented with 15% heat-inactivated FBS, 25 mM HEPES, 1X GlutaMAX and 1X Antibiotic-Antimycotic.

Flow Cytometry and Fluorescence-Activated Cell Sorting

The following antibodies were used for flow cytometry and fluorescence-activated cell sorting (FACS): APC anti-CD96 (NK92.39, BioLegend), BV-421 anti-CD96 (NK92.39, BioLegend), PE anti-NKG2A (REA110, Miltenyi Biotec), PE anti-DNAM-1 (11A8, BioLegend), APC anti-NKp46 (9E02, BioLegend), APC anti-NKG2D (1D11, BioLegend), PerCP-Cy5.5 anti-CD16 (3G8, BioLegend), APC Mouse IgG1κ Isotype (MOPC-21, BioLegend), BV-421 Mouse IgG1κ Isotype (MOPC-21, BioLegend), and PE REA control (REA293, Miltenyi Biotec). All the experiments were performed in CytoFLEX (Beckman Coulter), FACSJazz or FACSAria IIIu (BD Biosciences). The data were analyzed with FlowJo (BD Biosciences) and CytExpert (Beckman Coulter). Ice-cold FACS buffer (DPBS supplemented with 2% FBS, 25 mM HEPES and 0.5 mM EDTA) was used for washing, cell resuspension and antibody dilution. Briefly, the cells were pelleted by centrifugation at 300 g for 5 min, washed once and stained in the antibody solution (diluted as per manufacturer's recommended ratios) in the dark for 20 min on ice. After staining, the cells were washed once, resuspended and kept on ice before analysis. After FACS, the enriched cells were pelleted at 300 g for 5 min, resuspended to 2×10^5 cells/ml in NK-92 culture medium and proceed to standard culture method.

Viability Assays by Zombie Dye and Precision Beads

Zombie Violet Fixable Viability Kit, Precision cell count beads and 7-Aminoactinomycin D (7-AAD) were purchased from BioLegend. In Zombie dye assay, cells were pelleted at 300 g for 5 min and followed by DPBS wash once. The cells were then

stained in 1,000-fold diluted Zombie dye in the dark for 20 min, washed once and resuspended in FACS buffer for analysis. In Precision beads assay, cells were gently resuspended by pipetting, and filtered through 35-μm nylon mesh cell strainer (Corning). Precision beads were resuspended thoroughly before use by vortexing for 40 s. Precision beads solution was added at 0.1 v/v ratio to each filtered cell sample and vortexed at low speed for 5 s. The samples were then stored on ice until analysis by flow cytometry. One thousand Precision beads were counted, by APC and PB450 signals, to serve as an internal standard to quantitate the cell density (gated as P1 and shown as the red rectangle in **Figure S1**). 7-AAD staining and an FSC/SSC scattering plot were used to set an electronic gate on viable cells (gated as P2 and shown as the black circle in **Figure S1**). The following equations were used to calculate the percentages of recovery of viable cells and viable GFP^+ cells.

$$\text{Recovery of viable cells (\%)} = \frac{\text{Counts of viable cells in the sample}}{\text{Counts of viable cells in the untreated control}} \times 100$$

$$\text{Viable GFP}^+\text{ cells (\%)} = \frac{\text{Counts of GFP}^+\text{ cells in the viable cells}}{\text{Total viable cells}} \times 100$$

Preparation of Cas9 Protein and sgRNA

Cas9 recombinant protein was over-expressed in *E. coli* BL21 (DE3) from plasmid pMJ915 (Addgene # 69090), and purified as described previously (21). Cas9 protein was stored at −80°C in Cas9 RNP buffer (20 mM HEPES at pH 7.5, 150 mM KCl, 10% glycerol and 1 mM β-mercaptoethanol). The sgRNAs were designed by the CRISPR Design tool on Benchling website (www.benchling.com) that provided predictions for on-target efficiency and off-target effect. The sgRNAs with high off-target scores (indicating high editing precision and low off-target effect) were selected and synthesized by *in vitro* transcription (IVT) using T7 RNA polymerase as described previously (22). The DNA oligonucleotides for IVT template assembly were listed in **Table S2**. The synthesized sgRNAs were purified by denaturing urea-PAGE. The RNA bands corresponding the full-length sgRNA were excised to remove truncated forms. Additionally, the PAGE-purified sgRNAs were treated with calf-intestine phosphatase to remove the 5′ phosphate group to prevent triggering innate immune responses (23). The final sgRNA products were dissolved in Cas9 RNP buffer, quantitated by NanoDrop Lite (Thermo Fischer Scientific) and stored as aliquots at −80°C.

Cas9 RNP Nucleofection

Cas9 RNP complexes were assembled immediately before nucleofection, by mixing equal volumes of 40 μM of Cas9 protein and 48 μM of sgRNA at molar ratio of 1:1.2 and incubating at 37°C for 15 min. The final concentration of Cas9 RNP was defined as 20 μM. An nucleofection reaction consisted of 4×10^5 of NK-92 cells in 20 μl of nucleofection buffer, 2 μl of Cas9 RNP (equivalent to 40 pmol) and 2 μl of HDR DNA at the indicated concentration. In RNP dosage experiment, 4 and 6 μl of Cas9 RNP were added to obtain 80 and 120 pmol. The nucleofection buffer was either P3 (Lonza) or Sol2, which was composed of 150 mM sodium phosphate buffer (pH 7.2), 5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES and 50 mM mannitol (24). Sol2 was stored at 4°C and replaced every month. Freezing of Sol2 is not recommended due to precipitation. The nucleofection mixtures were then transferred into 16-well strip for nucleofection in Lonza 4D Nucleofector using the buffer and pulse code specified. Pipetting must be careful to prevent air bubbles trapped between the electrodes. Immediately after nucleofection, 100 μl of pre-warm NK-92 culture medium was added to each well for cell recovery in 37°C incubator for 15 min. The cells were then transferred to the culture plate filled with pre-warm culture medium. All analyses were performed 72 h after nucleofection unless otherwise stated.

Gene Editing Analyses by DNA Sequencing

The cells were pelleted at 300 g for 5 min and washed with DPBS once. Genomic DNA was extracted by lysing the cell pellet in QuickExtraction solution (Lucigen) at 65°C for 15 min and then 98°C for 5 min. The extracted genomic DNA was stored at −20°C. PCR amplification of the target sequences was performed using KAPA HiFi HotStart PCR kit. The primer sequences and PCR conditions were listed in **Table S3**. The PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and eluted in molecular-grade water. Fifty microgram of PCR DNA was used for Sanger Sequencing. The percentages of indel and HDR were analyzed online by Inference of CRISPR Edits (ICE) tool (https://www.synthego.com/products/bioinformatics/crispr-analysis).

Deep Sequencing Analysis of On-Target and Off-Target Sites

Off-target sites were predicted by the CRISPR Design tool on the Benchling website (www.benchling.com) based on the published algorism (25). The genomic sequences of the on-target site and two of the top predicted off-target sites were PCR amplified by the primer sets and conditions (**Table S3**). Briefly, target amplicons were amplified by 30 cycles of PCR from 300 ng of genomic DNA in QuickExtraction solution (Lucigen) using KAPA HiFi HotStart DNA Polymerase kit (KAPA Biosystems). The PCR amplicons were purified with Qiagen Gel Purification Spin Column, and subjected to QC assessment with Qubit DNA quantification (Thermo) and size profiling using Fragment Analyzer (Agilent). To add the dual-barcoded adaptor to the amplicons, Nextera XT Index Kit v2 (Illumina) was applied for indexing PCR with 5 μl of the amplicon template in 50 μl reactions, and amplified for 8 cycles using 2X KAPA HiFi Mastermix (KAPA Biosystems). The PCR products were cleaned up by AmPure beads (Beckman Coulter), and subjected to QC with Qubit and Fragment Analyzer as well as qPCR for molar concentration normalization using Kapa Illumina Library Quantification Kit (KAPA Biosystems) prior to library pooling. High throughput sequencing of PE2*151 bp was carried out on a MiSeq sequencer (Illumina), and obtained a total of 19.41 millions of pass-filter clusters at PF of 84.6% and >Q30 bases at 96 and 92% for Read1 and Read2, respectively. The dataset was generated and demultiplxed with BclToFastq 2.18 pipeline (lllumina). FASTQ reads were first processed by 30-bp HEADCROP trimming using Trimmomatic to eliminate the low-quality bases at the 5′ and 3′ ends. After

trimming, the files were analyzed by CRISPresso2 against human reference genome GRCh38 with default parameters. Random single nucleotide substitutions were discarded as amplification and sequencing errors. Deep sequencing data is available at the NCBI Sequence Read Archive (PRJNA608597). The % Indel of on-target and off-target sites were calculated by the following equation:

$$\%\ \text{Indel} = \frac{\text{Number of insertion reads+number of deletion reads}}{\text{Total number of reads}-\text{number of substitution reads}} \times 100$$

We detected a single-nucleotide variant in *KLRK1* on-target region in 97% of the reads. We didn't filter out this single nucleotide substitution. The following equation was used for *KLRK1* calculation:

$$\%\ \text{Indel} = \frac{\text{Number of insertion reads+number of deletion reads}}{\text{Total number of reads}} \times 100$$

Chromosomal Translocation Assay

The triple-negative population of edited NK-92 cells was isolated by FACS, and the genomic DNA was extracted by QuickExtraction solution (Lucigen). Chromosomal translocation was detected by an end-point PCR assay as described previously (26). Genomic amplification was performed using KAPA HiFi HotStart DNA Polymerase kit (KAPA Biosystems) and 15 combinations of forward and reverse primers for *CD96*, *KLRC1* and *NCR1* target loci (**Figure S2A** and **Table S3**). The thermocycler setting consisted of 30 cycles of 98°C for 10 s, 65°C for 10 s, and 72°C for 20 s, except for the combinations with *CD96* forward primer, which consisted of 30 cycles of 98°C for 10 sec, 70°C for 10 s and 72°C for 20 s. The DNA products were resolved in 2% agarose gel in TAE buffer and post-stained with SYBR Safe (Thermo Fischer Scientific) for visualization.

Construction and Preparation of HDR Templates

The HDR templates were constructed by Gibson Assembly using NEBuilder HiFi DNA Assembly kit (NEB). The constructs were composed of DNA fragments as described below, and were cloned into Sph1-BamH1 double-digested pUC19 vector. Left homologous arm of *CD96* (Fragment 1), right homologous arms of *CD96* (Fragment 2) and *mCherry* gene (Fragment 3) were assembled to CD96-mCherry HDR template. Left homologous arm of *FCGR3A* (Fragment 4), right homologous arms of *FCGR3A* (Fragment 5) and SFFV promoter (Fragment 6) were assembled to SFFV-CD16 HDR template. Left homologous arm of *CD226* (Fragment 7), right homologous arms of *CD226* (Fragment 8), and SFFV promoter (Fragment 9) were assembled into SFFV-CD226 HDR template. Fragment 1, 2, 4, 5, 7, and 8 were PCR amplified from NK-92 genomic DNA. Fragment 3 was amplified from pTR144 (Addgene # 112013). Fragment 6 and 9 were amplified from LeGO-iT2 (Addgene # 27343). Fragment PCR was performed using KAPA HiFi HotStart DNA Polymerase kit (KAPA Biosystems).

To prevent targeting of HDR template by Cas9 RNP, mutations were introduced into the HDR templates by Round-the-horn site-directed mutagenesis. In the *FCGR3A* template, the sgRNA16 PAM sequence in the left homology arm was mutated from AGG to ATT. In the *CD226* template, the sgRNA22 seed region sequence in the right homology arm was modified to silent mutations. Illustrations of *mCherry*, *FCGR3A,* and *CD226* HDR templates are in **Figures S3–S5**, respectively. The PCR conditions and the primer sequences are listed in **Table S3**. All constructs were validated by Sanger sequencing at the DNA sequencing core facility at the Institute of Biomedical Sciences at Academia Sinica. For restriction sequences KI experiments, the ssDNA ultramers were purchased from IDT DNA. Complete HDR template sequences are provided in **Table S4**.

For HDR experiments, linear dsDNA PCR templates were amplified from the plasmid constructs using KAPA HiFi DNA Polymerase kit (KAPA Biosystems) and the primers listed in **Table S3**. The PCR reaction mixture was purified by AMpure XP beads (Beckman Coulter) at 0.7 v/v ratio as per manufacturer's protocol. The dsDNA was eluted in molecular H_2O and precipitated by isopropanol at −20°C overnight. The precipitated dsDNA pellet was then washed three times by 70% ethanol, dried under vacuum and resuspended in molecular H_2O. The concentration of dsDNA was determined by NanoDrop Lite, adjusted to 1 μg/μl and stored at −20°C.

Construction of Cas9:sgRNA Dual Expressing Plasmids

The sgRNA guide sequences were inserted into a modified version of pX330 (Addgene # 42230), carrying an extended sgRNA scaffold for improved activity (5′ GTTTAAGAGCTA TGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAG TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT GCTTTTTT 3′). The sgRNA cloning method was as described previously (27). Briefly, complementary ssDNA oligonucleotides, which encoded the guide sequences, were purchased from IDT DNA and annealed to create dsDNA with overhangs. The overhangs directed the ligation of annealed dsDNA with BbsI-digested pX330 vector, inserting the guide sequences downstream of U6 promoter sequence for expression. The sequences of complementary DNA oligonucleotides were as follow. For *CD96* targeting: 5′ CACCGTGCAGATGCAATG GTCCA 3′ and 5′ AAACTGGACCATTGCATCTGCAC 3′. For *TIGIT* targeting: 5′ CACCGCCTCCTGATCTGGGCCCAG 3′ and 5′ AAACCTGGGCCCAGATCAGGAGGC 3′. For *KLRC1* targeting: 5′ CACCGAACAGGAAATAACCTATG 3′ and 5′ AAACCATAGGTTATTTCCTGTTC 3′. BbsI digestion and T4 ligation were performed according the manufacturer's protocol (NEB). The plasmids were validated by Sanger sequencing.

Plasmid DNA Nucleofection

NK-92 cells were prepared as described above. Four hundred ng of pmaxGFP (Lonza) per 4×10^5 of cells was used for condition screening. GFP expression and cell viability were analyzed by flow cytometry at 4 and 24 h after nucleofection. For plasmid-based gene editing, Cas9:sgRNA dual expression plasmids were purified by

Plasmid Midi Kit (Qiagen) and 2 μg of DNA was used per nucleofection. The reduction in target protein expression was analyzed by immunostaining and flow cytometry at 72 h after nucleofection. For HDR experiments, 100 pmol of ssDNA ultramer or 2 μg of dsDNA PCR template was used per nucleofection.

Calcein-AM Cytotoxic Assay

Trypsin should not be used for cell dissociation in the cytotoxicity assay to prevent digestion of NK cell-targeting ligands. Adherent cells were detached by enzyme-free cell dissociation buffer (Gibco), neutralized by culture medium and pelleted at 200 g for 3 min. The supernatant was aspired and the target cells were wash by DPBS. After washing, 1×10^6 cells were resuspended in 1 ml DPBS containing 10 μM Calcein-AM (BioLegend) and incubated at 37°C for 30 min. The target cells were then washed by culture media for three times and resuspended at the cell density of 8×10^5 or 1.6×10^6 cells/ml in RPMI-1640 (ATCC modification). NK-92 cells were pelleted at 90 g for 10 min and resuspended at the cell density of 1×10^5 cells/ml in RPMI-1640 (ATCC modification). One hundred microliter of NK-92 cells per well were added to U-bottom 96-well plates and serial dilution was performed for different NK-92-to-target ratios. One hundred microliter of the stained target cells were directly added into each well. For ADCC assay, BT-474 and Raji cells were incubated with Herceptin (Trastuzumab, Roche) and Rituxan (Rituximab, Roche), respectively, for 30 min in 37°C incubator prior to being added into each well. The 96-well plate was the centrifuged at 120 g for 1 min to accelerate the contact between NK-92 and target cells. The cells were allowed to co-culture for 4 h at 37°C incubator. Spontaneous release of Calcein-AM was measured in the absence of NK-92 cells. Maximal release was determined by complete lysis of target cells in RPMI-1640 (ATCC modification) containing 2% Triton-X100. After co-culture, the plates were centrifuged at 120 g for 1 min, and 100 μl of the supernatant was transferred to 96-well Opti-plates (PerkinElmer). The 488/520 values were recorded by M1000 pro (Tecan). The following equation was used for cytotoxicity calculation:

$$\text{Target lysis } (\%) = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Statistical Analyses

Except for the screening experiments, all data were collected from three independent experiments to determine mean values ± SD as shown. Two-tailed Welch's unequal variances *t*-test was used to test for significant differences between two groups. *P*-values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 8.

RESULTS

Gene KO by Cas9 RNP Nucleofection Is Efficient and Viable

We used a Lonza 4D Nucleofector for NK-92 nucleofection because of its non-toxic carbon-based electrodes and semi high-throughput and scalable capability. However, the trouble is that Lonza nucleofection solutions and pulse codes are propriety, and condition screening is therefore necessary to optimize payload delivery. To enable robust gene knockout (KO) and knock-in (KI) in NK-92, we identified a combination of nucleofection buffer and pulse code for optimal co-delivery of Cas9 RNP and DNA repair template. The genotype and phenotype of genome edited NK-92 were assessed by Sanger sequencing and Inference of CRISPR Edit (ICE) tool, next generation sequencing (NGS), flow cytometry and cytotoxicity assay (**Figure 1A**).

To screen for Cas9 RNP nucleofection conditions, we programmed Cas9 to target *CD96* gene, encoding a highly expressed NK cell inhibitory receptor. Cas9 cleavage at *CD96* triggers DNA double-stranded break repair by predominantly the error-prone Non-homologous End Joining (NHEJ) pathway (28). NHEJ frequently results in random insertion or deletion (indel), leading to frame-shift mutation and premature termination of protein synthesis. The reduction in CD96 expression was analyzed by flow cytometry as the phenotypic readout of gene KO efficiency. Cell viability was simultaneously monitored by Zombie dye.

We built upon the reference conditions from primary NK cells and performed new rounds of screening. We tested 16 pulse codes in two different nucleofection buffers: Lonza P3 and Solution2+mannitol (Sol2). Sol2 was originally formulated for T cells and later adopted for primary NK cells (19, 24). The 32 combinations contained three reference conditions for primary NK cells: (1) Lonza P3 with EN-138 pulse code (20), (2) P3 with EH-115 (18), and (3) Sol2 with CM137 (19) (**Figure 1B**). For parallel comparison, we standardized the NK-92 cell density (4×10^5 cells) and RNP concentration (40 pmol). Overall, Sol2 performed significantly better than P3, with condition 3 producing higher *CD96* KO efficiency and cell viability than conditions 1 and 2 (**Figure 1C**). These results were unexpected, because condition 1 yielded ∼75% KO at *TGFBR2* gene in the original report (20). Condition 2 was described for KI of *GFP* gene, but no KO efficiency was mentioned (18). We also identified a new condition 4 (Sol2 with CA-137) that further improved the KO efficiency to ∼90% while preserving similar viability (**Figure 1C**). The pulse codes, buffers, and raw data are summarized in **Table S1**.

Precision Cell Count Beads Improves the Accuracy of Viability Assay

The recovery of viable and gene-edited cells is also an important consideration. In some conditions, we noticed substantial cell debris after nucleofection, which was difficult to collect by centrifugation. This led to the loss of dead cells and overestimation of cell viability. To improve the accuracy of the viability assay, we adopted a flow cytometric method to calculate cell density using Precision cell count beads and to determine

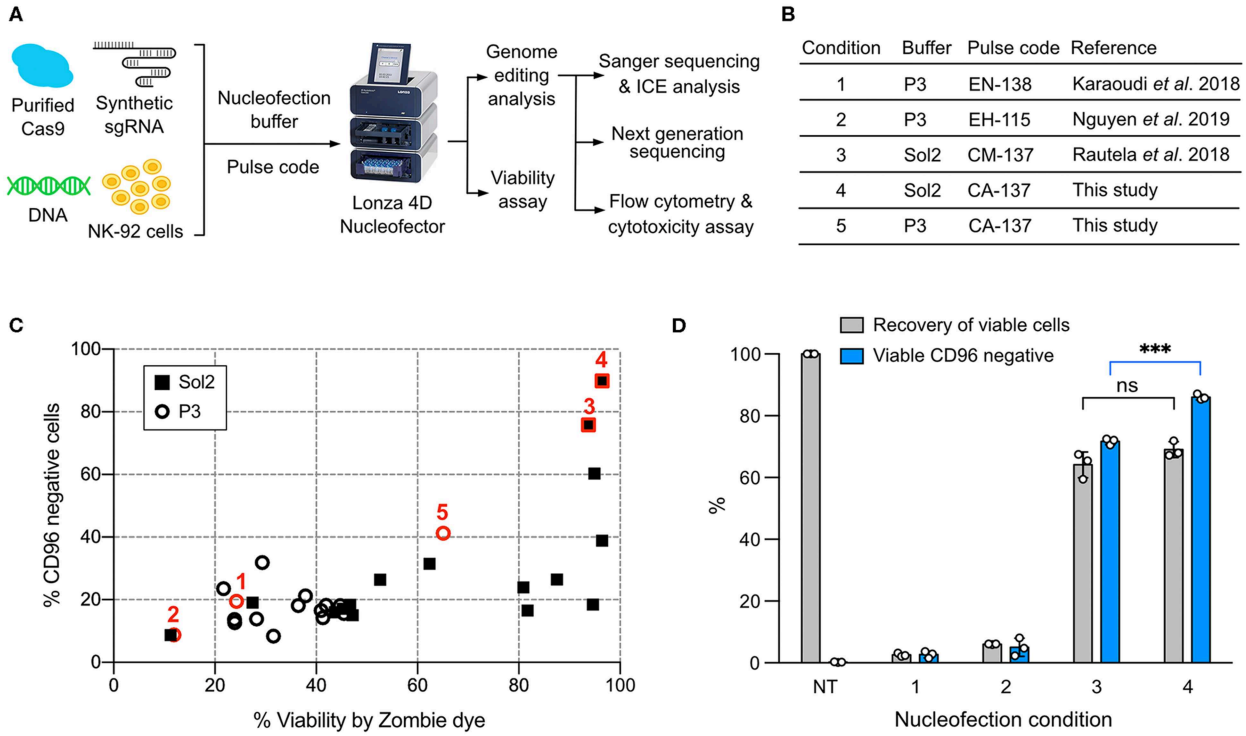


FIGURE 1 | Screening of the nucleofection conditions for Cas9 ribonucleoprotein (RNP) delivery. **(A)** Workflow of NK-92 genome editing by Cas9 RNP and DNA nucleofection using a Lonza 4D Nucleofector system. **(B)** A list of the reference and newly identified nucleofection conditions. **(C)** Sixteen pulse codes in combination with two buffers, Sol2 and P3, were tested to deliver Cas9 RNP to knockout (KO) *CD96* gene. Conditions 1–5 are highlighted in red to show their relative *CD96* KO efficiencies and cell viability. **(D)** Conditions 1–4 were re-examined by a more accurate viability assay. Recovery of viable cells was determined by Precision beads assay and normalized to untreated cells (NT). $CD96^-$ cells in the viable population were quantitated. Data are shown as mean ± SD of three independent experiments. Statistics by two-tailed Welch's unequal variances *t*-test; ns, not significant and $^{***}p \leq 0.001$. The pulse codes, buffers and raw data are summarized in **Table S1**.

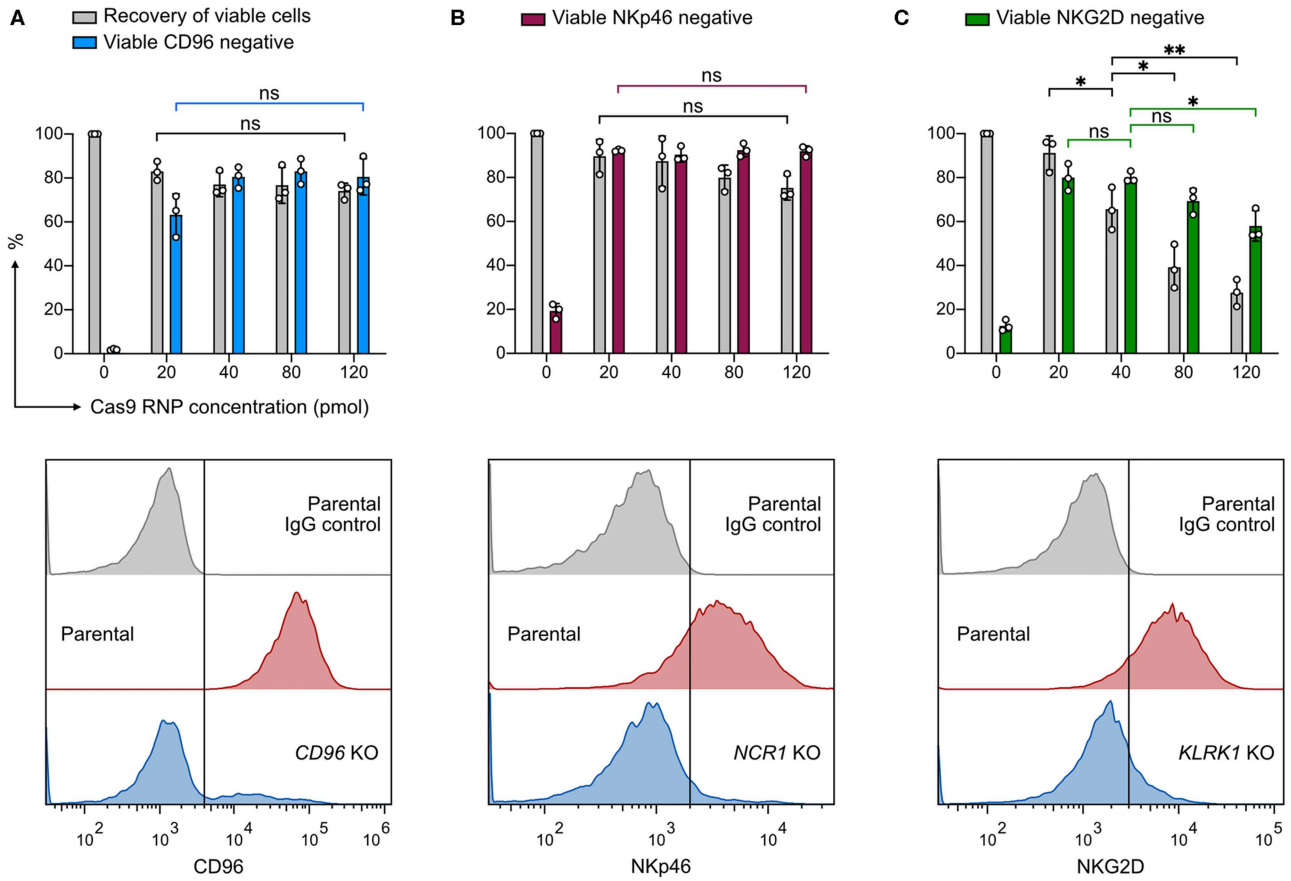
viable cells by light scattering and 7-Aminoactinomycin D (7-AAD) counterstaining (**Figure S1A**). This method eliminated the need for centrifugation and cell washing altogether and allowed retention of both the live cells and dead debris. Viable cells were 7-AAD negative and displayed normal dimensions as the untreated cells (black circle, **Figure S1B**).

We re-examined conditions 1–4 using this more rigorous viability assay. Both conditions 3 and 4 outperformed conditions 1 and 2, confirming that Sol2 was better than P3 in maintaining viable NK-92 cells (**Figure 1D**). Condition 3 and 4 had a similar recovery of viable cells, but within the viable population, condition 4 produced more $CD96^-$ cells (86 ± 0.9%) than condition 3 (71.7 ± 1.1%). The results show that high KO efficiency and cell viability can be achieved by Cas9 RNP nucleofection, but that NK-92 requires specific nucleofection conditions different from primary NK cells.

High Cas9 RNP Dosage Maintains Targeting Precision

We wanted to know whether Cas9 RNP concentration would impact KO efficiency, recovery of viable cells, or off-target cleavage. To probe this, we nucleofected increasing concentrations of *CD96*-targeting RNP ranging from 20 to 120 pmol. Higher concentrations of RNP (80 and 120 pmol) did not increase the *CD96* KO efficiency beyond that of 40 pmol RNP (**Figure 2A**). At 20 pmol, we observed a slight decrease in KO efficiency to 63%, but the difference was not statistically significant ($P = 0.07$). Similarly, no significant improvement in KO efficiency was observed at *NCR1* (**Figure 2B**). In both *CD96* and *NCR1* experiments, the recovery of viable cells remained high, at ~70–90% across the RNP concentrations. The outcome of *KLRK1* KO was different (**Figure 2C**). This gene encodes the NKG2D activating receptor that is important for NK cell proliferation and survival (29). Increasing the RNP dosage led to the reduction in viable cells that was also accompanied by decreasing KO efficiency. Our results indicate that *KLRK1* KO cells cannot survive, and suggest essential roles for NKG2D in NK-92.

To analyze off-target effects, we focused on the *CD96*- and *KLRK1*-targeting sgRNAs, and selected the top two off-target sites as predicted by the CRISPR Design tool on the Benchling website. This web tool provides *in silico* prediction of on-target (editing efficiency) and off-target (editing precision) scores using the algorithms developed by Doench et al., and Hsu et al., respectively (25, 30). All sgRNAs in this work were designed using the same tool and selected for high off-target scores to ensure targeting precision. As a result, we did not detect any off-target



D

Locus	Target site	Chr	Gene?	Sequence	PAM	% Indel at RNP concentration 0	40	120 (pmol)
CD96	on-target	3	yes	CGTGCAGATGCAATGGTCCA	AGG	0.12	86.91	91.31
	off-target 1	8	no	CTGGTAGATGCAATGGTCCA	GGG	0.11	0.10	0.09
	off-target 2	2	no	TTTGCAAATCCAATGGTCCA	GGG	0.12	0.06	0.11
KLRK1	on-target	12	yes	AATAATGAAACGGATTCCCA	TGG	2.76	68.10	57.93
	off-target 1	20	no	AATGGTGAAAAGGATTCCCA	GGG	0.19	0.19	0.18
	off-target 2	4	no	CATAATCAAACGGATTTCCA	AAG	0.13	0.14	0.13

FIGURE 2 | Effect of Cas9 RNP dosage to KO efficiency, cell viability, and off-target cleavage. **(A)** Recovery of viable cells and viable CD96$^-$ cells were determined at increasing dosages of Cas9 RNP by Precision beads assay. Representative flow cytometry plots using 40 pmol of Cas9 RNP are shown with the mean percentages of viable negative cells. **(B)** KO results of *NCR1*, which encodes the NKp46 receptor. **(C)** KO results of *KLRK1*, which encodes the NKG2D receptor. **(D)** Analysis of *CD96* and *KLRK1* on-target and off-target editing by next generation sequencing. Mismatches between the on- and off-target sequences are labeled in red. Sequence variations were determined by CRISPResso2 and presented as % indel. Data are shown as mean ± SD of three independent experiments. Statistics by two-tailed Welch's unequal variances *t*-test; ns, not significant, $^*p \leq 0.05$, $^{**}p \leq 0.01$.

mutation at frequencies higher than the untreated control at 40 or 120 pmol of RNP (**Figure 2D**). ICE and NGS analyses also revealed comparable on-target editing efficiencies. We observed a single thymidine indel at 2.76% frequency in the *KLRK1* on-target region at chromosome 12 position 10379779, even in the untreated cells. This thymidine indel happened in a poly-T track and was likely due to sequencing error. The off-target analysis shows that, with careful sgRNA design, genome editing by RNP nucleofection has high on-target efficiency and no detectable off-target cleavage at the predicted sites at up to 120 pmol of RNP. The dosage experiment suggests that 20–40 pmol of Cas9 RNP per 4×10^5 cells is an effective dosage for robust and precise KO in NK-92 cells.

In silico Prediction Does Not Guarantee sgRNA Performance in Cells

Gene KO is a popular approach to delineate gene functions, and Cas9 RNP nucleofection is a rapid platform to identify robust

sgRNAs and accessible target regions. To demonstrate this, we tested a series of sgRNAs and discovered that the performance of sgRNAs in the cells did not always agree with the *in silico* prediction of on-target score by Benchling CRISPR Design. For example, sgRNA4 and 6 of the *KLRC1* locus had similar on-target scores, but their editing efficiencies differed by more than 4-fold at 84 ± 3% and 18 ± 5%, respectively (**Figure 3**). At the *TIGIT* locus, exon 3 appeared inaccessible to Cas9 targeting regardless of on-target scores. Several other examples are shown in **Figure 3**. These data underscore the importance to experimentally validate sgRNAs in the target cells. Using Cas9 RNP nucleofection, we could quickly test sgRNA performance in NK-92 cells and identify the suitable sgRNA for KO and KI experiments.

Cas9 RNP Nucleofection Enables Robust Multiplex KO

Knocking out multiple genes one at a time is laborious and time-consuming. Multiplex KO is more straightforward to set up using the Cas9 RNP platform than the plasmid approach, which is restricted by the available copies of sgRNA expression cassette and cloning site on the plasmid. Cas9 RNPs of distinct targeting specificities can be pooled together *in vitro* at precise molar ratios for nucleofection. This approach eliminates repeated KO procedure and allows simultaneous disruption of multiple genes to study the combinatorial effect. To demonstrate this, we combined the best sgRNAs in a single nucleofection reaction for double and triple KO of NK-92 cell surface receptors.

We first targeted *CD96* and *KLRC1* (encoding NKG2A), and quantitated the target protein expression by flow cytometry as a measure of KO efficiency. Parental NK-92 cells were 92.5 ± 0.9% double positive for CD96 and NKG2A with only 2.8 ± 3.4% of double negative cells. After editing, the cell population shifted to 47.9 ± 1.2% double negative, 37.3 ± 3.2% CD96 single negative and 4.8 ± 0.7% NKG2A single negative, leaving only 10 ± 1.8% of double positive cells (**Figure 4A**). The uneven sgRNA efficiencies likely reflected the difference between the ratios of $CD96^-$ and $NKG2A^-$ cells. Next, we targeted *CD96* and *NCR1* (encoding NKp46), and again observed high double KO efficiency. The parental cells were 5.3 ± 0.2% double negative, and became 81.3 ± 1.9% double negative after editing (**Figure 4B**). Finally, we combined all three sgRNAs to disrupt *CD96*, *KLRC1*, and *NCR1* at one time. The triple negative cells increased from 1.2 ± 0.7% in the parental cells to 50.9 ± 2.1% after editing (**Figure 4C**). In all three experiments, the cell viability was maintained at ∼80% as seen in the RNP dosage experiment (**Figure 2**), suggesting that the cumulative dosage of multiple Cas9 RNPs were well-tolerated. These results demonstrate the efficiency of Cas9 RNP nucleofection for multiplex KO in NK-92.

The risk of chromosomal translocations is a concern when multiple DSBs are simultaneously induced by Cas9 and misligated by NHEJ. After the triple KO experiment, we adopted a PCR-based assay to detect chromosomal translocations between the Cas9-edited sites (26). We used specific PCR primer sets to probe 12 possible translocation patterns between the *CD96*, *KLRC1*, and *NCR1* target sites on chromosome 3, 12, and 25, respectively (**Figure S2A**). In only the triple KO cells, we detected evidence of translocations in eight out of 15 patterns, but at much lower levels than the wild-type sequences (**Figures S2B,C**). Our results indicate that chromosomal translocation can occur between multiple Cas9-edited sites, giving rise to unexpected mutations that are easily missed by standard gene editing analyses.

DNA Nucleofection Leads to Rapid Decline in Cell Viability

Cas9-mediated HDR requires the co-delivery of synthetic DNA repair templates to mediate DNA sequence exchange or gene insertion. Because the physicochemical and nucleofection properties of Cas9 RNP are distinct from those of DNA (31), we repeated the screening procedure for DNA nucleofection, aiming to find a suitable condition for co-delivery of Cas9 RNP and a DNA template. We tested the same 32 conditions as in Cas9 RNP nucleofection to deliver 0.4 μg of pmaxGFP plasmid, encoding turboGFP protein for detection. The expression of GFP was detected in as little as 4 h by flow cytometry (**Figure 5A**). Strikingly, cell viability declined rapidly in 24 h, with condition 4 being the most balanced condition with 17% GFP expression and 28% viability (**Figure 5B**).

DNA nucleofection appeared highly toxic to NK-92 cells. We performed the Precision beads assay to quantitate viable GFP^+ cells in conditions 1–4. We did not pursue condition 5 because it was inefficient for RNP nucleofection (**Figure 1C**). Most of the cells died in condition 1 and 2 (**Figure 5C**). Condition 3 and 4 produced comparable percentages of viable GFP^+ cells at 7.6 ± 2.5% and 11.5 ± 1%, respectively, but condition 4 yielded higher recovery of viable cells at 8.3 ± 0.6% (**Figure 5C**). Higher pmaxGFP concentrations at 1 and 2 μg helped increase viable GFP^+ cells; however, 4 μg of pmaxGFP showed no further improvement (**Figure 5C**). Overall, DNA nucleofection led to significantly more cell death than by RNP nucleofection. Collectively, 1–2 μg of DNA in condition 4 is the best combination.

Plasmid-Based CRISPR Gene Editing Is Ineffective

Plasmid-based CRISPR gene editing was reported to be ineffective in NK cells (19). We were curious whether this was also a problem for NK-92. We thus constructed three Cas9+sgRNA dual expressing plasmids, each encoding the best *CD96*, *TIGIT*, or *KLRC1* targeting sgRNA. We tested the three plasmids in NK-92 cells and also HEK293T cells as a control. We detected ∼80% indel in HEK293T cells across all three loci, confirming robust editing of the three sgRNAs (**Figure 5D**). In stark contrast, there was no detectable indel in NK-92 cells in any target locus. We performed Western Blotting and reverse-transcription PCR to check Cas9 and sgRNA synthesis, respectively (**Figure 5E**). We detected both Cas9 and sgRNA in HEK293T cells, but no Cas9 and only a hint of sgRNA in NK-92 cells, reflecting the lack of gene editing. These results emphasize the superiority of Cas9 RNP gene editing in NK-92 over the plasmid approach.

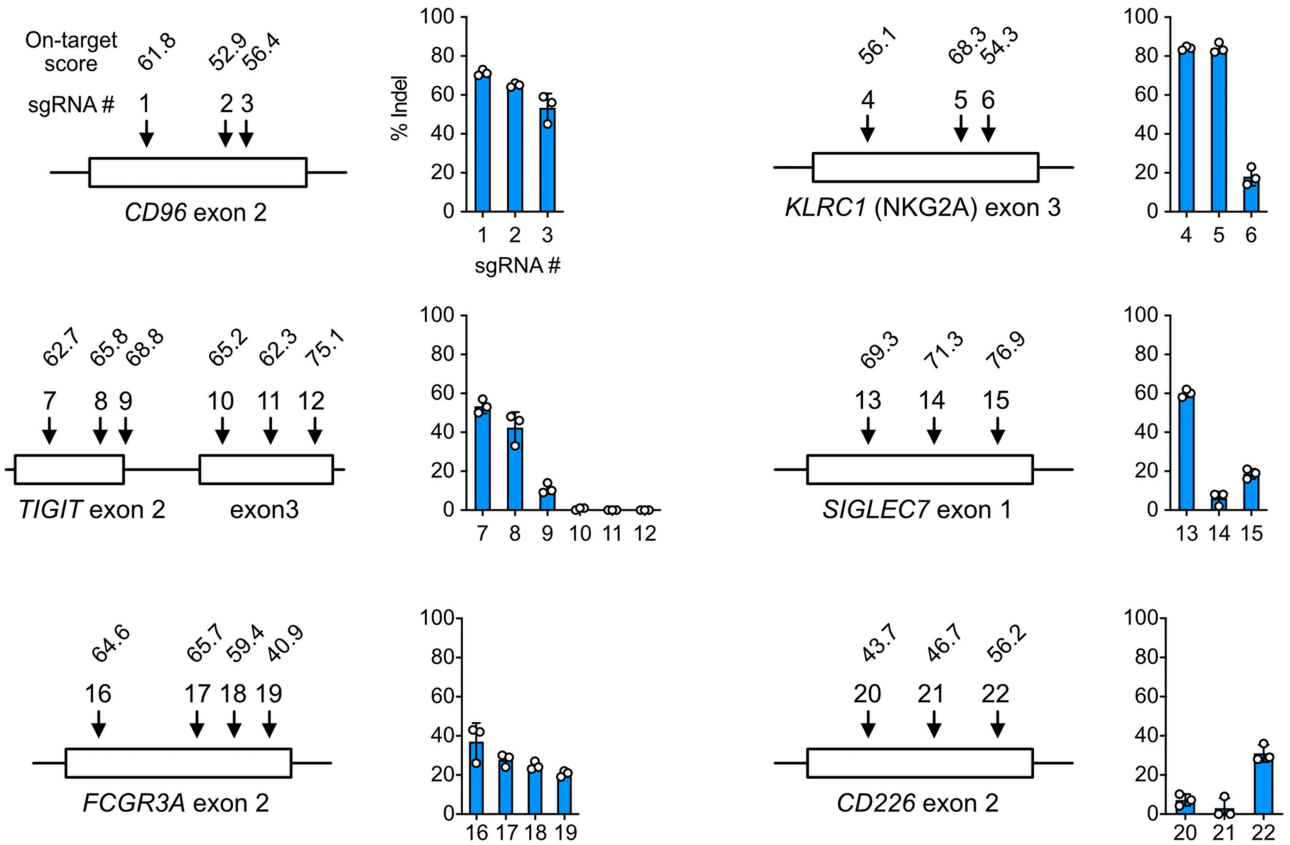


FIGURE 3 | Cell-based screening of efficient sgRNA. sgRNAs (22 in total) were synthesized to target the *CD96*, *KLRC1*, *TIGIT*, *SIGLEC7*, *FCGR3A*, and *CD226* genes at the indicated exons. On-target scores of the sgRNAs were predicted *in silico* by the CRISPR Design tool on the Benchling website, and are indicated above the sgRNA number. A higher on-target score predicts higher editing efficiency. Percentages of insertion-or-deletion (indel) were determined by Sanger sequencing and ICE analysis. Data are shown as mean ± SD of three independent experiments. The sgRNA position and exon length are relative and not to scale.

Cas9-Mediated HDR Allows Non-viral Gene KI

Cas9-mediated HDR allows precise sequence modifications and targeted gene insertion; however, this approach is not effective in all cell types. To test whether NK-92 cells are capable of HDR, we inserted restriction sites into the *CD96* and *TIGIT* loci and *hROSA26* genome safe harbor (**Figure 6A**). The restriction sites were encoded on synthetic DNA oligonucleotides (DNA ultramer) and flanked by 90-nt homology arms. We nucleofected NK-92 cells with 100 pmol (equivalent to 0.06 μg due to low molecular weight) of DNA ultramer using condition 4. We detected 20–30% HDR efficiencies across the three target loci by ICE analysis (**Figure 6B**). Although the percent total indel at *hROSA26* was lower than those at *CD96* and *TIGIT*, the HDR efficiency at *hROSA26* was comparable to the others. Cell viability was maintained at 60–80% at all three loci, because the concentration of DNA ultramer was much lower than that of pmaxGFP. Our results demonstrate that NK-92 can utilize DNA ultramer as HDR templates for short sequence modifications, opening the possibility to introduce point mutations and protein fusion tags.

Next, we integrated the *mCherry* reporter gene in-frame into *CD96* exon 2, by adopting a similar strategy from Roth et al. (32) The *mCherry* sequence was encoded on a PCR-synthesized double-stranded DNA (dsDNA) and flanked by 300-nt homology arms (**Figure 6C**; full DNA sequence in **Table S4**). The expression of mCherry was driven by the *CD96* endogenous promoter and quantitated by flow cytometry. After editing, the viability was 42.7 ± 6.7%, indicating that the 1,695-nt *mCherry* template was moderately toxic as compared to 186-nt DNA ultramer and 3,487-nt pmaxGFP (**Figure 6D**). About 3% of the edited cells were mCherry$^+$. The *mCherry* template-only control had 0.6%, likely due to non-specific genomic integration (**Figure 6E**). The KI efficiency of *mCherry* was significantly lower than that of restriction sites, revealing the challenge of introducing gene-sized modifications in NK-92 cells by Cas9-mediated HDR.

Being a lymphoma cell-type, NK-92 cells are capable of clonal expansion. We took advantage of this and isolated the mCherry$^+$ cells by FACS for expansion. To ensure accurate insertion, we validated the genomic junctions flanking the *mCherry* insert by Sanger sequencing (**Figure S3**). In the second round of FACS, the mCherry$^+$ population was further separated into low-expressing and high-expression groups (**Figure 6F**).

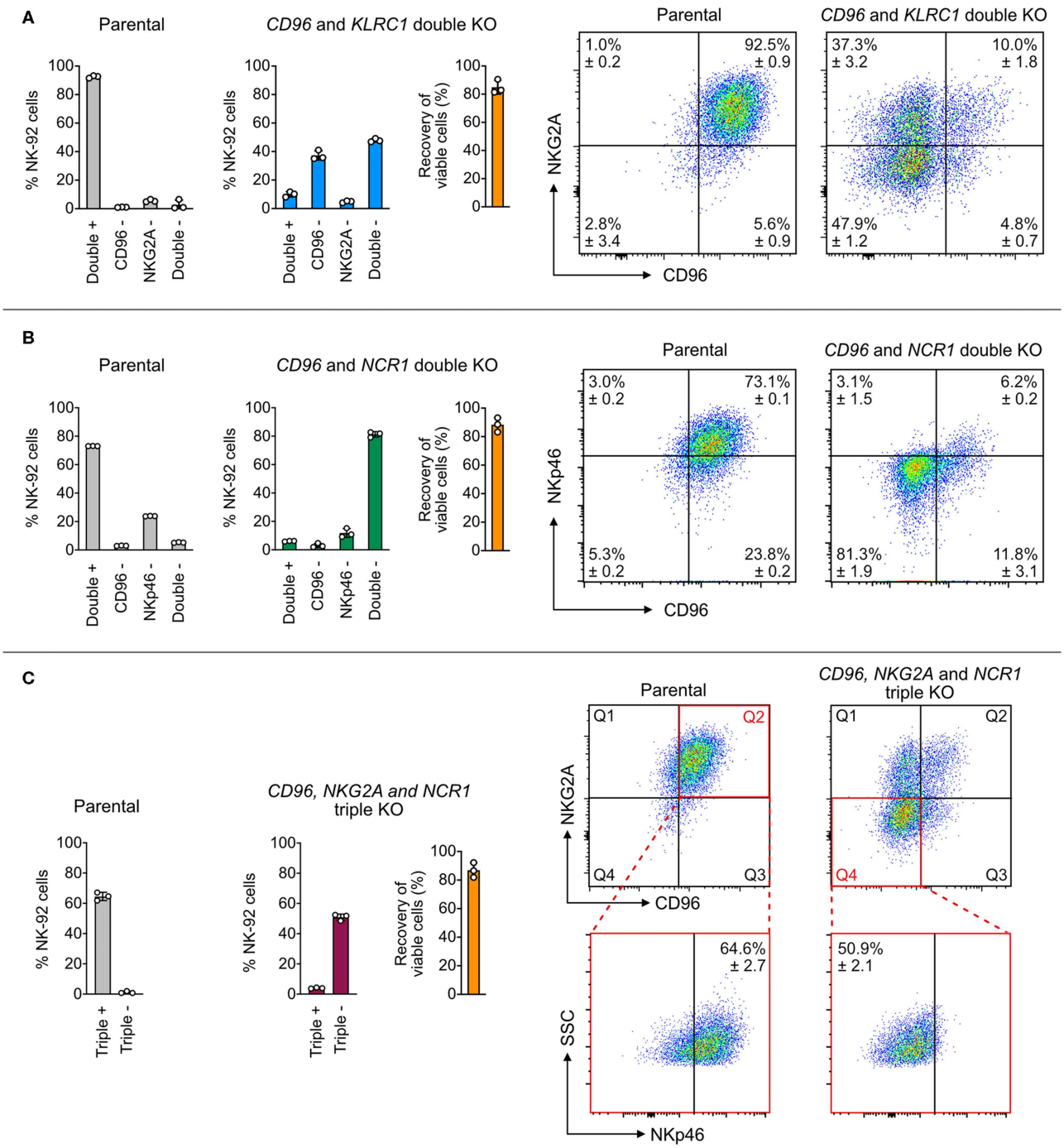


FIGURE 4 | Double and triple KO in a single nucleofection. **(A)** A mixture of two Cas9 RNPs, consisting of sgRNAs targeting both *CD96* and *NKG2A*, was nucleofected into NK-92 cells to simultaneously KO *CD96* and *NKG2A*. Representative flow cytometry plots show the expression levels of target proteins in the parental and double KO cells. **(B)** Double KO of *CD96* and *NCR1* (encoding NKp46). **(C)** Triple KO of *CD96*, *NKG2A*, and *NCR1* by nucleofection of a mixture of three Cas9 RNPs. To increase readability, only the percentages of triple positive and negative cells are shown in the bar graph. In the representative flow cytometry plots, Q2 of the parental plot was gated to determine triple positive cells. Q4 of the triple KO cells was gated to determine triple negative cells. Cell viability was determined by the Precision beads assay and normalized to untreated cells. Data are shown as mean ± SD of three independent experiments.

The enriched cells stably retained their mCherry expression levels after multiple passages and cryopreservation. Our results show that Cas9-mediated HDR coupled with FACS enrichment is a useful strategy to enable precise genome editing and overcome low HDR efficiency. Unfortunately, we could not isolate single clones because NK-92 did not grow from a single

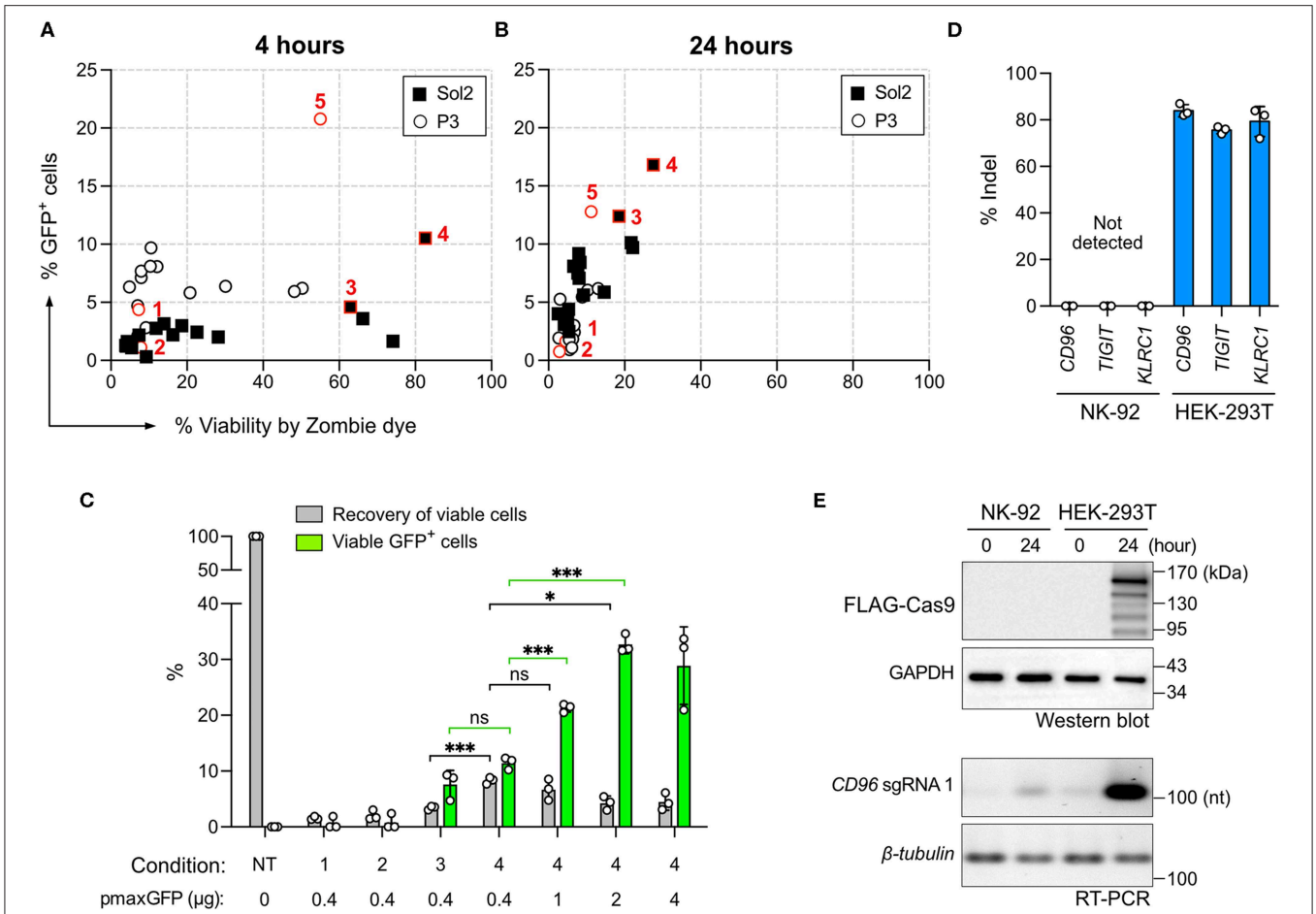


FIGURE 5 | Screening of DNA nucleofection condition. The same 32 conditions used for Cas9 RNP nucleofection were tested for the nucleofection of the pmaxGFP plasmid. GFP expression and cell viability were determined by flow cytometry with Zombie dye staining at 4 h **(A)** and 24 h **(B)** post nucleofection. **(C)** A comparison of conditions 1–4 was performed using Precision beads assay. Increasing concentrations of the pmaxGFP plasmid were added to improve GFP expression in condition 4. Recovery of viable cells was normalized to the untreated condition (NT). GFP^+ cells in the viable population were quantitated. **(D)** Plasmid-based CRISPR gene editing was performed to target *CD96*, *TIGIT*, and *KLRC1* genes in NK-92 and HEK-293T cells. No indel was detected in NK-92 by Sanger sequencing and ICE analysis. **(E)** Confirmation of Cas9 and sgRNA expression from the CRISPR plasmid by Western blotting and reverse-transcription PCR (RT-PCR). Cas9 protein carries a FLAG tag for detection. Cell samples were analyzed immediately (time = 0) and 24 h after nucleofection. GAPDH protein and *β-tubulin* mRNA serve as internal controls. Data are shown as mean ± SD of three independent experiments. Statistics by two-tailed Welch's unequal variances *t*-test; ns, not significant, $^*p \leq 0.05$ and $^{***}p \leq 0.001$. The pulse codes, buffers and raw data are summarized in **Table S1**.

cell. Overcoming this technical hurdle is necessary to create genetically defined NK-92 clones for therapeutic applications.

Cas9-Mediated Promoter Insertion Reactivates Silenced Genes

NK-92 cells is less potent than primary NK cells because some cytotoxicity-related genes are silenced. The common approach to restore these gene activities is to insert the cDNA of the silenced genes into NK-92 genome using viral transduction. However, the genomic insertion of viral vectors occurs at random sites, producing a heterogeneous population of cells with mixed genotypes and potentially uneven activities. To demonstrate that non-viral, site-specific genome editing is a better approach, we designed Cas9-mediated HDR to reactivate the endogenous genes by replacing the silenced promoter with a spleen focus-forming virus (SFFV) promoter (33). We targeted the *FCGR3A* and *CD226* genes, which encode CD16 and DNAM-1, respectively (**Figure 7A**). CD16 is a surface receptor that binds to the Fc region of IgG antibodies and is essential for antibody-dependent cellular cytotoxicity (ADCC) in NK cells (34). DNAM-1 binds to the tumor-associated antigens CD155 and CD112 and synergizes cytotoxicity with other activating receptors (35).

Parental NK-92 cells do not express CD16 and therefore cannot mediate ADCC. We programmed Cas9 RNP to target near the start codon of *FCGR3A*, in order to insert the SFFV promoter and Kozak sequence immediately upstream of the start codon (**Figure 7B**). The sgRNA selection was limited in this region, because most of the sgRNA candidates had low off-target scores and low editing efficiencies, except sgRNA16 (**Figure 3**).

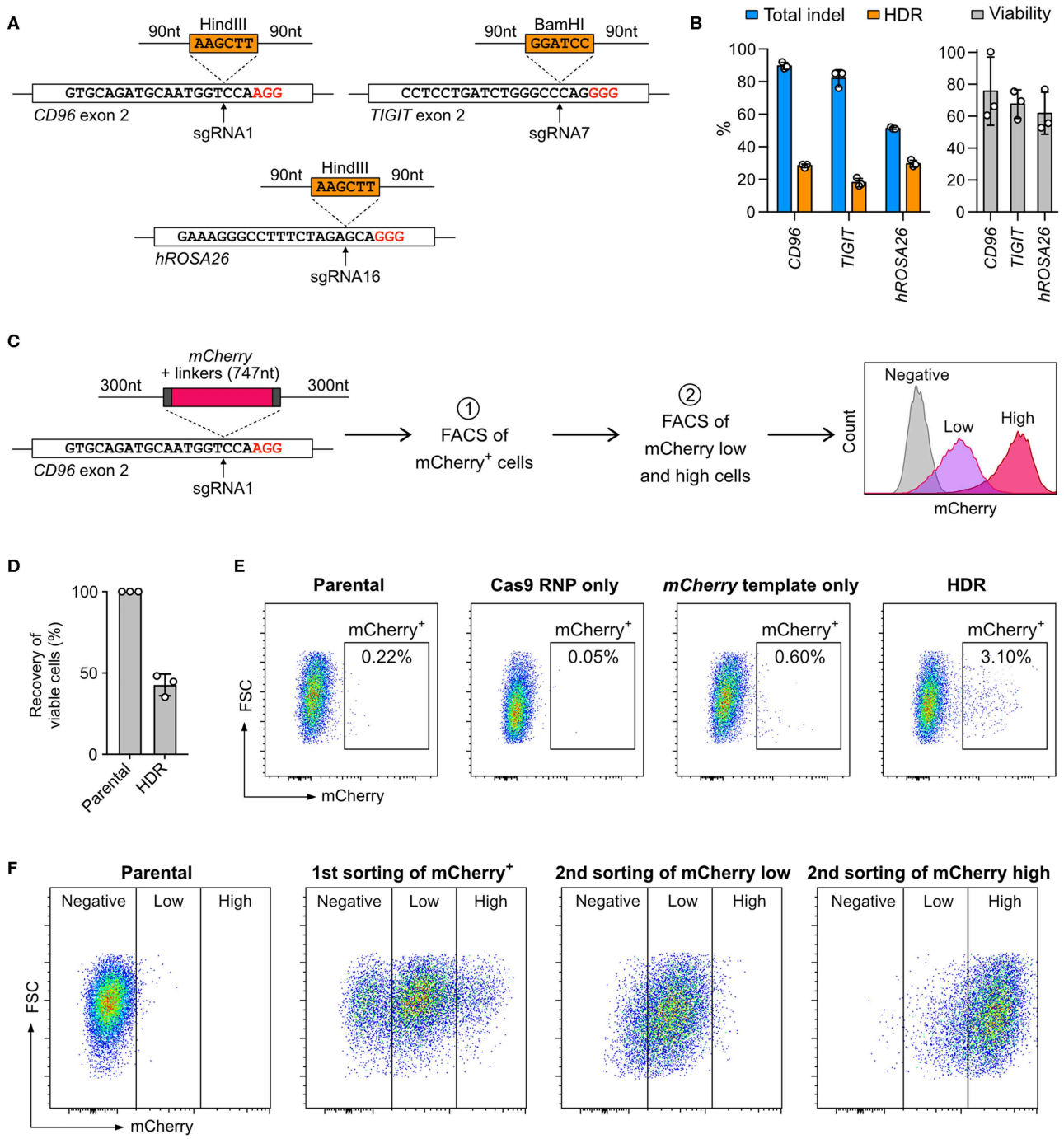


FIGURE 6 | Knock-in (KI) by Cas9 RNP-mediated homology-directed repair (HDR). **(A)** Co-nucleofection of DNA ultramer, as HDR templates, allows the insertion of either BamHI or HindIII restriction sites at the indicated sgRNA target sites. The upstream and downstream homology arms are 90-nt long. PAM sequences are marked in red. **(B)** Total indels and HDR frequencies were determined by Sanger sequencing and ICE analysis. The normalized cell viability shows that single-strand DNA is less toxic than pmaxGFP to NK-92. Data are shown as mean ± SD of three independent experiments. **(C)** A double-stranded PCR template was used to facilitate the insertion of a promoter-less *mCherry* reporter gene in-frame into the *CD96* exon 2 at the sgRNA1 target site. The homology arms are 300-nt long. The expression of mCherry, as driven by the endogenous *CD96* promoter, was analyzed by flow cytometry. Two rounds of fluorescence-activated cell sorting (FACS) were conducted to first enrich mCherry$^+$ cells and then further isolate mCherry low-expressing and mCherry high-expressing cells. **(D)** Viability assay reveals moderate level of toxicity of double-stranded PCR templates. **(E)** Flow cytometry plots show the percentages of mCherry$^+$ cells at 72 h after nucleofection. **(F)** Distributions of mCherry low-expressing and high-expressing cells are shown after each round of cell sorting.

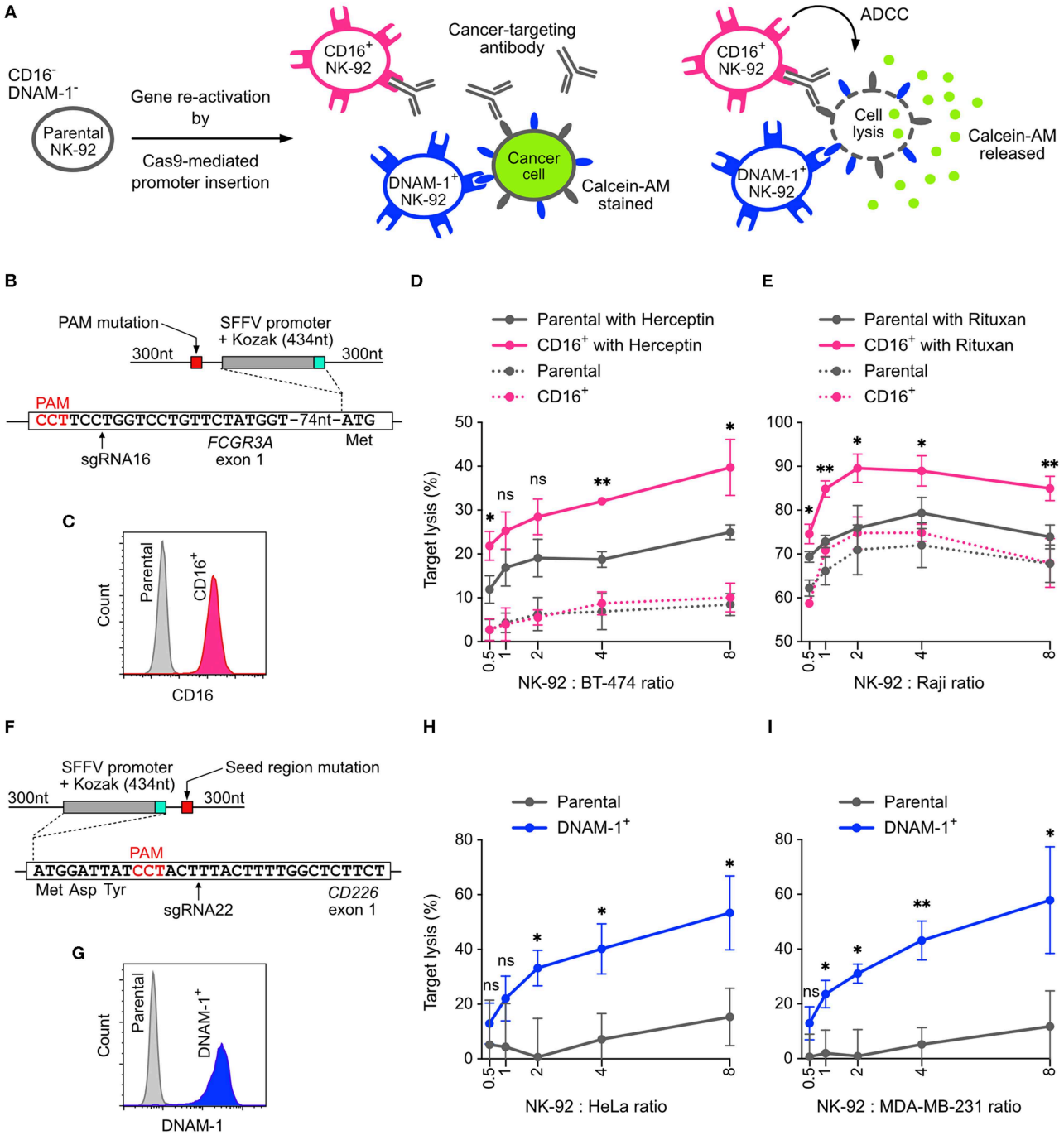


FIGURE 7 | Reactivation of CD16 and DNAM-1 by Cas9-mediated promoter insertion. **(A)** Parental NK-92 cells do not express CD16 and DNAM-1. Reactivation of CD16 enables NK-92 cells to execute antibody-dependent cellular toxicity (ADCC) in combination with a cancer-targeting antibody. Reactivation of DNAM-1 allows NK-92 to recognize CD155 or CD112 ligand on cancer cells and initiates cytotoxicity. In the cytotoxicity assay, lysis of the cancer cells is measured by the release of Calcein-AM. **(B)** A double-stranded HDR template facilitates the insertion of SFFV and Kozak sequences immediately upstream of the *FCGR3A* start codon. The sgRNA16-guided Cas9 cleavage site is marked by arrow. The amino acids indicate the immediate N-terminal peptide sequence. The PAM sequence of the sgRNA16 target site was mutated in the HDR template to avoid targeting by Cas9. **(C)** Flow cytometry plot shows enriched CD16$^+$ cells. **(D)** Lysis of BT-474 cells in Herceptin-mediated ADCC in CD16$^+$ NK-92 cells vs. parental cells. **(E)** Lysis of Raji cells in Rituxan-mediated ADCC. **(F)** In a similar setup, SFFV and Kozak sequences were inserted before the *CD226* start codon, at the cleavage site defined by sgRNA22 (arrow). The seed region of sgRNA22 target site was modified by silent mutations in the HDR template to avoid targeting by Cas9. **(G)** Flow cytometry plot shows enriched DNAM-1$^+$ cells. Lysis of HeLa **(H)** and MDA-MB-231 **(I)** cancer cells by DNAM-1$^+$ NK-92. Data are shown as mean ± SD of three independent experiments. Statistics by two-tailed Welch's unequal variances *t*-test; ns, not significant, $^*p \leq 0.05$ and $^{**}p \leq 0.01$. DNA sequences of HDR templates are in **Table S4**.

We anticipated low HDR efficiency because the cleavage site of sgRNA16 was 91-nt away from the intended insertion site. After HDR, we obtained 1.2% of $CD16^+$ cells. We were able to isolate the $CD16^+$ cells by FACS and expanded to 97.4% purity (**Figure S4A**). The expression of CD16 was stable after clonal expansion and cell passage (**Figure S4** and **Figure 7C**). We also verified the genomic junctions flanking the SFFV cassette by Sanger sequencing (**Figure S4**).

$CD16^+$ NK-92 was then assayed *in vitro* for ADCC against cancer cell lines. In the presence of Herceptin (anti-HER2 antibody), $CD16^+$ NK-92 showed 2-fold enhancement in cytotoxicity against BT474 ($HER2^+$ ductal carcinoma) compared to the parental cells at various NK-92-to-cancer ratios (**Figure 7D**). The combination of Herceptin and parental NK-92 also led to some increase in cancer lysis, likely due to the direct action of Herceptin against BT474. Similar levels of enhancement were also observed with Rituxan (anti-CD20 antibody) against Raji, a $CD20^+$ B cell lymphoma (**Figure 7E**). The results demonstrate that the cytotoxicity of NK-92 benefits significantly from reactivation of CD16 and ADCC.

Using the same HDR strategy, we also reactivated DNAM-1 expression by SFFV insertion, validated the genomic junctions, FACS enriched and clonal expanded the DNAM-1^+ cells (**Figures 7F, G** and **Figure S5**). We then assayed DNAM-1^+ NK-92 against HeLa and MDA-MB-231 cancer cell lines, which express high levels of CD155 and are challenging to kill by parental NK-92 cells. The expression of DNAM-1 activating receptor significantly boosted the cytotoxicity by 4-fold against both cancer cells across various NK-92-to-target ratios (**Figures 7H,I**). In summary, our results demonstrate that both CD16 and DNAM-1 enhance the cytotoxicity of NK-92 cells against cancer cell lines *in vitro*. The promoter-insertion strategy proves the possibility to site-specifically reactivate any endogenous genes by Cas9-mediated HDR, creating improved versions of NK-92 cells with well-defined genotypes.

DISCUSSION

NK-92 cells are a clinically valuable cell line that can help overcome the shortage of primary NK cells for adoptive immunotherapy. A robust and site-specific genome-editing tool is vital for functional study and therapeutic engineering of NK-92 cells. We describe a CRISPR genome editing platform for these cells based on the nucleofection of Cas9 RNP. This approach offers fast action, high efficiency, multiplex capability, low toxicity, and does not involve exogenous genetic materials in the forms of plasmid or viral DNA. Co-nucleofection of the DNA repair template allows synchronization of Cas9-mediated DNA cleavage and HDR to integrate exogenous DNA sequences into the targeted genomic loci. This is a popular approach for genome editing in a variety of human cells, and now a robust platform is also established for NK-92 cells.

Several interesting observations were made during the optimization of Cas9 RNP and DNA nucleofection. First, parallel comparison reveals that primary NK and NK-92 cells require different a nucleofection protocol. Overall, Sol2 is better than P3 for NK-92. Sol2 is more affordable, composed of common laboratory chemicals, and can potentially be fine-tuned to further improve cell viability and Cas9 RNP and DNA delivery. It is also possible to scale up the Lonza nucleofection reaction, using larger nucleofection cuvettes, to increase NK-92 cell production.

Second, DNA toxicity is a major hurdle in the introduction of exogenous DNA sequences. The pmaxGFP experiment reveals that, while it is possible to increase DNA delivery and transgene expression by using higher DNA dosages, NK-92 cells die rapidly in response to DNA nucleofection. Toxicity levels appear to correlate with DNA concentration and length, judging from the HDR experiments using a DNA ultramer, a mCherry dsDNA PCR template and a pmaxGFP plasmid. While the toxicity of DNA ultramer is the lowest, the length limitation (200 nt) of the synthetic oligonucleotides excludes the possibility of incorporating a promoter sequence or a gene. We have not compared the cell viability or HDR efficiency using single-stranded vs. double-stranded template of the same length. It would be interesting to convert a long dsDNA template into a single-stranded form, using the commercially available kits, to see if the toxicity problem is alleviated.

We are currently investigating intracellular DNA immunity in primary NK and NK-92 cells to understand the mechanism of DNA toxicity. Because NK cells are a pivotal component of innate immunity against viral infections, they likely possess a comprehensive set of intracellular sensing and defense mechanisms against DNA of foreign origins. The activation of intracellular DNA immunity, for example the cGAS-STING pathway, is known to induce rapid inflammatory responses, pyroptosis and apoptosis (36). Similar observations were made previously in primary lymphocytes, where the nucleofection of exogenous DNA induced inflammatory responses and apoptosis (37, 38). Whether or not intracellular DNA immunity is responsible for DNA-induced NK-92 cell death awaits experimental confirmation. Elucidation of DNA toxicity could offer valuable insight into the design of HDR templates that can evade immune detection in NK cells.

In contrast to DNA, nucleofection of high dosages of Cas9 RNP (up to 120 pmol per 4×10^5 cells) seems well-tolerated in NK-92 cells. No significant increase in KO efficiency was observed beyond 40 pmol at the *CD96* and *NCR1* loci, suggesting that higher RNP dosage is unnecessary for single KO. On the other hand, targeting essential genes is expected to have a detrimental effect on cell viability, especially when high KO efficiency obliterates completely two alleles. We suspect this is the case in the *KLRK1* KO. When the essential NKG2D receptor, encoded by *KLRK1*, is eliminated, NK-92 cells can no longer survive. However, we cannot completely rule out the possibility that off-target effects at high RNP dosage contribute to the death of *KLRK1* KO cells. Our amplicon-based NGS did not detect any sequence deviation at the two predicted off-target sites, but a more extensive investigation is necessary to confirm the integrity of genome.

Cas9 RNP nucleofection is a robust approach to perform double and triple KO in NK-92. When coupled with FACS, KO mutants can be isolated, expanded and assayed for the loss of gene functions. The molar ratio of the RNP pool can

be adjusted without difficulty *in vitro* to compensate for low KO efficiency of certain sgRNAs. Such adjustment cannot be made in plasmid-based editing because neither nucleofection nor chemical transfection can guarantee precise expression levels of the cargo genes. In fact, plasmid-based editing was completely ineffective in NK-92. The lack of Cas9 and sgRNA expression was the main reason that our three independent KO attempts failed to produce detectable indels.

Although multiplexed gene KO by Cas9 RNP is highly efficient, we have shown that chromosomal translocation can happen in NK-92 between Cas9 edited sites on different chromosomes due to mis-ligation by NHEJ pathway. Chromosomal translocations can produce unexpected mutations and phenotypes that are not easily detected by the standard amplicon-based gene editing analyses and functional assays, respectively. The ability to isolate clonal NK-92 cells is therefore necessary to obtain a homogeneous genotype of the edited cells and verify whole genome integrity. This process is not yet possible for NK-92 because single cell expansion remains a challenge.

Cas9-mediated HDR was successful in NK-92 cells, although the HDR efficiency decreased as the length of the DNA insert and HDR template increased. Insertion of restriction sites and the *mCherry* gene occurred at ∼20 and 3%, respectively, indicating that NK-92 cells are capable of HDR using both ssDNA ultramer and dsDNA PCR templates. Moreover, Cas9-mediated promoter insertion effectively reactivated the endogenous *FCGR3A* and *CD226* enhanced NK-92 cytotoxicity. This strategy demonstrates the feasibility of site-specifically reactivating endogenous genes by Cas9-mediated HDR, and offers an attractive alternative to viral transduction, where viral integration may perturb genome integrity and produce cell-to-cell variation. The length of the SFFV promoter is also shorter than a full transgene and can be encoded in a shorter HDR template. We anticipate that by optimizing the homology arms, switching to ssDNA template, or incorporating NHEJ and cell cycle regulators, HDR efficiency may be further improved.

In summary, we describe a highly efficient CRISPR platform for genome engineering of NK-92 cells. The nucleofection protocols, the multiplex KO and KI strategies, and functional analyses are robust and readily adaptable for the development of NK-92 therapeutics. However, DNA toxicity still remains a major obstacle that interferes with the HDR process and the recovery of viable KI cells. Overcoming this toxicity would improve HDR and allow engineering more novel functions into NK-92 cells. Such functions are expected to match or exceed the primary level of competency.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive (PRJNA608597).

AUTHOR CONTRIBUTIONS

R-SH, H-AS, M-CL, and SL conceived and designed this study. R-SH, H-AS, and M-CL performed the experiments. R-SH and Y-JC analyzed the NGS results. R-SH and SL wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01008/full#supplementary-material

Figure S1 | Cell viability assay by precision cell count beads. **(A)** Workflow of precision beads assay. **(B)** Flow cytometry data of the untreated control and nucleofected cells.

Figure S2 | Detection of chromosomal translocation in the CD96-KLRC1-NCR1 triple KO cells by a PCR-based assay. **(A)** Schematic representation of the three target loci showing the PCR primers, Cas9 RNP cleavage positions, and the expected size of PCR fragments. **(B)** Nine possible combinations of forward and reverse primers were used to detect different arrangements of chromosomal translocation. The presence of DNA bands indicates evidence of chromosomal translocation in the triple KO cells. **(C)** Six possible combinations of forward primers were used to detect chromosomal inversion. All four DNA gels contained the same amount of DNA ladder for parallel comparison.

Figure S3 | Validation of *mCherry* knock-in by Sanger sequencing using PCR primer sets that were specific for genomic DNA, but not the HDR template. The genomic junctions upstream and downstream from the insert were sequenced to confirm precise HDR.

Figure S4 | (A) Flow cytometry analysis of CD16 expression after HDR and FACS enrichment. **(B)** The insertion of SFFV promoter was validated by Sanger sequencing using PCR primer sets that were specific for genomic DNA, but not the HDR template. The PAM sequence of sgRNA16 target site was mutated in the HDR template to avoid targeting by Cas9.

Figure S5 | (A) Flow cytometry analysis of DNAM-1 expression after HDR and FACS enrichment. **(B)** The insertion of SFFV promoter was validated by Sanger sequencing using PCR primer sets that were specific for genomic DNA, but not the HDR template. The seed region of sgRNA22 target site was modified to silent mutations in the HDR template to avoid targeting by Cas9.

Table S1 | Raw data of Cas9 RNP and pmaxGFP nucleofection screening.

Table S2 | sgRNA list and gene editing efficiencies.

Table S3 | PCR primers for genomic DNA amplification, plasmid construction and NGS.

Table S4 | Full DNA sequences of the HDR templates.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Overcoming Chimeric Antigen Receptor (CAR) Modified T-Cell Therapy Limitations in Multiple Myeloma

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Multiple myeloma (MM) remains an incurable disease regardless of recent advances in the field. Therefore, a substantial unmet need exists to treat patients with relapsed/refractory myeloma. The use of novel agents such as daratumumab, elotuzumab, carfilzomib, or pomalidomide, among others, usually cannot completely eradicate myeloma cells. Although these new drugs have had a significant impact on the prognosis of MM patients, the vast majority ultimately become refractory or can no longer be treated due to toxicity of prior treatment, and thus succumb to the disease. Cellular therapies represent a novel approach with a unique mechanism of action against myeloma with the potential to defeat drug resistance and achieve long-term remissions. Genetic modification of cells to express a novel receptor with tumor antigen specificity is currently being explored in myeloma. Chimeric antigen receptor gene-modified T-cells (CAR T-cells) have shown to be the most promising approach so far. CAR T-cells have shown to induce durable complete remissions in other advanced hematologic malignancies like acute lymphocytic leukemia (ALL) and diffuse large B-cell lymphoma (DLBCL). With this background, significant efforts are underway to develop CAR-based therapies for MM. Currently, several antigen targets, including CD138, CD19, immunoglobulin kappa (Ig-Kappa) and B-cell maturation antigen (BCMA), are being used in clinical trials to treat myeloma patients. Some of these trials have shown promising results, especially in terms of response rates. However, the absence of a plateau is observed in most studies which correlates with the absence of durable remissions. Therefore, several potential limitations such as lack of effectiveness, off-tumor toxicities, and antigen loss or interference with soluble proteins could hamper the efficacy of CAR T-cells in myeloma. In this review, we will focus on clinical outcomes reported with CAR T-cells in myeloma, as well as on CAR T-cell limitations and how to overcome them with next generation of CAR T-cells.

Keywords: CAR T-cell, myeloma, toxicities, antigen escape, soluble protein, allogeneic CAR T-cell

INTRODUCTION

Multiple myeloma (MM) is an hematological malignancy characterized by the clonal proliferation of malignant plasma cells (1). Myeloma develops from a pre-malignant monoclonal proliferation of plasma cells (monoclonal gammopathy of undetermined significance) which progresses to smoldering myeloma and finally to symptomatic disease (1, 2). With an incidence of 5.6 cases per 100.000 people/year in Western countries it accounts for 1% of all cancers and around 10% of hematological malignancies (3). Diagnosis of MM is based on the presence of clonal plasma cells plus monoclonal protein in serum or urine and clinical manifestations including hyper*c*alcemia, *r*enal impairment, *a*nemia and/or *b*one lesions (acronym: CRAB) (4, 5). Levels of albumin, β2microglobulin and LDH together with the presence or not of high risk cytogenetic abnormalities, including del(17p), and/or t(4;14) and/or t(14;16), allows to identify subgroups of patients with very different outcomes varying from 82% overall survival (OS) at 5 years for the low risk, 62% for the intermediate risk and 40% for the high risk subgroups (6).

Great advances have been achieved in the last decade in the treatment of MM with the discovery of new therapeutic agents such as immunomodulatory drugs (thalidomide, lenalidomide, pomalidomide), proteasome inhibitors (bortezomib, carfilzomib), monoclonal antibodies (daratumumab and elotuzumab), and the use of hematopoietic stem cell transplantation (1, 4, 7). However, MM remains an incurable disease as patients almost invariably relapse upon treatment and the probabilities to obtain disease response decrease after each relapse and the time to progression does shorten in every relapse. It is therefore necessary to develop more efficient MM therapies (8).

Engineered T-cells expressing chimeric antigen receptors (CARs) have demonstrated encouraging results in the treatment of relapsed/refractory hematological malignancies (9–12). CARs are synthetic receptor proteins integrated by an extracellular antigen-binding domain derived from a single-chain variable fragment (scFv) of a monoclonal antibody linked to a T cell receptor (TCR)-derived CD3ζ chain, subsequently redirecting cytolytic T-lymphocytes to cells expressing this specific antigen in a human leukocyte antigen (HLA)-independent manner (3, 13). Second- and third-generation CARs present further costimulatory domains such as CD28, 4-1BB, or OX40 to potentiate T-cell activation. Fourth-generation CAR T-cells may include controllable on-off switch proteins, a suicide gene or molecules to potentiate T-cell function, expansion and reduce exhaustion (3, 14).

Two anti-CD19 CAR T-cell products, Tisagenlecleucel (Kymriah) and Axicabtagene Ciloleucel (Yescarta), have been approved by US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma (DLBCL). Sustained durable complete remissions have been accomplished with CD19 CAR T-cell products in relapsed/refractory ALL patients which have prompted attention to a possible alternative to overcome actual treatment limitations in MM (10, 12).

As far as MM is concerned, numerous CAR T-cell products are under development. B-cell maturation antigen (BCMA) is the predominantly used target against MM based on its high expression in the surface of malignant plasma cells and restricted expression in normal tissues/cells except for a low-level expression in mature B-cells. BCMA is vital for the survival and proliferation of MM cells, it is expressed in most MM patient samples (60–100%) and its efficacy as a MM antigen for targeted immunotherapy has been tested in several clinical trials. Other targets under development include CD38, CD138, CD19, or immunoglobulin kappa light chain (Ig-Kappa) (3, 15, 16). Despite the promising results achieved by CAR T-cell administration in MM in terms of response rates, the absence of a plateau corresponding with the absence of durable remissions is common to all studies. Clinical experience with CAR T-cell therapy has pointed out several limitations of this technology such as lack of effectiveness, toxicities, antigen loss, interference with soluble proteins or manufacturing issues (15, 17).

In this review, we will report clinical outcomes achieved so far with CAR T-cells for the treatment of MM, as well as focus on their limitations and how to overcome these restrictions with next generation CAR T-cells.

CAR T-CELLS IN CLINICAL TRIALS FOR MM

Selection of a suitable antigen is essential for the development of an optimal CAR T-cell product. As the recognition of an antigen by the CAR is HLA-independent, the target must be expressed in the cell surface. Besides, the antigen must be homogeneously expressed in tumor cells and have an essential role in their proliferation and survival to avoid escape from CAR T-cells recognition. It is also essential that the chosen antigen is not expressed in vital healthy tissues to avoid undesired on-target, off-tumor toxicities (15). Although BCMA is the predominantly used target for CAR T-cell products for the treatment of MM, several other antigens have been studied and some are being evaluated in clinical trials.

CD38

CD38 is a transmembrane glycoprotein implicated in calcium regulation, signal transduction and cell adhesion. Among the hematological cell lineages, CD38 is highly expressed on precursor B-cells, plasma cells, NK cells and myeloid precursors. CD38 is also expressed on gut, prostate cells, pancreas, nervous system, muscle cells and osteoclasts (18). Since the FDA approval of several anti-CD38 monoclonal antibodies for the treatment of MM in 2015 (daratumumab, istuximab) (19, 20) the generation of CD38 CAR T-cells has been extensively studied preclinically (21). The wide expression of CD38 among hematopoietic cells might be a critical inconvenient for its clinical application due to possible on-target, off-tumor toxicities. To date CD38 CAR T-cells are under clinical investigation in several trials.

TABLE 1 | Characteristics of T-cell products generated in each clinical trial.

Target	Identifier (ref)	Costimulatory domain	Selection of PBMCs from apheresis	Expansion cytokines	Transfer method	% Transduction efficiency (means)
CD138	NCT01886976 (26)	4-1BB	No selection	IFN-Y + IL-2	Lentiviral vector	32
CD19	NCT02135406 (27)	4-1BB	No selection	NR	Lentiviral vector	10.1
kappaLC	NCT00881920 (28)	CD28	No selection	IL-2/IL-7 + IL-15	Retroviral vector	82 (IL-2)/ 89 (IL-7 + IL-15)
BCMA	NCT02215967 (29)	CD28	No selection	IL-2	Retroviral vector	44.38
BCMA	NCT02546167 (30)	4-1BB	NR	IL-2	Lentiviral vector	17.47
BCMA	NCT02658929 (31)	4-1BB	No selection	IL-2	Lentiviral vector	85 CD4+ (42–98) 13 CD8+ (2–47)
BCMA	NCT03090659 (32)	CD28	T cell selection	IL-2	Lentiviral vector	NR
BCMA	NCT03430011 (33)	4-1BB	NR	NR	Lentiviral vector	NR
BCMA	NA (34)	4-1BB	NR	NR	Retroviral vector	NR
BCMA	NCT03338972 (35)	4-1BB	Positive selection CD4/CD8	NR	Lentiviral vector	NR
BCMA	NCT03288493 (36)	4-1BB	NR	NR	Transposon-based piggy- Bac system	NR
BCMA	NCT03274219 (37, 38)	4-1BB	No selection	IL-2, PI3K inhibitor	Lentiviral vector	NR

PBMCs, peripheral blood mononuclear cells; INF-γ, interferon gamma; IL, interleukin; NR, not reported; NA, non-applicable; PI3K, phosphoinositide 3 kinase.

In the study NCT03464916, a CD38 CAR T-cell product is being used as monotherapy for relapsed/refractory myeloma to evaluate its efficacy and safety although no outcomes have been posted yet. Moreover, CD38 is also being evaluated within clinical trials in combination with other target antigens including a dual specificity CD38 and BCMA CAR T-cell product (NCT03767751), and a combination CAR T therapy with CD19 (NCT03125577). Other approaches targeting several MM antigens including CD38, BCMA, CD138 and CD56 are being explored (NCT03271632, NCT03473496). A fourth generation CAR T-cell product targeting multiple antigens, including CD38, and expressing simultaneously interleukin-7 (IL-7) and chemokine (C-C motif) ligand 19 (CCL19), is also under clinical investigation for the treatment of relapsed/refractory MM patients (NCT03778346). Nevertheless, results from these CD38-targeted CAR T therapies have not been published to date.

CD138

CD138 belongs to the syndecan family type I transmembrane proteoglycans and it is implicated in wound healing, cell adhesion and endocytosis. CD138 is expressed on the surface of mature epithelial cells, nonetheless its expression is restricted within the hematopoietic system to normal and tumor plasma cells (22). It has been correlated with survival and disease progression of MM, and its inhibition promotes apoptosis of myeloma cells (23, 24). CD138 has been proven to be an effective target antigen for the treatment of MM in preclinical studies (25). To date, there is only one published clinical trial for the study of autologous CD138 CAR T-cells in relapsed/refractory MM patients pretreated with chemotherapy and stem cell transplantation. Five patients were treated with a single average dose of 0.756×10^7 cells/kg of CD138 CAR T-cells. The CAR gene was detectable in peripheral blood in all patients and high levels were persistent for at least 4 weeks after infusion. No severe toxicities were observed apart from infusion-related fever (grade 3) and nausea and vomiting (grade 2). Four patients experienced myeloma regression after CD138 CAR T-cells infusion for 3–7 months, while the other patient progressed despite the presence of CAR in bone marrow until day 90 post-infusion. No complete responses (CR) were achieved in this clinical trial (26) (**Tables 1**, **2**).

CD19

CD19 is a B lymphocyte-specific surface protein which constitutes a component of the B-cell co-receptor complex and belongs to the immunoglobulin superfamily. It is expressed throughout B-cell differentiation, from pre- to mature B-cells (39, 40). Although expression of CD19 is rare in plasma cells, there is a small population of $CD19_{positive}$ myeloma cells which has been discovered to be more pre-mature and might constitute the myeloma-initiating or myeloma-stem cells. They have been associated with high-risk disease, poor prognosis, relapses and reduced survival (41, 42). CD19 is the most widely studied target antigen for the development of CAR T therapies with two products (Kymriah and Yescarta) approved for the treatment of ALL and DLBCL, and multiple published and ongoing clinical trials (43). Therefore, targeting CD19 in MM represents an interesting strategy to focus on this $CD19_{positive}$ myeloma cell subset. In the study NCT02135406, 10 refractory MM patients were infused with autologous CD19 CAR T-cells after autologous stem cell transplantation (ASCT). All patients included received previously a first ASCT resulting in poor response with progression-free survival (PFS) of < 1 year. CD19 expression in myeloma cells was assessed by flow cytometry and, as expected, the predominant myeloma population was

TABLE 2 | Published clinical trials of CAR T-cell therapy in multiple myeloma.

Target	Identifier (ref)	Phase	*N*	Pre-conditioning regimen	CAR- T dosage (cells/kg)	Prior treatments (mean)	Median follow-up (months)	Side effects	Clinical effects	Progression-free survival	BCMA$^-$ relapse
CD138	NCT01886976 (26)	1/2	5	PCD/CP/VAD	0.756×10^7 (median)	10	NR	80% fever (G3)	SD (4) PD (1)	NR	NA
CD19	NCT02135406 (27)	1	10	Mel + ASCT	$1–5 \times 10^7$	6	NR	CRS (G1) (1) Intestinal GVHD (1) Mucositis (1)	sCR (1) VGPR (6) PR (2)	200.8 days	NA
kappaLC	NCT00881920 (28)	1	7	Cy (4) or none (3)	$0.92 \times -1.9 \times 10^8$ cells/m^2	4	NR	Lymphopenia (G3) (1) No CRS	SD (4) NR (3)	NA	NA
BCMA	NCT02215967 (29)	1	24	Cy + Flu	$0.3 \times -9 \times 10^6$	9.5	NR	38% CRS (grade 3–4) 44% CRS (grade 1–2) Neurotoxicity (1)	81% ORR sCR (2) VGPR (9) PR (4)	31 weeks	1 BCMA$^-$progression
BCMA	NCT02546167 (30)	1	25	Cy or none	$1–5 \times 10^7$ or $1–5 \times 10^8$	7	NR	88% CRS (G $\geq$ 3: 8 patients) 32% neurotoxicity	ORR (48%) cohort 1 (44%), cohort 2 (20%), cohort 3 (64%)	65, 57, 125 days (cohort 1, 2, or 3)	No BCMA- clones found
BCMA	NCT02658929 (31)	1	33	Cy + Flu	$50 \times 150 \times 450 \times$ and 800×10^6	7-8	11.3	70% CRS (grade 1–2) 6% CRS (grade 3) 42% neurotoxicity	ORR (85%), $\geq$ CR (45%) sCR (36%)	11.8 months	NR
BCMA	NCT03090659 (32)	1	57	Cy	$0.07–2.1 \times 10^6$	3	12	83% CRS (grade 1–2) 7% CRS (grade 3) Neurotoxicity (grade 1) (1)	88% ORR (68% CR 5% VGPR 14% PR)	15 mo (<40% BCMA$^+$) 11 mo (>40% BCMA$^+$)	NR
BCMA	NCT03430011 (33)	1/2	44	Cy + Flu	$50 \times$ or 150×10^6	7	2.6	80% CRS (G$\geq$3 9%) 25% neurotoxicity (G$\geq$3 7%)	82% ORR (27% CR)	NA	No relapses reported
BCMA	NA (34)	1	11	Cy or Flu + Cy	$72 \times 137 \times 475 \times 818 \times 10^6$	6	NR	40% CRS (G1-2) 20% CRS (G3) 10% neurotoxicity (G2)	64% ORR	NA	No relapses reported
BCMA	NCT03338972 (35)	1	7	Cy + Flu	$5 \times$ or 15×10^7	8	3.7	86% CRS (G $\leq$2) No neurotoxicity	100% ORR	NA	1 BCMA$^-$ relapse
BCMA	NCT03288493 (36)	1/2	23	Cy + Flu	$0.75 \times -15 \times 10^6$	6	137 days	9,5% CRS (G1-2) 4.8% neurotoxicity (G2)	63% ORR	NA	NR
BCMA	NCT03274219 (37, 38)	1	22	Cy + Flu	$150 \times 450 \times 800 \times 1200 \times 10^6$	7	23 weeks	59% CRS (5G1, 7G2, 1G3) 23% neurotoxicity (1G1, 2G2, 1G3, 1G4)	83% ORR	NR	NA

PCD, pomalidomide-cyclophosphamide-dexamethasone; CP, chlorambucil-prednisone; VAD, vincristine-doxorubicin-dexamethasone; PD, partial disease; Cy, cyclophosphamide; Flu, fludarabine; G, grade; Mel, melphalan; ASCT, autologous stem cell transplantation; NR, not reported; NA, non-applicable.

$CD19_{negative}$ in all patients. However, seven out of nine evaluable patients presented a small $CD19_{positive}$ subset (from 0.04 to 1.6%) (27). Patients were infused with 1–5 x 10^7 cells/kg CD19 CAR T-cells (CTL019) 2 weeks after high-dose melphalan and a second ASCT. In 2015, the clinical outcome of the first treated patient was reported with a sustained complete remission lasting for at least 12 months in spite of CD19 expression-absence in most of the myeloma cells (44). Six out of 10 patients infused obtained a very good partial response (VGPR) at day 100 post-transplantation. To find out whether CTL019 infusion did increase PFS after ASCT, they compared PFS from each subject after prior ASCT alone vs. ASCT+CTL019 treatment. Two patients significantly increased PFS after CTL019 treatment (479 vs. 181 days; 249 vs. 127 days). These results highlight the recognition of target antigen by the CAR even when it is present in very low intensity or non-detectable by flow cytometry (45) (**Tables 1**, **2**). The same group conducted a phase II clinical trial (NCT02794246) to study the efficacy of CD19 CAR T-cells infusion 60 days post-ASCT in 5 MM patients. No results have been published yet. The combination of autologous/allogenic CD19 CAR T-cells and BCMA CAR T-cells has also been explored.

Immunoglobulin Kappa Light Chain

Despite the success achieved with CD19 CAR T-cells in hematological malignancies, sustained clinical responses need long-term *in vivo* CAR persistence which is linked to B-cell aplasia and therefore impaired humoral immunity. This toxicity occurs due to the expression of CD19 in normal B-lymphocytes as it is a pan-B-cell expression marker. New antigens with more restricted distribution need to be explored to reduce cytotoxicity and allow normal humoral immunity recovery even with *in vivo* CAR persistence (10, 28). Expression of surface immunoglobulin, with either kappa (κ) or lambda (λ) light chain, is limited to mature B-cells and mature B-cell malignancies. Although normal plasma cells do not maintain immunoglobulin expression, a clonogenic MM-initiating population has been described which expresses surface immunoglobulin (46). Directing CAR T-cells to a certain type of immunoglobulin light chain (κ or λ) would eliminate the MM-monoclonal cells expressing the target light chain while avoiding cytotoxicity against normal mature B-cells expressing the remaining one. Therefore, targeting immunoglobulin kappa light chain (IgkLC) might be a feasible strategy to direct CAR T therapy to MM while being more restrained within the whole B-cell subset. In Ramos et al. (28) a clinical trial (NCT00881920) is described to evaluate safety and efficacy of κCAR T-cells in chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma (NHL) and MM patients. Seven relapsed/refractory MM patients were infused with 0.92–1.9 × 10^8 cells/m^2 after cyclophosphamide preconditioning. No serious CAR-related adverse events were reported excluding a patient with grade 3 lymphopenia. According to clinical responses, four out of seven patients reached stable disease (SD) from 6 to 24 months. The other three patients did not respond to the therapy (28) (**Tables 1**, **2**).

B-Cell Maturation Antigen

B-cell maturation antigen is the ultimate target studied for the development of CAR T therapies for MM with up to 53 clinical trials worldwide. BCMA is a transmembrane glycoprotein which constitutes part of the tumor necrosis factor receptor (TNFR) superfamily. It participates in the B-cell differentiation into plasma cells and in its long-term survival and proliferation (16, 47). Besides, expression of BCMA was confirmed by Friedman et al. (48) in malignant MM cells in 100% of the patients analyzed, though levels were variable. Several clinical trials have assessed the benefits of targeting BCMA for the treatment of MM either with anti-BCMA bispecific T-cell engagers (BiTE) or anti-BCMA antibody-drug conjugates (49, 50). Indeed, multiple clinical trials have explored the effect of BCMA CAR T-cells in the treatment of MM (51).

The first clinical trial designed with anti-BCMA CAR T-cells was carried out in the National Cancer Institute (NCT02215967) and results were presented by Brudno et al. (29) in 2018. They enrolled 24 patients, 10 in a dose escalation phase (0.3 × −3 x 10^6 cells/kg) and 16 were infused with the highest dose (9 × 10^6 cells/kg). They reported an overall response rate (ORR) of 81% among the 16 patients treated with the highest dose with 2 patients achieving a stringent complete response (sCR), 8 a VGPR, 3 a partial response (PR) and 3 non-responding to treatment. Peak CAR+ cell levels in peripheral blood, occurring 7 days-post-infusion, were associated with anti-myeloma responses. Cytokine release syndrome (CRS), resulting from T-cell activation after CAR T engagement, and neurotoxicity are the major CAR T-related adverse events described to date (52). CRS grade 3–4 was reported in 5 out of 16 patients infused with the highest dose (38%) and mild CRS was present in 7 patients (44%). Neurotoxicity was not reported or limited to delirium or confusion except for patient 15 who presented encephalopathy. Higher levels of bone marrow plasma cells were also associated with a more severe CRS. BCMA expression was also assessed in myeloma cells pre- and post-treatment and patient 11 was found to have $BCMA_{negative}$ myeloma cells at week 56 post-infusion followed by myeloma progression at week 68 with mixed BCMA expression (29) (**Tables 1**, **2**).

Cohen et al. (30) reported a phase I clinical trial (NCT02546167) to evaluate safety and efficacy of BCMA CAR T-cells in relapsed/refractory MM patients. Three different cohorts were studied, cohort 1: 1–5 × 10^8 BCMA CAR T-cells/kg infused (9 patients), cohort 2: 1–5 × 10^7 cells/kg + Cyclophosphamide as preconditioning (5 patients), and cohort 3: 1–5 × 10^8 cells/kg + Cyclophosphamide as preconditioning (11 patients). In this trial, all 25 patients were infused in a 3-dose-split protocol over 3 days (30). CRS was reported in 22 out of 25 patients (88%) (grade 3–4) and neurotoxicity was observed in 8 out of 25 patients (32%). Three patients presented severe neurotoxicity (grade 3–4) which correlated with high tumor burden, a dose of 5 × 10^8 cells/kg and grade 3-4 CRS. The objective responses within the cohorts were: 44% in cohort 1, 20% in cohort 2 and 64% in cohort 3 (1 CR, 5 VGPR and

1sCR). The ORR was 48% (12 out of 25), with 1-5 x 10^8 cells/kg being the most effective dose (11 responding-patients) (30) (**Tables 1**, **2**).

In the Bluebird study NCT02658929, 33 patients with relapsed/refractory MM were treated with anti-BCMA CAR T-cells (bb2121) in a dose/escalation study (50–800 × 10^6 cells/kg) (31). In this study, 25 patients presented CRS, among them 23 (70%) had grade 1–2 and 2 (6%) had grade 3–4. Neurologic toxicities were also remarkable occurring in 14 patients (42%). The ORR was 85% with 15 complete responses (45%), and all responding-patients had negative minimal residual disease (MRD). However, six out of the 15 patients with CR finally relapsed (31) (**Tables 1**, **2**).

In Zhao et al. (32) the authors developed a BCMA-directed CAR T-cell containing two heavy chain-only antibodies (VHH) which targets two different BCMA epitopes (LCAR-B38M). The safety and efficacy of this CAR T-cell product were studied in a clinical trial with 57 patients enrolled (NCT03090659) (32). They reported 51 (90%) patients who developed CRS on variable grades (**Table 2**). One patient presented neurotoxicity grade 1 with seizure-like activity, agitation and aphasia. The authors described an ORR of 88% with 68% CR, 5% VGPR and 14% PR. They analyzed BCMA expression on all patients but no correlation among BCMA expression, progression-free survival, overall survival or clinical response was found. Correlation between CAR T-cell dose and clinical response was not found either (32) (**Tables 1**, **2**).

The Memorial Sloan Kettering Cancer Center (MSKCC) has developed different CAR T-cell products containing human-derived or fully human scFv antibodies against BCMA named JCARH125, MCARH171, and FCARH143. All CAR T-cell products have been included in phase 1 clinical trials. JCARH125 is an anti-BCMA CAR T-cell product studied in a multicenter clinical trial in the United States (EVOLVE, NCT03430011). In this study, 44 patients were infused with two different dose levels (50 × 10^6 or 150 × 10^6 cells/kg). With a 2.6 months median follow-up, 82% overall responses were reported with 27% CR. CRS was present in 80% of patients and neurotoxicity in 25% (33, 51) (**Tables 1**, **2**). MCARH171 expresses a different human-derived scFv antibody than JCARH125 and T-cells were transduced with γ-retrovirus instead of using a lentiviral vector. Safety and efficacy were evaluated in a cohort of 11 patients with an ORR of 64%. CRS grade 1–2 occurred in 40% of patients and 20% had CRS grade 3. Neurotoxicity was only experienced by one patient with grade 1 encephalopathy (34) (**Tables 1**, **2**). It has been described that defined ratios of CD4+:CD8+ in the T-cell product might benefit expansion and function (53). FCARH143 employs the same construction as JCARH125 but $CD4^+$ and $CD8^+$ T-cells were cultured separately *ex vivo* and infused in a defined 1:1 ratio. Seven patients have been reported to date with an ORR of 100% at 28 days post-infusion. CAR^+ T-cells were detectable 90 days after infusion. One patient relapsed at day 60 and tumor biopsy demonstrated the presence of a $BCMA_{negative}$ plasma cell population (35) (**Tables 1**, **2**).

A novel CAR T-cell product designed with a non scFv antibody but a different BCMA-specific antibody-mimetic binding domain has been evaluated in a clinical trial. Besides, a different non-viral transfection method was used, named transposon piggy-bac system, which allows more cargo capacity and preferential transfection of stem cell memory T-cells with a lower cost (**Table 1**). To date, 23 patients have been treated in 5 different dose groups (**Table 2**). Two patients experienced CRS (grade 1–2) (9.5%) and neurotoxicity was reported in one patient (grade 3) (4.8%). ORR goes from 50% to 100% depending on dose group with a median of 63% in all patients (36, 51) (**Table 2**).

In Shah et al. (37) the authors designed a clinical trial with a next-generation CAR T-cell (bb21217) using the same construct as bb2121 (31) but with a novel approach by employing phosphoinositide 3 kinase (PI3K) inhibitor bb007 during *ex vivo* expansion to enrich the product in memory-like T-cells (**Table 1**). In the update presented at the American Society of Hematology Annual Meeting 2019, they reported 22 infused patients with an ORR of 83% (15/18 evaluable patients). CRS occurred in 59% of patients and neurotoxicity in 23% (38) (**Table 2**).

CAR T-CELL THERAPY LIMITATIONS IN MM

With an increasing number of CAR T-cell-treated patients, observations of therapy-related toxicity and disease relapse are showing the current limitations of this therapeutic modality (17, 54, 55). Key challenges related to CAR T-cell therapy include toxicities, antigen escape, suboptimal activation and persistence of CAR T-cells.

On/Off-Tumor Toxicity: Treatment-Related Toxicities

Immunotherapy with adoptive T-cells targeting myeloma-associated antigens are at various stages of development and have brought a new hope for cure (31, 38, 56, 57). Nevertheless, severe toxicities accompany this promising technology as it has been reported with the increasing clinical experience with CAR T-cell therapy (58–63). CAR T-cell related toxicities can be divided into two categories: (1) general toxicities due to T-cell recognition and activation against tumor cells and followed by uncontrolled release of high levels of cytokines (on-target, on-tumor toxicities); and (2) toxicities appearing from specific binding between CAR T-cell and its target antigen expressed in normal cells (on-target, off-tumor toxicities) (17, 64).

On-Target, On-Tumor Toxicity

Severe and sometimes lethal increases in systemic cytokine levels have been observed in patients treated with CAR T-cells in many clinical trials. Robust interactions between CAR modified T-cells either with tumor or host immune cells may result in CAR T-cell activation and expansion. In some cases, this immune cell activation and uncontrolled cytokine release can be toxic for patients (64). Typically observed CAR T-cell related toxicities are cytokine release syndrome (CRS) and neurotoxicity. CRS is the most-common toxicity of cellular immunotherapy and it appears as a consequence of accelerated expansion and activation of CAR T-cells. This pronounced activation provokes an extreme release of serum levels of interferon

gamma, interleukin 6, among other inflammatory cytokines. CRS commonly appears within the first 2 weeks post-infusion of CAR T-cells. Clinical symptoms of this on-target, on-tumor toxicity goes from mild fevers, malaise and flu-like symptoms to severe sepsis-like indications such as high-grade fevers, hypotension, hypoxemia, organ dysfunction, coagulopathy, and pancytopenia which may require intensive care admission (64–66). Treatment with corticosteroids could reduce CRS but to the detriment of effectiveness of CAR T-cells due to suppression of T-cell function and/or induction of T-cell apoptosis (67, 68). Interleukin 6 blockade by anti-interleukin 6 receptor antibodies, such as tocilizumab, is the most commonly used treatment of severe CRS. Besides, tocilizumab does not seem to have a major impact on CAR T-cell efficacy (64–66). Another common adverse event of CAR T-cells is neurotoxicity. This event is observed in up to 42% of patients receiving anti-BCMA CAR T-cells and usually overlaps or appears shortly after CRS (**Table 2**). Neurotoxicity is commonly restricted to low grade symptoms such as few days of mild confusion, somnolence, and/or word-finding difficulties. Eventually, neurotoxicity can evolve to more severe immune effector cell-associated neurotoxicity syndrome (ICANS) (66) which includes focal deficits, seizures, and fatal cerebral edema (69). Pathophysiology of neurotoxicity is not well-described, but high levels of inflammatory cytokines within central nervous system associated with an increase in endothelial cell activation and vascular permeability have been observed (65). Some factors associated with a higher risk of neurotoxicity include high tumor burden, and more rapid and severe CRS; however, more studies are needed to better understand this toxicity (69).

On-Target, Off-Tumor Toxicity

CAR T-cell infusion can also be associated to on-target, off-tumor toxicities due to their uncontrolled growth and excess cytokine release after recognition of target antigen expressed on non-malignant cells (58, 70, 71). The challenge to find tumor-restricted antigens that are not expressed on normal cells, is the major concern in CAR T-cell development (51, 72). Current target antigens of CAR T-cells are usually present on the surface of non-malignant cells, even though at low level. However, this low level could eventually lead to severe off-tumor toxicities (73). B-cell aplasia after CD19 CAR T-cells infusion, is an excellent example of on-target, off-tumor toxicity. The functionality of CD19 CAR T-cells is always followed by B-cell aplasia as a result of the depletion of $CD19_{positive}$ B-cell progenitors. As a consequence, reduced immunoglobulin level is observed in serum of these patients (74). However, while this side effect can be addressed by immunoglobulin replacement (27, 75, 76), damage to more essential organs or tissues can be fatal (61, 73). Among MM target antigens, CD38 has been reported to have medium-expression level on normal hematopoietic cells and non-hematopoietic tissues including prostate epithelial cells. The potency of CAR T-cells does not guarantee safety when targeting this widely expressed protein despite the currently use of anti-CD38 monoclonal antibodies clinically approved (77). Moreover, expression of CD38 on activated T-cells may suppose a detrimental effect because of fratricide cytotoxicity among CD38 CAR T-cells (78). Therefore, the affinity of the scFv antibody used to develop CD38 CAR T-cell product, needs to be accurately optimized to avoid on-target, off-tumor side effects (21, 71). The same happens with CD138 target antigen. Its widely expression leads to concerns with regard to off-tumor toxicities using CD138 CAR T-cells in myeloma patients. Although no severe epithelial toxicities have been noted at present (25), more studies evaluating CD138 CAR T-cells should include strategies to avoid off-tumor toxicities while maintaining anti-tumor effect. BCMA is the most commonly targeted antigen in CAR T-cell therapies for MM. BCMA plays a fundamental role in long-term plasma cell survival and B-cell differentiation into plasma cells (47) with an increasing expression during B-cell differentiation. It is found only in late memory B cells and normal and malignant plasma cells at varying intensities (79–81). Therefore, B-cell depletion is not anticipated when targeting BCMA, as the majority of normal B-cells are $BCMA_{negative}$. Although the toxicity profile of BCMA CAR T-cells seems to be manageable (80–82), alternative approaches have been developed to overcome this CAR T-cell therapy limitation.

Antigen Escape: Down-Regulation or Loss of Target Antigen

Antigen escape post-infusion of modified CAR T-cells is an emerging issue (17, 83, 84). Relapses have been observed in long term follow-up studies, but the mechanisms for these relapses need to be better described. The most common reason for relapse after CAR T-cell therapy is the emergence of tumor cells with loss or downregulation of target antigen expression. The use of CD19 CAR T-cells in ALL patients has highlighted the loss of CD19 antigen expression due to therapeutic pressure. Consequently, $CD19_{negative}$ tumor cells, have been reported in relapses. This can be caused by lymphoid to myeloid trans-differentiation, alternative splicing leading to target epitope loss, or selection of pre-existing antigen-negative leukemia cell clones (85–87). In the myeloma setting, BCMA loss or downregulation on residual MM cells after BCMA CAR T therapy has been reported in several clinical trials to date (29, 30, 88). Antigen escape in MM is more likely to occur due to the coexistence of numerous tumor subclones in treated patients resulting in a potential advantage to emerging or preexisting $BCMA_{low}$ or $BCMA_{negative}$ subclones during treatment (51). In the NCI trial, one patient presented persisting $BCMA_{negative}$ myeloma cells in the bone marrow 56 weeks after BCMA CAR T-cell infusion and at the time of myeloma progression (29). In the UPENN trial, 12 out of 18 (67%) evaluable patients had significantly diminished levels of BCMA expression intensity commonly 1 month after CAR T-cell administration (30). This reduction in BCMA intensity was more frequent on residual myeloma cells from patients responding to therapy than non-responder patients. Regarding to FCARH143 trial, the authors reported a patient who relapsed presenting a $BCMA_{negative}$ myeloma subclone and an overall reduction of 70% BCMA expression level in the $BCMA_{positive}$ myeloma cell population (35). The observed loss and downregulation of BCMA expression on the surface of myeloma cells after CAR T-cell infusion in several clinical trials, highlights an imperative

need to investigate the mechanism of resistance to BCMA CAR T-cell therapy.

Soluble Protein: Hampering CAR T-Cell Function

A potential limitation for the clinical application of CAR engineered T-cells would be the presence of soluble protein antigens in the serum of patients. Target antigens can be cleaved from the cell membrane and released into blood circulation, therefore CAR T-cell therapy could be limited due to binding to soluble target antigens. Thus, the functionality of CAR T-cells could be abrogated by soluble antigens (89). In the myeloma setting, CD138 extracellular domain has been reported to be cleaved from cell surface, which could abrogate CAR T-cells function by blocking their antigen-binding domain and, hence, resulting in immune escape (90). Cleavage of the target antigen is not exclusive to CD138 protein, but it has been well-described that BCMA can be released from myeloma cells into the serum of patients. BCMA expression on the surface of myeloma cells can be modified by a protease called γ-secretase (GS), which mediates BCMA shedding within the transmembrane domain, leading to the release of a soluble fragment of BCMA (sBCMA) (91). Consequently, recognition of tumor cells by CAR T-cells could be hampered by soluble BCMA as a result of reducing BCMA density on the tumor cell surface and/or blocking the antigen-binding domain of the CAR. Thus, the presence of sBCMA in the serum of MM patients is being actively discussed as an obstacle for BCMA CAR T-cell therapy. In this sense, controversial data have been described. Several studies have reported that BCMA CAR T-cells were not abrogated by soluble BCMA protein (48, 80). However, alternative BCMA CAR T-cells have recently shown that sBCMA in concentrations of 10 ng/mL decreases the frequency of cytokine-producing BCMA CAR T-cells, at the same time that did not compromise CD19 CAR T-cells function. Moreover, high concentrations (333–1,000 ng/ml) of soluble BCMA protein affected cytotoxic capacity of CAR T-cells against 1 out of 3 $BCMA_{positive}$ cell lines (92). These contradicting observations might be due to the use of different BCMA CAR T-cells directed to distinct epitopes, therefore it could happen that the BCMA-CAR target-epitope is not accessible in the soluble BCMA conformation.

Quality of Harvested T-Cells: Insufficient Persistence of CAR T-Cells

Response rates of 64–85% have been achieved in myeloma patients treated with BCMA CAR T-cells (29, 30). However, only 8-39% of patients had a sustained VGPR or CR/sCR and this, together with the fact that most of the current clinical trials to date have reported $BCMA_{positive}$ relapses, highlights a loss of efficacy of BCMA CAR T-cells against malignant plasma cells. This loss of efficacy might be a consequence of limited persistence of the CAR T-cells *in vivo*. Accordingly, long-term responses (>2 years) were unusually seen after BCMA CAR T-cell infusion (29–31). Nowadays, most of the CAR T-cell products are generated from autologous T-cells (17). Although this personalized cellular therapy has reported notable success in clinical trials (30, 31), the generation of CAR T-cells from patients could limit their application for multiple reasons. The first limiting factor is the harvesting of an adequate T-cell number from cancer patients who commonly present lymphopenia due to disease or previous treatments (17). Besides, the generation of autologous CAR T-cells might be a long procedure and progression of the disease could happen during manufacturing in advance-stage cancer patients (63, 93). Finally, intrinsic characteristics of apheresis products may be another limitation factor for the generation of autologous CAR T-cells (94). In the myeloma setting, harvesting the sufficient number of T-cells from patients seems to be feasible to produce CAR T-cells [e.g., the bb2121 clinical trial has shown that in 100% of patients who underwent leukapheresis, BCMA CAR T-cells were successfully generated (31)]. However, the quality of harvested T-cells in myeloma patients is likely a significant limiting factor due to deterioration of the immune system in these patients. Thus, the most solid indicator associated with response in the myeloma arena is the level of *in vivo* expansion and persistence of infused CAR T-cells (95). On the other hand, patient age (96, 97), number of prior lines of treatment (98), and the disease itself (99, 100) may limit the number and quality of patient-derived T-cells, potentially influencing the potency and variability of the CAR T products. Furthermore, the logistics of clinical manufacturing using patient-derived T-cells limits the accessibility of these therapies.

NEXT GENERATION CAR T-CELLS IN MM

To overcome CAR T therapy limitations in MM, new strategies have been developed in order to create next generation CAR T-cells to treat myeloma patients (**Figure 1**).

Overcoming On/Off-Tumor Toxicities of CAR T-Cells

Overcoming On-Target, On-Tumor Toxicity

To achieve clinical efficacy while avoiding on-target, on-tumor toxicities, CAR T-cells activation and cytokine release must remain under a controlled level. The affinity of the antigen-binding domain for the tumor epitope, tumor burden, antigen density on the surface of cancer cells, and costimulatory domains present in the CAR, along with other factors, are implicated in the kinetics of CAR T-cell activation (101, 102). Dose-escalation schedules in phase I clinical trials are needed to determine the therapeutic window of CAR T-cell activation for each CAR due to differences in CAR design. In order to optimize this therapeutic window, diverse regions of the CAR gene can be accurately modified (17). In this sense, the costimulatory domain is considered one of the key points to optimize CAR design to reduce on-target, on-tumor toxicities. The most common costimulatory domains used in CAR T-cells are CD28 and 4-1BB. The use of CD28 costimulatory region has been correlated with a high and pronounced CAR T-cell activation and, hence, immune exhaustion phenotype. On the other hand, CAR T-cells with 4-1BB costimulatory region present lower peak of expansion,

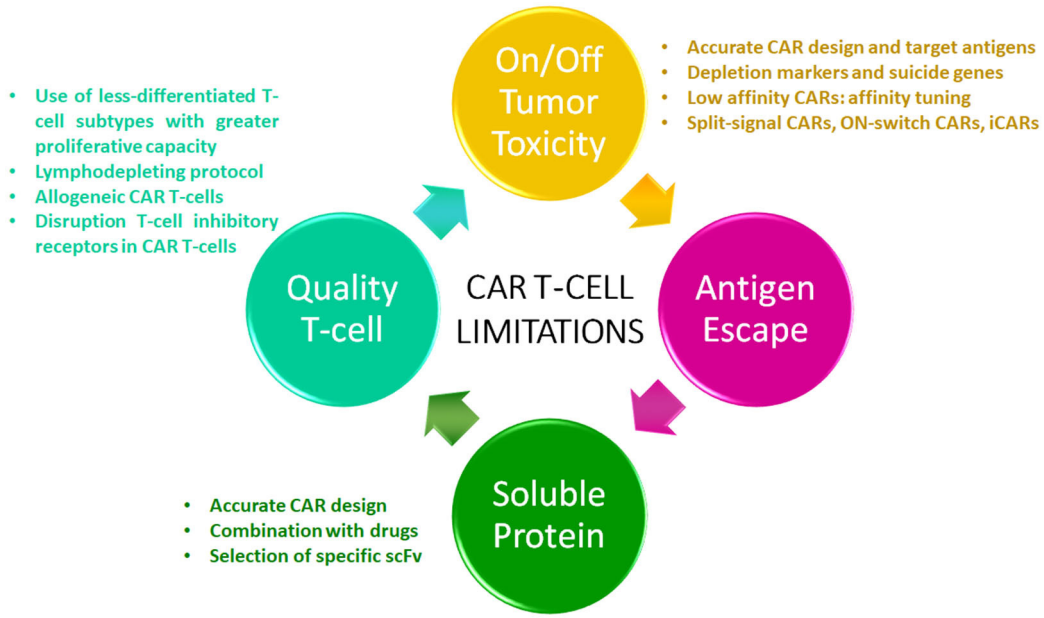

FIGURE 1 | CAR T-cell limitations. The diagram shows the current limitations of this therapeutic modality in multiple myeloma. Strategies to overcome these limitations are listed.

resulting in prolonged persistence and a lower risk of cytokine-mediated toxicities (103). Therefore, the costimulatory domain chosen in the CAR design may explain, at least in part, the toxicity-pattern differences observed in patients treated with CD28 containing CAR T-cells in which earlier onset CRS is more commonly observed as compared to patients treated with 4-1BB containing CAR T-cells (66). Some studies have also reported significant neurotoxicity and death from cerebral edema when CD28 containing CAR T-cell products were used (62). However, other clinical trials described no difference in the rate or grade of CRS and/or ICANS between CAR T-cells containing CD28 vs. 4-1BB costimulatory domains (63, 104, 105). Further studies are needed to better elucidate the link between costimulatory domain and toxicity events in patients receiving CAR T-cells. Others aspects of the CAR design such as the extracellular hinge region and/or transmembrane domain can also be optimized to reduce on-target, on-tumor toxicities. In fact, Ying et al. (86) generated a new anti-CD19 CAR variant [CD19-BBz (86)], which released lower levels of cytokines, expressed higher levels of anti-apoptotic molecules and proliferated more slowly than the prototype CD19-BBz CAR T-cells, while retained robust cytolytic activity. A phase I clinical trial was developed to evaluate this new variant and no significant CRS or ICANS events were reported while achieving a CR rate of 54.5% (106). In addition, novel strategies to control CAR T-cells toxicity include the engineering of depletion markers [e.g., truncated epidermal growth factor receptor [EGFRt]] and suicide genes (e.g., iCasp9) into the CAR design, providing a way to delete CAR T-cells if on-target, on-tumor (and/or on-target, off-tumor) toxicities appear. Accordingly, co-expression of the CAR gene together with the epitope recognized by clinically approved-monoclonal antibodies has been explored. For example, CD20 and EGFRt, which are targetable with rituximab and cetuximab, respectively (107–109). Furthermore, both depletion markers can be used to monitor T-cell transduction. Likewise, apoptosis of CAR T-cells can be induced by caspase pathway activation in iCasp9 next-generation CAR T-cells after the addition of the dimerization drug (109, 110).

Overcoming On-Target, Off-Tumor Toxicity

Multiple strategies to reduce off-tumor toxicities are now under development and are likely to offer novel clinically effective CAR T-cell products (111). Some of these approaches include the restriction of the recognition of normal cells by the CAR optimizing the specific interaction with tumor cells, either by (1) including the recognition of more restricted tumor-antigens or recognition of multiple antigens; and/or (2) limiting the spatial and temporal activity of CAR T-cells. In this sense, a novel target antigen has been reported for the immunotherapy of MM, GPRC5D. GPRC5D is a human orphan family C G protein-coupled receptor recently described to be expressed on 98% of $CD138_{positive}$ cells (112, 113). The expression pattern of GPRC5D has proven to be very restricted in non-plasma tissues with the only exception of hair follicle cells. Consequently, GPRC5D CAR T-cells were generated by Smith et al. (113) showing anti-tumor efficacy against myeloma cells both *in vitro* and in a mouse model. Of note, GPRC5D CAR T-cells were also effective in eradication of myeloma after BCMA CAR T-cell treatment in a murine model which might be an option to overcome BCMA antigen escape. These data suggest that GPRC5D CAR T-cells could be an attractive alternative treatment in MM.

A distinct approach to limit off-tumor toxicities when a tumor-restricted antigen is not identified, could be the "affinity-tuning," which is based on differences in density of antigen level among tumor and normal cells. Low-affinity CAR T-cells (low affinity scFv antibodies) could be generated to target antigens expressed at higher density on tumor cells than on normal cells. This is based upon published observations that T-cell activation initiated through the endogenous αβ TCR may result from reaching an activation threshold level, which can be triggered by binding a few high-affinity TCRs or greater number of low-affinity TCRs (114, 115). Several studies have demonstrated that a CAR with reduced affinity rendered T-cells preferentially activated by high, but not low, density of target antigen (116–119). However, immune escape has also been observed if downregulation of target antigen occurs (116, 120). In the myeloma setting, off-tumor toxicity profile of low affinity CD38 CAR T-cells has been evaluated. Drent et al. (71) showed that CD38 CAR T-cells with up to 1,000-fold decreased affinity were effective against $CD38_{positive}$ myeloma cells, whereas $CD38_{positive}$ healthy hematopoietic cells resulted unaffected.

Novel approaches to prevent off-tumor toxicities are the generation of CAR T-cells which require the recognition of two tumor antigens for their activation. In this regard, "split-CARs" co-expressing two different modules, one containing a scFv antibody along with CD3ζ (signal 1) and the second containing a different scFv antibody along with costimulatory domain (CD28, 4-1BB) have been explored (121, 122). This strategy may result in suboptimal CAR T-cell activation and limited off-tumor toxicity where only one antigen recognition occurs, which may be the case in normal cells. On the other hand, when both antigens are present, as in tumor cells, robust CAR T-cell activation is reached after double recognition (122). An alternative strategy consists of "ON-switch CARs" in which the antigen binding domain is dissociated from the signaling domain (CD3ζ), and CAR T-cell activation is controlled by a small molecule that induces dimerization (123). Subsequently, the intensity of CAR T-cells responses relies on the dose of dimerization molecule. Recently, "AND-gate CARs" has arisen as a promising technology. In this approach, the recognition of the first antigen by the synthetic Notch receptor induces the excision of the transcription factor which allows the expression of the CAR gene targeted against the second antigen. The expression of the CAR on the cell surface allows T-cell activation after antigen recognition (124). Therefore, CAR expression and CAR T-cell activation and hence, tumor elimination, can only happen when both target antigens are present. Nevertheless, the loss of the first antigen targeted by the synthetic Notch receptor which may result in immune escape, as well as slow activation kinetics, are the major limitations of this approach.

Another next-generation technology is the "inhibitory CAR" (iCAR) which incorporates the signaling region of an immunoinhibitory receptor (i.e., PD-1 or CTLA-4) to limit on-target, off-tumor toxicity. This novel strategy consists on the expression of a conventional CAR together with an iCAR in the same T-cell (125). The recognition of the target antigen by the iCAR restricts T-cell activation while the absence of this antigen allows CAR T-cell activation. Again, the challenge of this technology is finding antigens with an optimal expression pattern. First, iCAR should recognize antigens that are strictly expressed on normal cells whereas conventional CAR should recognize antigens that are specifically express on tumor cells.

Overcoming Antigen Escape

CAR engineered T-cells targeting multiple antigens can be generated to address the antigen loss and therefore, reduce relapse rates (55). Next generation CAR T-cells can be developed by (1) combining different CAR T-cell products directed against single target antigens pre-infusion (co-administration of two or more different CAR T-cell products) (**Figure 2A**) or (2) transducing the same T-cell with two different complete CAR constructs ("bicistronic CAR T-cells") (**Figure 2B**). Alternatively, bi-specific CAR T-cells, called also "tandem CAR T-cells," can be generated by designing a single CAR construct with two (or more) different binding domains against two different antigens (126, 127) (**Figure 2C**). One important factor in the CAR design is the length of the transgene. In this sense, "tandem CAR T-cells," which have smaller transgene length, show an advantage compared to "bicistronic CAR T-cells." On the other hand, "tandem CAR T-cells" need design optimization including linker sequence, spacer size and VH-VL orientation between both scFv antibodies to accomplish desired antigens recognition and CAR T-cell activation (128). In 2019, Yan et al. (129) evaluated the efficacy and safety of the co-infusion of CD19 and BCMA CAR T-cells in relapse/refractory MM patients (NCT 03455972). CAR T-cell products were co-administrated on day 14–20 after autologous transplantation. The authors reported an ORR of 92.6% with 11/28 (40.7%) CRs or sCR, 8/28 (29.6%) VGPR and 5/28 (18.5%) PR. The median OS was 16 months. CRS was grade 1–2 in 19 (67.9%) patients (129). Although the exact mechanism of co-infusion remains unclear, expanding the coverage of MM cell targets might lead to better depletion of MM cell clonogenicity improving the duration of responses avoiding antigen escape. Co-infusion of two CAR T-cell products could likely reduce antigen escape, at the expense of a possible increase in toxicities as a result of simultaneous targeting of two or more antigens. Further studies with longer follow up and larger cohort of patients are needed in order to make conclusions regarding the use of combinatory CAR T-cell products. Another possible combination to overcome antigen escape limitation in MM is targeting both BCMA and TACI on myeloma cells. However, it has not been described whether BCMA-directed therapy affects TACI expression on the surface of residual myeloma cells. APRIL, a proliferation-inducing ligand, is the natural ligand of BCMA and TACI, and it is secreted in a trimeric form (130–132). In the preclinical setting, APRIL CAR T-cells have been successfully developed targeting both BCMA and TACI (133). Consequently, a clinical trial phase I/II is ongoing to evaluate the safety and efficacy of APRIL CAR T-cells in myeloma patients (NCT03287804) (134). Relapsed/refractory MM patients were infused with 15×10^6 (1/11 pts), 75×10^6 (3/11 pts), 225×10^6 (3/11 pts), 600×10^6 (3/11 pts), and 900×10^6 (1/11 pts) APRIL CAR T-cells. The ORR was 43% (28 PRs and 14% VGPRs) in patients receiving $\geq 225 \times 10^6$ CAR T-cells (134). Long-term follow up is needed to assess the efficacy of this

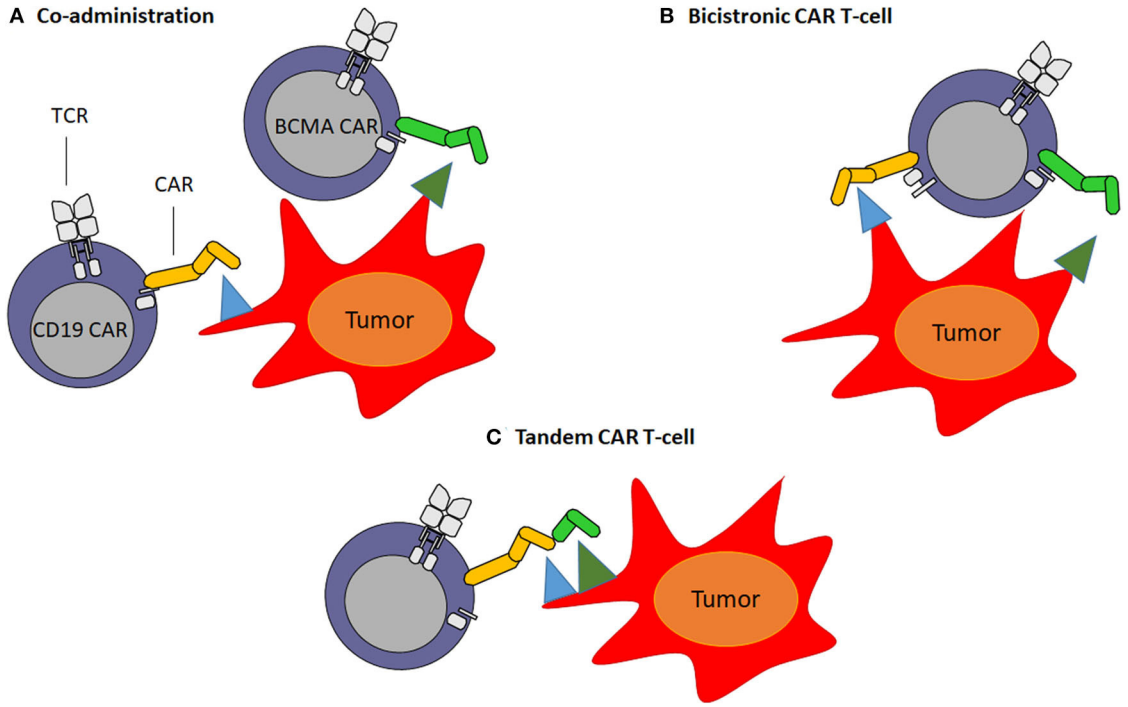

FIGURE 2 | Multi-targeted CAR T-cell approaches. **(A)** Co-administration: involves production of two separate CAR T-cell products infused together or sequentially. **(B)** Bicistronic CAR T-cells: consist of the expression of two different CARs on the same T-cell. **(C)** Tandem CAR T-cells: consist of encoding two different scFv antibodies on same chimeric antigen receptor protein using a single vector.

dual-targeting CAR. Recently, Maus et al. (135) designed a novel human APRIL CAR T-cell product preserving its trimeric conformation (TriPRIL) showing enhance killing of both BCMA myeloma cell lines and primary myeloma cells. More recently, bicistronic BCMA/GPRC5D CAR T-cells are being explored and, preclinical data demonstrated that BCMA/GPRC5D CAR T-cells can limit BCMA antigen escape-mediated relapse in a murine model (136).

Overcoming Abrogation of CAR T-Cells by Soluble Protein

Blocking the cleavage of target antigens from myeloma cells could be an interesting strategy to avoid the release of these target antigens as soluble proteins. In this sense, Pont et al. (92) have recently published that exposure to γ-secretase inhibitors (GSIs) efficiently blocks the release of BCMA from myeloma cells. Consequently, BCMA surface expression on MM cells is increased leading to a higher anti-tumor capacity of BCMA CAR T-cells, as well as improved cytokine production and proliferation in preclinical models (92). Relapsed myeloma patients with downregulated target antigen expression after BCMA-directed treatments could benefit from this novel GSI strategy. However, an optimal dose of GSI should be defined in order to avoid adverse effects on CAR T-cell function since the inhibition of GS did not impair viability or cytolytic activity but reduced IL2 production and proliferation of BCMA CAR T-cells (92). An alternative strategy might be the use of scFv antibodies to generate CAR T-cells directed against epitopes which are not accessible in the soluble protein conformation or belong to the extracellular region of the target protein that remains after cleavage. Thus, it has been reported that sBCMA folds and participates in the formation of high-molecular-weight complexes under physiological conditions (130, 137). Furthermore, data from three trials uncover the absence of correlation between sBCMA concentrations and extent of response (29, 56, 57). Therefore, the selection of the antigen-binding domain is a key factor in CAR design to overcome abrogation of CAR T-cells by soluble protein.

Increasing CAR T-Cell Persistence

A promising approach to increase CAR T-cell persistence is the use of T-cell products containing a higher frequency of less-differentiated T-cell subtypes such as naïve T-cells (TN), stem cell memory T-cells (TSCM) and central memory T-cells (TCM), which have a superior proliferation capacity showing a delayed exhaustion or senescence immunophenotype (138–140). Compared with conventional CAR T-cell products, less-differentiated CAR T-cells have shown a greater proliferation and killing capacity in preclinical studies. To generate less-differentiated CAR T-cells, several strategies have been developed such as previous pre-selection of TN/TSCM subtypes or manufacturing in the presence of kinase inhibitors (138, 141, 142). In this sense, Shah et al. (37) designed a clinical trial

with a next-generation CAR T-cell (bb21217) using the same construct as bb2121 but with a novel approach by employing phosphoinositide 3 kinase (PI3K) inhibitor bb007 during *ex vivo* expansion to enrich the CAR T-cell product in memory-like T-cells. To date, similar ORR and toxicity profile were observed in eight myeloma patients treated with the lowest dose of bb21217 (150×10^6 CAR T-cells). However, longer follow-up is required. Clinical trials are currently ongoing using CAR T-cell products selectively generated from CD8+ TCM cells to evaluate safety and efficacy (NCT01087294) (142). Another strategy could be to define the CD4:CD8 ratio into the CAR T-cell product. In this sense, increased CAR T-cell expansion has been observed in patients after the infusion of a defined 1:1 (CD4:CD8) CAR T-cell ratio (35, 105, 143).

In most clinical trials conventional CAR T-cells derive from autologous T-cells. Generation of autologous CAR T-cells is a lengthy and elaborated process, time-consuming and logistically challenging, which comprises multiple steps including T-cell isolation and selection, transduction, expansion and infusion into patients. Along with that, the majority of patients are in relapsed/refractory stage and have already received numerous lines of toxic treatments, which further weaken T-cells quality and hence, reduce their immune response against tumor cells. Therefore, the quality/fitness of T-cells from which CAR T-cells are generated might also have an important role in CAR T-cell expansion and persistence, and anti-tumor capacity. An allogeneic CAR T-cell approach might have the potential to circumvent all these challenges by using healthy donor-derived T-cells to produce CAR T-cells which can be available as an off-the-shelf product (144–146). However, the use of allogeneic CAR T-cells could induce graft-versus-host disease (GVHD). In this context, host allo-antigens can be recognized via TCR by allogeneic CAR T-cells. On the other hand, the infused allogeneic CAR T-cells could be attacked by the host immune system owing to the HLA disparity resulting in CAR T-cell elimination. In consequence, further genetic modifications are needed in order to create next generation off-the-shelf allogeneic CAR T-cells. These genetic modifications consist of disrupting the endogenous TCR using gene-editing technology such as CRISPR/Cas9 and TALEN to limit the risk of GVHD, and the addition of a safety element to limit toxicities (14, 147, 148). Moreover, suppression of HLA class I expression by disrupting the HLA-A or b2-microglobulin genes is a novel strategy that is being evaluated to avoid allogeneic CAR T-cell elimination by the host immune system (149). Alternative approaches to prevent early allogeneic CAR T-cell rejection have been explored for instance, the use of more intensive lymphodepletion protocols. Nevertheless, the later immune system reconstitution of the lymphodepleted patient may be an obstacle for the allogenic CAR T-cell persistence (51). Advances in gene-editing techniques are leading to a new scenario in CAR T-cell therapy resulting in the development of universal CAR T-cells from allogeneic healthy donors. In this regard, preclinical results of allogenic second-generation BCMA CAR T-cells, called ALLO-715, were reported (150). In this study the authors, after CAR-transduction, transfected CAR T-cells with messenger RNA of TALEN. In particular, both CD52 and T-cell receptor α-chain genes were specifically disrupted resulting in CAR T-cell resistance to anti-CD52 lymphodepletion treatment, as well as preventing GvHD, respectively, without compromising CAR-mediated cytotoxicity. Moreover, this ALLO-715 BCMA-CAR incorporates a rituximab-sensitive safety switch. Currently, a phase I trial is ongoing to evaluate the safety and efficacy of the ALLO-715 product.

CRISPR/Cas9 gene-editing tool constitutes a promising technology to create next-generation CAR T-cells. This technology offers a wide range of CAR T-cell products, such as more potent CAR T-cells by disrupting inhibitory genes, allogeneic CAR T-cells by endogenous TCR and HLA elimination and novel CAR T-cells by knock-out of the targeted antigens to avoid fratricide effect. However, off-target toxicity is the major barrier of CRISPR/Cas9 gene-editing technology. These off-target toxicities can provoke non-desired deleterious consequences such as activating oncogenes or disrupting tumor-suppressor genes (151). To overcome this limitation, several approaches have been explored such as optimized sgRNA design and Cas9 activity, prior off-target detection assays, and careful selection of the target site to reduce off-target effects (152, 153). Therefore, a deeply understanding of the potential toxic effects of gene-editing in CAR T-cells is required.

Finally, to improve T-cell persistence and, hence T-cell response, we might target several T-cell inhibitory receptors such as PD-1, TIM-3, LAG-3, and CTLA-4 which send inhibitory signals into the T-cell. The expression of these inhibitory receptors on CAR T-cells leads to T-cell exhaustion. Recent studies show that tumor cells can take advantage of these T-cell inhibitory receptors in order to evade the immune response. For example, tumor cells upregulate PD-1 ligand (PD-L1) which causes reduced immune responses through PD1/PD-L1 pathway (154). Therapeutic strategies specifically designed to inhibit these inhibitory signals by immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4, have been described showing promising results in the treatment of solid tumors in addition to hematological malignancies (155, 156). This background has led to design directed-CRISPR/Cas9 technology in order to disrupt immune checkpoints. Some studies suggest an improvement in the anti-tumor efficacy and clinical outcome using these next generation modified CAR T-cells (157–159).

FUTURE DIRECTIONS AND CONCLUSIONS

CAR T-cell therapy is an outbreaking technology to treat hematologic cancers, however, several limitations need to be overcome in order to reach optimal patient response. Promising strategies have been proposed to optimize conventional CAR T-cells increasing safety and efficacy and improving manufacturing feasibility. Fundamental modifications in CAR design can be a promising strategy to reduce CAR T-cell toxicities. Although it is still not clear the influence of soluble antigens in CAR T-cell therapy, the existence of soluble proteins in serum of myeloma patients is being actively discussed as an obstacle for this immunotherapy. Therefore, the selection of the antigen-binding domain is a key factor in CAR design to overcome

abrogation of CAR T-cells by soluble protein. In the myeloma setting, CAR T-cell treatment will probably become soon a key strategy for the treatment of relapsed/refractory patients. Likewise, resistance mechanisms to CAR T-cell therapy, such as antigen loss, need to be explored and strategies to overcome these limitations will be essential to ensure optimal efficacy in myeloma treatment. Development of novel strategies to increase long-term responses by combining CAR T-cell therapy with different drugs which increase antigen density specifically in myeloma cells avoiding antigen escape and toxicities is mandatory. Nowadays, CAR T-cells in myeloma are usually administered in patients who are refractory after numerous previous lines of treatment. Consequently, finding an adequate bridging therapy for these patients while CAR T-cell products are generated can be difficult. Can we obtain a better efficacy of CAR T-cells in earlier lines of treatment? Ongoing trials are looking at this question. Moreover, a new era is coming in CAR T-cell therapy in myeloma: allogeneic CAR T-cells derived from healthy donors can overcome some limitations as compared to conventional autologous CARs like manufacturing labor, production time and costs.

BCMA CAR T-cells have already proven safety and efficacy in relapsed/refractory patients and CAR T-cell therapy in myeloma is in great development with major efforts to optimize this approach all over the world. Even though there are more unsolved issues regarding the use of CAR T-cell therapy in MM, the field is still arising, and its full potential is about to discover.

AUTHOR CONTRIBUTIONS

EG-G wrote and revised the manuscript, figures and references, and supervised the tables. BS-M wrote the manuscript and tables, assisted in the elaboration of the references list. JP-S supervised the manuscript, figures and references.

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REVIEW
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CAR-T Cells Hit the Tumor Microenvironment: Strategies to Overcome Tumor Escape

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Chimeric antigen receptor (CAR) T cell therapies have demonstrated remarkable efficacy for the treatment of hematological malignancies. However, in patients with solid tumors, objective responses to CAR-T cell therapy remain sporadic and transient. A major obstacle for CAR-T cells is the intrinsic ability of tumors to evade immune responses. Advanced solid tumors are largely composed of desmoplastic stroma and immunosuppressive modulators, and characterized by aberrant cell proliferation and vascularization, resulting in hypoxia and altered nutrient availability. To mount a curative response after infusion, CAR-T cells must infiltrate the tumor, recognize their cognate antigen and perform their effector function in this hostile tumor microenvironment, to then differentiate and persist as memory T cells that confer long-term protection. Fortunately, recent advances in synthetic biology provide a wide set of tools to genetically modify CAR-T cells to overcome some of these obstacles. In this review, we provide a comprehensive overview of the key tumor intrinsic mechanisms that prevent an effective CAR-T cell antitumor response and we discuss the most promising strategies to prevent tumor escape to CAR-T cell therapy.

Keywords: chimeric antigen receptors (CAR), solid tumors, immunotherapy, immunosuppressive tumor microenvironment, adoptive cell transfer (ACT), inhibitory receptors

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INTRODUCTION

T cells that are genetically modified to express chimeric antigen receptors (CAR-T) constitute a potent new cancer therapy with curative potential (1, 2). CAR-T cell therapy has produced impressive response rates in patients with certain B-cell malignancies, resulting in the recent approval of two CAR-T cell products targeting CD19 (3, 4). Numerous CAR-T cell therapies targeting a variety of antigens are under clinical investigation, with anti-BCMA CAR-T cells showing very promising results for the treatment of multiple myeloma (5). Despite the impressive responses in patients with hematologic malignancies, early clinical trials using CAR-T cells in patients with solid tumors have reported limited antitumor activity, with objective responses observed only in a minority of patients (6–8).

The potential of T cells to induce complete responses in patients with solid tumors has been demonstrated by the success of immune checkpoint therapy (9). Also, objective responses to adoptive T cell therapy with tumor infiltrating lymphocytes (TILs) and T cells that are genetically

engineered to express a transgenic T cell receptor (TCR) have been reported in patients with melanoma, sarcoma, cholangiocarcinoma, and breast cancer (10). While only a proportion of patients exhibit long term, durable responses, these results suggest that T cells have the potential to eliminate solid tumors under adequate conditions. However, to date only anecdotes of CAR-T cell mediated response have been reported (6, 8). Understanding the mechanisms that limit CAR-T cell efficacy in solid tumors is essential to design the next-generation of CAR-T cell therapies with increased therapeutic index.

Some of the key factors limiting the applicability of CAR-T cells for the treatment of solid tumors include: the lack of truly tumor-specific target antigens (11); tumor heterogeneity and plasticity that can lead to tumor escape due to loss of antigen expression (12); T cell dysfunction driven by CAR-mediated tonic signaling (13–15) or chronic antigen exposure (16); and the immunosuppressive tumor microenvironment (TME) (17). In this review, we summarize the key challenges that CAR-T cell encounter in the TME, with a particular emphasis on tumor intrinsic factors, such us hypoxia, extracellular matrix (ECM) and stromal and immune cells. We also discuss some of the efforts that are underway to overcome these challenges and expand the therapeutic window of CAR-T cells for the treatment of solid tumors (**Table 1**).

TABLE 1 | Main challenges for CAR-T cell therapy in solid tumors and emerging strategies to address them.

Factors harnessing CAR-T cell therapy efficacy in solid tumors	CAR-T cell-based approaches proposed to overcome limitations
TUMOR PENETRATION	
Endothelial barriers	
• Tumor vasculature	• Disrupt tumor vasculature with CAR-T cells (18–22)
T cell exclusion from tumors	
• Extracellular matrix (ECM) • Cancer-associated fibroblasts (CAFs)	• Express matrix-degrading enzymes (23, 24) • Target CAFs with CAR-T cells (25–34)
TUMOR MICROENVIRONMENT (TME)	
Hypoxic tumor conditions	• Chose appropriate costimulatory domains (35–37) • Restrict CAR expression to hypoxic conditions (38) • Target antigens upregulated in hypoxic conditions (39)
Immunosuppressive immune cells	
• Regulatory T cells (T_{regs})	• Combine CAR-T cells with antibodies that reduce T_{reg} frequencies (40–43) • Target T_{regs} with CAR-T cells (44) • Use lymphodepleting regimens to eliminate T_{regs} (45) • Reduce IL-2 availability for T_{regs} by: ○ Choosing appropriate costimulatory domains (46–51) ○ Mutating costimulatory domains (52) ○ Using alternative cytokines to support engineered CAR-T cells (49, 53, 54)
• Tumor-associated macrophages (TAMs)	• Target TAMs with CAR-T cells (55–57) • Reeducate TAMs toward antitumor phenotype (58–64)
• Myeloid-derived suppressor cells (MDSCs)	• Combine CAR-T cells with agents that reduce MDSC content (43, 65–71) • Target MDSCs with CAR-engineered T/NK cells (72, 73)
Immunosuppressive soluble factors	
• TGF-β, IL-4, IDO	• Confer resistance to immunosuppressive factors by engineering CAR-T cells to express: ○ Dominant-negative receptors (74) ○ Switch receptors (75, 76) ○ Disrupt inhibitory cytokine receptors by genome editing (77) • Engineer CAR-T cells to release support cytokines (78–84) • Combine CAR-T cells with inhibitors (85)
IMMUNE EVASION AND SUPPRESSION	
Expression of inhibitory receptors and ligands by tumor and/or stromal cells	
• PD-1/PD-L1, CTLA-4, LAG-3, TIM-3, TIGIT	• Combine CAR-T cells with immune checkpoint blockade antibodies (71, 86–90) • Combine CAR-T cells with oncolytic viruses releasing immune checkpoint inhibitors (91) • Engineer CAR-T cells to express: ○ Blocking antibodies (92, 93) ○ Dominant negative receptors (86) ○ Switch receptors (94) • Disrupt T cell inhibitory receptors by genome editing (95–100)

PHYSICAL BARRIERS

Hypoxia

Defined as a shortage in oxygen availability, hypoxia is a prominent feature of solid tumors that results from an aberrant vascularization and rapidly proliferating tumor cells. Tumor hypoxia has been correlated with poor patient prognosis (101), resistance to neoadjuvant therapy (102, 103), and metastatic success (104). Importantly, reduced oxygenation can also influence antitumor immune responses (105).

Cellular adaptations to oxygen levels are governed by the hypoxia pathway and mediated by hypoxia-inducible factors (HIF). When oxygen is available, prolyl hydroxylase domain proteins (PHDs) are active and hydroxylate HIF, leading to HIF ubiquitination by Von-Hippel Lindau (VHL), and HIF degradation in the proteasome. When oxygen levels drop, hydroxylases become inactive leading to HIF stabilization and translocation to the nucleus, where it forms a transcriptional complex that directly binds to specific regions, termed hypoxia response elements (HREs). HREs are present in the promoters of several genes that encode for important proteins that mediate the cellular adaptation to hypoxia, such as glycolytic enzymes and the vascular endothelial growth factor-A (VEGF-A) (106). This family of transcription factors is mainly comprised of two isoforms: HIF-1α and HIF-2α (107), with HIF-1α being the main isoform expressed by activated T cells (108). HIF-1 accumulation in T cells promotes antitumor immunity in mouse models of solid tumors and metastases (109, 110).

After activation, T cells increase glucose uptake and glycolytic rate to support proliferation and the acquisition of effector functions (111). This process is supported by HIF stabilization after TCR engagement and augmented under hypoxia. A consequence of the T cell adaptation to hypoxia

is metabolic rewiring, a process in which the reduced rate of oxidative phosphorylation (OXPHOS) is compensated by enhanced glycolysis. Competition for nutrients, persistent antigenic stimulation and immunosuppressive networks in the TME can lead to T cell exhaustion (112). Another consequence of metabolic adaptation in T cells is the accumulation of metabolites that impact epigenetic landscapes that influence the fate and function of T cells (113). One example is the increased production of the oncometabolite 2-hydroxyglutarate (2-HG) by hypoxic T cells. 2-HG inhibits 2-oxoglutarate-dependent epigenetic enzymes (114) resulting in the modulation of the T-cell terminal differentiation and favoring a central memory phenotype (115). Certain histone demethylases, such as KDM6A and KDM5A, can also be directly inhibited by a shortage of oxygen in a HIF- and 2-HG independent manner, leading to the control of gene expression and cell fate (116, 117).

The level of oxygenation impacts several aspects of CAR-T therapies (**Figure 1**). *In vitro*, hypoxia decreases the expansion capacity of CAR-T cells, blocking their differentiation into effector memory cells, and enriching the cultures with T cells with a central memory cell phenotype (118). Culturing and expanding CAR-T cells under controlled physiological oxygen concentrations might be an approach for enriching the cultures with memory-like T cells, which are known to have better persistence and efficacy than terminally differentiated effector T cells (119).

After infusion, CAR-T cells must infiltrate solid tumors and carry out their cytotoxic activities. How hypoxia influences these processes remains largely unexplored. Recent development of *in vitro* tools will support the study of CAR-T function in relevant oxygenation conditions (120). In this context, the use of organoids and 3D tumor models (121–123) will support the preclinical development of CAR-T cells for the treatment of solid tumors.

The hypoxia pathway offers several opportunities for the design of CAR-T cells (**Figure 1**). The choice of the optimal costimulatory domains in the CAR might be influenced by oxygen availability in the TME, given that the metabolic consequences of signaling downstream of CD28 and 4-1BB are different (35–37). Another attractive approach is the design of CARs that are active in the TME, but inactive in better oxygenated environments in an attempt to reduce off-site toxicities. Novel strategies to confine CAR expression to the TME consists of introducing HRE regions on the promoter of the construct, or fusing HIF domains to the intracellular domain of the CAR to promote the hydroxylation and degradation of the CAR when oxygen is available (38). Both approaches rely on the endogenous T cell oxygen-sensing machinery to control the expression of the CAR. Alternatively, CAR-T cell activity can also be targeted to antigens that are known to be upregulated under hypoxic conditions in solid tumors, such as carbonic anhydrase IX (39).

Hypoxia also promotes immunosuppressive pathways in the TME that offer combinatorial therapeutic strategies with CAR-T cell approaches. Hypoxia and HIF promote the expression of program death ligand 1 (PD-L1) (86, 124) and adenosine levels (125, 126), as well as the recruitment of regulatory T (T_{reg}) cells in the TME (127), all of which are known to inhibit T cell responses.

Extracellular Matrix

The ECM is an integral constituent of the tumor stroma composed of different macromolecules including fibrous proteins, glycosaminoglycans, and proteoglycans. The ECM is produced by tumor cells themselves as well as by cancer-associated fibroblasts (CAFs) and play an important role in cancer progression. Increased deposition of collagen or hyaluronan, constituents of the ECM, in tumors correlate with poor prognosis in different cancer types (128–131).

In addition, the ECM represents a physical barrier to various anticancer therapies, preventing their penetration and infiltration of tumors. Agents such as collagenase or hyaluronidase can degrade distinct components of the ECM and improve antitumor efficacy of diverse cancer therapies, including chemotherapy, oncolytic viruses, monoclonal antibodies, or checkpoint blockade (132–142).

While the role of ECM in resistance to adoptive T cell transfer therapies remains underexplored, some studies demonstrate that peritumoral ECM collagen fibers limit T cell access to tumors, and indeed, tumors with high-collagen density present lower levels of infiltrating T cells (142, 143). Here, the use of the matrix-degrading agents that facilitate T cell infiltration of tumors provides a rationale for matrix degradation as a means to improve efficacy of CAR-T cell therapy (140–142). In this regard, CAR-T cells engineered to express heparanase (HPSE), which degrades heparan sulfate proteoglycans, better infiltrated tumors and had increased antitumor activity in mouse models (23). Since matrix metalloproteinases (MMPs), mainly produced by macrophages, also regulate synthesis and degradation of most of the ECM components, an alternative strategy is to leverage the capacity of macrophages to secrete MMPs and remodel the ECM in order to clear the way for T cells to infiltrate tumors (24). This has been demonstrated in the context of endogenous T lymphocytes, but it could be hypothesized that the use of CAR-macrophages might benefit tumor infiltration of CAR-T cells, although it has not been experimentally tested yet.

Tumor Vasculature

Aberrant tumor vasculature is required for tumor survival, progression, and metastasis, but also provides a physical barrier for T cell extravasation and infiltration into tumors (144). CAR-T cells capable of destroying tumor vasculature have been developed targeting molecules such as VEGFR-2 (18), VEGFR1 (19), PSMA (20), TEM8 (21), or the fibronectin splice variant EIIIB (22). All of these target antigens are also expressed by a range of tumor cell types, and some of them by immunosuppressive cell populations such as regulatory T cells (T_{regs}) and myeloid-derived suppressor cells (MDSCs, i.e., VEGFR2) (145, 146) or by the ECM (i.e., EIIIB), which may improve the outcome of the therapy in patients. Unfortunately, a clinical trial on metastatic cancer patient treated with VEGFR-2 CAR-T cells was terminated due to lack of objective responses (NCT01218867).

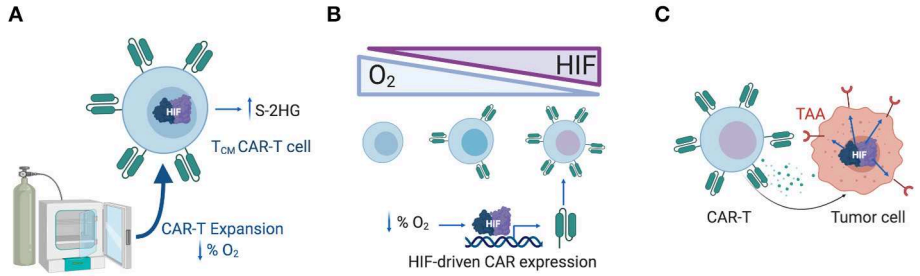

FIGURE 1 | Exploiting the hypoxia response pathway for CAR-T therapy. **(A)** Expanding CAR-T cells *ex vivo* under reduced oxygen concentrations (1–5% O_2) might support the enrichment of memory-like T cells, a process mediated by S-2HG. **(B)** CAR expression can be gradually modulated by increasing levels of HIF-1α in T cells, generating a hypoxia-responsive CAR-T with increased CAR expression in hypoxic tumors and reduced CAR expression in the periphery. **(C)** Selection of TAAs that are upregulated under hypoxic conditions in solid tumors might limit off-tumor CAR-T cell activity. HIF-1α, Hypoxia-inducible factor 1 alpha; S-2HG, S-2-hydroxyglutarate; TAA, tumor associated antigen.

FIBROBLASTS

CAFs can contribute to up to 90% of the solid tumor mass in carcinomas (147) and represent a complex barrier to entry and activity of endogenous and adoptively transferred immune cells.

CAFs signal in a paracrine fashion with tumor cells and other components of the TME. Tumor promoting CAFs secrete factors, including VEGFs, that induce angiogenesis to improve oxygen and nutrient availability in the tumor. CAFs can also directly provide cancer cells with nutrients, growth factors and immunosuppressive cytokines such as transforming growth factor beta (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF2), and serve as a physical barrier to T cell infiltration (148, 149). CAFs heavily contribute to the survival, proliferation, metastasis initiation and, even, de-differentiation of tumor cells into more stem cell-like phenotype (150, 151).

Given their powerful and diverse protumoral effects, an attractive therapeutic approach could be generating CAR-T cells that target CAFs. In addition to eliminating their multiple negative effects, an advantage to targeting fibroblasts would be that they are more genetically stable than tumor cells, so they are less likely to lose antigen expression via immunoediting. Moreover, since mesenchymal tumoral stromal cells are present in almost all human adenocarcinomas, therapies against CAFs could potentially be used for multiple types of tumors (152).

In the setting of solid tumors, different subtypes of CAFs have been proposed to have disparate effects on tumor establishment, growth and progression, as well as in metastatic capacity (25, 153). Therefore, when choosing a CAR-targeted protein, it is important to consider which fibroblast cell subpopulation is going to be depleted (154). With this thought in mind, fibroblast activation protein (FAP) has been proposed as a potentially good target. FAP is a surface peptidase that also has gelatinase activity and is widely expressed in a subset of protumoral fibroblasts in many cancer types (155–157). FAP expression in pancreatic cancer (158, 159) and non-small cell lung cancer (160) is associated with worse clinical outcome. Depletion of FAP+ cells using genetic depletion strategies appeared to enhance T cell mediated antitumor activity in preclinical models of melanoma and pancreatic ductal adenocarcinoma (161–163). Antibodies against FAP have confirmed the suitability of FAP as a target by demonstrating efficient tumor stroma targeting capabilities in clinical trials (157). However, no therapeutic responses were observed, prompting the development of alternative strategies such as FAP antibody conjugates including immunostimulatory antibodies (164) and immunocytokines (165). One of those, an anti-FAP-IL-2v fusion protein, is currently being tested in clinical trials (NCT02627274, NCT03386721) (166). Alternatively, CAR-T cell therapy targeting FAP might be a more potent and efficacious strategy.

CAR-T Cells Targeting Fibroblasts: A Potential Double-Edged Sword

A number of groups have generated CAR-T cells targeted to mouse FAP and tested their ability to inhibit tumor growth. To date, eight studies have demonstrated antitumor activity of FAP-targeting CAR-T cells in several preclinical models including mesothelioma, lung, mammary, colon, pancreatic cancers (25–32), with a key measure of these studies being the potential for toxicity.

A key concern of targeting FAP is that, while it is highly expressed by CAFs and in wound healing, it is also expressed at low levels in healthy tissues including muscle, adipose tissue, bone marrow mesenchymal stem cells (BMMSCs), skin, and pancreas (167, 168). Complete ablation of FAP-expressing cells in mice using genetic approaches resulted in body weight loss, anemia, bone marrow hypoplasia and pancreatic toxicity (167). With these toxicities in mind, it is of interest to review the studies in which CAR-T cells targeting mouse FAP were tested, however, it is important to recognize that each study used a different single-chain fragment variable (scFv) antibody targeting FAP, different cytoplasmic domains, and different types of T cells (murine vs. human T cells).

Tran and colleagues observed minimal antitumor effect using a CAR with the FAP-5-scFv coupled with mouse CD28, 4-1BB, and CD3ζ intracellular signaling domains, but did observe severe toxicity indicated by significant cachexia and anemia (30). In

contrast, Kakarla et al. showed that a FAP-CAR, using the MO35-scFv with human CD28 and CD3 derived domains, controlled tumor burden in a systemic lung carcinoma model without toxicity observed 2 days after T cell injection (27). However, this time point may be too early to see the negative effects exerted by the T cells.

The group at the University of Pennsylvania developed a FAP-CAR containing a scFv from the 73.3 anti-mouse FAP antibody and the human 41BB and CD3ζ intracellular domains (25, 28, 31, 32). These CAR-T cells slowed tumor growth in an immune-response dependent and independent manner in several tumor models in mice. Despite 73.3-FAP-CAR initial efficacy, CAR-T cells isolated from xenograft tumors became hypofunctional (28). Function was augmented by either using mouse T cells from mice lacking the inhibitory enzyme diacylgycerol kinase zeta (DGKZ) (32) or human T cells using the 73.3-CAR linked to the DAP12 signaling domain from natural killer (NK) cells (FAP-KIR CAR) (31). There was a link between enhanced CAR activity and toxicity: while no major toxicities were observed using the "basal" 73.3-FAP CAR-T cells, treatment with the more active DGKZ CAR-T cells resulted in a lymphocytic infiltrate observed in the pancreas (32). Likewise, treatment with the highly active FAP-KIR-CAR resulted in anemia, body weight loss and bone marrow hypoplasia (31). The "basal" 73.3-FAP-CAR targets cells with high FAP densities, like CAFs, while sparing low FAP expressing cells, which may provide a therapeutic window to obtain efficacy in the absence of toxicity. Unfortunately, the 73.3–FAP-CAR is mouse specific and cannot be used in the clinical setting.

There has been one reported clinical trial in which FAP CAR-T cells have been locally injected into the pleural effusion of mesothelioma patients (NCT01722149) (33). The authors reported the route of administration and the therapy to be safe in one patient (34) and, another patient showed stable disease for 1 year (26). Unfortunately, at the time of closure of the clinical trial in mid-2019 only 4 patients had been recruited.

In summary, FAP targeted CAR-T cells have clearly shown some antitumor activity in preclinical models, but they have also demonstrated the potential for toxicity. There does appear to be a viable therapeutic window, however. For this reason, it is likely that the role of FAP CAR-T cells will be in combination therapies. Combining FAP CAR-T cells with tumor-targeted CAR-T cells or with vaccines can result in additive or even synergistic effects (27, 32). Other target proteins like CD10 and GPR77 which identify a newly described CAF subpopulation with protumorigenic functions (169) provide alternative option for CAR development.

TUMOR-INFILTRATING IMMUNE CELLS AS BARRIERS TO EFFECTIVE CAR-T CELL THERAPY

Solid tumors are highly infiltrated with immune cells such as T_{regs}, tumor-associated macrophages (TAMs) or MDSCs that contribute to the establishment of a hostile and immunosuppressive TME capable of limiting the efficacy of CAR-T cell therapy. In this section, we review the obstacles imposed by each of these cell populations and the different strategies that have been utilized in order for CAR-T cells to be efficacious in such context, as illustrated in **Figure 2**. These include strategies to directly target and deplete the immunosuppressive immune cell populations as well as indirect approaches consisting of genetically engineering the CAR-T cells to endow them with transgenes capable of modulating the TME or to confer them with resistance to immunosuppression.

Regulatory T Cells

T_{regs} are a subset of T cells (phenotypically defined as $CD4^+CD25^+FoxP3^+$) which play a crucial role in maintaining immune tolerance to self-antigens but can also suppress antitumor immunity (170). Cancer patients have increased numbers of T_{regs} in peripheral blood (171–173), and their presence in tumors is associated with poor prognosis in a variety of cancers (174–178).

T_{regs} suppress antigen-specific $CD8^+$ T cell cytotoxicity by using different mechanisms: competitive consumption of IL-2; secretion of immunosuppressive cytokines such as IL-10 or TGF-β; CTLA-4-mediated suppression of antigen presenting cells (APCs); prevention of optimal T cell activation; or lysis of effector cells through the action of granzyme and/or perforin (170).

In the TME, T_{regs} play an inhibitory role in the antitumor efficacy of adoptively transferred tumor-targeted effector T cells (179). The frequency of T_{regs} in the blood of responder patients was lower than in samples obtained from non-responders in a combined analysis of multiple trials of adoptively transferred TILs (180). In a first-in-human study of an epidermal growth factor receptor variant III (EGFRvIII)-specific CAR in glioblastoma, the analysis of tumor specimens from patients who had post-treatment surgery revealed an increased influx of immunosuppressive T_{reg} cells, which might have limited the antitumor effect of the CAR-T cell therapy (181). In addition, the importance of the effector to regulatory T cell balance in predicting responses to immunotherapy treatments has been highlighted (182).

A first and obvious way to address this limitation is to specifically eliminate T_{regs}. The combination of CAR-T cells with antibodies targeting GITR or 4-1BB (whose expression has been reported to be specific for tumor T_{regs}) has been explored in mice, resulting in decreased T_{reg} frequencies and enhanced antitumor efficacy (40–43). The idea of directly depleting T_{regs} with a CAR has also been proposed by targeting the C-C chemokine receptor 4 (CCR4), which is expressed on T cell malignancies but also in T_{regs} (44).

Many clinical trials of CAR-T cells have failed to provide significant clinical benefit in the absence of prior lymphodepleting preconditioning (183–188). This lack of success may be explained, at least in part, by the fact that such preparative treatments are known to eradicate T_{regs}, which otherwise might suppress infused T cells (189). Illustrating this, the efficacy of CD19 CAR-T cells in a mouse model of lymphoma was completely abolished when T_{reg} cells were previously injected and restored by preparative treatment with cyclophosphamide (45). Unfortunately, preconditioning regimens carry with them toxicities, which in some cases

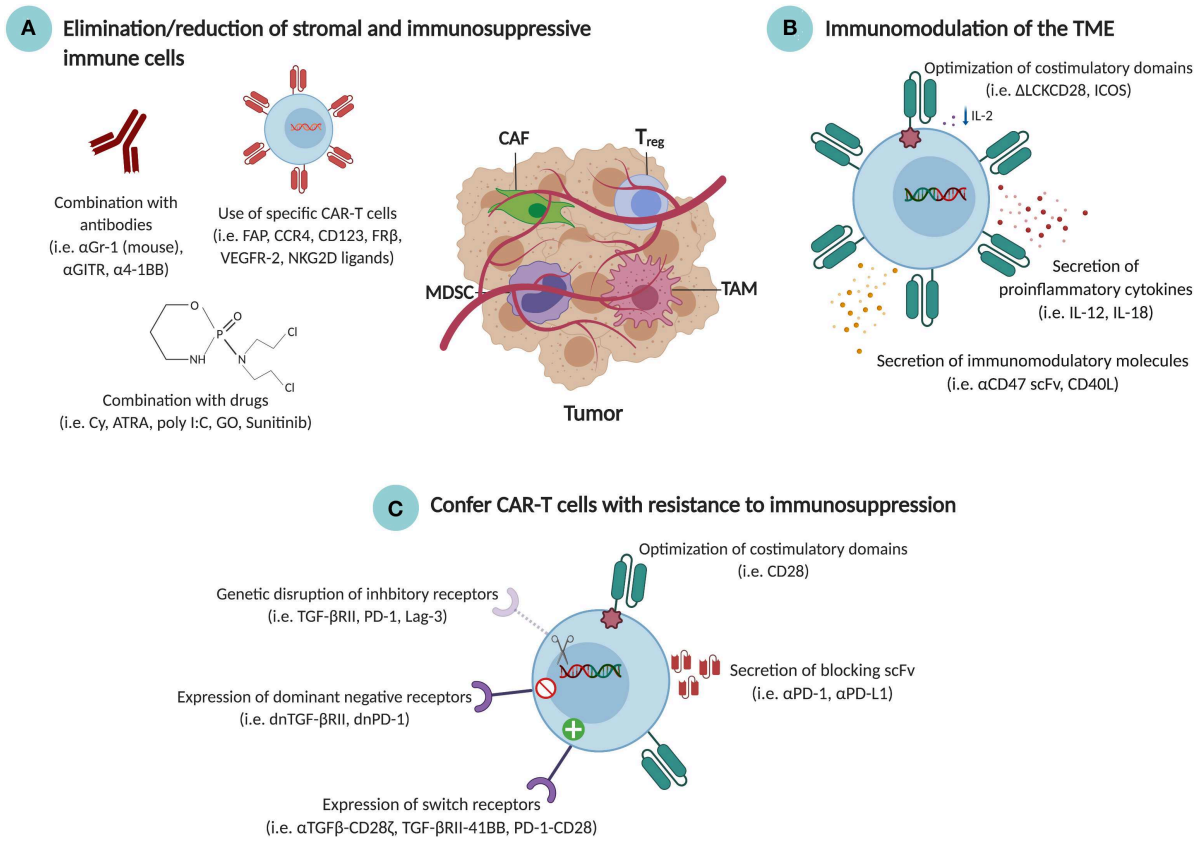

FIGURE 2 | Therapeutic strategies to overcome the immunosuppressive TME. Tumors are infiltrated with stromal cells, such as cancer-associated fibroblasts (CAFs), and immune cells, including regulatory T cells (T_{regs}), tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) which support cancer progression and promote immunosuppression. Therapeutic strategies utilized to hit these components to enhance the efficacy of CAR-T cell therapy can be categorized in three classes, and the most relevant examples are represented in this figure. **(A)** *Elimination or reduction of stromal and immunosuppressive immune cells:* the combination of CAR-T cells with agents such as antibodies or drugs has resulted in decreased frequencies of T_{regs} and/or MDSCs. Alternatively, CARs have been designed to target antigens expressed on CAFs, T_{regs}, TAMs and MDSCs to directly deplete them. **(B)** *Immunomodulation of the TME:* this group of strategies aims at manipulating the TME to create a favorable environment that allows a better performance of the CAR-T cells. Some examples include the modulation of the tumor cytokine milieu by the expression of proinflammatory cytokines by CAR-T cells or by the optimization of costimulatory signaling domains in order to reduce IL-2 secretion and therefore impair T_{reg} expansion and tumor infiltration. Immunomodulatory molecules that are able to polarize M2 TAMs into an antitumor M1 phenotype can also be expressed from CAR-T cells. **(C)** *Confer CAR-T cells with intrinsic resistance to immunosuppression:* CAR-T cells can be modified to be resistant to immunosuppression by endowing them with dominant-negative receptors (to disrupt signaling) or chimeric switch receptors (to convert negative signaling into positive), or by abrogating the expression of inhibitory receptors using genome-editing tools. Alternatively, antibodies blocking inhibitory receptors or ligands can be secreted by CAR-T cells. Also, it has been reported that the incorporation of particular costimulatory domains or the expression of some proinflammatory cytokines by CAR-T cells confer intrinsic resistance to T_{reg}-mediated immunosuppression.

create a need for alternative strategies. In this line, several studies have demonstrated that IL-12 can help to overcome T_{reg}-mediated immunosuppression and, therefore, the need for prior preconditioning. In mice, CAR-T cells engineered to constitutively produce IL-12 acquire intrinsic resistance to T_{regs} and are more efficacious in the absence of preconditioning (78, 79). Considering the potential clinical toxicity of constitutive IL-12 expression, safer approaches might involve the use of inducible systems to drive IL-12 production upon antigen recognition or the incorporation of elimination genes (80, 81). Constitutive IL-12-secreting mucin-16 ectodomain (MUC-16^{ecto})-specific CAR-T cells, which also express a truncated form of the human epidermal growth factor receptor (EGFRt) as a safety system, are currently being tested in an ovarian cancer phase I clinical trial (NCT02498912) (82, 83). CAR-T cells expressing alternative cytokines with safer clinical profiles such as IL-18 have also been tested in preclinical models with a similar impact on reducing tumor-infiltrating T_{reg} numbers and improving antitumor activity (84).

A second, indirect approach to overcome CAR-T cell suppression by T_{regs} is to restrain their proliferation and survival by modulating the cytokines in the TME, specifically IL-2. IL-2 sustains the survival and function of both regulatory and effector T cells (190). In fact, IL-2 is often used to improve persistence of adoptively transferred T cells, albeit its administration leads to the expansion of T_{regs} in cancer patients (191). CAR-T cells release high levels of IL-2 upon antigen engagement, becoming a main source of this cytokine. It could be expected, then,

that the use of CAR-T cells with reduced levels of secreted IL-2 would improve the antitumor efficacy of these engineered T cells as IL-2 would be no longer available to sustain T_{reg} persistence. Cytokine levels can be modulated by selecting an appropriate co-stimulatory endodomain such as ICOS, which has been reported to generate CAR-T cells with increased IL-17 production and reduced secretion of IL-2 (46). Alternatively, more conventional co-stimulatory domains such as CD28 can be mutated for the same purpose. It is known that IL-2 secretion is initiated by CD28-mediated LCK recruitment and phosphorylation, therefore, the mutation of the LCK binding domain abolishes IL-2 secretion by CAR-T cells (52). This modification improved antitumor efficacy of CAR-T cells in the presence of previously inoculated T_{reg} cells, which persisted less, compared to mice treated with CAR-T cells containing the wild type CD28 endodomain (52).

In apparent contradiction with this study, several groups report that the incorporation of a CD28 co-stimulatory domain in different CAR platforms provides increased resistance to T_{reg}-mediated immunosuppression, and more specifically, to TGF-β-mediated suppression of T cell proliferation (47–50). Conversely, while sustaining T_{reg} survival and function, IL-2 induced by CD28 activation of LCK and autocrine signaling through IL-2 receptor on tumor-specific effector T cells appears to be crucial to counteract the inhibitory effects of TGF-β (48, 49). In fact, the deletion of the LCK binding domain in CD28 reverted resistance to TGF-β-mediated suppression (49). One strategy proposed to compensate for the detrimental effect of LCK mutation while maintaining the benefits of abrogating IL-2 secretion is the addition of a 4-1BB co-stimulatory signaling domain (51). A different approach is the use of alternative cytokines to replace the CD28-induced IL-2 autocrine loop. For instance, CAR-T cells can be engineered to express IL-7Rα so that IL-7 can support their function (53). In a more sophisticated approach, CAR-T cells with a disrupted IL-2 axis can be engineered to release transgenic IL-7 and to co-express an IL-7Rα/IL-2β hybrid receptor to provide cell-intrinsic IL-2 signaling through IL-7 (49). Alternatively, cytokine stimulation can be provided by IL-15 through the expression of a tethered membrane-bound IL-15, which has been shown to favor the persistence and survival of CAR-T cells with a clinically desirable immature state of differentiation (54). This interesting approach avoids undesirable effects of soluble IL-15 coadministration or constitutive secretion by CAR-T cells such as o toxicity (192) or promotion of T_{regs} (193). In addition, there is great excitement on the use of engineered IL-2 mutants designed to preferentially signal into effector T cells but not T_{regs}, although this strategy has not yet been tested in the context of CAR-T cells (194–196).

Besides suppressing T cell proliferation, TGF-β induces a T_{reg}-like phenotype on CAR-T cells (77). Therefore, conferring CAR-T cells with intrinsic resistance to TGF-β represents an opportunity for improvement. TGF-β signaling in CAR-T cells can be abrogated by knocking out TGF-βRII through CRISPR/Cas9 technology (77). In the same line, CAR-T cells can be endowed with a TGF-β dominant-negative receptor (dnTGF-βRII). A first-in-human trial in patients with refractory castration-resistant metastatic prostate cancer has been initiated with a prostate-specific membrane antigen (PSMA)-specific CAR incorporating this receptor (NCT04227275) (74). Alternatively, switch receptors can be created by fusing the extracellular part of the TGF-βRII to the endodomain of 4-1BB or by linking a TGF-β-specific scFv to the CD28-CD3ζ intracellular signaling domains, rendering CAR-T cells capable of converting the immunosuppressive signal from soluble TGF-β into an immunostimulatory one (75, 76).

Tumor-Associated Macrophages

TAMs are the most abundant immune cells infiltrating human cancers and their accumulation in tumors correlates with poor prognosis in a broad range of tumor types (197, 198). TAMs can sustain cancer progression by secreting growth factors which stimulate tumor cell proliferation, proteolytic enzymes that promote matrix remodeling and facilitate metastasis, proangiogenic factors which support angiogenesis, or reactive oxygen species (ROS) and nitric oxide (NO) that induce genetic instability on tumor cells (199). Furthermore, TAMs can suppress T cell-mediated antitumor immunity by releasing IL-10 and TGF-β, amino acid-depleting enzymes such as arginase 1 or indoleamine 2,3-dioxygenase (IDO) which cause metabolic starvation on T cells or prostaglandins with immunosuppressive effects, or by expressing immune checkpoint ligands like PD-L1, PD-L2, B7-H4, or VISTA. Moreover, TAMs can promote the recruitment and immunosuppressive activity of T_{regs} (199). TAMs can also prevent T cell-mediated antitumor immune responses by physically creating long-lasting interactions with $CD8^+$ T cells, thus excluding them from tumors (200).

There is overt preclinical evidence of that TAMs can mediate resistance to immunotherapy, including adoptive cell transfer therapy. For instance, the depletion of TAMs through the administration of a CSF-1R inhibitor improved the efficacy of adoptively transferred tumor-specific T cells in syngeneic mouse models of melanoma (200). Superior antitumor activity of the combined treatment correlated with a decrease in the number of intratumoral macrophages, which subsequently facilitated an increase in expansion, intratumoral accumulation and functionality of the adoptively transferred T cells (201).

In the field of CAR-T cell therapy, the infusion of GD2-specific CAR-T cells in neuroblastoma patients provoked a striking expansion of circulating macrophages with immunosuppressive phenotype suggesting a role of macrophages limiting the antitumor efficacy (88).

Despite their overall tumor-promoting functions, certain subpopulations of TAMs can sustain antitumor activities including phagocytosis, antigen-presenting, or the release of proinflammatory cytokines such as TNF-α and IL-12. Indeed, in certain contexts, macrophages have been proven crucial for the development of effective immunotherapy (202–204). Several strategies have been proposed to either reprogram immunosuppressive "M2-like" TAMs into an antitumor "M1-like" phenotype which could cooperate with CAR-T cells to induce tumor regression, or to directly deplete TAMs to facilitate productive antitumor immunity.

One strategy of TAM reeducation consists in making them more phagocytic. CD47 is expressed on tumor cells and interacts

with SIRPα expressed on macrophages to deliver a "don't eat me" signal. CAR-T cells can be engineered to express CD47-blocking antibodies in order to prevent that interaction, thus stimulating phagocytosis of tumor cells and improving engagement of the innate immune system (58, 59). A clever approach to hijack the phagocytic capacities of TAMs and redirect them toward tumor-associated antigens is to engineer macrophages themselves to express a CAR. Interestingly, macrophage transduction with chimeric adenoviral vectors promoted a gene expression change toward a proinflammatory M1 phenotype, which subsequently converted bystander M2 TAMs into an M1 phenotype and boosted endogenous antitumor T cell responses (60).

TAMs can also be manipulated to become more functionally activated. CD40 is expressed in antigen presenting cells (APCs) including dendritic cells (DCs), B cells, monocytes and macrophages. Interaction of CD40 with its ligand, CD40L, is known to induce activation and IL-12 secretion by APCs. Preliminary studies using a bispecific antibody to mediate the interaction between a c-myc tag on CAR-T cells and CD40 on APCs demonstrated enhanced CAR-T cell function (61). Constitutive expression of CD40L by CAR-T cells improved their therapeutic efficacy in part through the induction of maturation and IL-12 secretion by monocyte-derived DCs and macrophages (62, 63). By means of a different pathway, the administration of the multikinase inhibitor sorafenib in combination with CAR-T cells also induced an increase in IL-12 production by TAMs which contributed to antitumor activity (64).

Not surprisingly, cytokines secreted by CAR-T cells upon antigen encounter can alter the TME and convert TAMs from immunosuppressive to immunostimulatory. For instance, secretion of GM-CSF and IFN-γ by CAR-T cells upon antigen engagement has been shown to elicit a recruitment of myeloid cells to the TME and to activate newly recruited as well as re-educate resident suppressive TAMs thus potentiating their IL-12 production, capacity of antigen presentation, and tumoricidal activity (205). Armoring CAR-T cells with additional cytokines can improve their capacity to modulate the TME. In mice, inducible IL-12 secretion by CAR-T cells resulted in the recruitment of activated TNF-α-producing macrophages which directly contributed to tumor elimination in a TNF-α-dependent manner (206). In addition, IL-12 secretion by CAR-T cells indirectly mediated the depletion of TAMs as a result of Fas engagement on TAMs by FasL on CAR-T cells and altered the phenotype of remaining TAMs toward a proinflammatory one (83). IL-18-secreting CAR-T cells also led to a reduction in "M2-like" macrophages in tumors as well as T_{regs} (84).

A different strategy to overcome immunosuppression in the TME is to develop CARs that target antigens expressed by TAMs to directly eliminate them. CAR-T cells targeting the antigen CD123, with shared expression in malignant cells and TAMs, have been proposed for the treatment of Hodgkin lymphoma which contains a highly immunosuppressive TME (55). Alternatively, rather than hitting all macrophages by using a pan-macrophage target, it would be desirable to design CARs that are able selectively deplete TAMs with protumor "M2-like" properties while sparing other TAM populations with antitumor "M1-like" functions. CAR-T cells targeting folate receptor β (FRβ), which is expressed only in the immunosuppressive TAM population, have been developed for that aim (56). Similarly, CAR-T cell targeting B7-H4, a molecule expressed by cancer cells and TAMs, mediated antitumor responses in a preclinical ovarian cancer model, but was also toxic due to possible targeting of tissue resident macrophages (57).

Finally, CAR-T cells can also be combined with agents that protect them from TAM-related immunosuppressive pathways, such as that mediated by IDO. IDO is produced by tumor cells and TAMs and mediates the metabolism of tryptophan into immunosuppressive metabolites that can suppress CAR-T cell function. The use of IDO inhibitors or preconditioning with fludarabine, which can inhibit IDO expression, are strategies that can be used to improve the activity of CAR-T cells in immunosuppressive microenvironments (85).

Myeloid-Derived Suppressor Cells

MDSCs are a highly diverse population of immature myeloid cells which include two major subsets: the mononuclear MDSCs (M-MDSCs), which are morphologically and phenotypically similar to monocytes and can differentiate into TAMs, and the polymorphonuclear MDSC (PMN-MDSCs), which resemble neutrophils and are precursors of tumor-associated neutrophils (TANs), as well as a small group of myeloid progenitors (207). MDSCs play a role in supporting tumor progression, and according to a meta-analysis of the literature, their accumulation is associated with poor clinical outcome in cancer patients (208). The hallmark feature of MDSCs is their strong capacity to inhibit immune responses, with T cells being the main targets of these effects. Mechanisms implicated in MDSC-induced immunosuppression are common to those reported for TAMs, including production of NO and ROS, elimination of key nutrition factors needed for T cell proliferation such as arginine, cysteine, or tryptophan, production of IL-10 and TGF-β, and induction of T_{regs} (209). MDSCs have also been implicated in limiting the effects of CAR-T cell therapy. In a clinical trial of third generation CD19 CAR-T cell therapy, low levels of M-MDSCs was associated with response in patients with lymphoma and leukemia (210).

The detrimental effect of MDSCs on CAR-T cell proliferation and cytolytic function has been demonstrated by using CARs targeting a number of different antigens (65–67). As a proof of concept, depletion of MDSCs with anti-Gr-1 antibody resulted in improved antitumor efficacy of CAR-T cells in mouse models (40, 65, 66). Unfortunately, the lack of a suitable marker for human MDSCs prevents their targeting by using a single antibody. It has been demonstrated that GM-CSF and STAT3 signaling through GM-CSF and/or IL-6 can drive the expansion of MDSCs and support PD-L1 expression by these cells, promoting suppression of CAR-T cells through the PD-1/PD-L1 axis. Therefore, GM-CSF neutralization, STAT3 inhibition or PD-L1 blockade might represent alternative targets to limit the impact of MDSCs in humans (65, 68). The combination of CAR-T cells with compounds such as polyinosinic-polycytidylic acid (poly I:C), all-trans retinoic acid (ATRA), gemtuzumab ozogamicin (GO) or sunitinib also resulted in improved antitumor efficacy attributed

to a reduction in the content and suppressive function of MDSCs (66, 67, 69, 70).

Interestingly, some studies combining CAR-T cell therapy with anti-PD-1 or anti-4-1BB antibodies have reported a decrease in the percentage of MDSCs in the TME, correlating with improved antitumor effects (43, 71). However, mechanisms underlying MDSC depletion mediated by immune checkpoint blockade are not fully understood.

A more direct approach of depleting MDSCs by using CAR-T cell therapy is to target antigens expressed on their surface. For instance, CAR-T cells targeting tumor vasculature through VEGFR-2 were able to reduce the frequency of MDSCs in the TME, which also expressed VEGFR-2 (72). Parihar and colleagues engineered NK cells to express a chimeric activating receptor comprised of the extracellular domain of NKG2D receptor fused to the T cell signaling domain CD3ζ (73). Engineered NK cells achieved efficient depletion of MDSCs, which express NKG2D ligands, and increased the recruitment and tumor infiltration of tumor-specific CAR-T cells when given in combination.

Neutrophils can also be immunosuppressive in the context of cancer, and their presence in tumors has been associated with poor outcome (211). In a CAR-T cell therapy trial targeting CEA, increased neutrophil to lymphocyte ratios correlated with poor responses in colon cancer patients with liver metastasis (212). Like TAMs, tumor-associated neutrophils (TANs) can be generally classified into antitumorigenic "N1" or protumorigenic "N2" phenotypes (213). Although strategies to target "N2" TANs have not been reported yet in the context of CAR-T cell therapy, some of the above-mentioned strategies could be used to counteract immunosuppressive pathways common with T_{regs}, TAMs, or MDSCs.

INHIBITORY RECEPTORS AND THEIR LIGANDS

Tumor cells, tumor-infiltrating immune cells and tumor-derived exosomes frequently express an array of ligands that bind to inhibitory receptors on T cells to suppress antitumor immunity. Blocking these interactions with therapeutic antibodies, known as immune checkpoint inhibitors, releases the brakes from suppressed T cells, allowing them to recover their antitumor activity. This therapeutic approach can mediate long-term responses, especially in a subset of tumors that are infiltrated with neoantigen-specific T cells. Therapeutic antibodies targeting the inhibitory receptors CTLA-4 and PD-1 or the PD-1 ligand PD-L1 have been approved for clinical use in patients with different solid cancer types (9). Checkpoint blockade has revolutionized cancer treatment, highlighting the tremendous power of T cells in controlling solid tumors.

Among the different immune checkpoints, the PD-1/PD-L1 axis has gained increasing attention. PD-1 is expressed in the surface of activated or dysfunctional T cells, while PD-L1 is frequently expressed in the surface of tumor cells and immune cells, and can also be found in extracellular forms (214, 215). PD-L1 upregulation is mainly associated with IFN-γ release in response to T cell activation (216); however more recent findings suggest that multiple cytokines found in the TME (including IL-10, IL-1α, IL-27, and IL-32γ) can induce PD-L1 expression (217). Of note, some cancer cells can constitutively express the PD-L1 gene due to hypomethylation of its promoter, while TAMs have been reported to also express PD-L1 naturally or via trogocytosis from tumor cells (218). Expression of PD-L1 in the tumor restrain tumor infiltrating lymphocytes from full and persistent activation. Moreover, PD-L1 expression in the stroma can prevent T cells from infiltrating the tumor, excluding them to the margin of the tumor (219). Blocking the PD-1-PD-L1 interaction can promote T cell proliferation and infiltration into the tumor, and results in durable antitumor responses (219).

The success of checkpoint immune therapies targeting CTLA-4 or the PD-1/PD-L1 axis has prompted intense investigation into new inhibitory receptors, including TIM-3, LAG-3, and TIGIT. A new wave of therapeutic agents targeting these receptors are being investigated in clinical trials, with encouraging initial results (220). However, little is known about the biology of these receptors and the interactions with their ligands. TIM-3 ligands include the cell surface ligands Ceacam-1 and Phosphatidyl serine-PTdSer (221) and the soluble factors, Galectin-9 (222) and HMGB1, that are released to the TME. LAG-3 also interacts with various ligands in the TME, including MHC class II expressed in APC and tumor cells; Galectin-3 (223) and LSECtin, expressed on tumor-associated stromal cells and tumor cells; and FGL-1, a soluble factor produced in some tumors (224). TIGIT interacts with the ligands CD112 and CD155, which are expressed on APCs and tumor cells. Expression of these ligands in tumors is associated with tumor progression and inhibition of antitumor T cell responses (224–227).

Releasing the Breaks on CAR-T Cells

A promising strategy to increase the antitumor efficacy of CAR-T cells is to prevent or revert T cell dysfunction driven by engagement of inhibitory receptors with their ligands in the tumor. Upon antigen recognition, CAR-T cells up-regulate different inhibitory receptors, similarly to endogenous tumor-specific T cells. CAR-T cells isolated from xenograft tumors typically express high PD-1 levels, with a fraction of these cells co-expressing TIM-3 and LAG-3 (86, 228). Overexpression of PD-L1 by tumor cells has been shown to inhibit CAR-T cell function, while combining CAR-T cell therapy with antibodies that block the PD1/PD-L1 interaction has proved to increase the antitumor effects of each therapy alone (71, 86, 87). One study using syngeneic mouse models showed therapeutic responses when combining CAR-T cells with PD1-blocking antibodies, which was correlated with a decrease in MDSCs (71). Several ongoing clinical trials are testing the combination of CAR-T cells with anti-PD-1/PD-L1 blocking antibodies in patients with hematologic malignancies or solid tumor (NCT02414269, NCT01822652, NCT03980288, NCT03726515), with some preliminary results with small groups of patients showing safety and encouraging efficacy results (88–90).

Novel alternative approaches to target the PD-1/PD-L1 axis include the genetic modification of CAR-T cells to release a PD-1- or PD-L1-blocking scFv in the tumor (92, 93), to

express PD-1 dominant negative receptors (86), or chimeric switch receptors (94). These strategies may avoid the toxicities associated with systemic delivery of checkpoint inhibitors and bypass the requirement for repeated antibody administration. Expression of chimeric switch receptors has the advantage of converting an inhibitory signal (PD-1) into a costimulatory signal (i.e., CD28) (94). Compared to PD-1 chimeric receptors, the delivery of PD-1 or PD-L1 blocking antibodies (by combination therapy or genetic modification) offers the possibility to re-invigorate endogenous tumor-specific T cells (92), which may be required to achieve complete responses in solid tumors. In this line, combination of CAR-T cells with oncolytic viruses releasing an anti-PD-L1 mini-body locally in the tumor resulted in enhanced therapeutic effects (91). Oncolytic viruses provide a danger signal able to diminish tumor immunosuppression while inducing tumor debulking, and may be ideal partners to combine with CAR-T cells and immune checkpoint inhibitors (229).

Another strategy to counteract tumor-induced T cell inhibition is to disrupt T cell inhibitory receptors by genome editing. Several studies have demonstrated that PD-1 gene editing, using TALEN or the CRISPR/Cas9 system, can augment T cell-mediated killing *in vitro* and enhance clearance of PD-L1$^+$ tumors *in vivo* (95–97). However, reported *in vivo* results testing this strategy seem to be contradictory and conflicting. Recent studies suggest that PD-1 ablation or knockdown can accelerate T cell exhaustion, prevent memory formation and reduce long-term antitumor efficacy (230, 231). Enhanced antitumor effects with PD-1 knockout (KO) CAR-T cells are usually observed in animal experiments using tumor cell lines genetically modified to express constitutive and uniform levels of PD-L1. So, it is possible that PD-1 disruption is only beneficial in tumors with high PD-L1 tumor densities. Different clinical trials are actively testing PD-1 KO engineered T cells for the treatment of solid tumors (NCT03747965, NCT03525782, NCT03706326, NCT03399448). A first-in-human phase 1 clinical trial has recently published the safety and feasibility of deleting three genes (TRAC, TRBC, and PDCD1, the gene encoding PD-1) using CRISPR-Cas9 in cancer-specific T cells for the treatment of patients with refractory cancer (98). Initial results in three patients demonstrated engraftment of PD-1–deficient T cells with no evidence of autoimmunity or T cell genotoxicity. Surprisingly, it was found that, in one patient, the percentage of tumor-specific T cells with mutations in the PD-1 locus decreased from 25% in the infusion product to 5% 4 months post-infusion. While further investigations are required to interpret these results, loss of PD-1 edited T cells would be consistent with mouse studies highlighting the role of PD-1 in preserving T cells from overstimulation and terminal differentiation. In this same line, initial reports have established the feasibly of knocking out other inhibitory receptors, such as CTLA-4 or LAG-3, but it remains unclear as to whether these modifications result in enhanced CAR-T cell activity (99, 100). A better understanding on the mechanisms by which inhibitory receptor negatively regulate T cell function together with preclinical models that better recapitulate the TME are required to design the next-generation CAR-T cell therapies.

CONCLUSIONS AND FUTURE DIRECTIONS

Unprecedented durable responses in cancer patients treated with checkpoint blockade antibodies or CAR-T cell therapy is generating considerable optimism. Augmenting the therapeutic outcome of CAR-T cell therapy in the context of solid tumors represents the next big challenge and opportunity for the field. Clearly, a major obstacle for CAR-T cells in solid tumors is the immunosuppressive TME. There is now an understanding that physical barriers and stromal and immune cells that express and release an array of immunosuppressive molecules limit CAR-T cell persistence and efficacy. In these hostile circumstances, strategies aimed at remodeling the tumor microenvironment or conferring intrinsic CAR-T cell resistance to immunosuppression may be more promising than targeting only one specific pathway. The cellular component of TME is characterized by considerable diversity and a high degree of plasticity (232, 233). Several strategies directed to regulating this plasticity and reversing immunosuppression are being explored. Armored CAR-T cells expressing proinflammatory cytokines or combination of CAR-T cells with oncolytic viruses could serve this purpose (234). Gene ablation technology will allow CAR-T cells to avoid immunosuppresssive signals in the TME. By a different approach, direct elimination of stroma or immune suppressive cells could revert immunosuppression, tackling different pathways simultaneously. Ongoing efforts seek to develop a new generation of CAR-T cell therapies targeting fibroblasts, T_{regs}, M2 macrophages or MDSCs.

Other factors such as the effect of gut microbiota on response to immune therapies might be also considered. It has been recently reported by many groups that microbiome composition modulates the antitumor response to immune checkpoint inhibitors. This effect is described to be mediated by IL-12 and to correlate with a decrease of T_{regs} and MDSCs in the TME (235). Similar observations have been made in preclinical mouse studies in the context of adoptive cell transfer therapy (236). In the field of CAR-T cell therapy, a preliminary study of microbiota composition in cancer patients prior to CAR-T cells infusion found a correlation between the presence of certain bacterial families and efficacy and toxicity of the therapy (237). This observation warrants future consideration of strategies such as the use of specific antibiotics or fecal microbial transplantation in combination with CAR-T cell therapy (238).

One of the greatest challenges in developing effective and safe CAR-T cells that tackle the TME is the lack of clinically relevant models that reflect the challenges of solid tumors. Currently available preclinical models have been unable to predict the toxicities observed in clinical trials and the lack of antitumor activity, especially in patients with solid tumors. Advanced preclinical models relevant to study the impact of tumor heterogeneity and the role of the TME in CAR-T cell efficacy are required to test the next-generation of CAR-T cells

as monotherapy or in combination with other agents. The testing of such CAR-T cell approaches in canines with spontaneous solid cancer represents a promising avenue of investigation (239). Current clinical studies will hopefully reveal information on the safety and efficacy of novel CAR-T cell approaches, including those addressing barriers of the TME. Lessons learned from these early-phase clinical trials will be important to continue to develop novel CAR-T cell therapies for the treatment of solid tumors.

AUTHOR CONTRIBUTIONS

SG and AR-G conceptualized, wrote, and edited the manuscript. AP and EN-O wrote and edited the manuscript. AR-G and AP designed the figures. DP edited the manuscript.

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Conflict of Interest: DP and SG are inventors on patents related to CAR-T cell therapy, filed by the University of Pennsylvania and licensed to Novartis.

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Next-Generation Manufacturing Protocols Enriching T_{SCM} CAR T Cells Can Overcome Disease-Specific T Cell Defects in Cancer Patients

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Chimeric antigen receptor (CAR) T cell expansion and persistence emerged as key efficacy determinants in cancer patients. These features are typical of early-memory T cells, which can be enriched with specific manufacturing procedures, providing signal one and signal two in the proper steric conformation and in the presence of homeostatic cytokines. In this project, we exploited our expertise with paramagnetic beads and IL-7/IL-15 to develop an optimized protocol for CAR T cell production based on reagents, including a polymeric nanomatrix, which are compatible with automated manufacturing *via* the CliniMACS Prodigy. We found that both procedures generate similar CAR T cell products, highly enriched of stem cell memory T cells (T_{SCM}) and equally effective in counteracting tumor growth in xenograft mouse models. Most importantly, the optimized protocol was able to expand CAR T_{SCM} from B-cell acute lymphoblastic leukemia (B-ALL) patients, which in origin were highly enriched of late-memory and exhausted T cells. Notably, CAR T cells derived from B-ALL patients proved to be as efficient as healthy donor-derived CAR T cells in mediating profound and prolonged anti-tumor responses in xenograft mouse models. On the contrary, the protocol failed to expand fully functional CAR T_{SCM} from patients with pancreatic ductal adenocarcinoma, suggesting that patient-specific factors may profoundly affect intrinsic T cell quality. Finally, by retrospective analysis of *in vivo* data, we observed that the proportion of T_{SCM} in the final CAR T cell product positively correlated with *in vivo* expansion, which in turn proved to be crucial for achieving long-term remissions. Collectively, our data indicate that next-generation manufacturing protocols can overcome initial T cell defects, resulting in T_{SCM}-enriched CAR T cell products qualitatively equivalent to the ones generated from healthy donors. However, this positive effect may be decreased in specific conditions, for which the development of further improved protocols and novel strategies might be highly beneficial.

Keywords: CAR T, CAR T cell manufacturing, CAR T cell fitness, CAR design, patient samples, B-ALL and PDAC

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INTRODUCTION

Chimeric antigen receptor (CAR) T cell therapy for B-cell tumors has so far gained impressive clinical results, leading to unprecedentedly high complete remission rates in patients resistant to standard treatments (1–4).

However, frequent relapses in treated patients, together with inability to achieve complete remission in other disease types (4–7), underline that additional efforts at the preclinical level are needed to improve the efficacy of this innovative therapeutic strategy (4, 8, 9). Recent clinical experience has clearly indicated that CAR T cells have to accomplish several features to sustain an effective and long-lasting anti-tumor response. In particular, CAR T cell expansion immediately after infusion and long-term persistence after initial tumor control represent crucial efficacy determinants (10). It has become increasingly evident that these properties can be enhanced by enriching early memory CAR T cell subsets, e.g., stem cell memory (T_{SCM}) and central memory (T_{CM}) T cells, by reducing the expression of inhibitory and exhaustion markers and by activating metabolic programs that foster oxidative phosphorylation and fatty acid oxidation (4, 11). In addition, especially when dealing with solid tumors, CAR T cells need to traffic to the tumor sites, recognize tumor cells and expand in an extremely immunosuppressive environment (9, 12). Therefore, proper attention should be dedicated to the creation of CAR T cell products capable of facing multiple challenges at a time, depending on tumor context and disease-specific factors. Hence, the capability of manufacturing protocols to shape the final CAR T cell product so that it could succeed in all these aspects currently represents one of the major goals of pre-clinical research in the field.

T cell fitness can be influenced by multiple factors, such as patients' features, like disease histology, age, prior treatments and the presence of a hostile microenvironment, which can compromise proper functionality of the T cell compartment (4, 13, 14). This is the case of chronic lymphoblastic leukemia (CLL), where baseline T cell dysfunction seems to be the primary cause of resistance to CAR T cell therapy, which proved effective in only 15–30% of patients (3, 5, 6) if not combined with other drugs, such as ibrutinib, which significantly increased response to treatment (15). In particular, it has been reported that T cells from non-responding CLL patients, either contained in the apheresis or in the final CAR T cell product, have a metabolic, phenotypic and transcriptomic signature associated with T cell exhaustion and late memory (11). Moreover, a reduction in the initial naïve T cell (T_N) content has been reported in several tumor contexts already at the diagnosis and even more pronounced after repeated chemotherapy cycles, resulting in the failure to generate productive CAR T cell formulations (16). Accordingly, ∼10–20% of therapeutic failures are still due to hurdles in the manufacturing process (17), with CAR T cell productions still skewed toward those patients displaying an absolute lymphocyte count around the physiological range, i.e. ∼1200 cells/uL (18, 19).

In keeping with this, procedures for T cell manufacturing have evolved over time in order to generate CAR T cells highly enriched in T_{SCM} and T_{CM} and endowed with improved *in vivo* fitness. This goal has been accomplished by providing both signal one and signal two in the proper steric conformation, e.g., through cell-sized beads (20) or polymeric nanomatrices (21), and in the presence of homeostatic cytokines, such as IL-7 and IL-15. More recently, it has been described that the activation of pre-selected T_N cells in the presence of specific cytokines (22, 23), Wnt agonists (13) or antioxidant molecules (24) could further improve the quality of the final T cell product (10).

Presently, standardized protocols for CAR T cell manufacturing are still missing, with the overall processes being extremely complex, as comprising multiple handling steps, each one capable of causing operator errors, compromising the overall reproducibility. On the contrary, efficient clinical translation of CAR T cell therapies would require the development of optimized and automated protocols, which allow to reduce costs and errors, while increasing standardization and reproducibility. In this regard, the TransAct T cell activation reagent, a polymeric nanomatrix agonist for CD3 and CD28, has been recently reported to be compliant to good manufacturing procedures (GMP) guidelines and compatible with the CliniMACS Prodigy device (17, 25), which permits the enrichment of cellular products under a closed and standardized system (17).

In this manuscript, we exploited the best-performing T cell activation protocol developed in our institution (20, 22, 23, 26), which is based on αCD3/αCD28 paramagnetic beads and IL-7/IL-15, for setting up a nanomatrix-based procedure compliant with automated CAR T cell manufacturing. We investigated the effect of this protocol on different T cell sources, either derived from healthy donors (HDs) or patients suffering from B-cell acute lymphoblastic leukemia (B-ALL) and pancreatic ductal adenocarcinoma (PDAC). These analyses revealed that CAR T cells generated with nanomatrix and paramagnetic beads are comparable and equally enriched in T_{SCM}, whose frequency in the manufactured product was found to positively correlate with anti-tumor activity in xenograft mouse models. Moreover, we observed that T cells derived from B-ALL and PDAC patients are differently responsive to the manufacturing procedure, indicating the existence of intrinsic T cell defects that, depending on tumor of origin, patients' age and previous treatments, require or not the development of additional strategies to be efficiently overcome.

MATERIALS AND METHODS

Primary T Cell Culture, Transduction and Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Lymphoprep, Sentinel Diagnostics). Peripheral blood lymphocytes (PBLs) were sorted with the CD4 and CD8 Isolation Kits (Miltenyi Biotec). PBLs were activated with αCD3/αCD28 beads (Dynabeads, Thermofisher CTS™ Cell Therapy Systems) or αCD3/αCD28 nanomatrix (T-Cell TransAct Reagent, Miltenyi Biotec) according to the following procedures. *αCD3/αCD28 beads*: PBLs were stimulated at the 3:1 bead:cell ratio and lentivirally (LV)-transduced at day 2 (multiplicity of infection, MOI: 5). Beads were removed by magnetic separation at day 6 and cells were expanded till day 14, when they were cryopreserved until

TABLE 1 | List of B-cell acute lymphoblastic leukemia (B-ALL) patient samples.

Patient#	Tumor	Age	Sex	CT cycles	Administered drugs
1	B-ALL	36	M	4	Metotrexate-Cytarabine
2	B-ALL	18	M	1	Vincristine-Idarubicin-Dexamethasone-Asparaginase
3	B-ALL	19	M	6	Vincristine-Idarubicin-Dexamethasone-Cyclophosphamide- Cytarabine-6Mercaptopurine
4	B-ALL	20	F	7	Clofarabine-Cyclophosphamide-Etoposide
5	B-ALL	62	M	6	Metotrexate-Cytarabine
6	B-ALL	21	F	9	Mitoxantrone-Cytarabine
7	B-ALL	37	M	7	Metotrexate-Cytarabine
8	B-ALL	55	F	11	Blinatumomab
9	B-ALL	29	M	5	Metotrexate-Asparaginase-6Mercaptopurine
10	B-ALL Ph+	54	M	8	Dasatinib-Vincristine-Idarubicin-Dexamethasone-Prednisone
11	B-ALL Ph+	58	M	8	Metotrexate-Cytarabine
12	B-ALL Ph+	43	M	3	Imatinib-Metotrexate-Cytarabine
13	B-ALL Ph+	35	M	3	Imatinib-Metotrexate-Cytarabine

B-ALL Ph+, B-ALL positive for Philadelphia chromosome translocation; CT, chemotherapy.

TABLE 2 | List of pancreatic ductal adenocarcinoma (PDAC) patient samples.

Patient#	Tumor	Age	Sex	Last treatment
1	PDAC	76	M	none
2	PDAC	N/A	N/A	none
3	PDAC	57	M	none
4	PDAC	N/A	N/A	none
5	PDAC	66	F	none
6	PDAC	47	M	none
7	PDAC	73	M	none

N/A, not available.

use. *αCD3/αCD28 nanomatrix*: PBLs were stimulated with the nanomatrix according to manufacturing instructions and LV-transduced at day 1 (MOI: 5). The nanomatrix was removed by centrifugation at day 2 and cells expanded till day 14, when they were cryopreserved until use. In both protocols, T cells were cultured in the TexMACS medium (Miltenyi Biotec) supplemented with 1% penicillin/streptomycin (100 U/ml and 0,1 mg/ml, Euroclone), IL-7 (25 U/ml, Miltenyi Biotech) and IL-15 (50 U/ml, Miltenyi Biotec) in the presence of 3% fetal bovine serum (FBS, Carlo Erba). Only experiments showed in **Supplementary Figure 1** were performed in serum-free conditions.

Buffy coats from healthy donors were obtained after written informed consent. All patients signed informed consent forms approved by the Ospedale San Raffaele Ethics Committee, in accordance with the declaration of Helsinki. B-ALL samples were selected on the basis of the disease classification (type B), a leukemic blast content inferior to 50% in peripheral blood (PB) and no prior transplantation. Patients' characteristics are summarized in **Tables 1**, **2**.

Cell Lines

Cell lines were cultured in RPMI 1640 (Lonza) supplemented with 10% FBS (Carlo Erba), 1% penicillin (100 U/ml, Euroclone)/streptomycin (0,1 mg/ml, Euroclone) and 1% L-glutamine (2 mM, Euroclone).

For *in vivo* experiments, RAJI, NALM-6 and BxPC-3 cell lines were transduced with a bidirectional lentiviral vector encoding for the secreted Gaussia luciferase Lucia (Invivogen) and the LNGFR selection marker, which allowed the isolation of transduced cells. The lentiviral bidirectional construct was kindly provided by Prof. Luigi Naldini. The Gaussia Luciferase is actively secreted by cells and detectable in the blood allowing the easy monitoring of tumor progression in mice (27).

Vector Constructs

The expression cassettes for both the CD19 and EGFR CARs are comprised in bidirectional lentiviral vectors provided by Miltenyi Biotech in the context of the European project horizon 2020-CARAT. The vectors contain the second-generation CAR linked to the selection marker NGFR by means of a sequence encoding a 2A element. The different CARs are composed by an extracellular domain derived from the single chain fragment variable (scFv) of a monoclonal antibody directed against the CD19 (FMC63) or EGFR (high affinity: Cetuximab, low affinity: Nimotuzumab) antigens, linked to the CD3z chain of the TCR complex by means of a CD8 spacer and transmembrane domain, together with the 4-1BB co-stimulatory domain.

Co-culture Assays

CAR T cells and un-transduced (CTRL) effector cells were co-cultured at different effector to target (E:T) ratios with tumor cells ($CD19^+$ RAJI, NALM-6 and BV173 cells; $CD19^-$ MM.1S cells; $EGFR^+$ BxPC3). After 4 days, CAR T cells and target cells were discriminated and counted by fluorescence-activated cell sorting (FACS) analysis, using cell-specific markers and Flow-Count Fluorospheres (BeckmanCoulter).

The elimination index (EI) was calculated as follows: 1–(number of residual target cells in presence of CAR T cells/number of target cells in presence of control T cells). Supernatants were collected after 24 h of co-culture to analyze cytokine release with the Th1/Th2 LEGENDplex assay (Biolegend), according to manufacturer's instructions. Data were analyzed with the software provided by the kit and subsequently with Prism software 8.1.1 (GraphPad).

In vivo Experiments

All mouse experiments were approved by the institutional animal care and use committee (IACUC) of San Raffaele University Hospital and Scientific Institute and by the Italian Governmental Institute of Health (Rome, Italy).

Six to 8-week-old female or male NOD.Cg-Prkdcscid IL-2rgtm1Wjl/SzJ (NSG) mice were infused intravenously with 0.5

$\times 10^6$ Lucia$^+$/LNGFR$^+$ RAJI or Lucia$^+$/LNGFR$^+$ NALM-6 cells and, after 7 and 4 days, respectively, treated with 4×10^6 and 3×10^6 CD19 CAR T cells from HDs, patients or CTRL. The same experimental setting was used by injecting 0.5×10^6 Lucia$^+$/LNGFR$^+$ BxPC3 cells. In this case, NSG mice were treated with 5×10^6 CTRL and EGFR CAR T cells obtained from PDAC patients. Tumor progression was monitored twice a week by bioluminescence, using the QUANTI-Luc detection reagent (InvivoGen) and expressed as relative light units (RLUs), according to the manufacturer instructions. Circulating human T cell counts were measured by FACS using Flow-Count Fluorospheres (BeckmanCoulter). Mice were sacrificed when tumor growth reached the threshold value of 10^6 RLU for NALM-6 and BxPC3 and 0.5×10^5 for RAJI, or when manifesting signs of suffering.

Flow Cytometry

T cell basal and post-activation phenotypes were characterized by flow cytometry staining with FITC, PE, PerCP, PeCy7, APC, APC-H7, Pacific Blue, BV510 conjugated antibodies, and analyzed with a FACS Canto II flow cytometer (BD Biosciences). CAR T cells and mouse samples were stained with one or more of the following conjugated monoclonal antibodies: anti-human CD3 PB (Biolegend, clone HIT3a), CD45 BV510 (Biolegend, clone HI30), CD271 PE-Cy7 (Biolegend, clone CD40-1457), CD271 PE (BD, clone C40-1457), CD4 FITC (Biolegend, clone SK3), CD14 APC (Biolegend, clone M5E2), CD19 APC/Cy7 (Biolegend, clone HIB19), HLA-DR APC/Cy7 (Biolegend, clone L243), CD45RA FITC (Biolegend, clone HI100), CD62L APC (Biolegend, clone DREG-56), CD8 PerCP (BD, clone SK1), CD57 APC/Cy7 (Milteny, clone TB03), CD127 PE (Biolegend, clone A019D5), EGFR PE (Biolegend, clone AY13), CD95 (Fas/APO-1) PE-Cy7 (Biolegend, clone DX2), CD279 (PD-1) PE-Cy7 (Biolegend, clone EH12.1), TIM-3 Alexa Fluor 488 (Biolegend, clone F38-2E2), and anti-mouse CD45 PerCP (Biolegend, clone 30-F11). 7-Aminoactinomicin D (7-AAD, Biolegend) and DAPI were used to discriminate viable and non-viable cells.

All data were analyzed with the Flow Jo_V10 software (Tree Star Inc.).

Statistical Analyses

Statistical analyses were performed with Prism Software 8.1.1 (GraphPad). Data are shown as Mean ± SEM with at least $n = 3$ replicates. Datasets were analyzed with paired/unpaired Student's *t*-test or one way/two-way ANOVA tests, depending on the experimental design considered. Differences with a P value < 0.05 were considered as statistically significant.

RESULTS

Nanomatrix- and Paramagnetic Beads-Based Protocols Generate Similar CAR T Cell Products

In order to verify the robustness of nanomatrix-based CAR T cell manufacturing, we compared it with the best-performing procedure developed in our laboratory, based on activation with paramagnetic beads and culture with homeostatic cytokines (20, 23, 26, 28). While in one platform the αCD3 and αCD28 antibodies are covalently coupled to cell-size magnetic beads, in the other they are rather embedded in a small, polymeric and biodegradable nanomatrix, leading thus to an easier and faster removal passage compatible with CliniMACS Prodigy device (29). Briefly, peripheral blood (PB) T lymphocytes from HDs were stimulated with either the nanomatrix or paramagnetic beads, transduced with a lentiviral vector encoding for a 4-1BB co-stimulated CD19 CAR (CD19.BBz CAR), and cultured in the presence of low concentrations of IL-7 and IL-15. To get close to GMP-grade manufacturing, T cells were kept in culture in serum-free TexMACS medium. Interestingly, both T cell populations expanded similarly up to day 14, while at later times points CAR T cells generated with paramagnetic beads acquired a significant proliferative advantage (**Supplementary Figure 1A**). Of notice, transduction efficiency was slightly but significantly higher after stimulation with paramagnetic beads than with the nanomatrix. This discrepancy could be explained by a different activation kinetic, which was anticipated and milder in the case of the nanomatrix compared to paramagnetic beads (**Supplementary Figure 1B**). Moreover, we observed that the addition of a small amount of FBS significantly increased fold expansion of CAR T cells generated with the nanomatrix (**Supplementary Figure 1C**). For these reasons, we modified both the manufacturing procedures by implementing 3% serum to the TexMACS medium and by anticipating lentiviral transduction of nanomatrix-activated T cells from day 2 to day 1.

Under these optimized conditions, CAR T cells generated with the nanomatrix expanded similarly to CAR T cells obtained with paramagnetic beads and achieved comparable transduction efficiencies (**Figure 1A**). An equivalent CD4/CD8 ratio was reached as well, together with a substantial enrichment of early memory T cell compartments, including both T_{SCM} (CD45RA$^+$CD62L$^+$CD95$^+$) and T_{CM} (CD45RA$^-$CD62L$^+$) in the absence of relevant PD-1 expression (**Figure 1B**). When challenged against CD19$^+$ targets *in vitro*, both CAR T cell products displayed a similar cytotoxic activity (**Figure 1C**), even though CAR T cells generated with the nanomatrix featured a higher proportion of IFN-γ$^+$/IL-2$^-$ cells compared to the ones obtained with paramagnetic beads, pointing toward a stronger effector signature of the formers (**Figure 1D**). Most importantly, when challenged in NSG mice against CD19$^+$ RAJI lymphoma cells, CAR T cells manufactured with either the nanomatrix or paramagnetic beads mediated a comparable anti-tumor activity and displayed similar expansion kinetics, resulting in super-imposable survival curves (**Figure 1E**).

Collectively, these results indicate that CAR T cells generated with reagents compatible with automated manufacturing via the CliniMACS Prodigy are phenotypically and functionally equivalent to CAR T cells generated with paramagnetic beads.

Optimized Manufacturing Can Rescue the Phenotype of T Cells Derived From B-ALL, but Not PDAC Patients

Afterwards, we investigated the ability of the nanomatrix-based manufacturing procedure to generate fully functional CAR T cells from patients suffering from B-ALL and PDAC. Patients' characteristics are listed in **Tables 1**, **2**.

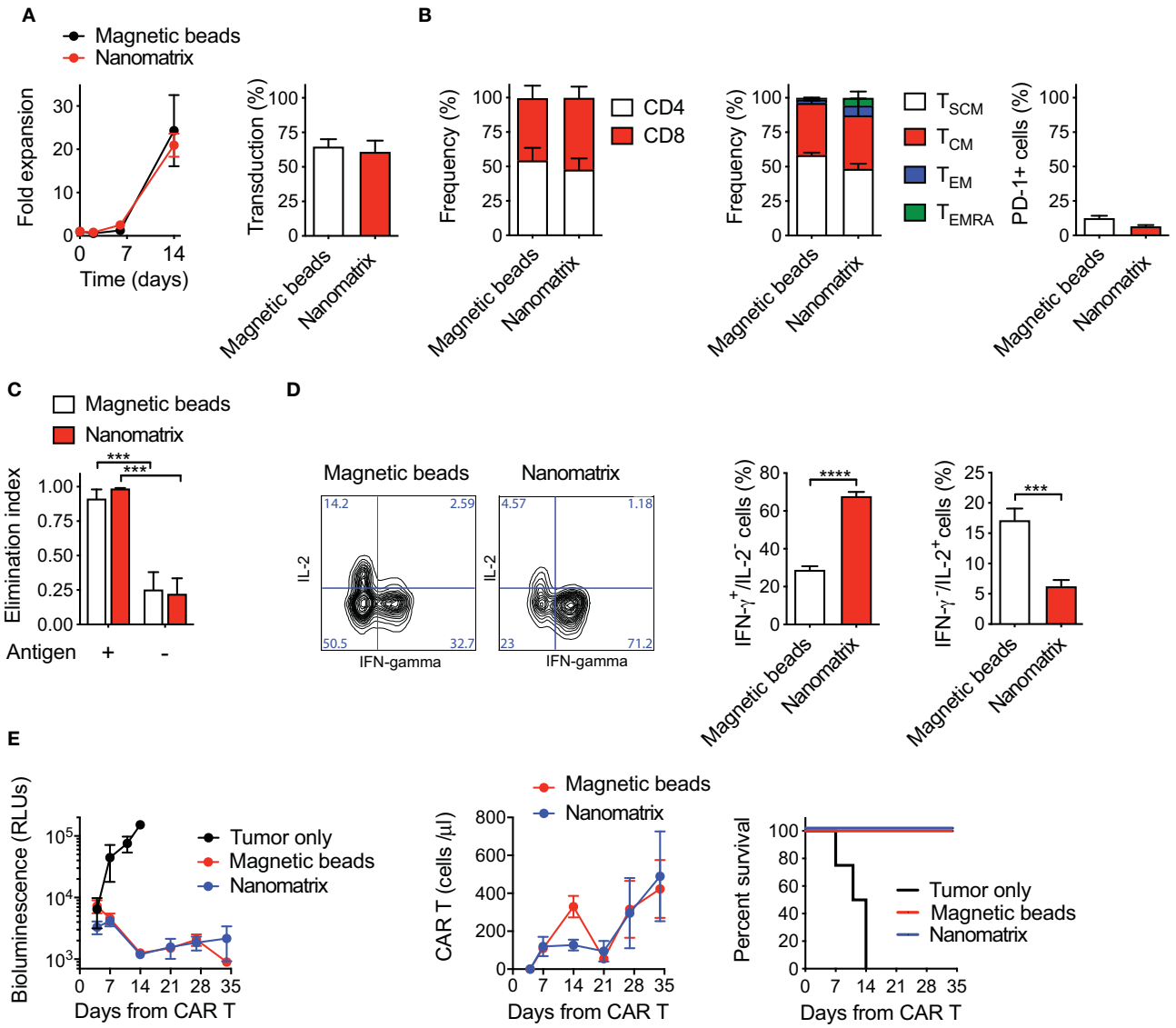

FIGURE 1 | CAR T cells generated with either the nanomatrix or paramagnetic beads are biologically equivalent. **(A)** Analysis of fold expansion in culture and transduction efficiency at day 8 of both nanomatrix- and paramagnetic beads-generated CD19.BBz CAR T cells ($n = 3$). **(B)** Phenotypical characterization of CAR T cells generated with both the nanomatrix and paramagnetic beads protocols at the end of the culture period, illustrating the CD4/CD8 ratio (left, $n = 5$), T cell memory compartments (middle, $n = 5$) and PD-1 expression (right, $n = 5$). T_{SCM}: $CD45RA^+CD62L^+$; T_{CM}: $CD45RA^-CD62L^+$; T_{EM}: $CD45RA^-CD62L^-$; T_{EMRA}: $CD45RA^+CD62L^-$. **(C)** Killing assay performed by co-culturing nanomatrix- and paramagnetic beads-generated CAR T cells with $CD19^+$ BV-173 cells and $CD19^-$ MM.1S cells for 4 days at the 1:5 effector to target (E:T) ratio ($n = 3$). **(D)** Intracellular staining depicting IFN-γ and IL-2 production by nanomatrix- and paramagnetic beads-generated CAR T cells after 24 h stimulation with PMA/Ionomicin. Left panel: representative dot plots. Right panel: mean ± SEM ($n = 5$). **(E)** Left panel: bioluminescence detection of $Lucia^+/NGFR^+$ RAJI systemic growth in NSG mice after treatment with nanomatrix- and paramagnetic beads-generated CAR T cells, together with Mock control. Middle panel: CAR T cell expansion kinetic in the peripheral blood (PB) of RAJI-bearing mice, together with Mock control. Right panel: survival curve of RAJI-bearing mice treated with different CAR T cell populations or Mock control. Data are reported as Kaplan–Meyer survival plots for mice and results of a Mantel–Cox two-sided log-rank test are reported when statistically significant ($n = 6$ mice/group; $n = 4$ mice for Mock control, 1 donor). Except for survival plots, data are reported as the result of mean ±SEM and paired *t*-test and two-way ANOVA statistical analyses are reported when statistically significant (***$p < 0.001$; ****$p < 0.0001$).

Analysis of T cell composition in starting PB samples revealed a similar CD4/CD8 ratio in patients' T cells, as compared to HDs (**Figure 2A**). However, patients' T cells featured lower proportions of T_N ($CD45RA^+CD62L^+CD95^-$) and T_{SCM} ($CD45RA^+CD62L^+CD95^+$) compared to HDs (**Figure 2B**, **Supplementary Figure 2A**), together with a lower frequency of T cells expressing the IL-7Rα (CD127) (**Figure 2C**, **Supplementary Figure 2B**). Such differentiated signature, typical of patients' T cells as opposed to HDs, was observed in both the CD4 and CD8 compartments (**Supplementary Figure 3A**).

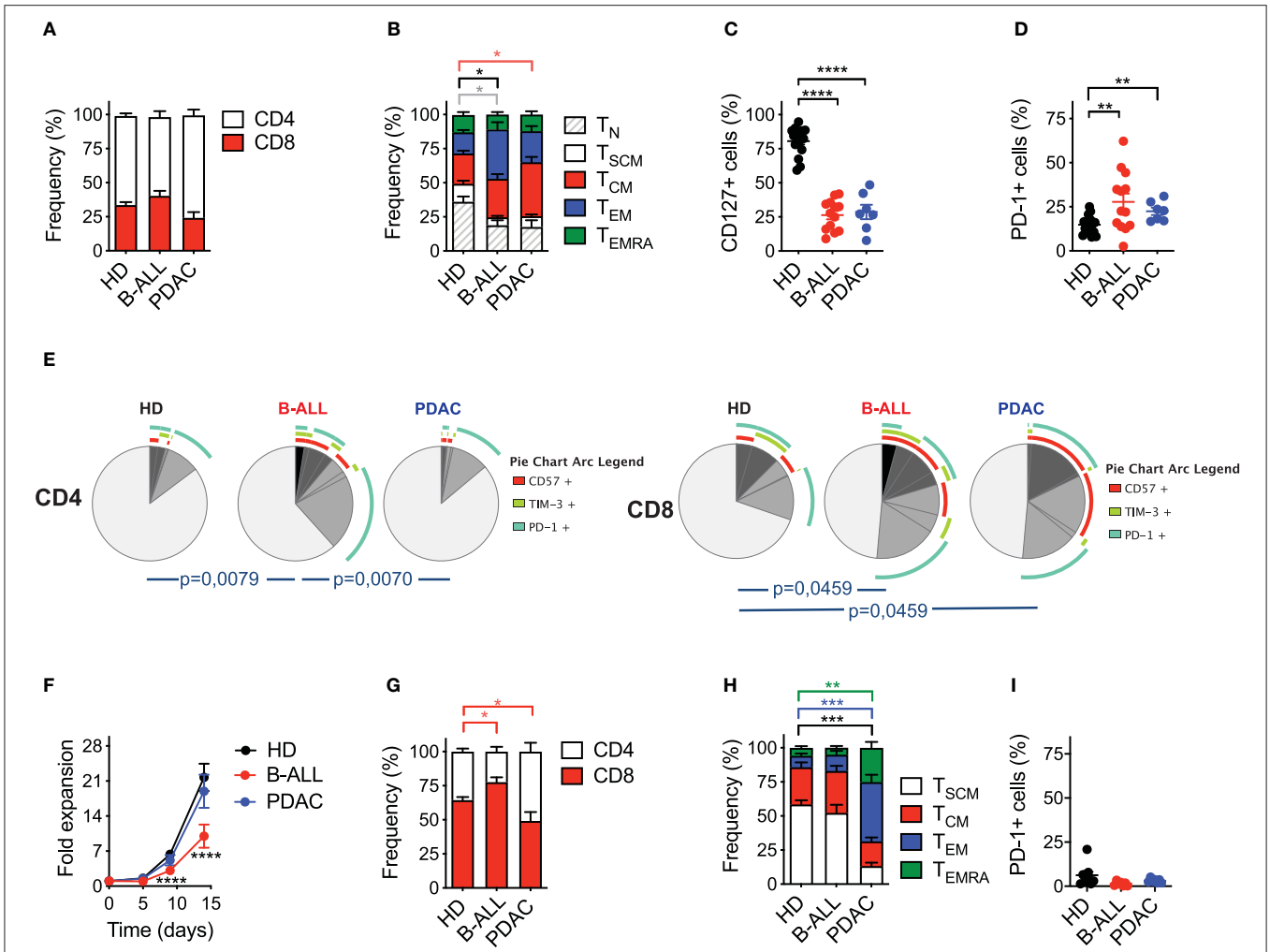

FIGURE 2 | Optimized CAR T cell manufacturing is able to rescue the phenotype of T cells derived from cancer patients in a disease-specific manner. **(A–E)** Phenotypical characterization of healthy donor- (HD), B-cell acute lymphoblastic leukemia- (B-ALL) and pancreatic ductal adenocarcinoma (PDAC)-derived T cells in PB prior activation with the nanomatrix protocol, illustrating **(A)** CD4/CD8 ratio ($n = 16$ for HD; $n = 12$ for B-ALL; $n = 7$ for PDAC), **(B)** T cell memory phenotype ($n = 7$ for HD; $n = 13$ for B-ALL; $n = 7$ for PDAC), T_N: $CD45RA^+CD62L^+CD95^-$; T_{SCM}: $CD45RA^+CD62L^+CD95^+$; T_{CM}: $CD45RA^-CD62L^+$; T_{EM}: $CD45RA^-CD62L^-$; T_{EMRA}: $CD45RA^+CD62L^-$, **(C)** CD127 expression ($n = 16$ for HD; $n = 13$ for B-ALL; $n = 7$ for PDAC) and **(D)** PD-1 expression ($n = 16$ for HD; $n = 13$ for B-ALL; $n = 7$ for PDAC). **(E)** Analysis of PB T cell exhaustion by means of SPICE algorithm calculated on both CD4 and CD8 compartments, depicting concomitant expression of inhibitory and senescence markers (PD-1, TIM-3 and CD57, $n = 7$ for HD; $n = 11$ for B-ALL; $n = 7$ for PDAC). **(F)** Fold expansion in culture of HDs and patient-derived T cells after activation with the nanomatrix protocol and transduction with a CD19.BBz CAR ($n = 10$ for HD; $n = 7$ for B-ALL; $n = 7$ for PDAC). **(G–I)** Phenotypical characterization of HDs and patient-derived CD19.BBz CAR T cells after activation with the nanomatrix protocol, illustrating **(G)** the CD4/CD8 ratio, **(H)** T cell memory phenotype and **(I)** PD-1 expression ($n = 12$ for HD; $n = 7$ for B-ALL; $n = 7$ for PDAC). Data are reported as the result of mean ±SEM and paired *t*-test and two-way ANOVA statistical analyses are reported when statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,****$p < 0.0001$).

Moreover, cancer patients were characterized by a higher frequency of T cells co-expressing one or more inhibitory receptors and senescence markers, such as TIM-3, PD-1 and CD57 (**Figures 2D,E** and **Supplementary Figures 3B**, **4A–C**). Interestingly, both $CD4^+$ and $CD8^+$ cells were phenotypically compromised in B-ALL patients, while the exhaustion signature was limited to the CD8 compartment in PDAC patients.

We then proceeded to assess the responsiveness of different T cell sources to the manufacturing procedure. PB T cells from both HDs and cancer patients were engineered according to the optimized protocol based on the nanomatrix reagent. To exclude potential variability deriving from different CAR constructs and target antigens, all T cell populations, including those from PDAC patients, were transduced with a CD19.BBz CAR. Interestingly, PDAC CAR T cells expanded similarly to HD CAR T cells, while B-ALL CAR T cells expanded less (**Figure 2F**). As expected, in all groups the CD8 compartment was significantly enriched (**Figure 2G**). Strikingly, however, while the optimized protocol succeeded in expanding early memory CAR T cells from B-ALL patients, it failed to enrich these subsets in PDAC patients (**Figure 2H**, **Supplementary Figure 5A**). Indeed, while more than 80% of B-ALL CAR T cells were either

T_{SCM} or T_{CM} ($CD45RA^-CD62L^+$) PDAC CAR T cells were significantly enriched in T_{EM} ($CD45RA^-CD62L^-$) and terminal effectors ($CD45RA^+CD62L^-$). On the other hand, the reduction in the frequency of PD-1^+ CAR T cells were observed in all conditions (**Figure 2I**, **Supplementary Figure 5B**), in line with the application of a protocol known to increase the overall CAR T cell fitness. Importantly, similar observations can be applied to both the CD4 and CD8 compartments (**Supplementary Figure 3C**).

Taken together, these results suggest that the employment of the nanomatrix-based manufacturing protocol, conceived for enriching early memory T cell subsets and their associated fitness, was able to rescue the phenotype of T cells derived from B-ALL but not PDAC patients, highlighting the existence of disease-intrinsic T cell defects that are differentially responsive to the manufacturing procedure.

Optimized Manufacturing Can Generate Fully Functional CAR T Cells From B-ALL, but Not PDAC Patients

To test the functional profile of CAR T cells generated from cancer patients and HDs, we challenged them in co-culture experiments against $CD19^+$ tumor cells. While B-ALL and HD CAR T cells performed similarly, the lytic activity of PDAC CAR T cells was significantly higher, possibly reflecting the more differentiated phenotype (**Figure 3A**). Interestingly, CAR T cell activation in response to target antigen encounter was superior for patients' CAR T cells compared to HDs, as observed by increased HLA-DR expression levels in these conditions (**Figure 3B**). Conversely, the production of pro-inflammatory cytokines was similar among all CAR T cell products (**Figure 3C**). The specificity of CAR T cell targeting was confirmed by lack of lysis, milder activation and minimal cytokine production in response to $CD19^-$ tumor cells (**Supplementary Figures 6A,B**).

Next, in order to assess *in vivo* activity in the B-ALL setting, we challenged the different CAR T cell populations in NSG mice infused with NALM-6 leukemia cells. Importantly, B-ALL CAR T cells were as effective as HD CAR T cells in mediating anti-tumor responses, significantly increasing the survival of treated mice. On the contrary, PDAC CAR T cells failed to efficiently counteract leukemia growth, resulting in only a mild survival prolongation (**Figures 3D,E**).

With the aim of confirming hypo-responsiveness of PDAC CAR T cells with other CAR specificities and in the pancreatic context, we generated two EGFR-targeting CARs incorporating the 4-1BB co-stimulatory domain and including either the high or low affinity scFv from cetuximab (CETU) or nimotuzumab (NIMO), respectively (30). Even in this setting, we confirmed that the optimized manufacturing procedure was not sufficient *per se* for rescuing the phenotype and functionality of CAR T cells derived from PDAC patients. Similar to what observed with the CD19 CAR, EGFR CAR T cells generated from PDAC patients significantly expanded in culture (**Figure 4A**) and were highly enriched in effectors while devoid of early memory subpopulations (**Figure 4B**), with also a similar proportion of CD4 and CD8 subsets (**Figure 4C**). Interestingly, while CETU CAR T cells exerted cytotoxic activity *in vitro*, NIMO CAR T cells did not (**Figure 4D**), suggesting that BxPC3 did not express EGFR at sufficient levels to be recognized by low-affinity CAR T cells (30, 31). This behavior was also confirmed in terms of cytokine release (**Figure 4E**). Finally, we observed that neither CAR T cell condition efficiently counteracted the growth of BxPC3 pancreatic cells in NSG mice (**Figure 4F**), supporting the notion that the activity of CAR T cells from PDAC patients is hampered by intrinsic functional defects.

Overall, these results suggest that in B-ALL optimized manufacturing procedures have the potential to overcome initial T cell defects, generating completely functional CAR T cells. Conversely, in PDAC additional improvements in the manufacturing procedures or combination with other therapeutics is needed to ameliorate the final outcome of CAR T cell therapy.

The Proportion of T_{SCM} Cells in the Final CAR T Cell Product Positively Correlates With *in vivo* Efficacy

Intrigued by the idea of better dissecting the features accounting for long-lasting CAR T cell anti-tumor responses, we retrospectively correlated CAR T cell features with therapeutic outcomes *in vivo*, collecting all data from leukemia-bearing mice treated with CD19 CAR T cells derived from HDs, B-ALL and PDAC patients. By doing this, it was possible to clearly identify two cohorts of short-term and long-term responders, according to the duration of remission after CAR T cell treatment (**Figure 5A**). Specific analysis of these cohorts revealed predominant differences between the two conditions, with long-term responders relying on superior CAR T cell expansion and higher frequencies of $CD8^+$ CAR T cells soon after infusion, followed by expansion of $CD4^+$ CAR T cells, as compared to short-term responders (**Figure 5B**).

The capability of CAR T cells to exert these functions is probably the result of intrinsic features of the final CAR T cell product. Therefore, in order to determine if a defined phenotype *in vitro* could predict a specific behavior *in vivo*, we sought to correlate this aspect with CAR T cell expansion in mice. Of relevance, the frequency of T_{SCM} in the final CAR T cell product positively correlated with CAR T cell expansion *in vivo,* supporting the notion that enriching early memory populations is a key requirement for a successful CAR T cell therapy (**Figure 5C**). In parallel, also higher frequencies of $CD8^+$ CAR T cells were found to correlate with CAR T cell expansion *in vivo,* possibly reflecting the fact that T_{SCM} are preferentially enriched in this T cell subset. On the contrary, the presence of high T_{EM} levels, as well as higher frequency of $CD57^+$ cells in the final CAR T cell composition negatively correlated with CAR T cell expansion *in vivo*.

These results confirm clinical evidences that the final therapeutic outcome strictly relies on high CAR T cell expansion

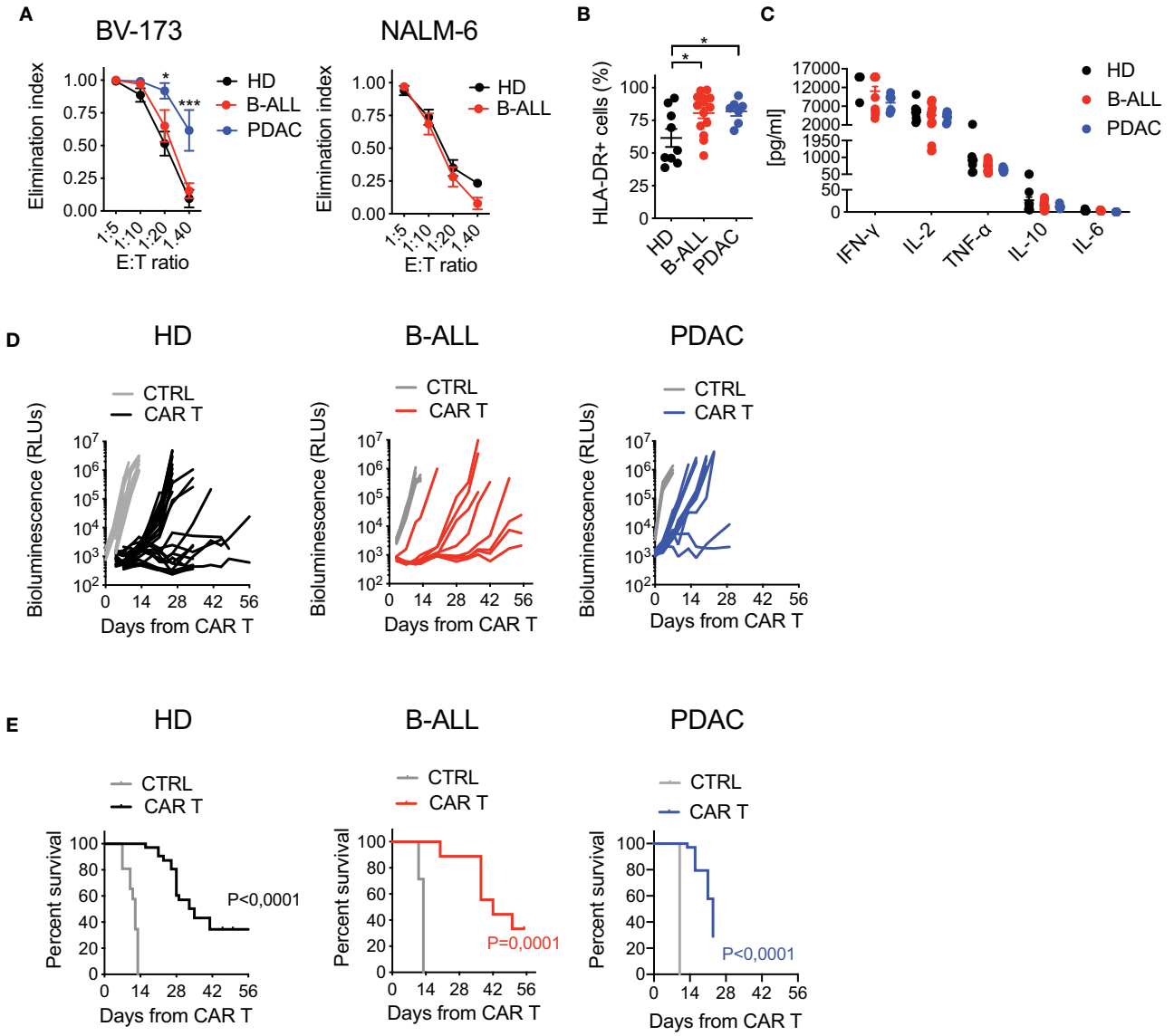

FIGURE 3 | Optimized CAR T cell manufacturing is able to generate fully functional CAR T cells from cancer patients in a disease-specific manner. **(A)** Killing assays performed by co-culturing HD- and patient-derived CD19.BBz CAR T cells with $CD19^+$ targets (BV-173 and NALM-6 cell lines) for 4 days at different E:T ratios ($n = 7$ donors/group). **(B)** Analysis of CAR T cell activation, by means of HLA-DR expression, after co-culture with $CD19^+$ target cells for 4 days at the 1:10 E:T ratio ($n = 9$ for HD; $n = 7$ for B-ALL; $n = 7$ for PDAC). **(C)** Quantification of pro-inflammatory cytokine production after 24 h co-culture of CAR T cells with $CD19^+$ targets at the 1:10 E:T ratio ($n = 9$ for HD; $n = 7$ for B-ALL; $n = 7$ for PDAC). **(D)** Bioluminescence detection of $Lucia^+/LNGFR^+$ NALM-6 systemic growth in NSG mice after treatment with 3×10^6 HD- and patient-derived CD19.BBz CAR T cells, together with Mock control ($n = 26$ mice for HD, 8 donors, and $n = 19$ mice for Mock control, 6 donors; $n = 9$ mice for B-ALL and $n = 7$ mice for Mock control, 3 patients: #5, #8, #9 in **Table 1**; $n = 12$ mice for PDAC and $n = 3$ mice for Mock control, 3 patients: #1, #2, #3 in **Table 2**). Data are reported as the result of mean ±SEM and paired *t*-test and two-way ANOVA statistical analyses are reported when statistically significant ($^*p < 0.05$, $^{***}p < 0.001$). **(E)** Survival curves of $Lucia^+/LNGFR^+$ NALM-6 leukemia bearing mice treated with HD- and patient-derived CAR T cells, together with Mock control ($n = 26$ mice for HD, 8 donors, and $n = 19$ mice for Mock control, 6 donors; $n = 9$ mice for B-ALL and $n = 7$ mice for Mock control, 3 patients: #5, #8, #9 in **Table 1**; $n = 12$ mice for PDAC and $n = 3$ mice for Mock control, 3 patients: #1, #2, #3 in **Table 2**). Data are reported as Kaplan–Meyer survival plots for mice and result of a Mantel–Cox two-sided log-rank test are reported when statistically significant.

after infusion and on a specific CD4/CD8 ratio over time (10, 32). Moreover, we observed that the frequency of T_{SCM} and $CD57^+$ cells in the final CAR T cell product can be potentially exploited as predictive positive and negative biomarkers for CAR T cell expansion and anti-tumor efficacy *in vivo*.

DISCUSSION

So far, retrospective analyses on overall response rates in patients receiving CAR T cell therapy pointed out that CAR T cell fitness is a crucial aspect for gaining therapeutic success, regardless of the tumor context (4, 10). Frequently, however,

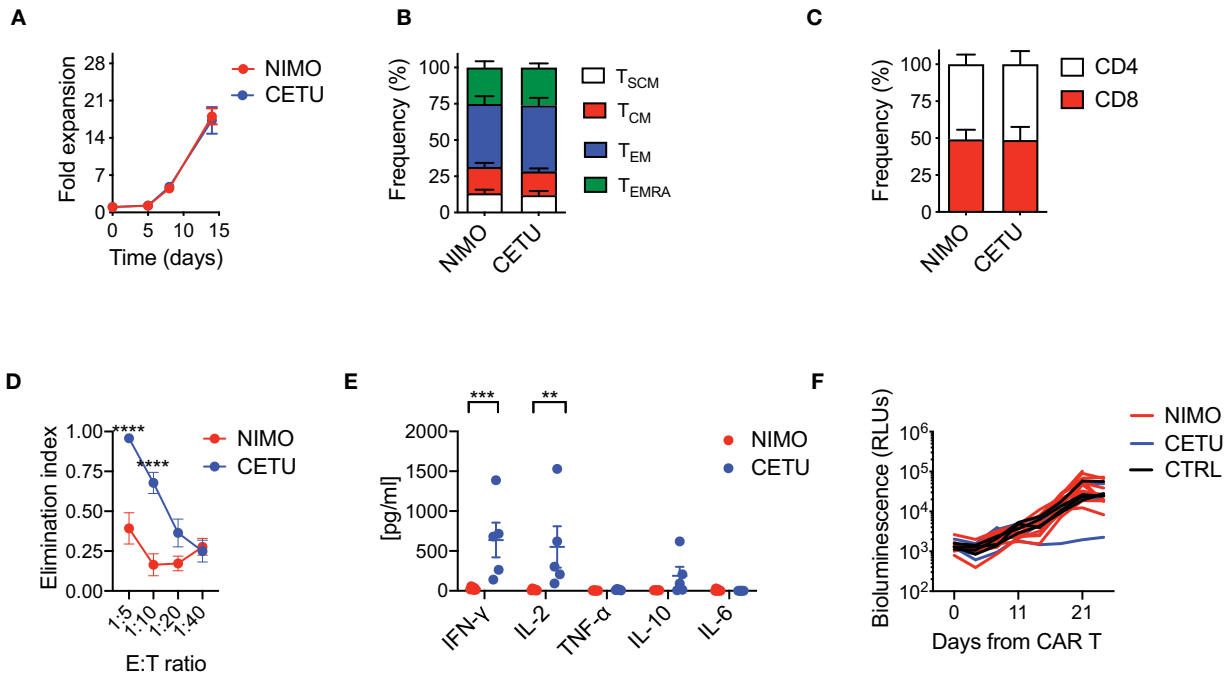

FIGURE 4 | Optimized CAR T cell manufacturing fails to generate EGFR CAR T cells effective against pancreatic cancer. **(A)** Fold expansion in culture of PDAC-derived T cells after activation with the nanomatrix protocol and transduction with EGFR.BBz CARs displaying high (CETU) and low (NIMO) affinity toward EGFR ($n = 5$ for NIMO; $n = 7$ for CETU). **(B,C)** Phenotypical characterization of PDAC-derived EGFR.BBz CAR T cells at the end of the culture period, showing T cell memory compartments and CD4/CD8 ratio ($n = 5$ for NIMO; $n = 7$ for CETU). T_{SCM}: $CD45RA^+CD62L^+CD95^+$; T_{CM}: $CD45RA^-CD62L^+$; T_{EM}: $CD45RA^-CD62L^-$; T_{EMRA}: $CD45RA^+CD62L^-$. **(D)** Killing assays performed by co-culturing PDAC-derived EGFR.BBz CAR T cells with BxPC3 $EGFR^+$ target cells for 4 days at different E:T ratios ($n = 5$ for NIMO; $n = 7$ for CETU). **(E)** Quantification of pro-inflammatory cytokine production after 24 h co-culture of CAR T cells with BxPC3 $EGFR^+$ cells at the 1:10 E:T ratio ($n = 5$ for NIMO; $n = 7$ for CETU). **(F)** Bioluminescence detection of $Lucia^+/LNGFR^+$ BxPC3 systemic growth in NSG mice after treatment with 5×10^6 EGFR.BBz CAR T cells, together with Mock control, respectively ($n = 10$ mice for NIMO, 3 patients: #2, #3, #7 in **Table 2**; $n = 4$ mice for CETU, 3 patients: #1, #3, #7 in **Table 2**; $n = 4$ mice for Mock control, patient #3 in **Table 2**). Data are reported as the result of mean ±SEM and paired *t*-test and two-way ANOVA statistical analyses are reported when statistically significant (**$p < 0.01$, ***$p < 0.001$; ****$p < 0.0001$).

the application of non-optimized manufacturing procedures failed to generate high-quality CAR T cell products, limiting the achievement of long-lasting anti-tumor responses. Moreover, long and individualized manufacturing is not always compatible for patients with high proliferative diseases and advanced stage cancers, due to the possibility of further progressing during the handling procedure (1, 8, 33, 34). Finally, as a result of limited scalability, high complexity and lack of standardization, the costs associated with the overall process still remain very high, limiting the applicability to a wide number of patients.

It has been previously reported that stimulation with αCD3/αCD28 paramagnetic beads and IL-7/IL-15 proved optimal to enrich early memory CAR T cells, especially T_{SCM}, which are endowed with improved ability to expand and persist *in vivo*, becoming particularly attractive for adoptive immunotherapeutic approaches (20, 22, 23, 26, 28). Currently, the CliniMACS Prodigy is the only technology worldwide for the clinical enrichment of cellular products within a closed system (17, 25). As a result, this device offers the unique opportunity to increase standardization and reduce the costs of CAR T cell manufacturing, by diminishing the need for highly experienced personnel and by mitigating clean room requirements. In this work, we proved that CAR T cell products generated with Prodigy-compliant reagents, including the TransAct nanomatrix, are comparable to those manufactured with paramagnetic beads and IL-7/IL-15. Indeed, both protocols proved capable to enrich CAR T cells with a stem memory phenotype, devoid of inhibitory receptors and able to mediate profound anti-tumor responses in xenograft mouse models.

To date, CAR T cell therapy was mainly applied in the autologous setting. However, autologous CAR T cell formulations suffer from several hurdles, which limit the number of patients who can effectively benefit from this therapeutic approach. First, due to a sequela of prior treatments, including chemotherapy, most patients are lymphopenic, challenging the collection of sufficient T cell numbers (8). In addition, reduced frequency of T_N has been reported in cancer patients, either at diagnosis and after chemotherapy cycles, resulting in poor performing CAR T cells and production failures (16, 35). Finally, having lived in a tumor-bearing host may have significantly compromised the fitness of patient-derived T cells, resulting in poor CAR T cell performances *in vivo* (4, 8, 9, 36). In this work, we reported initial T cell defects in both B-ALL and PDAC patients, as compared to HDs, including a reduced frequency of T_N and T_{SCM}, as well as a clear signature of T cell exhaustion, characterized by high expression of inhibitory

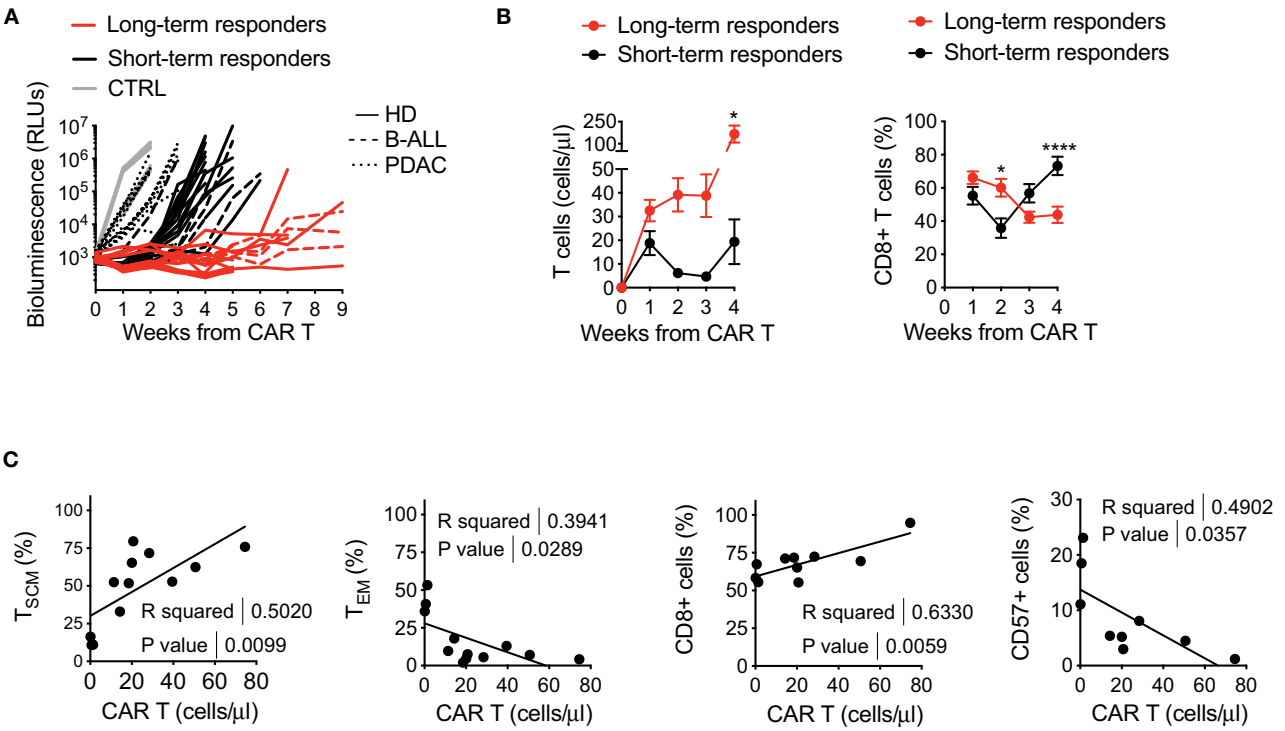

FIGURE 5 | Retrospective correlation of CAR T cell features with therapeutic outcomes *in vivo*. **(A)** Bioluminescence detection of Lucia$^+$/LNGFR$^+$ NALM-6 systemic growth in NSG mice after treatment with 3×10^6 CD19.BBz CAR T cells of HD- and patient-derived origin, retrospectively clustered as short- and long-term responders ($n = 18$ for long-term responders, red lines; $n = 29$ for short-term responders, black lines. More in detail: $n = 26$ mice for HD, 8 donors, continuous lines; $n = 9$ mice for B-ALL, 3 patients: #5, #8, #9 in **Table 1**, dashed lines; $n = 12$ mice for PDAC, 3 patients: #1, #2, #3 in **Table 2**, dotted lines; $n = 10$ mice for Mock control, 3 donors, gray lines). Data are represented as single lines identifying individual mice treated with CD19.BBz CAR T cells of HD- and patient-derived origin and differently clustered on the basis of CAR T cell anti-leukemia activity. **(B)** Left panel: analysis of CD19.BBz CAR T cell expansion kinetic (cell count/µL) of short- and long-term responders in the peripheral blood of Lucia$^+$/LNGFR$^+$ NALM-6 bearing mice; Right panel: frequency of CD8$^+$ CAR T cells during *in vivo* expansion. Data are reported as the result of mean ± SEM and two-way ANOVA statistical analyses are reported when statistically significant (*$p < 0.05$; ****$p < 0.0001$). **(C)** Relationships between CAR T cell phenotype and *in vivo* growth after leukemia encounter, correlating the initial proportion of T_{SCM}, T_{EM}, CD8$^+$ and CD57$^+$ CAR T cells with *in vivo* expansion levels at day 7 ($n = 12$ for T_{SCM}, T_{EM}, CD8 and CD57). *R* and *P*-values for each correlation are reported.

receptors and senescence markers. Strikingly, however, while in B-ALL the manufacturing procedure was able to rescue initial dysfunctions, expanding T_{SCM} CAR T cells that performed similarly to those from HDs, CAR T cells from PDAC patients were preferentially T_{EM} and failed to exert significant anti-tumor activity *in vivo*, using different CAR designs and specificities. Unfortunately, the small size of our patients' cohorts impedes to draw definitive conclusions on the reasons accounting for what observed. Reasonably, multiple factors could have been involved.

It is known that patients' age *per se* can shape T cell differentiation and senescence, resulting in an overall reduction in lymphocyte collection efficiencies and T cell fitness (14, 37). Although CAR T cell therapy in B-ALL has offered spectacular promise in children and young adults, definitive data on its effectiveness in older individuals are still awaited (38–40). Of notice, our analysis revealed that, despite initial T cell defects, the optimized manufacturing procedure succeeded in generating fully functional CAR T cell products from adult individuals suffering from B-ALL (mean age: 37, range: 18–58). Of relevance, patients with PDAC were much older (mean age: 64, range: 47–76), possibly contributing to the disappointing results obtained with PDAC samples.

Previous studies have indicated that chemotherapy-related depletion of early lineages, especially T_N, decrease the rate of successful *ex vivo* stimulation responses (16, 35). Notably, even though heavily pretreated and T_N-deficient, our cohort of B-ALL patients successfully responded to the optimized manufacturing procedure, supporting the notion that proper activation in the presence of IL-7 and IL-15 can rescue initial T cell defects (35). Conversely, our PDAC patients were chemotherapy-naïve, supporting a prominent impact of the tumor itself. Despite the lack of post-treatment PDAC samples to use as comparison, this hypothesis finds some clues in the literature. Indeed, it has been described that CD8$^+$ T cells infiltrating solid tumors exist in two dysfunctional states, either reversible or permanent, depending on the respective chromatin arrangements (41). Whether this signature is equally ascribed to solid tumor-derived T cells circulating in the PB is still a matter of debate (16, 41). Moreover, it has been recently reported that, despite already "imprinted" in the early phases of solid tumor development, T cell dysfunctions are further amplified with disease progression and severity,

observation that can be particularly relevant in the case of PDAC (42, 43). Of relevance, such dysfunctional state was only initially therapeutically reversible, then evolving in a fixed state (44). In perspective, it would be interesting to expand our analysis to other solid malignancies and hematological tumors characterized by severe intrinsic T cell defects, like CLL and acute myeloid leukemia (AML) (11, 36, 45, 46).

It has been recently described that patients with AML relapsing after hematopoietic stem cell transplantation have a higher proportion of early memory T cells, including $T_{SCM,}$ expressing multiple inhibitory receptors compared to patients achieving complete remissions (47). These evidences suggest that the exhaustion of specific T cell memory compartments could be critical to define response to the manufacturing procedures. Moreover, it is known that "quorum sensing" mechanisms between memory and naïve T cells culminate in the synchronization of T_N cell behavior to that of memory T cells, resulting in an accelerated differentiation at the transcriptional, metabolic and functional level (48). Consequently, the presence of dysfunctional antigen-experienced T cells may negatively shape the differentiation of T_N, compromising the overall quality of CAR T cell products.

Finally, even though we cannot formally rule out a role of regulatory T cells (Tregs) in poor-performing CAR T cell products, culture in the presence IL-7, which has been reported to inhibit both Treg expansion and suppressive activity (49), and the clear effector signature characterizing our cell products, suggest that Tregs are not crucially involved in our setting.

Our data point out that additional strategies are required to customize fully functional CAR T cells in specific disease conditions. One possibility is to exploit allogeneic T cell sources, which have the advantage of being unaffected by prior treatments or by the tumor itself (33). Even though this option is limited by the risk of graft versus host reactions (GVHD), gene-editing approaches aimed at eliminating the endogenous TCR are currently becoming more common (8, 33). However, further preclinical investigation is still required to improve their safety profile, therefore imposing caution. An alternative strategy can be to deeper investigate and overcome initial T cell defects in the autologous setting. For example, it is possible to further optimize the manufacturing procedure by supplementing compounds known to expand T_{SCM}, e.g., N-acetylcysteine (NAC), a reagent able to inhibit the metabolism of reactive oxygen species (24) or to pre-select definite T cell subsets as source material to get rid of more differentiated T cells. Directly inhibiting T cell exhaustion is another valuable option that can be achieved by combining CAR T cell therapy with checkpoint inhibitors (50, 51) or by additional genetic engineering of CAR T cell products (8). In particular, a recently proposed innovative strategy relies on CAR T cells over-expressing C-Jun, a transcription factor belonging to the AP-1 family, that resulted in resistance to exhaustion, enhanced *in vivo* expansion, reduced terminal differentiation and higher anti-tumor potency (52).

In this work, we also tried to identify informative efficacy biomarkers. By retrospectively analyzing CAR T cell-mediated anti-leukemia responses *in vivo*, we identified two cohorts of short- and long-term responders, characterized by relapses occurring at different interval times. Specific examination of these cohorts revealed that *in vivo* CAR T cell expansion represents a potent determinant of anti-leukemia efficacy, in accordance with clinical evidences (4, 10, 32). Moreover, in long-term responders, we observed CD8 prevalence soon after infusion, followed by predominance of $CD4^+$ CAR T cells at later time points. These peculiar dynamics can be related to the need for rapid tumor de-bulking at early phases, provided by $CD8^+$ CAR T cells, followed by the need for $CD4^+$ CAR T cell help to achieve long-term anti-tumor control. Lastly, we observed that the frequency of T_{SCM} and $CD8^+$ T cells in the final CAR T cell product positively correlated with T cell expansion *in vivo*, as opposed to the presence of more differentiated effector memory and $CD57^+$ CAR T cells, which accounted as negative contributors. These observations indicate that such features need to be fostered during CAR T cell manufacturing and can be used as predictive biomarkers for the biological quality of CAR T cell products.

Overall, our work indicates that optimized manufacturing protocols can overcome initial T cell defects typical of cancer patients, resulting in CAR T cell products qualitatively equivalent to the ones generated from healthy donors. However, our results also highlight that the rescue of proper T cell functions cannot be achieved for specific tumor types, for which the development of further improved protocols and new strategies might be highly beneficial. Moreover, the crucial role of early memory T cells, especially T_{SCM}, to achieve profound and durable anti-tumor responses *in vivo* was confirmed. Indeed, our data point out that next-generation CAR T cell manufacturing processes need to foster the enrichment of this T cell compartment, in order to significantly widen CAR T cell efficacy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the IACUC: Institutional Animal Care and Use Committee (IACUC #646). The protocol received approval by the Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

SA designed and performed experiments, analyzed data, and wrote the manuscript. LF and BC designed and performed the experiments. FD and MB performed experiments and analyzed the data and FD helped with the preparation of the figures. FG provided patient information. FC, CB, and AB actively contributed to the scientific discussion and manuscript revision. MC designed the study, analyzed and interpreted the data, wrote the manuscript, and acted as senior author of the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01217/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Engineering Cytoplasmic Signaling of CD28ζ CARs for Improved Therapeutic Functions

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Chimeric antigen receptor modified T cells (CAR-T) have yielded impressive clinical outcomes in treating hematopoietic malignancies. However, relapses have occurred in a substantial number of patients and limited the development of CAR-T therapy. Most underlying reasons for these relapses can be attributed to poor persistence and rapid exhaustion of CAR-T cells *in vivo*. Despite multiple strategies having been developed, how to improve CAR-T persistence or resist exhaustion while maintaining sufficient cytotoxic functions is still a great challenge. Here we discuss engineering cytoplasmic signaling as an important strategy for CAR optimization. This review summarizes recent advances showing that the anti-tumor function of CAR-T cells can be improved by optimizing the CD3ζ domain or downstream signaling of CD28ζ CAR.

Keywords: chimeric antigen receptor, CD3ζ, ITAM, signal strength, cytotoxicity, persistence

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INTRODUCTION

Chimeric antigen receptor modified T cells (CAR-T) therapy achieved great success against hematological malignancies. Through genetic engineering with a chimeric antigen receptor (CAR), the modified T cells can be activated by specific antigens on the surface of target cancer cells and produce anti-tumor toxicity. As the recognition of antigens or activation of downstream signals does not depend on MHC molecules, CAR-T cells are anticipated to overcome the immune escape route of cancer cells through their down-regulated expression of MHC molecules (1). CAR-T therapy achieved a milestone in 2017 when two CD19 targeted CAR-T therapies (Yescarta and Kymriah) were approved by the US Food and Drug Administration (FDA). Nowadays, many emerging preclinical and clinical studies on multiple malignancies are demonstrating the significant anti-tumor potential of CAR-T therapies (2, 3).

Despite impressive clinical outcomes, there are still limitations of CAR-T therapies. Previous clinical studies indicated that although a large proportion of patients could achieve a complete remission (CR), some of them suffered disease relapse. For relapsed/refractory B-cell acute lymphoblastic leukemia (ALL) patients, although CR rate after initial anti-CD19 CAR-T therapy is higher than 70%, ∼30 to 50% of these CR patients suffered disease relapse within 1 year of treatment (4). Among many strategies used to solve the problem of relapse, improving the persistence and attenuating exhaustion of CAR-T cells is regarded as a critical requirement for long-term tumor remission (5).

Strategies to optimize each module, especially the costimulatory domain of CARs, have been extensively reviewed elsewhere (6, 7). In this review, we will discuss recent novel strategies of CAR

optimization. Particularly we will focus on the optimization of CD3ζ domain of CD28ζ CAR and its downstream signaling in an effort to improve CAR-T cell functions.

DEVELOPMENT OF DIFFERENT CAR DESIGNS

As a CAR is designed to activate T cells in response to specific antigens, the essential CAR structure is the extracellular antigen recognition domain and the intracellular signal transduction domain. In the late 1980s, Eshhar et al. designed the first-generation CARs, which included an antibody derived single-chain variable fragment (scFv) as the antigen recognition domain, and a Fc receptor γ chain (FcRγ) or CD3ζ derived signal transduction domain (8). T cells equipped with first-generation CARs were demonstrated to recognize antigens in an MHC independent manner. However, these T cells only exhibited a weak anti-tumor activity and tended to be anergized or exhausted *in vivo* (9). The reason was attributed to the lack of costimulatory signals, which are the essential "second signal" to fully activate T cells (10). The second-generation CARs solved this problem by including an intracellular domain derived from costimulatory molecules such as CD28, ICOS, 4-1BB, OX40, or CD27. The incorporation of a costimulatory domain was a breakthrough for CAR-T therapy as it equipped CAR-T cells with potent *in vivo* anti-tumor activity (11–13). Subsequent studies have engaged in optimizing costimulatory domains for enhanced T cells activation. These studies led to the development of third-generation CARs (containing multiple costimulatory domains). In some preclinical studies, the incorporation of multiple costimulatory components has been demonstrated with improved anti-tumor functions. However, their therapeutic outcomes in recent clinical trials have shown modest benefits compared to second-generation CARs (14).

ANTI-TUMOR FUNCTIONS OF CD28ζ AND 4-1BBζ CARS

Until now, the most successful application of CAR-T therapy has been CD19-targeted CARs toward B cell malignancies such as non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL), and ALL (15). Despite different CAR designs, manufacturing, and clinical regimens, accumulated clinical trials have shown that CD19 CARs achieved 70–90% CR rates among children and adults with relapsed/refractory B-cell ALL (16).

In these clinical studies, most CARs contain either a CD28 or 4-1BB cytoplasmic domain as the costimulatory element. In the treatment of ALL, both CD28ζ and 4-1BBζ CARs achieved similar outcomes. However, in CLL and NHL, clinical trials indicated the superior efficacy of 4-1BBζ CAR compared to that of CD28ζ CAR (17). In a recent clinical study of NHL, a parallel comparison showed that both CD28ζ and 4-1BBζ CAR-T cells displayed similar anti-tumor efficacies within 3 months. However, CD28ζ, but not 4-1BBζ, CAR-T cells induced severe cytokine release syndrome (CRS) and neurotoxicity (18). These distinct clinical outcomes may be attributed to the different downstream signaling cascades invoked by the CD28 or 4-1BB cytoplasmic domain.

Endogenous CD28, as a member of the CD28 family, is known to induce signals via PI3K, NF-κB, Akt, Erk, and NFAT to regulate expression of T-bet, Eomes, and GATA3 (19). 4-1BB, as a member of the tumor necrosis factor receptor super family (TNFRSF), is known to activate downstream signals through the recruitment of TRAF proteins. Corresponding to their different signaling and regulation patterns, *in vitro* functional assays showed that CD28ζ CAR induced higher levels of released cytokines such as IL-2, IFNγ, and TNFα, and an enhanced cytotoxic effect than 4-1BBζ CAR (20). Kinetics and protein phosphorylation profile studies showed that CD28ζ CAR was more prone to activate effector T cell-associated genes, while 4-1BBζ CARs preferentially activated memory T cell-associated genes (21). In accordance with these findings, *in vivo* 4-1BBζ CAR was shown to promote the differentiation of central memory T cells, while CD28ζ CAR was more prone to promote the differentiation of effector memory T cells (17). As a result, 4-1BBζ CAR-T cells have been shown to have a superior *in vivo* persistence than CD28ζ CAR-T cells. As reported, the persistence of CD28ζ CAR-T cells is about 30 days, while 4-1BBζ CAR-T cells may exceed 4 years in some patients (17). The long persistence of 4-1BBζ CAR may be responsible for its comparable clinical efficacy with CD28ζ CAR in ALL despite its weaker cytotoxic effect (15). Collectively, the clinical outcomes of CD28- or 4-1BB-based CAR-T therapies suggest that cytotoxic effects and persistence properties are crucial factors affecting CAR-T therapeutic functions.

BALANCING EFFECTOR AND MEMORY DIFFERENTIATION DETERMINING CAR-T CELL FUNCTIONS

To understand the functions of engineered CAR-T cells, we may first review what happens to un-engineered native T cells. During T cell response to infection, recognition of a specific antigen induces naïve T cells activation, leading them into rapid proliferation and differentiation. This response will create a highly diverse T cell pool, in which the cooperation of effector and memory T cell subpopulations is indispensable for efficient antigen clearance (22). On one hand, the cytotoxic effect is mainly performed by effector T cell subsets. By producing cytokines and cytotoxic molecules, effector T cells can directly kill target cells. On the other hand, following antigen clearance most activated T cells die while a small pool of memory T cells can persist for a rapid response to the antigen re-challenge (23, 24). Particularly, in response to cancers or chronic infection, T cells have to deal with persistent antigen stimulation. In that circumstance, T cells may fail to differentiate into a memory subset and become

Abbreviations: CAR, chimeric antigen receptor; CAR-T, chimeric antigen receptor modified T cells; FDA, Food and Drug Administration; CR, complete remission; scFv, single-chain variable fragment; FcRγ, Fc receptor γ chain; NHL, non-Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; CRS, cytokine release syndrome; AICD, activation-induced cell death; ITAM, immunoreceptor tyrosine-based activation motifs; T_{CM}, central memory T cells; T_{EFF}, effector cells; T_{SCM}, stem cell memory T cells.

exhausted. The exhausted T cells lose effector functions and are unable to efficiently clear target cells (22).

The response of CAR-T cells to tumors could follow similar processes. For rapid and efficient tumor clearance, CAR-T cells need to perform effective cytotoxicity, which rely on the effector T cell subset. However, overpowering cytotoxicity can induce certain issues and impair therapeutic functions. One issue is the severe side effects including CRS, which is characterized by massive synchronized T cell activation and the release of large amounts of cytokines, and immune effector cell-associated neurotoxicity syndrome. Even though macrophages have been identified as a major source for CRS (25, 26), CAR designs that reduce effector functions of CAR-T cells have been shown to decrease CRS in patients (27). Another issue is activation-induced cell death (AICD) and exhaustion of T cells. The enhanced differentiation toward effector T cell subsets inevitably attenuates the development of a memory T cell pool (28–30). The weak memory T cell subset attenuates T cell persistence and is responsible for tumor relapse. Therefore, balancing the effector and memory function of CAR-T cells is critical for effective relapse-free anti-tumor efficacy.

As the weak persistence of CD28ζ CAR-T cells is an important reason for unfavorable clinical outcomes, improving the persistence of CD28ζ CAR-T cells while keeping their potent effector functions is a good strategy to improve their therapeutic functions. For this purpose, the signal transduction of CD28ζ CAR can be properly modified to balance T cell differentiation in response to antigen stimulation.

TCR AND CD28-BASED SIGNAL TRANSDUCTION

Currently, the precise molecular mechanism of CAR-induced T cell activation is still not well-understood. As the signal transduction of CARs largely depends on the signaling domains of the original immunoreceptors, it is assumed that the CARs transduce intracellular signals similar to endogenous TCRs and costimulatory molecules (**Figure 1**).

The TCR complex consists of a TCRαβ or TCRγδ heterodimers and a CD3 complex containing the CD3γε, CD3δε, and CD3ζζ dimers. While TCRαβ (or TCRγδ) subunits recognize antigens through their specific extracellular regions, the CD3

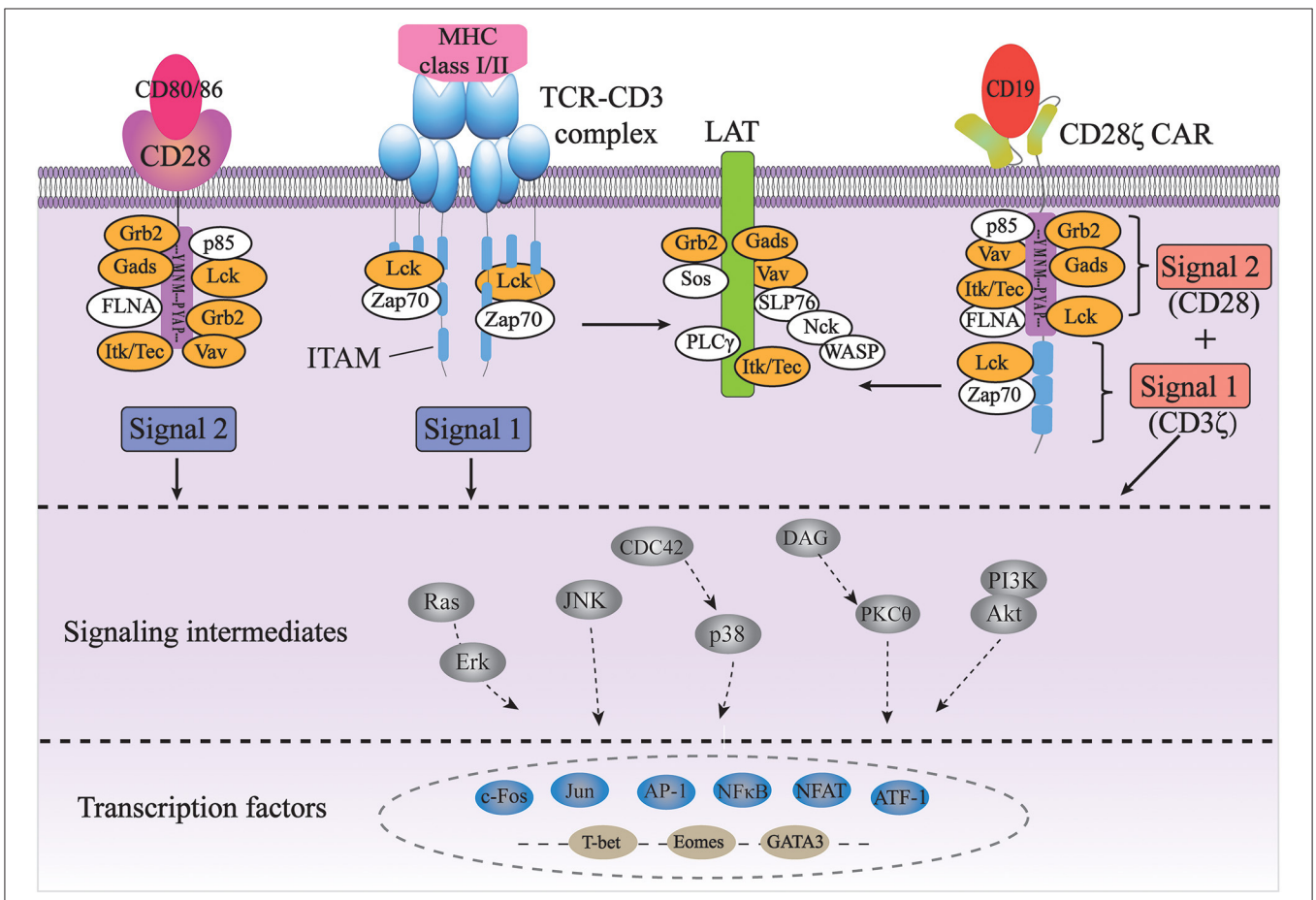

FIGURE 1 | Signal transduction of TCR and CAR for T cell activation. The full activation of T cells requires TCR signaling through the CD3 complex (signal 1) and costimulatory CD28 signaling (signal 2); CD28ζ CAR integrates with the CD3ζ and CD28 domains, transducing two signals together in an antigen-dependent manner (orange color indicates common players shared by TCR/CD3 and CD28 pathways).

complex mainly carries out signal transduction functions in the complex through its well-conserved immunoreceptor tyrosine-based activation motifs (ITAMs) (31). First identified based on their sequence homology, ITAMs consist of two consecutive YxxL/I motifs separated by a defined number of amino acids (YxxL/I-X_{6-8}-YxxL/I) (32). ITAMs are usually found in receptors expressed in hematopoietic cells and are especially well studied in the context of TCR signaling. The CD3γ, CD3δ, and CD3ε chains each contain one ITAM, while the CD3ζ chain contains three ITAMs. TCR binding to peptide-MHC leads to the activation of a Src family kinase Lck, which phosphorylates two tyrosine residues in each of the ITAMs in CD3 (33). Each bisphosphorylated ITAM then gains the ability to bind to the two tandem SH2 domains of a Syk family kinase, ZAP-70. This interaction brings ZAP-70 in close proximity to Lck, resulting in the phosphorylation and activation of ZAP-70 by Lck. Activated ZAP-70 further phosphorylates its downstream targets, such as adaptor protein LAT and SLP-76. Phosphorylated LAT and SLP-76 provide scaffolds for many other proteins, such as PLC-γ, Grb2/Sos, Gads and Itk, Vav, and Nck, eventually leading to calcium mobilization, Ras/Erk activation, actin cytoskeletal rearrangement, and ultimately activation of gene expression (31). Therefore, the ITAMs in CD3 are the major if not the only signaling moieties in TCR signaling.

Besides TCR signaling (Signal 1), full activation and expansion of T cells also requires signaling through costimulatory receptors such as CD28 (Signal 2). Interaction of CD3 with CD28 has been demonstrated to play a crucial role in modifying the endogenous TCR signal (34, 35). CD28, on the other hand, doesn't contain any ITAM. Instead, its cytoplasmic domain contains a YMNM motif that gets phosphorylated upon CD28 binding to its ligand CD80/CD86, which can bind to the p85 subunit of PI3K and Grb2/Gads. Additionally, proline-rich regions of CD28 can interact with Itk, Tec, Lck, Grb2/Vav, and FLNA (36, 37). Therefore, antigen-binding initiated TCR signaling through CD3 and CD80/86-binding initiated CD28 signaling share many common players, such as Grb2, Vav, Gads, Lck, and Itk. In addition, the activation of both pathways occurs in the signaling complexes assembled near the plasma membrane at the immunological synapse, physically bringing signaling molecules from two pathways together in space (38, 39). Last but not least, CD28-induced calcium signaling occurs seconds after TCR-initiated intracellular calcium increase, if not sooner, suggesting the temporal proximity/closeness of the two pathways (40). All of these suggest a synergetic spatiotemporal collaboration between TCR-CD3 and CD28 signaling, which contributes to the highly ordered signal transduction of T cells.

STOICHIOMETRY IMBALANCE OF CD3ζ AND CD28 SIGNALING IN CD28ζ CAR

In CD28ζ CAR, CD28 and CD3ζ domain are fused together to implement signal transduction. Therefore, it is assumed that the synergetic effect of CD3 and CD28 signaling also plays a crucial role in regulating downstream signaling and affecting T cell function. Nevertheless, when comparing CD28ζ CAR signaling to TCR signaling, some major differences are obvious due to the fusion of two cytosolic domains. First is that in CAR, CD28 signaling domain is *in cis* with CD3ζ signaling domain, while endogenous CD28 is recruited into the immune synapse and co-localized with CD3ζ *in trans*. Second, CD28 activation is concurrent with CD3ζ activation in CAR while CD28 costimulation occurs seconds after TCR ligation. Third, in human T cells, CD28 and TCR are normally expressed at $\sim 6 \times 10^4$ and $\sim 2 \times 10^4$ molecules per cell (41). Therefore, three molecules of CD28 can provide signaling support for one molecule of TCR/CD3. However, second-generation CD28ζ CAR has the design of fused CD28 and CD3ζ cytosolic domains, fixing the stoichiometry ratio of them to be 1:1. The redundancy of CD3ζ signaling may disturb the signaling homeostasis of CAR and result in improper enhancing of T cell stimulation, which accounts for the poor persistence of CAR-T cells. Balancing the costimulation signaling and activation signaling may help solve the existing overstimulation problem of CD28ζ CAR.

FINE-TUNING ITAM NUMBERS AND POSITIONS OF CD3ζ DOMAIN IN CD28ζ CAR

Although the detailed mechanisms regulating the formation of effector and memory T cell pools are still elusive, it is generally considered that signal strength is an important determinant for T cell fate (42). In native T cells, the TCR complex binds to antigens and transduces the binding across the plasma membrane to intracellular signals. It has been reported that weak TCR signals favor memory T cells differentiation, whereas strong TCR signals promote the formation of effector T cell subsets (43, 44).

Multiple ITAMs in CD3 and TCR complexes have been proposed to amplify TCR signals (31, 45). So, the number and type of signaling domains matter in TCR signal strength. In animal models, mice with fewer than seven CD3 ITAMs developed a lethal multiorgan autoimmune disease (46). Further analysis demonstrated that the efficiency of Notch1 induced c-Myc expression was reduced in T cells expressing CD3 with two or four ITAMs, which resulted in impaired cellular proliferation (47). These studies suggested a linear relationship between the number of ITAMs and the proliferative ability of naïve T cells. The type of ITAMs also matters. The three ITAMs in CD3ζ differ in their primary amino acid sequences as well as their positions relative to the plasma membrane (namely ITAM1, ITAM2, and ITAM3 from membrane proximal to distal), therefore their ability of being phosphorylated by Lck or binding to ZAP70 upon phosphorylation are different (48). Mutations of ITAM1 and ITAM2 in CD3ζ significantly impaired signal transduction and induced cell death. However, mutation of ITAM3 in CD3ζ did not induce cell death but rather increased IL-2 secretion and MAPK phosphorylation (49). Therefore, ITAMs in CD3ζ are functionally different in regulating T cell activation.

In CAR-T cells, the CAR molecules are responsible for antigen recognition and signal transduction. Therefore, it is logical to modulate T cell differentiation potentials by controlling the signal strength of CARs. Nowadays, many strategies have

been developed to optimize CAR designs. Modification of the extracellular scFv, the hinge, the transmembrane domain, and the costimulatory domains of CAR have been evaluated by multiple studies (6). However, limited works have focused on the modification of the CD3ζ domain of CAR (50, 51).

Actually, the first-generation CAR was designed to have either a CD3ζ chain or a FcRγ as the intracellular signaling component. T cells with first-generation CD3ζ CAR were later demonstrated to have a greater cytotoxicity and anti-tumor functions than those with FcRγ CAR (52). And the greater cytotoxicity was attributed to the presence of three ITAMs in CD3ζ compared to the one ITAM in FcRγ. Subsequently, the CD3ζ instead of FcRγ chain was preferentially used in the next generation CAR designs. However, in second-generation CD28ζ CARs, incorporation of CD28 costimulatory domains provides a quantitative support for downstream signaling and the signal transduction of CD3ζ domain may be significantly affected by the synergetic effect with CD28 signaling (41). Therefore, further evaluation may be required to ascertain the suitability of CD3ζ with three ITAMs in the context of CD28ζ second-generation CAR.

Meanwhile, several *in vitro* studies have indicated the significant effect of CD3ζ modification on CAR functions. A study of CD28-based ErbB2 CAR with only one ITAM at second position showed reduced apoptosis upon T cell activation *in vitro* (53). Increasing the number of ITAMs from three to six was shown to increase the efficiency of T cell activation (50). Interestingly, decreasing the ITAM number from three to two showed equivalent T cell activation for target cells expressing CD19 with high density (50). Although limited to *in vitro* evaluations, these studies informed the importance of ITAMs on CAR-T cell functions.

In a recent study, Feucht and colleagues designed CD19-targeted CD28ζ CAR (1928ζ CAR)-based new CARs with a defined ITAM number and position to see whether these CARs could overcome some of the adverse issues (54). The study first showed that CAR with only ITAM1 (referred as 1XX CAR) or only ITAM2 (referred as X2X CAR) induced comparable *in vitro* cytotoxicity with the original CD28ζ CAR, while CAR with only ITAM3 (referred as XX3 CAR) led to impaired cytotoxic function (54). In an *in vivo* animal model, the study further showed that 1XX CAR achieved durable and complete tumor remission. However, X2X CAR and XX3 CAR both failed to achieve complete tumor remission. More importantly, 1XX CAR treatment significantly increased the mice survival rate even better than the original CD28ζ CAR (54).

The improved therapeutic function of 1XX CAR can be attributed to the increased persistence *in vivo*. Firstly, in 1XX CAR treated mice, there was a higher accumulation of CAR-T cells at the tumor site, and both $CD4^+$ and $CD8^+$ CAR-T cell subsets showed a higher percentage of $CD62L^+CD45RA^-$ central memory T cells (T_{CM}) and a lower fraction of terminally differentiated $CD62L^-CD45RA^+$ effector cells (T_{EFF}) (54). Secondly, results from antigen re-exposure assay and exhaustion markers detection collectively demonstrated that exhaustion was rapidly acquired in CD28ζ CAR-T cells but largely attenuated in 1XX CAR-T cells. Thirdly, following tumor rechallenge, 1XX CAR achieved complete tumor control, while CD28ζ CAR failed to control tumor rechallenge, correlating the long persistence with low relapse (54).

Finally, at transcriptional level, the study revealed that wild type CD28ζ CAR-T cells were similar to T_{EFF} cells with the highest expression of effector differentiation related genes such as T-bet, PRDM1, and ID-2. XX3 CAR-T cells, on the other hand, were more similar to naïve T cells with the most significant down-regulation of T cell differentiation related genes and up-regulation of naïve/memory-associated genes such as TCF7, BCL6, LEF1, and KLF2. However, 1XX CAR-T cells exhibited a greater similarity to stem cell memory T cells (T_{SCM}) with a more balanced expression of differentiation and memory related genes (54).

Taken together, CAR modified with 1XX achieved a superior anti-tumor efficacy, making it a great candidate for next step clinical trials (**Figure 2A**). By modifying the ITAM configuration of CD3ζ domain, the persistence of CAR-T cells can be improved while keeping the desired cytotoxic effect.

MODIFYING DOWNSTREAM SIGNALING OF CD28ζ CAR

With improved understanding of CAR signaling mechanisms, additional strategies can be used to modify downstream signaling of CD28ζ CAR. A recent study by Sun and colleagues found that CD28ζ CAR had higher basal phosphorylation of CD3ζ domain and higher antigen-dependent T cell activation than 4-1BBζ CAR. To tune down its phosphorylation state, they introduced an FRB element into the intracellular domain of CD28ζ CAR and designed a fusion protein linking FKBP and SHP1 phosphatase. The administration of small molecule AP21967 will induce the heterodimerization of FKBP with FRB, recruiting SHP1 to the CD3ζ domain and promote its dephosphorylation (**Figure 2B**). In a humanized mouse model, they demonstrated that this design effectively suppressed tumor growth without significant weight loss of the mice. The reduced cytokine release in the plasma after AP21967 administration indicated that toxicities such as CRS could be ameliorated by this strategy (55). Therefore, the cytotoxicity of CAR-T cells can be precisely controlled by small molecules to prevent possible severe side effects.

Cytokine signaling is generally considered important for optimal T cell activation as signal 3. Kagoya et al. showed that adding signaling modules from cytokine receptors can also be beneficial to CD28ζ CAR function. They inserted an IL-2Rβ domain between CD28 and CD3ζ, and a YXXQ motif at the distal region of CD3ζ domain (**Figure 2C**). The engineered CAR recruited JAK, STAT3, and STAT5 to activate the JAK-STAT pathway upon antigen stimulation. Gene expression analysis showed that the incorporation of these modules not only preferentially activated IL21-induced genes and STAT3 targets, but enriched genes associated with cytolytic activity. Compared to the original CD28ζ CAR, the engineered version achieved a greater proliferation ability and maintained more memory T cells *in vitro* even after repeated stimulation. More importantly, the engineered CAR showed superior anti-tumor functions in

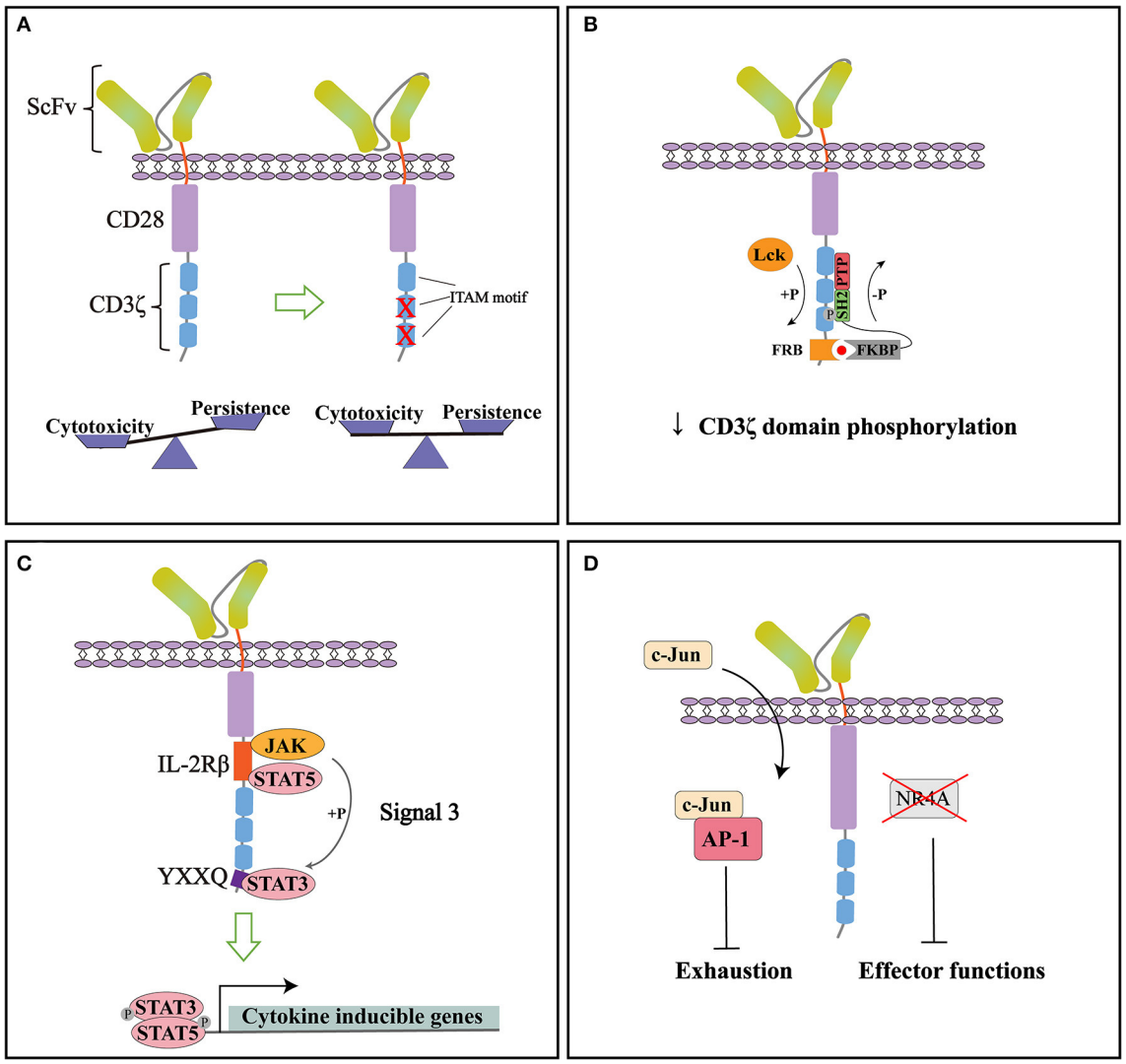

FIGURE 2 | Representative strategies to optimize cytoplasmic signaling of CD28ζ CAR. **(A)** By mutating the two membrane-distal ITAMs while keeping the membrane-proximal ITAM intact (1XX), the cytotoxicity and persistence of CARs can be balanced to improve therapeutic functions. **(B)** Small molecule-induced SHP1 phosphatase binding can tune down the basal phosphorylation level of CD28ζ CAR and reduce its antigen-dependent response. **(C)** Incorporation of signaling modules from cytokine receptor (signal 3) specifically activates the JAK-STAT pathway and improves the persistence and anti-tumor effect of CD28ζ CAR. **(D)** CAR signaling can be optimized through direct manipulation of transcription factors. NR4A knockout or c-Jun over-expression can counteract the exhaustion of CAR-T cells and improve their anti-tumor efficacy.

multiple mouse models, with a high percentage of $CD8^{+}$CAR-T cells in peripheral blood, significantly reduced tumor growth, and the prolonged overall survival of mice. These data suggested that combining signals from cytokines can enhance persistence and promote the anti-tumor effect of CAR (56).

Moreover, activation and exhaustion of CAR-T cells can be balanced by targeting transcription factors. In a recent study, Chen et al. revealed that NR4A1, NR4A2, and NR4A3 are the key transcription factors that drive T cell dysfunction. NR4A triple knockout down-regulated the expression of PD-1 and TIM3. CAR-T cells lacking three NR4A proteins showed an enhanced effector function and anti-tumor effect (57). On the other hand, Lyn and colleagues developed a CAR-T exhaustion model and identified that the abnormal expression of JunB, BATF, and IRF4 disrupted the functions of AP-1 and are responsible for T cell exhaustion. Further genetic analysis revealed that in exhausted T cells, AP-1 was prone to interact with JunB, BATF3, IRF4, etc. This interaction antagonized the binding of AP-1 with its canonical factor c-Jun and resulted in exhaustion gene expression. To counteract this effect, they overexpressed c-Jun in CAR-T cells, which significantly promoted IL-2 and IFNγ expression, increased the frequency of memory T cells subsets, and improved tumor-free survival of mice. Therefore, c-Jun overexpression could be an effective method to rescue

exhausted CAR-T cells and enhance their anti-tumor functions (**Figure 2D**) (58).

CONCLUSION

Although CAR-T therapy has shown impressive potential in the treatment of previously uncurable malignancies, some barriers still need to be overcome. Among them, the high rate of tumor relapse is a critical concern. To accomplish complete tumor elimination without relapse, CAR-T cells need to persist with sufficient cytotoxicity and limited exhaustion. This partly relies on the fine-tuning modification of CAR signaling. Herein, we have discussed strategies to optimize the CD3ζ domain or downstream signaling of CARs for improved anti-tumor efficacy. CD3ζ chain has been widely used in CAR designs since the first-generation CARs. However, very few studies have evaluated its suitability in second-generation CARs. This review highlighted CD3ζ domain modification as one important strategy to optimize CAR functions. Particularly, we provided evidence showing that the cytotoxicity and persistence of CAR-T cells can be balanced by modifying ITAM motifs of the CD3ζ domain. Moreover, recent advances in CAR signaling provide exciting new strategies to optimize CAR function. These studies highlighted that modifying cytoplasmic signaling of CAR is effective in improving CAR-T efficacy. This review has focused on the optimization of CD28-based CAR. As the signaling pathway of 4-1BB is significantly different from CD28, it will be interesting to see whether these strategies are applicable to 4-1BBζ CAR. Further studies are encouraged to investigate whether modifying ITAM configurations or downstream signaling contributes to improved functions of other antigen-targeted and costimulatory domain-based CARs.

AUTHOR CONTRIBUTIONS

XM, RJ, and LQ collected data and wrote the manuscript. JS and CZ wrote the manuscript and supervised the research. All authors read and approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Third Generation Anti-HER2 Chimeric Antigen Receptor Mouse T Cells Alone or Together With Anti-PD1 Antibody Inhibits the Growth of Mouse Breast Tumor Cells Expressing HER2 *in vitro* and in Immune Competent Mice

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Chimeric Antigen Receptor (CAR)-T cells have great efficacy against $CD19^{+}$ leukemia but little success for solid tumors. This study explored the effectiveness of third generation anti-HER2 CAR-T cells alone or in combination with anti-PD1 antibody on breast tumor cells expressing HER2 *in vitro* and in immune competent mouse model. The PDL1-positive mouse mammary tumor cell line 4T1 engineered to express luciferase and human HER2 was used as the target cell line (4T1-Luc-HER2). Anti-HER2 CAR-T cells were generated by transducing mouse spleen T cells with recombinant lentiviruses. ELISA analysis showed that IL-2 and IFN-γ secretion was increased in CAR-T cells co-cultured with the target cells, and the secretion of these two cytokines was increased further with the addition of anti-PD1 antibody. Lactate dehydrogenase assay revealed that CAR-T cells displayed a potent cytotoxicity against the target cells, and the addition of anti-PD1 antibody further enhanced the cytotoxicity. At the effector: target ratio of 16:1, cytotoxicity was 39.8% with CAR-T cells alone, and increased to 49.5% with the addition of anti-PD1 antibody. In immune competent syngeneic mouse model, CAR-T cells were found to be present in tumor stroma, inhibited tumor growth and increased tumor apoptosis significantly. Addition of anti-PD1 antibody further enhanced these anti-tumor activities. Twenty-one days after treatment, tumor weight was reduced by 50.0% and 73.3% in CAR-T group and CAR-T plus anti-PD1 group compared with blank T group. Our results indicate that anti-PD1 antibody can greatly increase the efficacy of anti-HER2 CAR-T against HER2-positive solid tumors.

Keywords: HER2, breast cancer, chimeric antigen receptor (CAR)-T cells, PD1, immunotherapy

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INTRODUCTION

Breast cancer has the highest incidence in women worldwide (1). About 20–25% of breast cancers are human epidermal growth factor receptor-2 (HER2) positive due to HER2 amplification or HER2 overexpression (2). Patients with HER2-positive breast cancer were not sensitive to chemotherapy and endocrine therapy, having a poor prognosis (3). Other malignant solid tumors with HER2 overexpression include ovarian cancer, non small cell lung cancer, prostate cancer, gastric cancer, colorectal cancer, renal cell carcinoma, and bladder cancer (4–6). HER2 is a significant target for cancer therapy (7–10). Herceptin (trastuzumab) is a humanized monoclonal antibody targeting HER2 and is widely used for the treatment of HER2-positive breast cancer. However, the efficacy of herceptin is limited as the resistance to its action frequently develops (11).

Treating solid tumors with chimeric antigen receptor (CAR)-T cells is being actively explored (12–16). In this immunotherapeutic strategy, T cells isolated from patients are genetically modified to express CAR on the cell surface. CAR contains the extracellular domain from the single-chain variable fragment (scFv) region of the antibody for tumor associated antigen, the transmembrane domain, and the intracellular domain from the signal activating regions for T cells. CAR-T cells recognize target antigen on the surface of tumor cells without the help of MHC molecules. The first generation CAR consists of an antigen recognition scFv and the CD3ζ signaling region whereas the second generation CAR includes an additional signaling region from one co-stimulatory molecule and the third generation CAR includes additional signaling regions from two co-stimulatory molecules (17).

Although CAR-T cell therapy has shown great success against hematological malignancies, it is less effective in the treatment of solid tumors (18–20). There are many factors limiting the anti-tumor effect of CAR-T cells (20–22). They includes the increased expression of inhibitory immune receptors such as programmed death-1 (PD1/CD279), which limits the strength and duration of CAR-T cell activation (23). PD1 expression is increased following the binding of T-cell receptor with its ligand. The ligand for PD1 is programmed cell death 1 ligand 1 (PDL1/CD274) that is also overexpressed in various cancers (24). The PD1/PDL1 pathway was regarded as a promising target for cancer therapy (25, 26). In recent years, it has been reported that CAR-T cell therapy and PD1 checkpoint blockade is one of the reasonable combinations in immunotherapy of solid tumor models (27–30).

We have previously designed and constructed a third-generation anti-HER2 CAR that carried two co-stimulatory molecules (CD28 and CD137). The third generation anti-HER2 CAR or anti-HER2 CAR-T cells in combination with anti-PD1 antibody have not been tested in clinical trials for breast cancer. We hypothesized that anti-HER2 CAR-T cells alone should have efficacy against $HER2^+$ breast cancer cells. Anti-HER2 CAR-T cells in combination with anti-PD1 antibody could overcome immunosuppression of anti-HER2 CAR-T cells induced by PD1/PDL1 pathway, and enhance the therapeutic efficacy. In this study, we generated the mouse breast cancer cell line 4T1-Luc-HER2 and used it as HER2 and PDL1 double positive target cells. We made anti-HER2 CAR-T cells by transducing mouse T cells with CAR-recombinant lentivirus. Using our tumor target cells and anti-HER2 CAR-T cells, we evaluated the anti-tumor effect of CAR-T cells *in vitro*, with or without anti-PD1 antibody. In addition, we established a new HER2 and PDL1 double positive breast cancer mouse model for the novel purpose of testing anti-tumor effect of CAR-T cells plus anti-PD1 in a complete tumor-immune system microenvironment *in vivo*.

MATERIALS AND METHODS

Cell Lines and Media

Mouse breast cancer cell line 4T1 cells were grown in RPMI-1640 medium (Gibco, California, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco). Human breast cancer cell line BT474 and SKBR3 cells, and human embryonic kidney cell line HEK 293T-17 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS. All the above cell lines were bought from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured with 5% CO_2.

Generation of the Mouse Breast Cancer 4T1-Luc-HER2 Cells Expressing Both Luciferase (Luc) and HER2

Since there is no HER2-positive/luciferase-positive mouse breast cancer cell line commercially available, we generated 4T1-Luc-HER2 cells from HER2-negative/luciferase-negative mouse breast cancer cell line 4T1 cells as stated below. The retroviral expression plasmid pLNCX2-GFP-Luc was kindly provided by Dr. Steve Anderson (University of Colorado, School of Medicine), in which GFP and luciferase was fused (31). For generating GFP-Luc-recombinant retrovirus, 293T-17 cells were co-transfected using polyethyleneimine (PEI) with four plasmids, including pLNCX2-CMV-GFP-Luc, pVSV-G, pJK3 and pTAT2 (SBI System Biosciences, Palo Alto, CA, USA) in the ratio of 2:1:1:1. The lentiviral expression plasmid pLEX-CMV-HER2 was kindly provided by Dr. Bolin Liu (University of Colorado, School of Medicine) (32). For generating humanized-HER2-recombinant lentivirus, 293T-17 cells were co-transfected using PEI with three plasmids, including pLEX-CMV-HER2, pSPAX2 and pMD2G (SBI System Biosciences, Palo Alto, CA, USA) in the ratio of 4:3:1. The retrovirus or lentivirus supernatants were collected at 48 and 72 h post-transfection, centrifuged at 3,000 rpm for 10 min and filtered through a 0.45 μm membrane before stored at −80°C.

The luciferase-negative/HER2-negative mouse breast cancer 4T1 cells were cultured with GFP-Luc-recombinant retrovirus supernatants supplemented with 8 μg/ml of polybrene (Sigma, St Louis, MO, USA) for 16 h. Twenty-four hours later, 4T1 expressing luciferase was selected with 600 μg/ml of G418 (Solarbio, Beijing, China) for 1 week. Then, the selected cells were incubated with HER2-recombinant lentivirus supernatants supplemented with 8 μg/ml of polybrene, and selected with 2.5 μg/ml puromycin (Invitrogen, San Diego, USA) for 4 days. Finally, the stable 4T1-Luc-HER2 cells expressing both luciferase and HER2 were generated.

Generation of the Third-Generation Anti-HER2 Mouse CAR-T Cells

The third generation anti-HER2 CAR was generated according to our published work (33). The recombinant lentivirus expressing the anti-HER2 CAR was produced by co-transfecting 293T-17 cells with recombinant lentiviral vector pLVX-EF1α-CAR-IRES-ZsGreen1 together with the packaging plasmids pSPAX2 and pMD2.G. The protocol for packaging, concentration and purification of the recombinant lentivirus was essentially as we described previously (33).

Splenocytes were from 6-week female BALB/c mice. Mouse spleen lymphocytes were isolated by density-gradient centrifugation (at 900 × g) using a lymphocyte separation medium Histopaque (Sigma). Mouse spleen lymphocytes were activated in 24-well plates at a density of 1×10^6/ml by 5 μg/mL of plate-bound anti-CD3 monoclonal antibody (Invitrogen) and 2 μg/mL of solution-state anti-CD28 monoclonal antibody (Invitrogen) for 3 days. Mouse T cells were cultured in RPMI 1640 medium with 10% FBS, 0.2 mmol/L L-glutamine (Gibco), 50 μmol/L β-mercaptoethanol (Gibco), 25 mmol/L HEPES (Gibco), and 500 IU/mL recombinant human interleukin 2 (IL-2) (PEPROTECH, Rocky Hill, NJ, USA).

On day 4, the $CD3^+$ mouse splenic T cells were mixed with CAR-recombinant lentiviruses at a multiplicity of infection (MOI) value of 20 in the presence of 8 μg/mL polybrene. The tissue culture plate was then centrifuged at 1,500 × g for 2 h at 32°C, incubated for 10–14 h at 37°C, replaced with fresh medium and cultured for an additional 3–4 days for expansion. Transduced cells were examined for proper GFP expression rate (∼40% GFP-positive cell ratio) under fluorescence microscope (Nikon Eclipse Ti), and subjected to flow cytometry to evaluate the GFP (CAR) positive cell percentage and western blot for CAR expression level, respectively.

Flow Cytometric Analysis

The expression of HER2 on 4T1-Luc-HER2 cells was detected using allophycocyanin (APC) labeled anti-human CD340 (erbB2/HER2) antibody, with APC mouse IgG1κ (both from BioLegend, San Diego, CA, USA) used as an isotype control. The expression of PDL1 on 4T1-Luc-HER2 cells was determined using PE/Cy7 anti-mouse CD274 (PDL1) antibody (BioLegend), with PE/Cy7 Rat IgG2bκ (BioLegend) used as an isotype control. The expression of CD3 in the activated mouse T cells was evaluated using APC anti-mouse CD3ε (BioLegend), with APC Armenian Hamster IgG (BioLegend) used as an isotype control. 7-aminoactinomycin D (7-AAD) solution (BioLegend) was used to determine the percentages of viable CAR-T cells. The expression of PD1 on anti-HER2 CAR-T cells was examined using PE-conjugated anti-mouse PD1 antibody (BD Biosciences), with PE mouse IgG2aκ (BD Biosciences) as an isotype control.

Western Blot Analysis

Western blot was used to detect the expression level of HER2 in 4T1-Luc-HER2 cells and the expression level of CAR in the CAR-transduced T cells. Cells were lysed in lysis buffer, and the protein concentrations of cell lysates were analyzed by the BCA kit (Beyotime Institute of Biotechnology, Shanghai, China). Lysates (10 μg per lane) were resolved by 10% SDS-PAGE, transferred to PVDF membranes (EMD Millipore). The blots were blocked with 5% non-fat dry milk in TBST, immunoblotted with anti-human HER2 antibody (dilution 1:1,000; Abcam) or anti CD3ζ polyclonal antibody (dilution 1:1,000; Affinity Biosciences, Jiangsu, China) and anti-rabbit HRP-conjugated secondary antibody (dilution 1:5,000; Santa Cruz Biotechnology, Inc. CA, USA), developed utilizing ECL reagent (Beyotime Institute of Biotechnology), and detected using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.). The expression level of HER2 or CAR (containing exogenous CD3ζ) was detected, respectively, using tubulin or endogenous CD3ζ as a loading control.

Detect the Expansion of CAR-T Cells

The blank T cells or anti-HER2 CAR-T cells were co-cultured with $HER2^-$ 4T1 or $HER2^+$ 4T1-Luc-HER2 tumor cells at an effector: target ratio of 4:1 in the absence or presence of 20 μg/mL anti-PD1 antibody. The T cells were counted using hemocytometer with trypan blue exclusion, respectively, at 24, 48, and 7 2h after co-cultured with tumor cells.

Detection of Cytokine Secretion by the CAR-T Cells

Anti-HER2 CAR-T cells or in the presence of 20 μg/ml anti-PD1 antibody were incubated with $HER2^+$ 4T1-Luc-HER2 cells at an effector: target ratio of 4:1 for 24 h in a 96-well plate. Anti-PD1 antibody (clone RMP1-14) was purchased from BioXCell (New Hampshire, USA). ELISA kits (R&D Systems, Inc. Minneapolis, USA) were used to analyze mouse IL-2 and IFN-γ concentrations in the supernatants of T cells according to the manufacturer's instructions. The co-cultures of $HER2^-$ 4T1 cells with anti-HER2 CAR-T cells (or in combination with anti-PD1 antibody), or $HER2^+$ 4T1-Luc-HER2 cells with the blank T cells (or in combination with anti-PD1 antibody) were used as negative controls.

In vitro Cytotoxicity Assay

The cytotoxicity assay was essentially as described (34). Briefly, anti-HER2 CAR-T cells (effector cells) in the absence or presence of 20 μg/mL anti-PD1 antibody were incubated with $HER2^+$ 4T1-Luc-HER2 cells (target cells) at the effector: target ratios of 2:1, 4:1, 8:1, and 16:1 for 18 h in a 96-well plate. The co-cultures of anti-HER2 CAR-T cells in the absence or presence of anti-PD1 antibody with $HER2^-$ 4T1 cells, the blank T cells in the absence or presence of anti-PD1 antibody with $HER2^+$ 4T1-Luc-HER2 cells were used as negative controls. Specific lactate dehydrogenase (LDH) released into the cell-free supernatant from the target cells was determined using the cytotoxicity LDH detection kit (Genmed, Addlestone, UK) according to the manufacturer's instructions. The amount of released LDH was used to assess the extent of target cell lysis, which can be translated into the effectiveness of effector

cells. Percent cytotoxicity was calculated according to OD values utilizing the following formula: Cytotoxicity (%) = (Experimental lysis − Effector spontaneous lysis − Target spontaneous lysis)/(Target maximum lysis − Target spontaneous lysis) × 100%.

Construction of the Syngeneic Mammary Tumor Model

Protocols for the animal studies were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. All animal experiments were performed in accordance with the relevant guidelines and regulations. BALB/c female mice with an intact immune system (6-week-old, weighed 17–20 g) purchased from GemPharmatech (Nanjing, China) were used for *in vivo* experiments. After 1 week of housing in the animal facility, 4×10^6 of 4T1-Luc-HER2 cells in 0.1 mL PBS mixed with 0.1 mL matrigel (Corning, Bedford, MA, USA) were injected subcutaneously into each of 24 mice at right back region on day 0. On day 14, when the diameter of the engrafted tumors reached about 6 mm, mice were randomized into four groups for treatments. The experiment was repeated for three times.

Anti-tumor Treatments *in vivo*

Twenty-four tumor-grafted mice were randomly divided into four groups ($n = 6$): blank T group (injection of blank T cells), anti-PD1 group (injection of anti-PD1 antibody), CAR-T group (injection of CAR-T cells), CAR-T plus anti-PD1 group (injection of CAR-T cells and anti-PD1 antibody). The tumor-grafted mice were administrated via caudal vein injection with blank T cells or CAR-T cells, 1×10^7 cells in 0.1 mL PBS/each mouse/each time, on day 14 and 21. The tumor-grafted mice were administrated intraperitoneally with anti-PD1 antibody, 250 μg in 0.1 mL PBS/each mouse/each time on day 14, 18, 22, and 26. All the mice were injected intraperitoneally with 20,000 IU IL-2 once every 2 days from day 14 to day 34.

Monitoring Tumor Growth and Collecting Tumor Tissues

Tumor growth was monitored on day 14 (just before anti-tumor treatment) and 28 (after anti-tumor treatment for 14 days) using Lumina Series III IVIS imaging system (PerkinElmer, MA, USA) as described (35). Briefly, on the day of IVIS imaging, mice were first anesthetized with isoflurane (RWD Life Science, Shenzhen, China) and then injected with 150 mg/kg luciferase solution (PerkinElmer) intraperitoneally. Images were captured using the IVIS system and analyzed with the Living Image @4.3.1. software.

During the above anti-tumor experiment *in vivo*, tumor volumes were measured once every 5 days using a caliper, and calculated using the equation $V\ (mm^3) = 1/2 \times L \times W^2$, where L (length) is the largest diameter and W (width) is the smallest diameter.

On day 35 (after anti-tumor treatment for 21 days), all of the mice were euthanized. All tumors were excised from tumor-bearing mice, weighed, fixed, and embedded for pathologic examination and immunohistochemical analysis. The inhibition ratio of tumor weight (%) = (tumor weight of group 1 − tumor weight of group 2)/tumor weight of group 1 × 100%.

Histological Examination and Immunohistochemical Analysis of Tumor Tissues

Tumor sections (three for each tumor) were stained with hematoxylin-eosin (HE) as described (36). HE stained tumor tissues were examined with a microscope at 400× magnification.

Tumor sections (five for each tumor) were immunostained with antibodies for cleaved caspase-3 and CD3 expression. Rabbit anti-mouse cleaved caspase-3 antibody and rabbit anti-mouse CD3ε antibody (1:200 dilution) were purchased from Cell Signaling Technology (Danvers, MA, USA). The sections were treated with antigen retrieval solution followed by 3% hydrogen peroxide treatment, blocked with 5% goat serum, incubated with primary antibody and with biotin-labeled secondary antibody (goat anti-rabbit; 1:1,000 dilution; Abcam, Cambridge, UK). The stained tissues were visualized by using the DAB chromogen (Abcam) reagent and counterstained with hematoxylin. For quantification of immunohistochemical staining, positive cell percentages were counted in five random 400× microscopic fields for each tissue section.

Statistical Analysis

Probability (P) values were calculated by using SPSS 22.0 software. Unpaired student T-test was used when comparing data between two groups. Comparisons among three or more groups were performed by one-way ANOVA. First homogeneity of variance was tested, if equal variances assumed, then P-values were calculated by Tukey; otherwise, P-values were calculated by Tamhane's T2. The difference with $P < 0.05$ was considered statistically significant.

RESULTS

Successful Generation of the Mouse Breast Cancer 4T1-Luc-HER2 Cells Expressing Both Luciferase and HER2

After the mouse breast cancer 4T1 cells were transduced with GFP-Luc-recombinant retroviruses and HER2-recombinant lentiviruses, the expression of both GFP (luciferase) and HER2 in 4T1-Luc-HER2 cells was detected by flow cytometry and western blot, using 4T1 cells as a negative control. The percentage of GFP $(Luc)^+$ or $HER2^+$ cells was 49.7 and 87.9%, respectively; the percentage of GFP $(Luc)^+/HER2^+$ double positive cells was 42.6%, detected by flow cytometry (**Figure 1A**). The expression level of HER2 in 4T1-Luc-HER2 cells was, at least, as same as that in two HER2-positive controls (human breast cancer BT474 and SKBR3 cells), detected by western blot (**Figure 1B**).

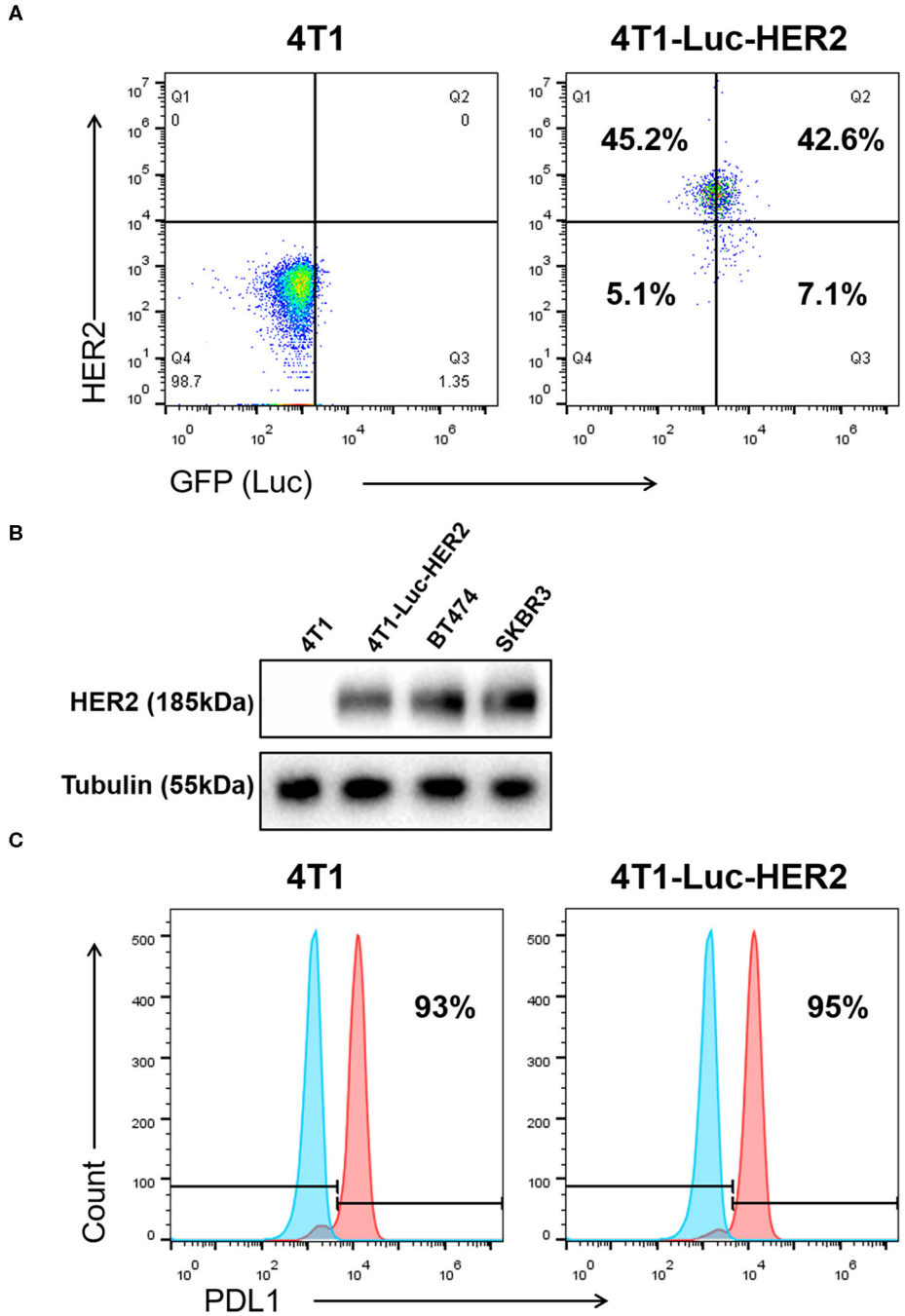

FIGURE 1 | Expression of HER2, GFP (Luc) and PDL1 in 4T1-Luc-HER2 cells. **(A)** The expression level of HER2 and GFP (Luc) in 4T1-Luc-HER2 cells was detected by flow cytometry (using anti-human APC-HER2). 4T1 cells were set as $HER2^{-}$/$GFP\ (Luc)^{-}$ control. The values indicated the percentages of positive or negative cells. **(B)** The expression level of HER2 in 4T1-Luc-HER2 cells was detected by western blotting, using tubulin as a loading control. 4T1 cells were set as HER2-negative control. BT474 and SKBR3 cells were set as HER2-positive controls. **(C)** The expression level of PDL1 in 4T1-Luc-HER2 and 4T1 cells was detected by flow cytometry (using anti-mouse PE/Cy7-PDL1). The values indicated the percentages of PDL1-positive cells. The left histogram was an isotype control. The experiments have been repeated for three times. Luc, luciferase.

Before testing the efficacy of anti-HER2 CAR-T cells together with anti-PD1 antibody against breast cancer cells, we checked the expression of PDL1 on both 4T1 and 4T1-Luc-HER2 cells. The percentages of PDL1-positive 4T1 and 4T1-Luc-HER2 cells were 93 and 95%, respectively, detected by flow cytometry (**Figure 1C**).

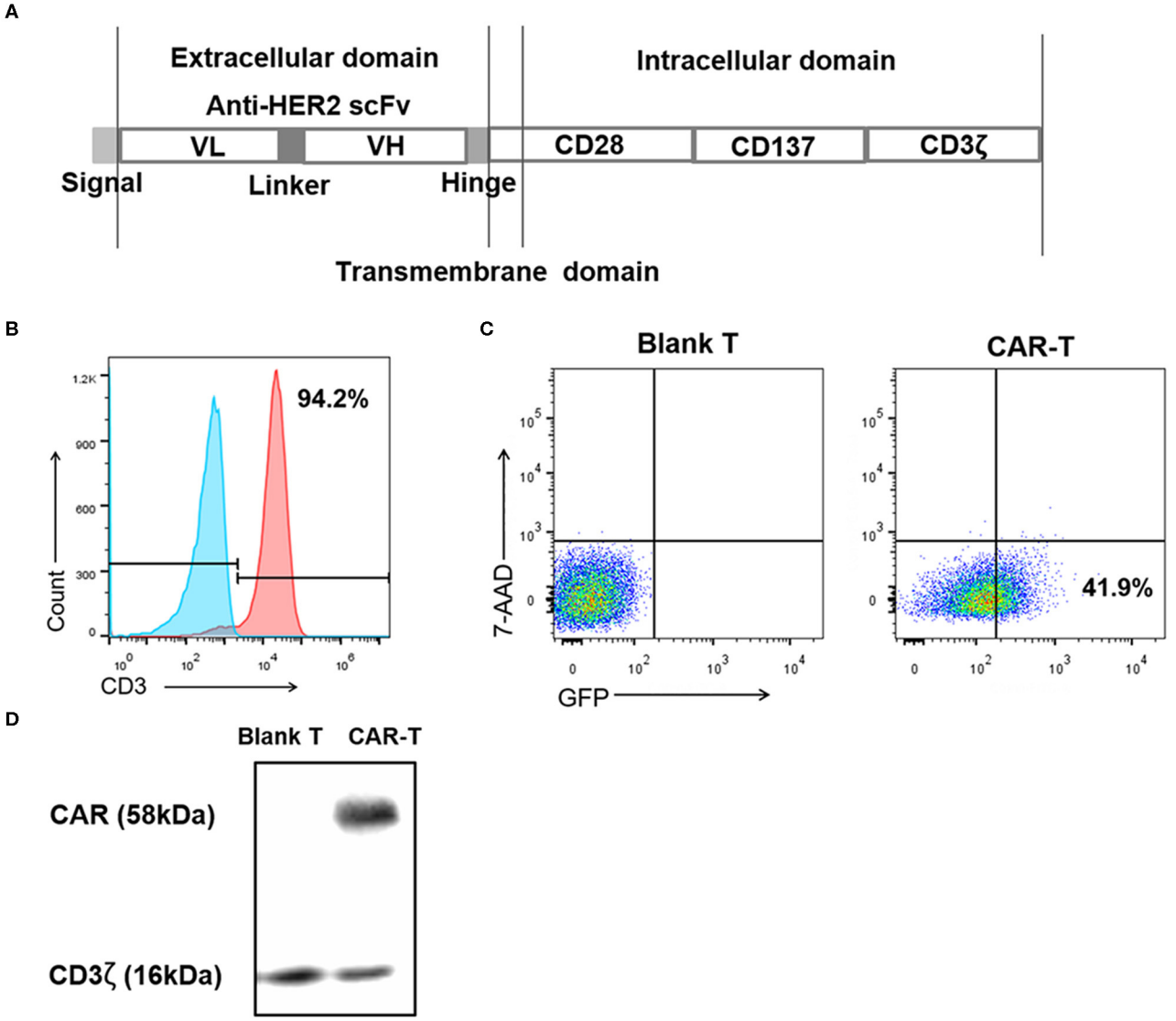

FIGURE 2 | Preparation of the third generation anti-HER2 CAR-T cells. **(A)** Diagram of anti-HER2 CAR. **(B)** After activation and separation, mouse spleen lymphocytes were detected by anti-CD3 monoclonal antibody using flow cytometry. The values indicated the percentage of CD3-positive mouse spleen T cells. The left histogram was an isotype control. **(C)** After the mouse spleen T cells were transduced with the recombinant lentiviruses at MOI 20 for 4 days, the percentages of 7-AAD-negative (viable) cells and GFP-positive (transduced) cells were analyzed using flow cytometry, with (untransduced) blank T cells as the control. The value indicated the percentage of transduced viable cells. **(D)** The expression level of CAR (containing exogenous CD3ζ) in CAR-T cells was detected by western blotting using an anti-CD3ζ antibody, with blank T cells as negative control, and endogenous CD3ζ as a loading control. The experiments have been repeated for three times. CAR, chimeric antigen receptor; scFv, single chain variable fragment; VH, heavy chain variable region; VL, light chain variable region; MOI, multiplicity of infection; 7-AAD, 7-aminoactinomycin D; Blank T, T cells not transduced; CAR-T, T cells transduced with CAR.

Generation of the Third Generation Anti-HER2 CAR-T Cells

The preparation of third generation anti-HER2 CAR-T cells started from the construction of anti-HER2 CAR as shown in **Figure 2A**, which was packed in lentivirus described in Materials and Methods above. The second step was preparation of mouse spleen T cells through activation of spleen lymphocytes and separation of spleen T cells. As detected by flow cytometry in **Figure 2B**, 94.2% of isolated cells were CD3-positive T cells. These cells were then transduced with CAR-recombinant lentivirus at a MOI of 20 for 4 days. GFP-positive (transduced) and 7-AAD-negative (viable) cells were counted by flow cytometer to evaluate the transduction efficiency. The transduction efficiency was 41.9% shown in **Figure 2C**. The expression level of CAR (containing exogenous CD3ζ) in transduced T cells was detected by an anti-CD3ζ antibody in western blotting. The endogenous CD3ζ was used as a loading control. CAR-T cells expressed the expected exogenous CD3ζ as

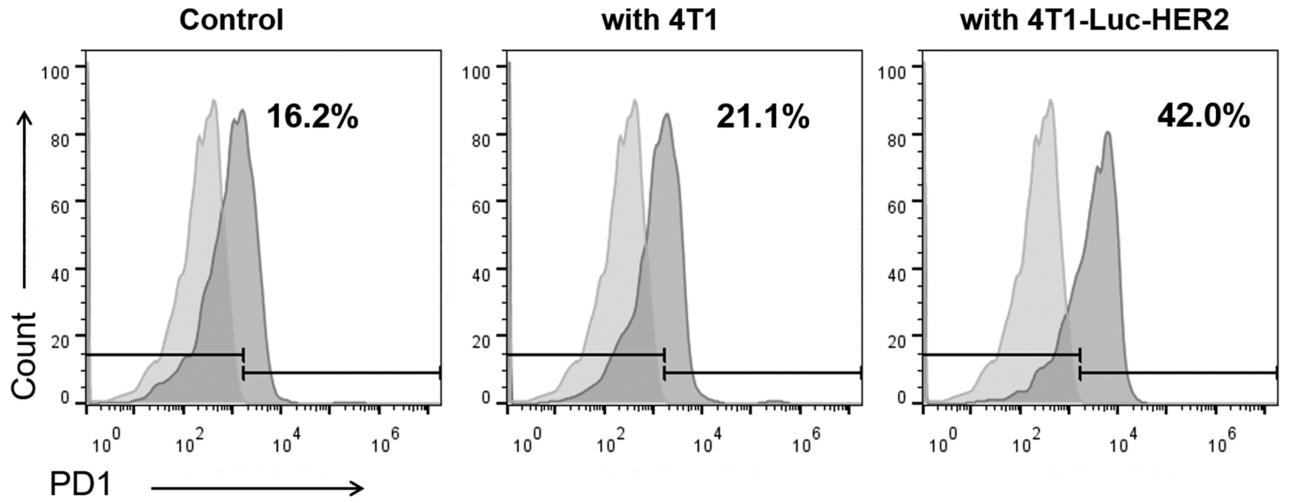

FIGURE 3 | PD1 expression is increased in anti-HER2 CAR-T cells when co-cultured with HER2-positive breast cancer cells. Anti-HER2 CAR-T cells were cultured alone as control, or co-cultured with HER2$^-$ 4T1 cells, or HER2$^+$ 4T1-Luc-HER2 cells at an effector: target ratio of 4:1 for 24 h. Then, anti-HER2 CAR-T cells were subjected to flow cytometry analysis using PE anti-mouse PD1 antibody (right histogram) and PE isotype control (left histogram). The experiments have been repeated for three times.

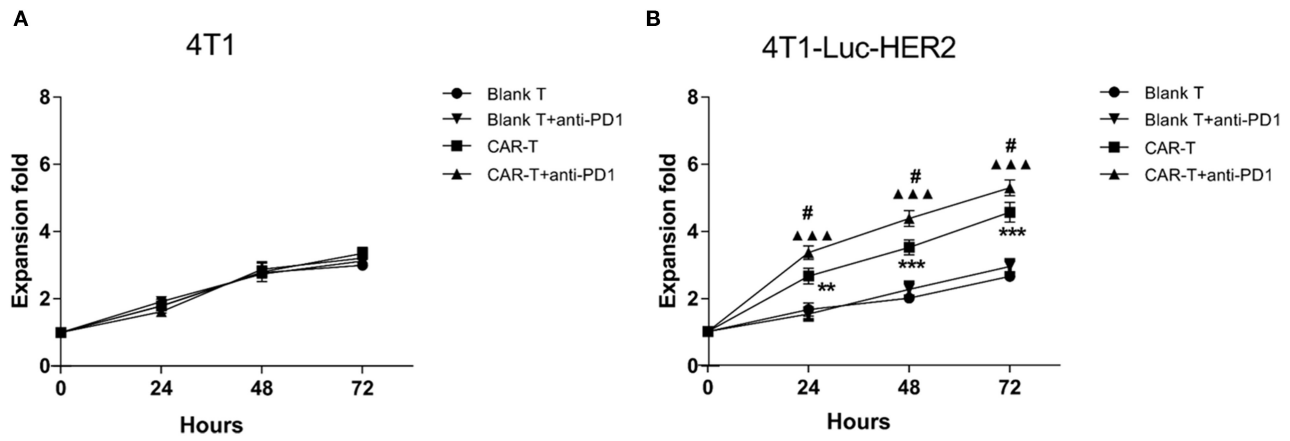

FIGURE 4 | HER2-positive breast cancer cells induced expansion of anti-HER2 CAR-T cells can be further enhanced with the addition of anti-PD1 antibody. Blank T cells or anti-HER2 CAR-T cells were co-cultured with **(A)** 4T1 or **(B)** 4T1-Luc-HER2 cells in the absence or presence of anti-PD1 antibody at an effector: target ratio of 4:1 for 24, 48 or 72 h. Viable cells were counted by trypan blue exclusion each day. The mean values from three different experiments were presented. CAR-T group was compared with blank T group, $^{**}P < 0.01$ and $^{***}P < 0.001$; or CAR-T plus anti-PD1 group was compared with blank T plus anti-PD1 group, ▲▲▲$P < 0.001$; or CAR-T group plus anti-PD1 group was compared with CAR-T group, $^{\#}P < 0.05$.

part of CAR (58 kDa), and its expression level was much more than endogenous CD3ζ (16 kDa) (**Figure 2D**).

PD1 Expression Is Increased in Anti-HER2 CAR-T Cells When Co-cultured With HER2-Positive Breast Cancer Cells

It is known that after activation of T cells, the expression of inhibitory receptors including PD1 is increased. Therefore, we examined the PD1 expression in anti-HER2 CAR-T cells activated by HER2-positive breast cancer cells. Flow cytometry results demonstrated that 21.1% of the anti-HER2 CAR-T cells were PD1$^+$ when co-cultured with HER2$^-$ 4T1 cells (**Figure 3**). In contrast, PD1$^+$ cells in anti-HER2 CAR-T cells were significantly increased to 42.0% when co-cultured with HER2$^+$ 4T1-Luc-HER2 cells (**Figure 3**).

HER2-Positive Breast Cancer Cells Induced Expansion of Anti-HER2 CAR-T Cells Can Be Further Enhanced With the Addition of Anti-PD1 Antibody

T cells can proliferate violently after being activated by antigens presented by MHC molecules. We examined the proliferation of

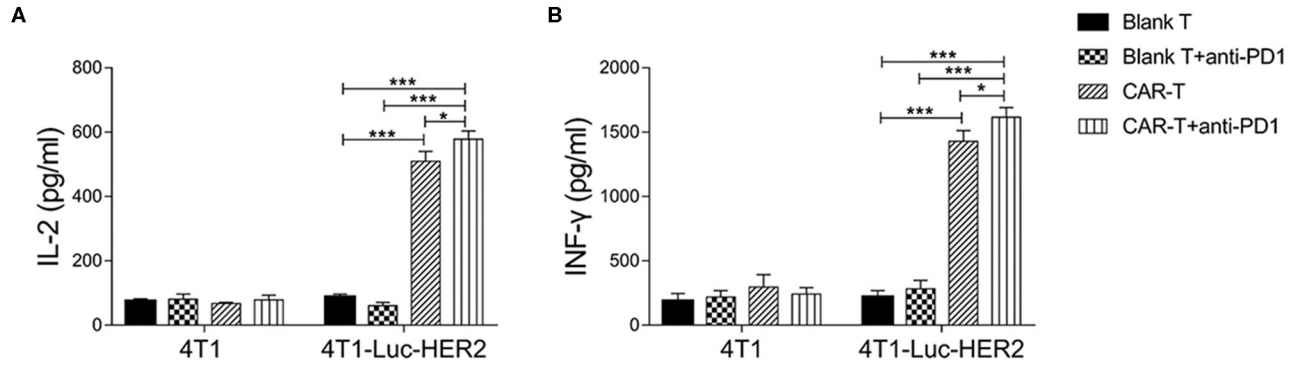

FIGURE 5 | Cytokine secretion of anti-HER2 CAR-T cells. After anti-HER2 CAR-T cells (effector cells) (or in combination with anti-PD1 antibody) were co-cultured with $HER2^+$ 4T1-Luc-HER2 cells (target cells) at an effector to target ratio of 4:1 for 24 h, supernatants were collected. ELISA kits for detecting **(A)** IL-2 and **(B)** IFN-γ were used to analyze supernatants. Supernatants from the co-culture of anti-HER2 CAR-T cells with $HER2^-$ 4T1 cells, as well as from the co-culture of (untransduced) blank T cells (or in combination with anti-PD1 antibody) with $HER2^+$ 4T1-Luc-HER2 cells were used as negative controls. Three repeated tests were performed and the mean values were presented. For **(A)** IL-2 or **(B)** IFN-γ secretion, CAR-T group was compared with blank T group; or CAR-T plus anti-PD1 group was compared with blank T plus anti-PD1 group; or CAR-T plus anti-PD1 group was compared with CAR-T group; or CAR-T plus anti-PD1 group was compared with blank T group (*$P < 0.05$; ***$P < 0.001$). CAR, chimeric antigen receptor; Luc, luciferase; CAR-T, T cells transduced with CAR; Blank T, T cells not transduced.

blank T or anti-HER2 CAR-T cells after they were co-cultured with $HER2^-/PDL1^+$ 4T1 or $HER2^+/PDL1^+$ 4T1-Luc-HER2 breast cancer cells at effector: target ratio of 4:1 in the absence and presence of anti-PD1 antibody for 24, 48, and 72 h. As a negative control, blank T cells or anti-HER2 CAR-T cells proliferated slowly when co-cultured with 4T1 cells regardless of the presence anti-PD1 antibody (**Figure 4A**). In contrast, the growth of anti-HER2 CAR-T cells was significantly higher than blank T cells when co-cultured with 4T1-Luc-HER2 cells (**$P < 0.01$ and ***$P < 0.001$) (**Figure 4B**). Importantly, the addition of anti-PD1 antibody further enhanced the growth of anti-HER2 CAR-T cells when co-cultured with 4T1-Luc-HER2 cells ($^{\#}P < 0.05$) (**Figure 4B**). It is suggested that the combination of CAR-T and PD1 blockade has an additive effect.

Anti-HER2 CAR-T Cell Activation Induced by HER2-Positive Breast Cancer Cells

To evaluate whether anti-HER2 CAR-T cells were activated by target cells in the absence or presence of anti-PD1 antibody, cytokine secretion of IL-2 and IFN-γ from anti-HER2 CAR-T cells was detected by ELISA. The results revealed that IL-2 and IFN-γ secretion were significantly increased in CAR-T group when compared with blank T group ($P < 0.001$) after co-cultured with 4T1-Luc-HER2 cells at an effector to target ratio of 4:1 for 24h (**Figure 5**). IL-2 increased to 5.8-fold, and IFN-γ increased to 6.6-fold. IL-2 and IFN-γ secretion of CAR-T group co-cultured with 4T1-Luc-HER2 cells, increased to 7.6- and 4.8-fold respectively, compared with CAR-T group co-cultured with 4T1 cells. These results indicated that CAR-T cells could specifically bind to 4T1-Luc-HER2 cells and be activated. This activation could be enhanced further by adding anti-PD1 antibody as indicated by more IL-2 and IFN-γ secretion of columns representing CAR-T cells plus anti-PD1 antibody ($P < 0.05$) (**Figure 5**).

Anti-PD1 Antibody Enhances the Cytotoxicity of Anti-HER2 CAR-T Cells Against HER2-Positive Breast Cancer Cells

In order to determine the efficacy of effector cells together with anti-PD1 antibody on target cells *in vitro*, anti-HER2 CAR-T cells were co-cultured with HER2-positive breast cancer cells (4T1-Luc-HER2) at effector: target ratios of 16:1, 8:1, 4:1 and 2:1 for 18 h in the absence or presence of anti-PD1 antibody. The cytotoxicity (%) of CAR-T group against the 4T1-Luc-HER2 cells was significantly higher than that of blank T group at every effector: target ratio (*$P < 0.05$ and ***$P < 0.001$) (**Figure 6B**). Addition of anti-PD1 antibody significantly enhanced the cytotoxicity (%) of CAR-T cells against 4T1-Luc-HER2 cells at the effector: target ratios of 16:1 and 8:1, as indicated by $^{\#}P < 0.05$ (**Figure 6B**). No significant cytotoxicity differences were observed when HER2-negative 4T1 cells were used as the target cells (**Figure 6A**). At the effector: target ratio of 16:1, cytotoxicity (%) of CAR-T cells against 4T1-Luc-HER2 cells reached 39.8%, while cytotoxicity (%) against HER2-negtive 4T1 cells was much lower, at just 15.2% (**Figure 6C**) ($P < 0.001$). When anti-PD1 antibody was added, cytotoxicity (%) of CAR-T cells against 4T1-Luc-HER2 cells reached 49.5%, whereas cytotoxicity (%) against 4T1 cells remained low at just 16.4% (**Figure 6C**) ($P < 0.001$).

Inhibition of Syngeneic 4T1 Tumor Growth by Anti-HER2 CAR-T Cells or Together With Anti-PD1 Antibody

To examine whether anti-HER2 CAR-T cells alone or in combination with anti-PD1 antibody could inhibit the growth of HER2-positive tumors, we treated mice carrying the syngeneic 4T1-Luc-HER2 tumors by anti-HER2 CAR-T cells without or with anti-PD1 antibody. Tumor growth was monitored by IVIS system on day 14 and day 28.

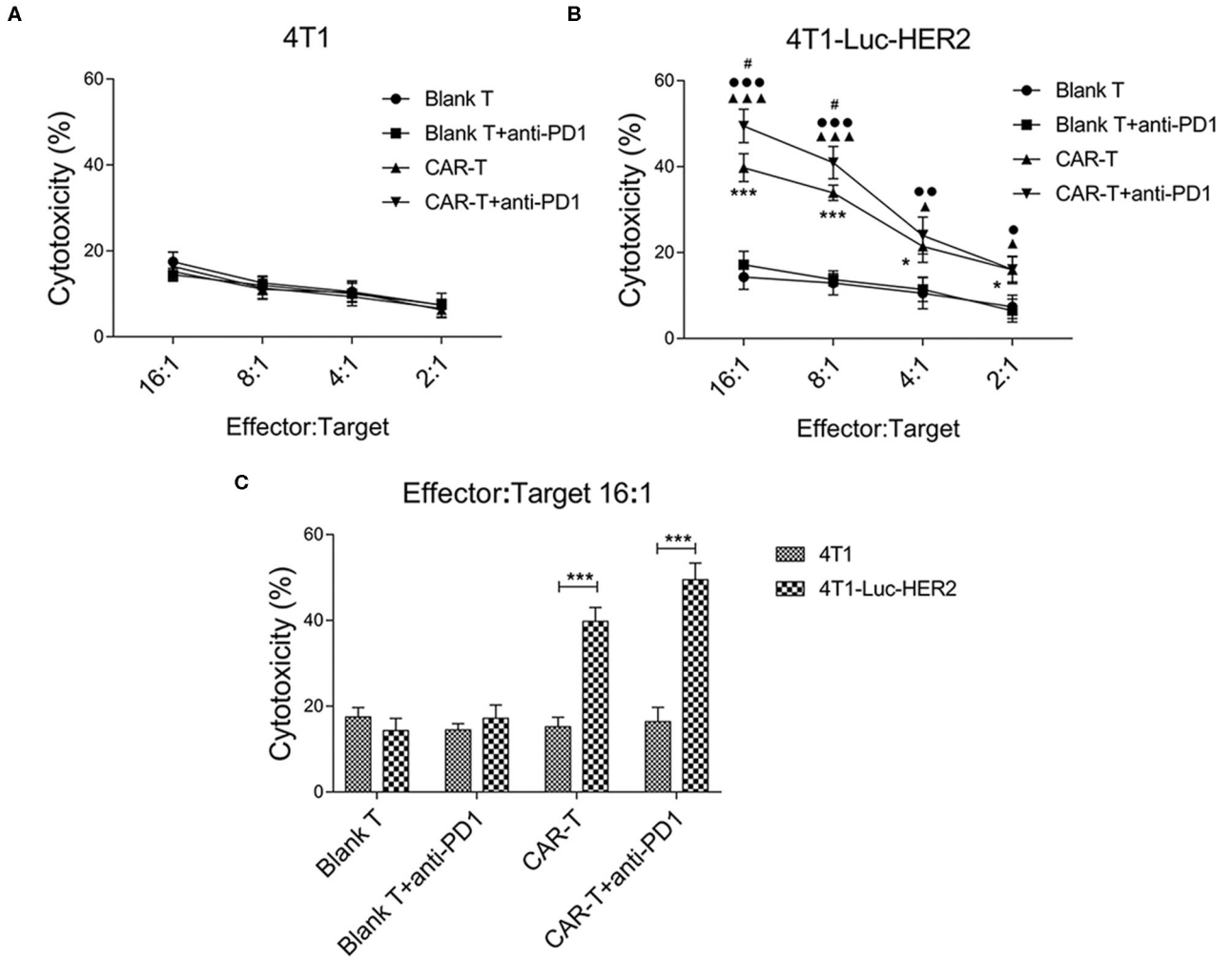

FIGURE 6 | The cytotoxicity of anti-HER2 CAR-T cells (or in combination with anti-PD1 antibody) against 4T1-Luc-HER2 cells. **(A)** As a negative control, anti-HER2 CAR-T cells were co-cultured with HER2$^-$ 4T1. **(B)** After anti-HER2 CAR-T cells (effector cells) were co-cultured with HER2$^+$ 4T1-Luc-HER2 (target cells) (or in combination with anti-PD1 antibody) at different ratios of effector: target for 18 h, supernatants were detected using a cytotoxicity LDH detection kit for LDH released from lysed target cells. Cytotoxicity (%) was calculated. At effector: target ratios of 2:1, 4:1, 8:1 or 16:1 respectively, the cytotoxicity (%) of CAR-T group was compared with blank T group, $^*P < 0.05$, $^{***}P < 0.001$; or CAR-T plus anti-PD1 group was compared with blank T plus anti-PD1 group, ▲$P < 0.05$, ▲▲▲$P < 0.001$; or CAR-T plus anti-PD1 group was compared with CAR-T group, #$P < 0.05$; or CAR-T plus anti-PD1 group was compared with blank T group, •$P < 0.05$, ••$P < 0.01$, •••$P < 0.001$. **(C)** At effector: target ratio of 16:1, the cytotoxicity (%) of 4T1-Luc-HER2 group was compared with 4T1 group, $^{***}P < 0.001$. Three repeated tests were performed and the mean values were presented. CAR, chimeric antigen receptor; Luc, luciferase; CAR-T, T cells transduced with CAR; Blank T, T cells not transduced; LDH, lactate dehydrogenase.

Bioluminescent imaging data revealed that there were not statistically significant among four groups on day 14 (just before anti-tumor treatment) (**Figures 7A,B**). On day 28 (after anti-tumor treatment for 14 days), CAR-T group significantly reduced the tumor growth compared with blank T group ($P < 0.05$) (**Figures 7A,B**). CAR-T plus anti-PD1 group had even higher inhibitory effect on tumor growth ($P < 0.05$) (**Figures 7A,B**).

The tumor volume of CAR-T group was reduced during the anti-tumor treatment compared with blank T group ($^{**}P < 0.01$, $^{***}P < 0.001$), and the significant difference started on day 24 (after anti-tumor treatment for 10 days) ($^{**}P < 0.01$) (**Figure 7C**). The tumor volume of CAR-T plus anti-PD1 group significantly reduced from day 29 (after anti-tumor treatment for 15 days) compared with CAR-T group ($^{\#}P < 0.05$) (**Figure 7C**).

The individual syngeneic tumor size of each group on day 35 was showed in **Figure 7D**, and the excised tumors were weighed. The tumor weight of CAR-T group was significantly less than those of blank T group ($P < 0.05$) (**Figure 7E**). CAR-T plus anti-PD1 group had the least tumor weight among four groups (**Figure 7E**). On day 35 (after anti-tumor treatment for 21 days), for the inhibition ratio of tumor weight, CAR-T group compared to blank T group

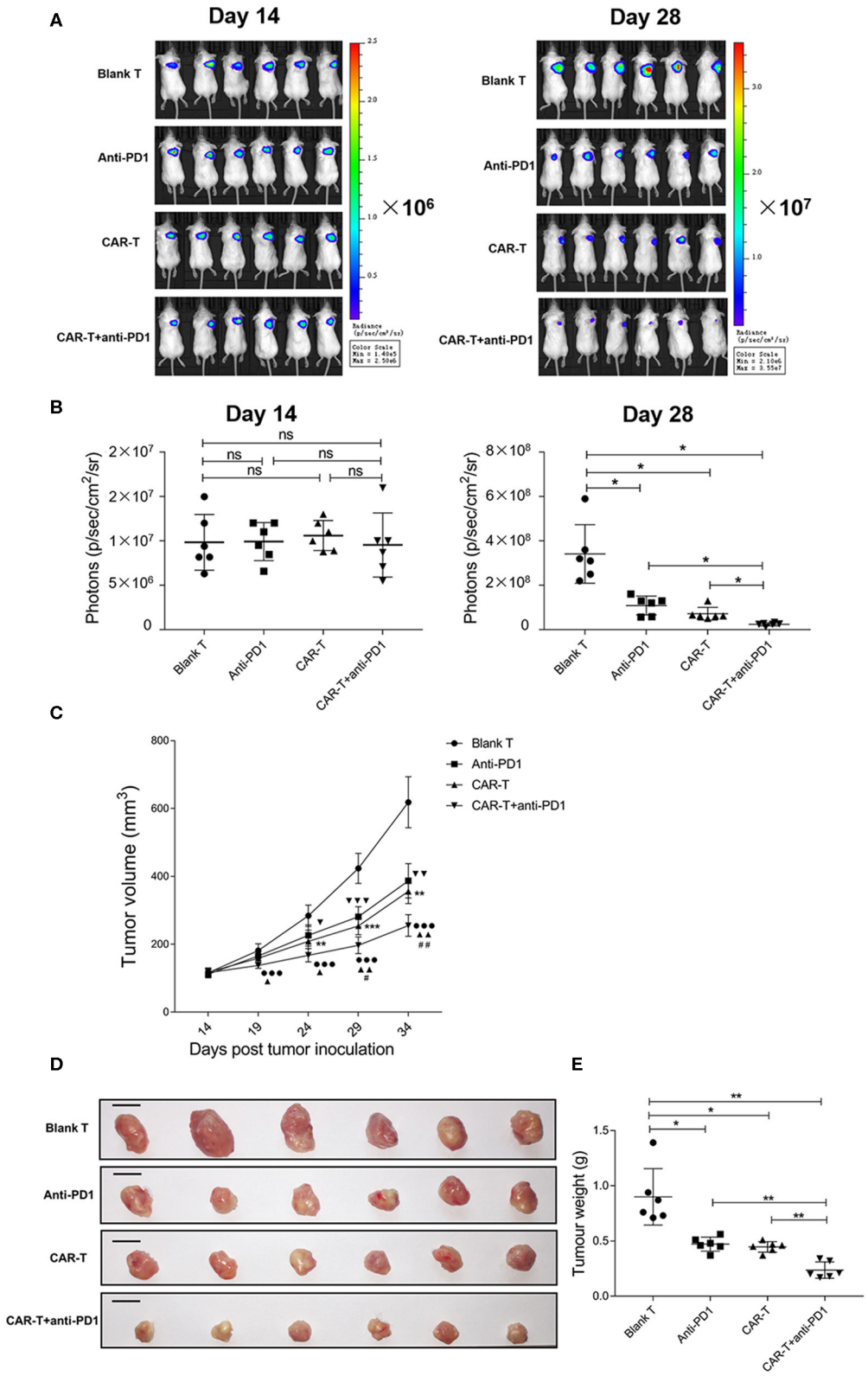

FIGURE 7 | The inhibition of syngeneic tumor growth by anti-HER2 CAR-T cells or in combination with anti-PD1 antibody. **(A)** Tumor growth of mice was monitored by IVIS image, after the mice were injected with 4T1-Luc-HER2 cells for 2 weeks before anti-tumor treatment (on day 14), and after anti-tumor treatment for 2 weeks

(Continued)

FIGURE 7 | (on day 28). **(B)** Bioluminescence photons were monitored by IVIS image, after the mice were injected with 4T1-Luc-HER2 cells for 2 weeks before anti-tumor treatment (on day 14), and after anti-tumor treatment for 2 weeks (on day 28). CAR-T group compared with blank T group, or CAR-T plus anti-PD1 group compared with anti-PD1 group, or anti-PD1 group compared with blank T group, or CAR-T plus anti-PD1 group compared with CAR-T group, or CAR-T plus anti-PD1 group compared with blank T group, *ns:* not significant, $^{*}P < 0.05$. **(C)** Tumor volumes (mm^3) were calculated as described in "Materials and methods." Three determinations were made and the mean values were shown. CAR-T group compared with blank T group, $^{**}P < 0.01$, $^{***}P < 0.001$; CAR-T plus anti-PD1 group compared with anti-PD1 group, $\blacktriangle P < 0.05$, $\blacktriangle\blacktriangle P < 0.01$; anti-PD1 group compared with blank T group, $\blacktriangledown P < 0.05$, $\blacktriangledown\blacktriangledown P < 0.01$, $\blacktriangledown\blacktriangledown\blacktriangledown P < 0.001$; CAR-T plus anti-PD1 group compared with CAR-T group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; CAR-T plus anti-PD1 group compared with blank T group, $\bullet\bullet\bullet P < 0.001$. **(D)** The tumors were excised from tumor-bearing mice after anti-tumor treatment for 3 weeks (on day 35), the individual syngeneic tumor size was shown. Scale bar = 1 cm. **(E)** The excised tumors were weighed. CAR-T group compared with blank T group, or CAR-T plus anti-PD1 group compared with anti-PD1 group, or anti-PD1 group compared with blank T group, or CAR-T plus anti-PD1 group compared with CAR-T group, or CAR-T plus anti-PD1 group compared with blank T group, $^{*}P < 0.05$, $^{**}P < 0.01$. The experiments have been repeated for three times. CAR, chimeric antigen receptor; Luc, luciferase; CAR-T, T cells transduced with CAR; Blank T, T cells not transduced.

was 50.0%, CAR-T plus anti-PD1 group compared to CAR-T group was 46.7%, CAR-T plus anti-PD1 group compared to blank T group was 73.3%. These results suggested that anti-HER2 CAR-T cells could shrink syngeneic tumors, and anti-PD1 antibody enhanced the anti-tumor activity of CAR-T cells *in vivo*.

Histomorphometric Analysis of Syngeneic 4T1 Tumor Tissue After Anti-tumor Treatments

HE stained slides of syngeneic tumor tissue were examined by an optical microscope (**Figure 8A**). The number of tumor cells was decreased significantly in the tissue sections from the CAR-T group and CAR-T plus anti-PD1 group compared with the blank-T group. Especially for CAR-T plus anti-PD1 group, nuclear/cytoplasm ratio was decreased; the cytoplasm mostly became transparent and filled with vacuoles, and more apoptotic cells had pyknotic nuclei. In contrast, in tumor sections from blank T group, there were more tumor cells that were densely organized. In addition, tumor cells were with significant larger and aberrant nuclei, and increased nuclear/cytoplasm ratio. Furthermore, the cytoplasm of tumor cells was rarely transparent and vacuous.

Immunohistochemical Analysis of Cleaved Caspase-3 and CD3 Expression in Tumors From Syngeneic Mice After Anti-tumor Treatments

The positive staining of cleaved caspase-3 (as shown in brown) was localized mostly on cytoplasm and nucleus (**Figure 8B**) and the positive staining of CD3 (as shown in brown) was localized mostly on plasma membrane and cytoplasm (**Figure 8C**). Tumors from mice treated with blank T cells consisted primarily of living tumor cells, rarely of apoptotic tumor cells (cleaved caspase-3 staining), with occasional T cells (CD3 staining) (**Figures 8B,C**). There were more apoptotic tumor cells in the tumor tissues from CAR-T group (37.0%) than in those from blank T group (8.18%) ($P < 0.01$) (**Figures 8B,D**). Tumors from mice treated with CAR-T cells together with anti-PD1 antibody showed the highest percentage of apoptotic tumor cells (63.28%) among the four groups (**Figures 8B,D**). There were significantly more T cells in tumors from CAR-T group (24.8%) than in those from blank T group (5.18%) ($P < 0.001$ or $P < 0.01$) (**Figures 8C,E**). The highest T cell numbers were observed (32.34%) in tumors from mice treated with CAR-T cells together with anti-PD1 antibody (**Figures 8C,E**).

DISCUSSION

This study was about evaluating the anti-breast cancer efficacy of anti-HER2 CAR-T in combination with anti-PD1 antibody. Many previous studies have indicated that tumor specific CAR-T has therapeutic effect on target tumor. However, this effect was compromised by inhibitory signal delivered to CAR-T from target tumor cells through PD1-PDL1 signal pathway. Our hypothesis was blocking this signal pathway could enhance therapeutic effect of CAR-T. To prove this hypothesis, we first generated the mouse breast cancer 4T1-Luc-HER2 cells (**Figure 1**) from PDL1-positive 4T1 cells. 4T1-Luc-HER2 cells were ideal $HER2^{+}/PDL1^{+}$ target cells, since the percentage of HER2-positive (87.8%) and PDL1-positive (95%) was high (**Figures 1A,C**), and the expression level of HER2 protein was at least as same as that of HER2-positive human breast cancer BT474 and SKBR3 cells (**Figure 1B**). We then developed third-generation CAR containing CD28-CD137-CD3ζ (**Figure 2A**), which has two co-stimulatory molecules CD28 and 4-1BB/CD137. We prepared anti-HER2 CAR-T cells by transducing mouse spleen T cells with CAR-recombinant lentivirus (**Figure 2**). After successfully making our own $HER2^{+}/PDL1^{+}$ target tumor cells (4T1-Luc-HER2) and third generation anti-HER2 CAR-T cells, we tested the effects of anti-HER2 CAR-T cells on target tumor cells *in vitro* and *in vivo*, with or without combination of anti-PD1 antibody.

Our results showed that PD1 expression was increased in anti-HER2 CAR-T cells when co-cultured with HER2-positive breast cancer cells (**Figure 3**). HER2-positive breast cancer cells induced expansion of anti-HER2 CAR-T cells can be further enhanced with the addition of anti-PD1 antibody (**Figure 4**). Our results also showed that the secretion of IL-2 and IFN-γ from CAR-T cells was increased when co-cultured with 4T1-Luc-HER2 cells. Addition of the anti-PD1 antibody further enhanced the secretion of these two cytokines (**Figure 5**). Anti-HER2 CAR-T cells possessed potent cytotoxicity against 4T1-Luc-HER2 cells, and the addition of anti-PD1 antibody further enhanced the efficacy of CAR-T cells (**Figure 6**). At an effector: target of

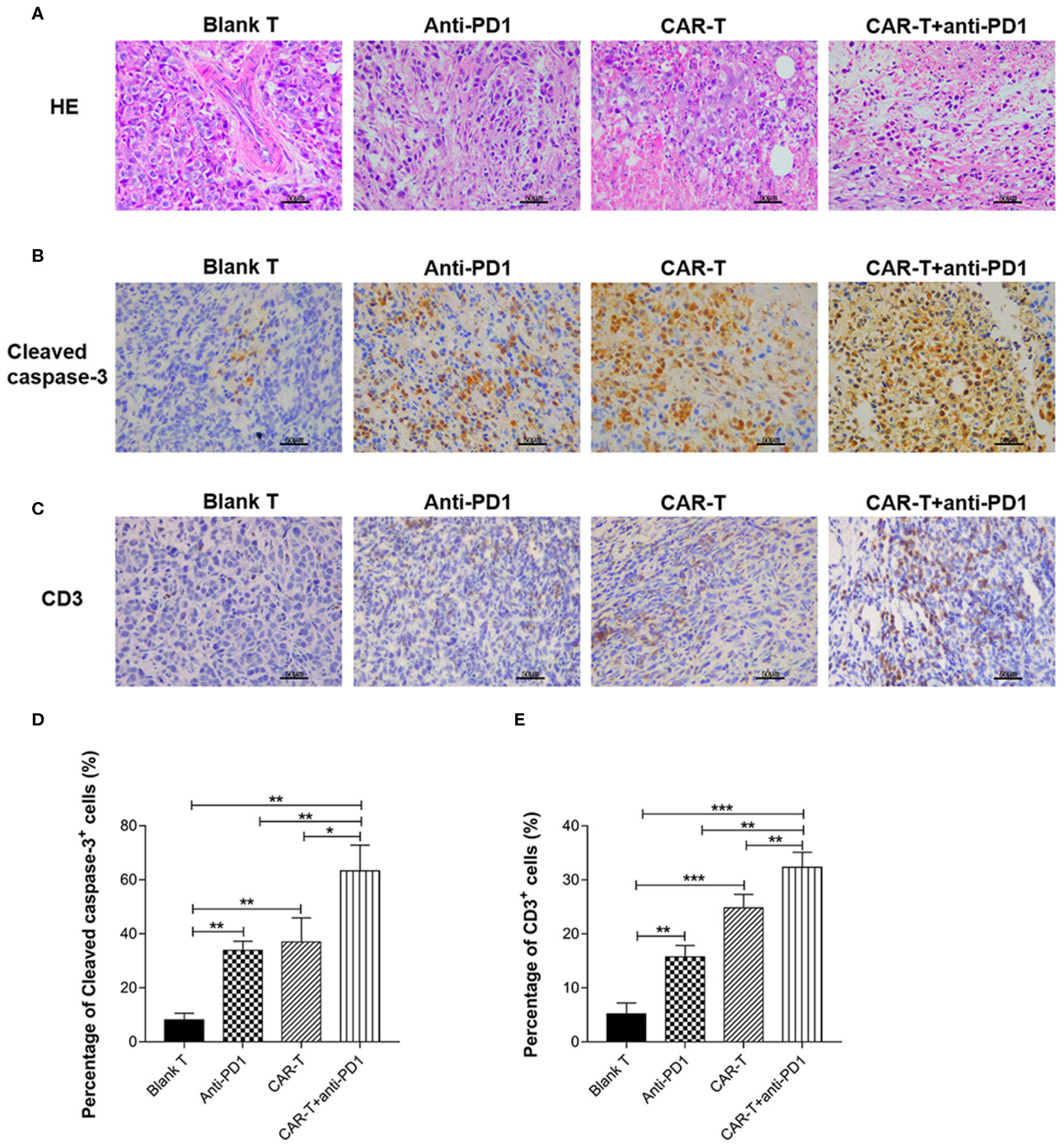

FIGURE 8 | The syngeneic tumor tissue observed by microscope after HE staining or immunohistochemical staining and the quantification of cleaved caspase-3 and CD3 expression. **(A)** The tissue sections of syngeneic tumors were stained by HE and observed by microscope (400×). Scale bar is 50 μm. **(B)** The tissue sections (five tissue sections for each mouse) of syngeneic tumors were stained by immunohistochemistry for cleaved caspase-3 expression and observed by microscope (400×). The slides were incubated with rabbit anti-mouse cleaved caspase-3 antibody. Scale bar is 50 μm. **(C)** The tissue sections (five tissue sections for each mouse) of syngeneic tumors were stained by immunohistochemistry for CD3 expression and observed by microscope (400×). The slides were incubated with rabbit anti-mouse CD3ε antibody. Scale bar is 50 μm. **(D)** For quantification of immunohistochemical staining, cleaved caspase-3 positive cells were counted in five random 400× microscopic fields for each tissue section. **(E)** For quantification of immunohistochemical staining, CD3ε-positive cells were counted in five random 400× microscopic fields for each tissue section. CAR-T group compared with blank T group, or CAR-T plus anti-PD1 group compared with anti-PD1 group, or anti-PD1 group compared with blank T group, or CAR-T plus anti-PD1 group compared with CAR-T group, or CAR-T plus anti-PD1 group compared with blank T group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The experiments have been repeated for three times. CAR, chimeric antigen receptor; Luc, luciferase; CAR-T, T cells transduced with CAR; Blank T, T cells not transduced.

16:1, after co-culture for 18 h, cytotoxicity (%) of CAR-T cells against 4T1-Luc-HER2 cells reached 39.8% (**Figure 6C**). With the addition of anti-PD1, cytotoxicity reached 49.5% (**Figure 6C**). All these results showed that addition of anti-PD1 antibody can enhance the efficacy of CAR-T cells against tumor cells *in vitro*. In the presence of the target tumor cells, CAR-T cells together with anti-PD1 antibody can secret more IL-2 and IFN-γ and eliminate tumor cells more efficiently in comparison with CAR-T cells alone. The results are consistent with our hypothesis that blocking PD1 signaling can rescue exhausted anti-HER2 CAR-T cells and can be a more effective treatment for cancer.

In most of the reported studies, NOD or severe combined immune deficiency (SCID) or NOD/SCID/γ-chain$^{-/-}$ mouse xenograft tumor model was used to evaluate the efficacy of CAR-T cell treatment alone (37, 38) or in combination of PD1 blockage (27, 28, 30). However, the lack of a complete immune system in these mice precludes a detailed understanding of the complex tumor microenvironment that affects the overall efficacy of CAR-T cells or in combination of PD1 blockage. In order to simulate tumor microenvironment, in this study, we established a syngeneic tumor model using immune competent mice so that an intact immune system was considered. By using this immune competent tumor model, we found that anti-HER2 CAR-T cells could likely infiltrate into tumors (**Figures 8C,E**), inhibit growth (**Figure 7**), change histomorphology (**Figure 8A**), and increase apoptosis (**Figures 8B,D**) of HER2$^+$ tumors. Adding anti-PD1 antibody could further enhance the anti-tumor activity of anti-HER2 CAR-T cells (**Figures 7**, **8**). Twenty-one days after treatment (on day 35), tumor weight was reduced by 50.0% in CAR-T group compared with blank T group, and was decreased by 73.3% in CAR-T plus anti-PD1 group compared with blank T group. The tumors from mice treated with CAR-T cells in combination with anti-PD1 antibody showed the highest percentage of apoptotic tumor cells (63.28%) among the four groups (**Figures 8B,D**). There were significantly more T cells in tumors from CAR-T group than in those from blank T group ($P < 0.001$ or $P < 0.01$) (**Figures 8C,E**). The highest T cell numbers were observed in tumors from mice treated with CAR-T cells together with anti-PD1 antibody (**Figures 8C,E**). Theoretically mouse CD3-positive cells in the tumor stroma include all mouse endogenous and exogenous T cells. However, the more amount of CD3-positive cells in the tumor stroma of CAR-T group and CAR-T plus anti-PD1 group compared with blank T group were likely due to the presence of more CAR-T cells, based on our result *in vitro* that anti-HER2 CAR-T cells could expand better after co-cultured with HER2$^+$ target cells, and the addition of anti-PD1 could further enhance their expansion.

Our study showed that the third generation anti-HER2 CAR-T cells can effectively eliminate HER2-positive breast tumor cells. The specificity of our anti-HER2 CAR-T cells relied on anti-HER2 scFv of CAR. The present study used the anti-HER2 scFv from specific anti-HER2 antibody 4D5 for CAR construction. The efficiency of our anti-HER2 CAR-T cells came from our selection of two co-stimulatory molecules CD28 and 4-1BB/CD137. The incorporation of additional co-stimulatory molecules into the CAR has greatly enhanced the efficacy of CAR-T cells. Various co-stimulatory molecules including CD28, 4-1BB/CD137, CD27, OX40 have been embedded in the CAR to explore their roles in antitumor immunity of CAR-T cells (39). In comparison with other co-stimulatory molecules, CD28 was more effective at increasing IL-2 production, enhancing clonal expansion and maintaining persistence of CAR-T cells. CD28 in combination with 4-1BB/CD137 is more effective than CD28 alone in eliciting cytotoxicity and IFN-γ production (40).

CAR-T cell therapy has become a promising method for the treatment of a variety of cancers (18–20, 41). However, the efficacy of CAR-T cell therapy for solid tumors was limited (18–20), one important reason was immunosuppressive microenvironment (20–22). PD1/PDL1 is one of the regulatory pathways that can serve as negative feedback loops after the initial immune response to switch off adaptive immunity (27). Currently, monoclonal antibody blocking PD1/PDL1 pathway has achieved encouraging results in patients in clinical trials (25, 26, 42). CAR-T cells in combination with immune inhibitors may improve the anti-tumor effect for solid tumors. The results in mouse models have indicated that anti-MSLN CAR-T (27) and anti-hPSMA CAR-T (28) cell therapy, in combination with anti-PD1 antibody, could improve the activity of CAR-T cells and enhance anti-tumor activity. The results from our *in vitro* and *in vivo* studies were consistent with these previous studies. PD1/PDL1 is only one of immunosuppressive pathways that affect the immune system against tumors. It will be interesting to see what will happen when we interfere with other immunosuppressive pathways.

In conclusion, our study demonstrated that third generation anti-HER2 CAR-T cells could specifically and efficiently eliminate HER2$^+$ breast cancer cells *in vitro* and *in vivo*. When combined with anti-PD1 antibody, CAR-T cells had a stronger therapeutic effect on HER2$^+$/PDL1$^+$ breast cancer cells *in vitro* and in mouse model with an intact immune system.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

AUTHOR CONTRIBUTIONS

PL, LY, TL, SB, BS, and YH performed experiments. PL, LY, and KY analyzed the data. HL and HG conceived and supervised the study. HL, DS, HG, and PL drafted and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Use of Cell and Genome Modification Technologies to Generate Improved "Off-the-Shelf" CAR T and CAR NK Cells

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The broad success of adoptive immunotherapy to treat human cancer has resulted in a paradigm shift in modern medicine. Modification of autologous and allogenic immune cells with chimeric antigen receptors (CAR) designed to target specific antigens on tumor cells has led to production of CAR T and CAR NK cell therapies, which are ever more commonly introduced into cancer patient treatment protocols. While allogenic T cells may offer advantages such as improved anti-tumor activity, they also carry the risk of adverse reactions like graft-versus-host disease. This risk can be mitigated by use of autologous immune cells, however, the time needed for T and/or NK cell isolation, modification and expansion may be too long for some patients. Thus, there is an urgent need for strategies to robustly produce "off-the-shelf" CAR T and CAR NK cells, which could be used as a bridging therapy between cancer diagnosis or relapse and allogeneic transplantation. Advances in genome modification technologies have accelerated the generation of designer cell therapy products, including development of "off-the-shelf" CAR T cells for cancer immunotherapy. The feasibility and safety of such approaches is currently tested in clinical trials. This review will describe cell sources for CAR-based therapies, provide background of current genome editing techniques and the applicability of these approaches for generation of universal "off-the-shelf" CAR T and NK cell therapeutics.

Keywords: chimeric antigen receptor, T cell, immunotherapy, genome editing, CRISPR-Cas9

INTRODUCTION

The clinical usefulness of cellular therapies was increasingly demonstrated through decades of successful hematopoietic stem cell transplantations (HSCT) in both autologous and allogeneic settings. In the case of allogeneic HSCT, some patients develop a complication called graft-versus-host disease (GVHD) due to cytotoxic alloreactivity of donor T cells that were transferred from the donor graft and which destroy tissues in the recipient. GVHD occurs due to immuno-incompatibility, e.g., human leukocyte antigen (HLA) mismatches between the donor and recipient. Although GVHD can result in increased transplant-related mortality, it was observed that

the cytotoxic T cells transferred in the graft also provided improved anti-cancer (leukemia) activity (1). These seminal discoveries led to investigation of cellular therapies in clinical modalities, including infusion of tumor infiltrating lymphocytes (TIL) for greater disease control [reviewed in (2)].

The cytotoxic activities of immune cells, like T and NK cells, can be exploited to generate more effective anti-cancer cell therapies. In the case of T cells, the T cell receptor (TCR) is activated upon recognition of and binding to "foreign" peptides presented by the major histocompatibility complex (MHC) class I on antigen presenting cells. A cascade of signaling events then ensues, including co-receptor binding that leads to activation of the SRC tyrosine kinase LCK, which phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3ζ complex. ZAP-70 is recruited to the phosphorylated CD3ζ and orchestrates downstream signaling events that lead to NFAT and AP-1 activation, resulting in T cell expansion, cytokine production (e.g., IL2, IFNγ) and stimulation of cytotoxic activity (3, 4). To avoid detection and subsequent elimination by T cells, transformed cells often exhibit repressed levels of MHC expression (5, 6). In contrast, NK cells become activated depending on the balance of activating and inhibitory signals that are generated by NK cell receptors during surveillance of cells that they contact (7). As loss of MHC on a tumor cell results in decreased inhibitory signaling in the NK cell, cancer cells must use other mechanisms to inhibit the cytotoxic function of NK cells. Alternative tumor immune escape mechanisms include upregulation of HLA-E on the tumor cell surface and release of soluble NKG2D ligands, such as MICA and MICB (8–10).

The idea of combining the anti-cancer activity of immune cells, such as T and NK cells, with the concept of antibody-specificity to redirect the cytotoxic activity of these cells to target tumor cells that express a particular antigen led to the development of chimeric antigen receptor (CAR) T and NK cells (11). Specifically, CARs are synthetic receptors that contain an extracellular antibody-like region designed to target a specific antigen called the single chain variable fragment (scFv), a hinge region that can be of different lengths, the choice of which may be guided by the proximity of the recognized epitope to the target cell surface, a transmembrane domain, one or more co-stimulatory domains and a signaling domain to induce cytotoxicity upon antigen binding (**Figure 1**). The choice of the co-stimulatory and signaling domains have been largely based upon components of the T cell receptor (TCR), i.e., containing CD28 and/or 4-1BB costimulatory domains and a CD3ζ signaling domain. Clinically approved second generation CARs contain a CD3ζ signaling domain in combination with either a CD28 (Yescarta®) or 4-1BB (Kymriah®) co-stimulatory domain. While the CD28-CD3ζ-containing CAR T cells were shown to exhibit more rapid and stronger signaling and to favor development of effector cell phenotypes, 4-1BB-CD3ζ-containing CAR T cells had a memory cell phenotype with greater persistence (12). Direct comparison of CD28 and 4-1BB co-stimulatory domains in anti-CD19-CARs showed that 4-1BB contributes to greater CAR T cell persistence and a more favorable toxicity profile in B cell non-Hodgkin's lymphoma (B-NHL) patients (13). The efficacy of CAR T cells, and recently CAR NK cells, has been shown for liquid tumors, most prominently in $CD19^+$ lymphoid-derived cancers and several clinical studies currently explore the translation of these promising results to solid tumors (link to these studies on clinicaltrials.gov). However, there are important clinical challenges that must be addressed to further improve CAR T cell approaches. For example, one major adverse event that commonly occurs during CAR T cell therapy is cytokine release syndrome (CRS), in which greatly elevated levels of inflammatory cytokines such as interleukin (IL)-6 are observed. Severity of CRS was correlated with patient IL-6 levels and the anti-IL-6 receptor antibody tocilizumab can be used to reverse CRS symptoms without interfering with CAR T cell anti-tumor activity (14). CRS was more severe in B-NHL patients treated with CD28-CD3ζ CAR T cells as compared to 4-1BB-CD3ζ CAR T cells, possibly due to the high immune response induced by CD28 stimulation (13). As it is not always possible to identify a neoantigen that is only expressed on the tumor cell to be targeted by a CAR-modified cell, healthy cells may also be eliminated by on-target-off-tumor activity. While this may be clinically manageable in some cases, e.g., loss of healthy B cells with CARs directed against CD19, the adverse events due to on-target-off-tumor activity may be more severe with other targets, such as unwanted destruction of lung tissue after administration of anti-ERBB2(HER2)-CAR T cells designed to treat metastatic $ERBB2^+$ cancer (15). The severity of on-target-off-tumor activity may be modulated by the dose of CAR T cells applied, as another study that tested HER2-CAR T cells in sarcoma patients showed this to be safe if administered up to 1×10^8 CAR T/m^2 compared to 10^{10} (or 6.25×10^{10} based on average female body surface area of 1.6 m^2) CAR T cells in the former study (16). Disease relapse due to lack of CAR T cell persistence has also been reported. Loss of anti-CD19 CAR T cells was found to result from $CD8^+$ immunity against the CAR T cells in some patients, which may have been due to the use of a murine scFv in the clinical CAR construct (17). To decrease potential immunogenic effects of CAR scFv sequences derived from mouse monoclonal antibodies, and, thus improve CAR T cell persistence, these should be humanized (18) (NCT02659943). To increase safety of CAR T cell therapies, vectors designed to deliver the CAR can be engineered to co-express suicide genes to allow removal of CAR T cells in case of uncontrollable severe adverse events. Examples of clinically available suicide gene strategies include the HSV-tk suicide gene (19), which makes the cells sensitive to ganciclovir-induced cytotoxicity or the inducible caspase 9 (iCasp9) gene cassette, which leads to rapid caspase-mediated apoptosis of expressing cells (e.g., CAR T cells) upon application of a synthetic inducer of dimerization, such as AP1903 or AP20187 (20, 21).

T and NK cells engineered to express CARs still eliminate target cells via the same cytotoxic mechanisms as unmodified T and NK cells, i.e., by release of perforins and granzymes, as well as death receptor interactions (22, 23). However, the cytotoxic activity is specifically amplified through binding of the scFv to the respective tumor-associated antigen. In addition, the concept of CAR T cell therapy is also applicable to other disease indications, incl. autoimmune diseases, in which CARs

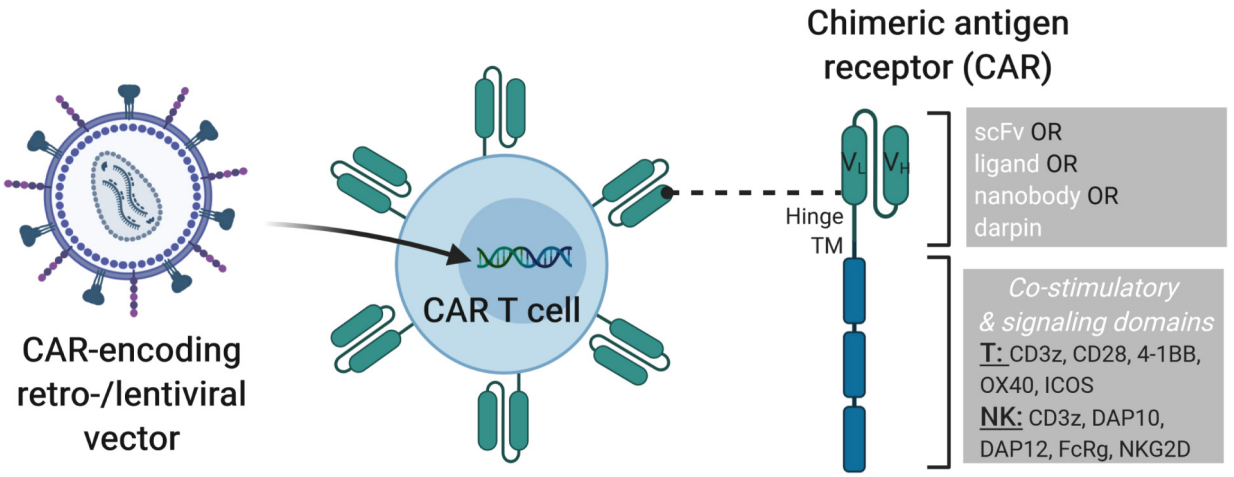

FIGURE 1 | Modification of T or NK cells with CAR-encoding retro- and lentiviral vectors. On the left, a lentiviral vector is shown that transfers the genetic cargo into the T or NK cells leading to the expression of a chimeric antigen receptor (CAR) on the cell surface. On the right, the structure of a 3rd generation CAR is depicted with single chain variable fragment (scFv, including V_H and V_L chains), hinge, transmembrane and signaling domains shown. CARs can be engineered with cell-type specific modules to enhance CAR T or CAR NK cell activity. Examples of cytoplasmic signaling domains that can be engineered into CARs for T and NK cells are shown. Combining such strategies with additional genome modification approaches described in later sections of this review will lead to improved "off-the-shelf" cell products.

are introduced into regulatory T cells (Tregs), which have anti-inflammatory activities [(24) and reviewed in (25)].

Development of cell-based immunotherapeutic treatment strategies is, at least partially, directed by the characteristics of the disease to be treated and the available technology or feasibility to generate the necessary technologies. In the case of generating new CAR therapeutics to treat cancer, one of the key decisions to be made is which tumor-associated antigens to target with the scFv design. This will largely determine the specificity of tumor targeting and the extent of on-target, but also off-tumor side effects. Another important consideration is the design of the remaining domains of the CAR, for example which transmembrane, co-stimulatory and signaling domains should be incorporated. This decision may also be influenced by the cell type (e.g., T cell, NK cell, other immune cells) to be used as the "living" drug as well as the temporal window in which these cell therapies should be active. Interestingly, CAR designs based on the T cell receptor also function in NK cells (26–28). However, this does not rule out the possibility to engineer immune cell type-specific CARs for optimal use in the chosen cell type (**Figure 1**). For example, modification of NK cells with a chimeric receptor consisting of the NK cell activating receptor NKG2D, DNAX-activation protein 10 (DAP10) and CD3ζ led to increased cytotoxic activity against cancer cell lines and improved activity in an osteosarcoma mouse model (29, 30). CAR NK cells designed to target the prostate stem cell antigen (PSCA) on prostate cancer cells were modified with a CAR vector in which the CD28 transmembrane and costimulatory domains as well as the CD3ζ signaling domain were exchanged for DNAX-activation protein 12 (DAP12) transmembrane and intracellular signaling domains, which resulted in specific cytotoxicity against PSCA-positive tumor cell lines as compared to PSCA-negative tumor cells *in vitro* and *in vivo*. NK cells modified with a chimeric receptor that fused NKG2D to CD3ζ (NKG2D.ζ) eliminated myeloid-derived suppressor cells (MDSC) and the anti-cancer activity of these modified NK cells was not suppressed by the tumor microenvironment (TME) (31). Of note, CAR T cells that were administered following NKG2D.ζ-NK cells had improved tumor infiltration and anti-cancer activity. While most studies to date collect and modify autologous T cells to produce CAR T cell therapies, use of allogeneic CAR NK cells derived from primary NK cells (e.g., cord or peripheral blood) or from NK cell lines derived from lymphoma patients (e.g., NK-92) is increasing and other "off-the-shelf" cell sources are also being tested.

In the following sections, important concepts of how to generate "off-the-shelf" CAR cell therapies, such as the source of immune cells to be modified, strategies to overcome tumor immune escape mechanisms and genome engineering approaches that can be applied to improve CAR T and CAR NK cell function will be considered.

T CELL SOURCES: AUTOLOGOUS, ALLOGENEIC, INDUCED PLURIPOTENT STEM CELL-DERIVED AND EXPANDED PROGENITOR-DERIVED

The most common source of CAR T cells currently applied clinically is patient-derived autologous T cells, which are then genetically modified to express the CAR of choice, expanded and re-infused into the patient. Lentiviral or gammaretroviral vectors are often used in clinical trials to deliver the CAR into the T cell genome (**Figure 1**) (32, 33), but also non-viral integrating technologies such as Sleeping Beauty transposons were shown to efficiently generate CAR T cells (34). While use

of autologous cells is enticing as this avoids challenges with immuno-incompatibilities, such as complications like GVHD, there are also disadvantages with autologous cell sources. For example, the immune cell populations may be adversely affected in heavily pre-treated patients so that the quality and number of cells for *ex vivo* modification and expansion may be suboptimal. Additionally, patients who have infections or rapidly advancing cancers might not survive the several weeks needed to produce autologous CAR T cells, as the cells have to be collected by apheresis, shipped to the facility site for genetic modification, expansion and formulation, before being shipped back to the hospital where the patient will be infused with the CAR T cells. Advantages of allogeneic CAR T cells include a lower risk of genetically modifying and re-infusing leukemic cells (35), and allogeneic cells can be prepared and stored for future use so that there is a shorter waiting period for infusion into the patient. Thus, "off-the-shelf" allogeneic cell sources could provide greater flexibility for treatment protocols, potentially lower overall costs if multiple patients can be treated from a single CAR T cell product and could be expected to allow broader access to these clinical procedures (36).

Therefore, methods for efficient and reliable production of "off-the-shelf" T cells remain highly sought goals in the field of cellular immunotherapy. Important conditions that these cells must meet include avoidance of rejection due to recognition by host T cells via HLA class I molecules or host NK cells by HLA class II receptors. "Off-the-shelf" immune cells should also lack alloreactivity to limit unwanted toxicities due to recognition and destruction of healthy host tissues. Several strategies to avoid host cell recognition have been explored, such as knock-down or knock-out of MHC molecules to block recognition by host T cells. However, this can result in elimination of the modified cells due to NK cell activity against cells lacking MHC expression (37–39). Expression of ligands that inhibit NK cell cytotoxicity, like HLA E or HLA G, can ameliorate elimination of the engineered cells, but since receptors for these ligands are not expressed on all NK cells, veritable "off-the-shelf" cell therapeutics will likely require genetic engineering strategies that address multiple layers of immune cell recognition patterns and cytotoxic mechanisms (40–42). Importantly, these modifications would ideally not negatively impact immune cell expansion that is necessary for clinical application or cell persistence and function, which are important for immune cell anti-cancer efficacy. Additionally, potential risks of genetically modified immune cells must be evaluated, although such risks are generally low in differentiated somatic cells like T cells.

NK CELL SOURCES

An alternative approach is to exploit the natural cytotoxic activity of NK cells to generate allogeneic "off-the-shelf" CAR NK cells to target cancer cells. One advantage of NK cells is that they were shown to not induce GVHD even in mismatched settings (43). However, an earlier study observed acute GVHD in five of nine patients who received donor-derived allogeneic NK-donor lymphocyte infusions (NK-DLI) after HLA-matched transplantation of T cell-depleted (for delivery of $\leq 2 \times 10^4$ T cells/kg) peripheral blood stem cells from matched sibling donors (1 of 5) or matched unrelated donors (4 of 4) (44). The authors propose that allogeneic NK-DLI may have contributed to the observed GVHD by aggravating an existing subclinical T cell-mediated GVHD. This is supported by the assessment of donor chimerism based upon CD3, which showed significantly higher donor chimerism in GVHD patients, and that allogeneic NK-DLI was accomplished shortly prior to detection of the high donor chimerism in three of the five patients who developed GVHD. The relative risk of GVHD following NK cell application will become clearer as more data accumulates with CAR NK cells, which are increasingly incorporated into clinical trials (**Table 1**).

Different NK sources have been used to generate pre-clinically and clinically tested CAR NK cells, including cell lines such as NK-92 cells (45), cord blood-derived NK cells (43, 46) and peripheral blood-derived NK cells (28). Of note, a recent landmark phase 1 and 2 study showed the feasibility of cord-blood-derived CAR NK cells to treat relapsed or refractory $CD19^+$ B-cell cancers (43). Eight of eleven (73%) patients responded rapidly (within 30 days after CAR NK cell infusion), including seven complete remissions. Of particular interest, the only major adverse events were related to the lymphodepletion strategy (i.e., neutropenia, lymphopenia) and no cytokine release syndrome, neurologic events or GVHD were observed, even with 2-5 HLA allelic mismatches (43). CAR NK cells persisted for at least 12 months after infusion, which may have been at least partially due to inclusion of an IL-15 expression cassette in the CAR construct, a cytokine known to enhance NK cell survival and proliferation (43). The same group previously showed that one cord-blood unit could be used to produce over 100 CAR NK doses, further highlighting allogeneic CAR NK cells as potential "off-the-shelf" drugs (47). While regulatory guidelines may vary depending on the country in which the study is performed, cell therapeutics should be viable (e.g., $\geq$70%) and demonstrated to be negative for endotoxin, mycoplasma or bacterial contaminations. For CAR NK cells, the cell product should contain mostly $CD56^+$ cells ($\geq$90%), and be free of $CD3^+$ cells (e.g., $\leq$0.2%) and $CD14^+$ cells (e.g., $\leq$5%). In the case that CAR NK cells are expanded via co-culture with irradiated feeder cells, for example, membrane bound IL-15 and 4-1BB ligand expressing K562 cells or membrane bound IL-21 expressing OCI-AML3 cells, the final CAR NK cell product should be demonstrated to be free from contamination of co-cultured cells (e.g., $\leq$1%) (48, 49). Contamination of primary NK cell therapeutics with feeder cells may be mitigated by alternative expansion methods, such as use of coated beads or cytokine combinations to expand NK cells. Primary NK cells can be activated and expanded with cytokines such as IL-2, IL-12, IL-15, IL18, and IL-21 (50–53). Similarly to expansion of primary T cells with CD3/CD28 beads, primary NK cells can also be expanded with CD335 (NKp46)/CD2 beads.

iPSC AND OTHER CELL SOURCES

Additional cell sources to produce "off-the-shelf" CAR cells include stem cell and progenitor cell populations such as induced pluripotent stem cells (iPSC) and precursor T cells.

TABLE 1 | Selected clinical trials testing potential "off-the-shelf" CAR cell therapies.

Cells	CAR	Diseases/Patients	1°/2° outcomes	References	
No genome modification					
Allogeneic T cells	CD19-CAR	Relapsed or refractory $CD19^+$ B cell malignancies	1°: DLT, CR 2°: ORR, DOR, safety, tolerability, TRM	NCT04384393	ThisCART19
Allogeneic T cells	CD19-CAR	Elderly relapsed or refractory B-ALL	1°: occurrence of adverse events 2°: overall response rate, DFS, OS	NCT02799550	
Allogeneic T cells	alloCART-19	Pediatric relapsed or refractory ALL	1°: DLT 2°: AE, ORR, BOR	NCT04173988	
Allogeneic T cells	NKG2D-based CAR-T plus inhibitory peptide T cell receptor (TCR) inhibiting molecule (TIM) to reduce signaling of the TCR complex through a non-gene edited approach	Metastatic colorectal cancer (mCRC)	1°: DLT, ORR 2°: AE, safety, ORR, BOR, kinetics, clinical activity, PFS, EFS, OS	NCT03692429	alloSHRINK trial
Genome modified					
Allogeneic T cells (TCRα/β disruption)	Anti-CS1 CAR (UCARTCS1A)	Relapsed or refractory MM	1°: safety	NCT04142619	MELANI-01
Allogeneic T cells (TCRα/β disruption)	Anti-CD123 (UCART123)	Relapsed or refractory AML	1°: safety, tolerability	NCT03190278	AMELI-01
Allogeneic T cells (TCRα/β disruption)	CD19-UCART	Relapsed or refractory B cell malignancies	1°: DLT 2°: ORR, CART persistence	NCT03229876	
Allogeneic T cells (TCRα/β disruption)	BCMA-UCART	Relapsed or refractory MM	1°: ORR 2°: safety, tolerability, CART persistence	NCT03752541	
Allogeneic T cells (TCRα/β disruption)	CD22-CAR (UCART22)	Relapsed or refractory $CD22^+$ B-cell B-ALL	1°: safety, tolerability	NCT04150497	BALLI-01
Allogeneic T cells (TCRα/β disruption)	CD19-UCART	Relapsed or refractory B-ALL	1°: DLT 2°: safety, tolerability, objective remission rate and duration, PFS, OS	NCT02746952	CALM
Allogeneic T cells (TCRα/β and B2M disruption)	UCART019	Relapsed or refractory $CD19^+$ leukemia and lymphoma	1°: safety, feasibility, persistence 2°: tumor response, test for humoral immunity against murine CD19 scFv	NCT03166878	
Allogeneic T cells (TCRα/β and B2M disruption)	CTX110 (CD19-CAR)	Relapsed or refractory B cell malignancies	1°: DLT, ORR 2°: DOR, PFS, OS	NCT04035434	
Allogeneic T cells (TCRα/β and B2M disruption)	CTX120 (BCMA-CAR)	Relapsed or refractory MM	1°: AE, DLT, ORR 2°: PFS, OS	NCT04244656	
Allogeneic T cells (TCRα/β and B2M disruption)	CTX130 (CD70-CAR)	Relapsed or refractory renal cell carcinoma	1°: AE, DLT, ORR 2°: PFS, OS	NCT04438083	
Allogeneic T cells (TCRα/β and B2M disruption)	Universal Dual CD19 + CD20-CAR or CD19 + CD22-CAR	Relapsed or refractory B-cell malignancies	1°: safety, feasibility, persistence 2°: anti-tumor response, test for humoral immunity against murine CD19 scFv	NCT03398967	
Donor T cells (CMV- or EBV-specific T cells derived from donor CD62L + TCM cells)	CD19-CAR	B cell malignancies after allogeneic transplant	1°: safety, feasibility 2°: persistence, trafficking to bone marrow, function, CMV/EBV reactivation, elimination of $CD19^+$ tumor cells	NCT01475058	
Allogeneic EBV specific cytotoxic T-lymphocytes (EBV-CTLs)	CD19-CAR	B cell malignancies after allogeneic transplant or high risk for relapse	1°: safety, persistence 2°: assess effects on leukemia progression, CAR-T cell survival and	NCT01430390	

(Continued)

TABLE 1 | Continued

Cells	CAR	Diseases/Patients	1°/2° outcomes	References	
			proliferation, long-term status of treated patients		
Allogeneic EBV specific T cells	Anti-CD30 CAR	Relapsed or refractory $CD30^+$ lymphoma	1°: DLT 2°: ORR, DOR, SD, PFS	NCT04288726	
Other "off-the-shelf" CAR cells					
NK-92 cell line	CD33-CAR (CD28-CD137 (4-1BB)-CD3ζ)	Relapsed or refractory $CD33^+$ AML	1°: safety, feasibility 2°: anti-leukemia response, *in vitro* anti-AML cytotoxicity, test for development of humoral immunity against the murine anti-CD33 scFv	NCT02944162	
NK-92 cell line	CD7-CAR (CD28-4-1BB-CD3ζ)	CD7 + leukemia and lymphoma	1°: AE, toxicity profile 2°: clinical response, persistence	NCT02742727	
NK-92 cell line	CD19-CAR	$CD19^+$ leukemia and lymphoma	1°: AE 2°: ORR	NCT02892695	
Allogeneic NKT cells	CD19-CAR + IL-15	Relapsed or refractory B cell malignancies	1°: DLT 2°: persistence of modified cells, overall response	NCT03774654	ANCHOR
Haploidentical/Allogeneic Gamma Delta (γδ) T cells	NKG2DL-targeting CAR	Relapsed or refractory solid tumors	1°: DLT 2°: AE, efficacy, PFS, DOR	NCT04107142	
Allogeneic Gamma Delta (γδ) T cells	CD19-CAR	High risk, relapsed $CD19^+$ B cell malignancies	1°: safety (adverse events) 2°: CAR γδ persistence, antitumor activity, MTD	NCT02656147	

AE, adverse events; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; BOR, best overall response; CR, complete remission; DFS, disease-free survival; DLT, dose limiting toxicities; DOR, duration of response; EBV, Epstein Barr virus; MM, multiple myeloma; ORR, objective response rate; PFS, progression-free survival; OS, overall survival; scFv, single chain variable fragment; SD, stable disease; TRM, treatment related mortality.

iPSC possess a nearly unlimited proliferative potential and can be differentiated into various cell types, including T and NK cells. Thus, iPSC offer a renewable source of potentially standardized cells for immunotherapies and can be easily genetically modified to generate immune cells with improved characteristics (54). The feasibility of producing CAR T cells from iPSC was demonstrated by transduction of peripheral blood lymphocyte-derived iPSC with a lentiviral vector encoding for a second-generation anti-CD19-CAR (55). After hematopoietic specification and expansion, the authors used a T-lymphoid commitment co-culture protocol to generate anti-CD19-CAR-T-iPSC-T. The authors directly compared the iPSC-derived CAR T cells with TCR-αβ and TCR-γδ peripheral blood lymphocytes from the same donor and transduced with the same CAR and demonstrated that the iPSC-derived CAR T cells showed a similar anti-cancer activity as the CAR TCR-γδ cells in an immunodeficient mouse xenograft tumor model using the $CD19^+$ Raji human Burkitt lymphoma cell line (55).

Adaptation of CAR designs to exploit the signal transduction pathways naturally used for cell activity may lead to improved CAR NK or other CAR-cell type activities. For example, an "NK-CAR" engineered to contain the NKG2D transmembrane domain, the 2B4 co-stimulatory domain and the CD3ζ signaling domain was used to modify iPSC cells, which were subsequently differentiated into NK-CAR-iPSC-NK cells (iPSC-derived NK cells equipped with an NK-CAR). The NK-CAR-iPSC-NK cells demonstrated superior anti-tumor activity when directly compared to T-CAR-iPSC-NK cells in an ovarian cancer xenograft model and had similar activity as observed for CAR T cells that expressed a typical CAR designed for T cells (CD28-CD3ζ) (56). Advantages of iPSC-derived CAR T/CAR NK cells include their enormous proliferative and expansion capacities as well as the relative ease of genomic modification, which provides the possibility to create cell banks with different CAR constructs as standardized "off-the-shelf" immunotherapies.

A recently described inducible transcription factor-mediated forward programming approach to efficiently produce large numbers of hemato-endothelial progenitor cells and hematopoietic progenitor cells may also become useful for generating "off-the-shelf" cell therapies, such as CAR NK cells (57). While this strategy led to sustained production of myeloid lineages, differentiation into the lymphoid lineages was less robust. However, RNAseq interrogation of gene expression patterns revealed several transcription factor targets whose expression could potentially be modulated to overcome this. Similarly, methods to produce conditionally immortalized murine lymphoid progenitors might be exploited to efficiently generate CAR T cells, although this remains to be tested and applied to human lymphoid progenitors (58, 59). Expression of an anti-CD19-CAR in lymphoid progenitors was shown to suppress T cell development with the generation of cells with NK cell-like characteristics that had strong cytotoxic activity

against CD19$^+$ leukemia cells across MHC barriers and without causing GVHD. Importantly, this shift in differentiation was dependent upon ongoing signaling activity of the respective CAR early during hematopoietic development (60). Most recently, CAR-macrophages (CAR-Ms) were shown to phagocytose tumor cells in an antigen-specific manner, decrease tumor burden in two solid tumor xenograft mouse models and to promote anti-cancer T cell activity by inducing a pro-inflammatory tumor microenvironment (61).

Thus, much progress has been made in identification of alternative "off-the-shelf" therapeutic CAR cells. As any manipulation of the genome, such as insertion of therapeutic CAR vectors (**Figure 1**), carries an inherent risk, these must be carefully evaluated. While possible genotoxic risks such as transformation of a healthy cell to a cancer cell are low in terminally differentiated somatic cells like T and NK cells, such modifications in stem cells (e.g., HSC, iPSC) or progenitor populations that can be differentiated into T or NK cells may carry higher risks, which should be assessed and mitigated as necessary. There are also differences in regulatory requirements for clinical use of primary lymphocytes and cell lines. For example, cell lines must be irradiated to minimize the risk of secondary lymphoma (e.g., due to uncontrolled proliferation of immortalized cell lines), and it has to be documented that culture conditions did not include animal based supplements (e.g., fetal bovine serum) or antibiotics (e.g., penicillin, streptomycin).

GENOME MODIFICATION TECHNIQUES AND APPLICATION TO CAR T CELLS

In addition to gene transfer technologies to improve CAR T cell function, genomic modification strategies have been used to advance "off-the-shelf" cell therapeutics. Zinc-finger nucleases (ZFN), transcription activator-like nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (CRISPR-associated protein 9) systems are currently the most commonly employed genome editing technologies. ZFN and TALEN technologies target specific genomic loci via protein-DNA interactions, and require protein engineering expertise that is not available in every laboratory. In contrast, the CRISPR-Cas9 system uses RNA-guided DNA recognition to define the genomic modification locus, which makes tailored design of CRISPR-Cas9 to target specific genes relatively easy and has led to wide-spread use of this technique in the scientific community. Genome editing occurs after double strand break (DSB) induction through two main DNA repair mechanisms, non-homologous end joining (NHEJ) and homology directed repair (HDR), with NHEJ active throughout all phases of the cell cycle and the less efficient HDR mainly confined to the S phase. The efficiency of targeted insertion by HDR can be improved by TP53 inactivation to block the TP53 damage response and interruption of the cell-cycle induced by DNA double-stranded breaks caused by CRISPR-Cas9 (62–64). Interestingly, NHEJ was further improved by a new method called CRISPR-HOT, which stands for CRISPR–Cas9-mediated homology-independent organoid transgenesis (65). This method enables the efficient generation of knock-in human organoids in different tissues and achieves precise integration of exogenous DNA sequences into desired loci, without the necessity to inactivate TP53.

ZFN "Off-the-Shelf" Cell Products

In order to produce "off-the-shelf" allogeneic CAR T cell products, it is necessary to disrupt adverse reactions such as GVHD due to endogenous TCR activation that occurs with HLA-mismatched donors and recipients. Possible gene editing strategies to circumvent this include elimination of endogenous TCR expression to generate universal donor T cells (66) and targeted insertion of CAR into the TCR alpha constant (TRAC) locus (67). Efficient genome editing of CD8 and CD4 T cells by HDR was accomplished using Adeno-associated virus (AAV) serotype 6 vectors (AAV6) to deliver the homologous donor template and electroporation of ZFN mRNA (68).

Electrotransfer was also used to deliver designer ZFN to delete TCR α or β chains in CD19-CAR T cells, and the TCR$^-$CAR$^+$ population maintained CD19 specificity without responding to TCR stimulation (69).

TALEN "Off-the-Shelf"Cell Products

Universal CAR19T (UCART19) cells were generated using TALENs to target the constant region of the *TCRα chain* (*TRAC*) and the *CD52* gene to make UCART19 cells resistant to Alemtuzumab (Campath®), which is an antibody used to eliminate CD52$^+$ lymphocytes in B-cell chronic lymphocytic leukemia (66). UCART cells led to rapid molecular remissions (28 days) in two infants with refractory high-risk B-ALL. One patient had grade 2 skin GVHD and the second patient had a possible mild skin GVHD that was quickly resolved with topical steroids (66). TALENs were also used to disrupt the TCRαβ locus to generate universal allogeneic CAR T cells directed against the tumor-associated antigen CS1 (UCARTCS1A), which are currently tested in relapsed and refractory multiple myeloma patients (NCT04142619) (**Table 1**). A similar approach was used to generate universally applicable anti-CD22 CAR T cells (UCART22) to treat patients with relapsed and refractory CD22$^+$ B-cell B-ALL (NCT04150497).

CRISPR-Cas9 "Off-the-Shelf"Cell Products and Developing Technologies

CRISPR-Cas9 RNPs and AAV6 were used to specifically deliver an engineered 2.3-kb-long TCR construct TCR25D6, which recognizes a peptide derived from myeloperoxidase as a tumor-associated antigen in myeloid neoplasia patients when presented on HLA-B7, into the *TRAC* locus (70). CRISPR-Cas9-mediated knockout of TCRα/β and B2M in combination with lentiviral delivery of an anti-CD19 CAR into allogeneic T cells resulted in universal CAR T cells (UCART019) that are clinically tested in relapsed or refractory CD19$^+$ leukemia and lymphoma patients (NCT03166878). CRISPR-Cas9-mediated TCRα/β and B2M knockout to generate "off-the-shelf" allogeneic CAR T cells is also evaluated in other clinical trials for CD19$^+$ leukemia and lymphoma patients (NCT04035434), multiple myeloma

patients (NCT04244656) and renal cell carcinoma patients (NCT04438083) (**Table 1**).

Multiplex CRISPR-Cas9 allows simultaneous editing of several genomic loci. The feasibility and safety of using multiplex CRISPR-Cas9 to engineer autologous T cells with enhanced anticancer activity was recently demonstrated in a phase I trial (NCT03399448) (71). CRISPR guide RNA was electroporated into T cells to delete endogenous TCRα and TCRβ chains as well as the *PDCD1* gene that encodes the programmed cell death protein 1 (PD-1). Endogenous TCR disruption was done to allow enhanced expression of the cancer-specific TCR NY-ESO-1, which was introduced by lentiviral transduction. In addition, PD-1 knockout was accomplished to improve activity of the engineered T cells by avoiding checkpoint inhibition through tumor-associated cells. This may be an important strategy as disruption of PD-1 on T cells may help avoid immune-related side effects observed upon systemic administration of anti-PD-1 monoclonal antibodies, while still improving CAR T cell anti-tumor activity.

Advances to Increase CRISPR Technology Specificity and Safety

While RNA-guided (sgRNA) programmable nucleases based on CRISPR-Cas9 are very versatile and useful tools and mostly generate accurate and precise DNA DSBs, potentially also off-target effects can occur. Furthermore, chromosomal translocations are rare unwanted side effects, especially in case of multiplexing (72). To decrease the risk of these unwanted events, further engineering, e.g., CRISPR-Cas9 systems with less off-target effects, and newer gene editing approaches are being developed as discussed below. Such advances will lead to more efficient and safer generation of genome modified "off-the-shelf" CAR T and CAR NK cell products (**Figure 2**).

CRISPR-Cas9 has two nuclease domains and introduction of inactivating mutations into one of these domains results in so-called nickases, which cleave only one strand of the targeted DNA (73). As a further improvement, "dead" Cas9 variants with inactivating mutations in both nuclease domains were created that could be fused to DNA modifying enzymes, such as Apobec-like nucleobase deaminase enzymes. These "base editors" lead to defined base alterations without the need to cut the DNA and thus further reduce the likelihood of side effects (74). More recently, a catalytically impaired Cas9 was fused to an engineered (Murine Leukemia Virus-derived) reverse transcriptase to enable "prime editing" as a new technology to write new genetic information into a specified DNA site (75). Accordingly, prime editing further expands the capabilities of gene editing to create new options for immunotherapeutics.

Additional approaches have been developed with the intent to minimize possibly deleterious activity of genome editing described above. For example, as long-term CRISPR-Cas9 expression is not required for efficient genome modification, transient RNA-protein (RNP) complexes can be delivered into the target cell population in place of viral vectors or DNA constructs. Alternatively, non-integrating lentiviral vectors can be engineered for transient delivery of CRISPR-Cas9 editing coupled with the possibility to target specific cell populations (76). High-fidelity CRISPR-Cas9 nuclease variants designed to have fewer interactions with non-specific DNA sequences, but maintaining on-target DNA activity were also developed (77). As mentioned above, novel Cas9 fusion proteins were engineered to create base editors, i.e., cytosine base editors (CBE) and adenosine base editors (ABE), capable of editing single bases (78, 79). CBE were generated by fusing a cytidine deaminase to a catalytically impaired Cas9 protein (i.e., that is unable to induce double-strand DNA breaks) and uracil glycosylase inhibitor. Since Cas9-independent off-target DNA editing was observed with CBEs largely due to cytidine deaminase activity, additional Cas9 fusion variants were generated and shown to have up to 100-fold less Cas9-independent off-target DNA editing, but retained 50–90% of on-target DNA editing (80). Approaches like these will make it easier to safely modify allogeneic T cells into universal CAR T cells via disruption of TCRα/β and B2M without the need to introduce double strand DNA breaks.

MECHANISMS TO IMPROVE IMMUNE CELL ANTI-CANCER ACTIVITY

In addition to enhancing immune cell recognition of tumor cells via CAR expression, additional modifications of CAR cells may be necessary to effectively overcome tumor cell resistance mechanisms. One mechanism tumor cells use to evade immune cell-mediated cytotoxicity is exploitation of immune checkpoint signaling, which is used to inhibit immunologic damage of "self" cells in the healthy state. Immune checkpoints are critical components of autoimmune tolerance to avoid autoimmune diseases like rheumatoid arthritis (81, 82), type I diabetes (83) and multiple sclerosis (84). Checkpoint receptors on immune cells recognize ligands expressed on cells being surveilled and activation of these immune checkpoint receptors by the ligands leads to inactivation of the immune cells. This mechanism is exploited by tumor cells, which may overexpress these ligands or induce other cells [e.g., tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs)] within the tumor microenvironment (TME) to express checkpoint ligands to create an immunosuppressive shield throughout the TME and thus help tumor cells evade immunosurveillance (85–87). Secretion of immunosuppressive factors like transforming growth factor-β (TGF-β) by cells in the TME can directly inhibit CAR T cell cytotoxic activity and even direct differentiation of effector T cells to regulatory T cells (88–90).

Immune checkpoint molecules include cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), PD-1 (PDCD1, CD279), lymphocyte activation gene 3 (LAG-3), and T cell membrane protein 3 (TIM3, HAVCR2) (91–94). Interaction of immune checkpoints with their cognate ligands results in suppression of immune cell function. Thus, tumor cells may express CD80/86 to suppress T cell activity via binding to CTLA4, or express PD-1 ligands PD-L1 (CD274) or PD-L2 (PDCD1LG2, PD-2 ligand). Accordingly, binding of LAG-3 to MHC class

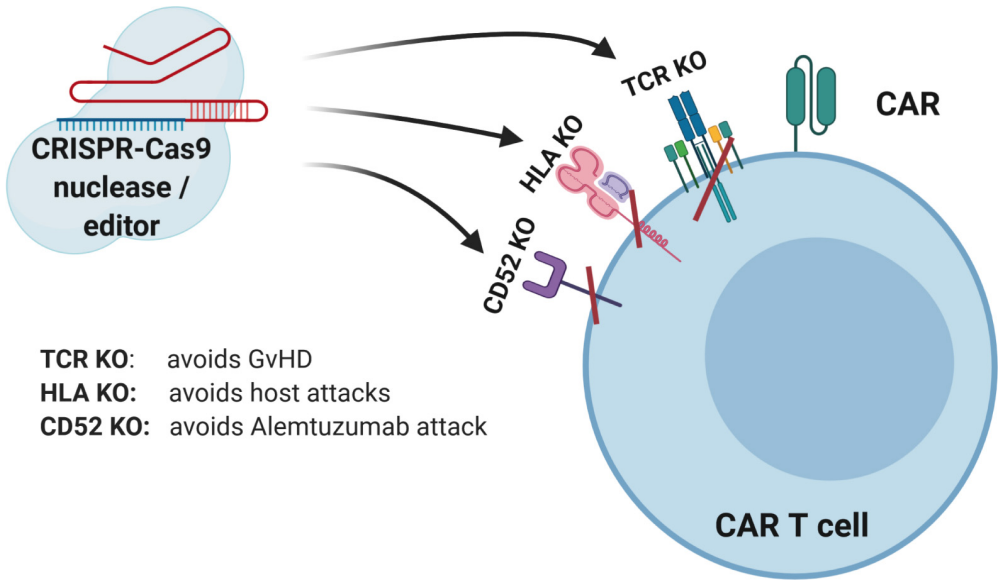

FIGURE 2 | CRISPR-Cas9-mediated gene editing of CAR T cells. The TCR can be knocked out to lessen the likelihood of graft versus host disease (GVHD). The HLAs can be knocked out to increase persistence of gene-modified cells. Knockout of receptors that can be targeted by other medications, such as antibodies, can be accomplished to allow selective survival of gene-modified cells, e.g., CD52.

II or fibrinogen-like protein 1 (FGL1), or ligation of TIM3 to galectin 9, carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), high-mobility group box protein 1 (HMGB1) or the non-protein ligand phosphatidylserine was shown to negatively regulate immune cell cytotoxic activity (95–97).

Several antibodies were developed to inhibit the activity of immune checkpoint molecules and clinical anti-cancer activity was demonstrated for some of these checkpoint inhibitors. Currently, most studies have investigated checkpoint inhibition of CTLA4 and PD-1 activities (98, 99). However, strategies to inhibit LAG-3 may be even more promising as antibodies that target LAG-3 were shown to enhance cytotoxic T cell activation and may inhibit Treg-induced immunosuppressive activity as elevated levels of a LAG-3^{+} subpopulation of Tregs was found at tumor sites and in peripheral blood mononuclear cells of patients with melanoma or colorectal cancer (100). Combination of the anti-LAG-3 antibody IMP321 with paclitaxel led to improved immune responses and greater antitumor activity in metastatic breast cancer patients (101). Currently, more than 240 clinical studies are evaluating the efficacy of checkpoint inhibitors in several different treatment modalities in cancer patients (link to respective studies on clinicaltrials.gov). As CAR-modified immune cells can become functionally inactivated or depleted due to tumor escape mechanisms such as immune checkpoints, checkpoint inhibition can help promote CAR T and perhaps CAR NK cell persistence and anti-tumor activity. The efficacy of CAR T cells directed against mesothelin with concomitant CRISPR-Cas9-mediated knockout of TCRαβ and PD-1 is currently tested in a clinical trial (NCT03545815) of patients with mesothelin positive solid tumors. Such studies will help elucidate the feasibility of combining checkpoint inhibition in an "off-the-shelf" CAR T cell setting.

In addition to application of immune checkpoint inhibitors, anti-tumor activity was demonstrated by CAR-mediated cytokine secretion at the tumor site using T cells redirected for universal cell killing (TRUCKs) (102–105). This strategy involves modification of T cells with a constitutively expressed CAR and a cytokine expression cassette that is controlled by an inducible promoter. The TRUCK concept uses the NFAT signaling pathway to produce pro-inflammatory cytokines upon activation of the CAR CD3ζ signaling domain after tumor antigen recognition. This results in modification of the TME via cytokine secretion and recruitment of additional anti-tumor immune cells to increase anti-cancer activity (see **Figure 3**). In the original design, TRUCKs were generated by retroviral vector-mediated transfer of two separate vectors – one for the CAR and the second for the inducible cytokine expression cassette. Recent work showed the feasibility to deliver the necessary genetic cargo on a single lentiviral vector (106), thus advancing the potential use of this technology for "off-the-shelf" immunotherapy.

DIRECT *IN VIVO* APPLICATION OF CAR-BASED PRINCIPLES USING TARGETED VIRAL VECTOR NANO-PARTICLES

As the whole genesis of CAR-harboring cellular products is highly demanding, future-oriented approaches for "off-the-shelf" applications are also considering the direct application of GMP-grade viral vector preparations to deliver the CAR-based principles directly into the target immune cells of choice, e.g., T and NK cells. This method entirely circumvents immunological

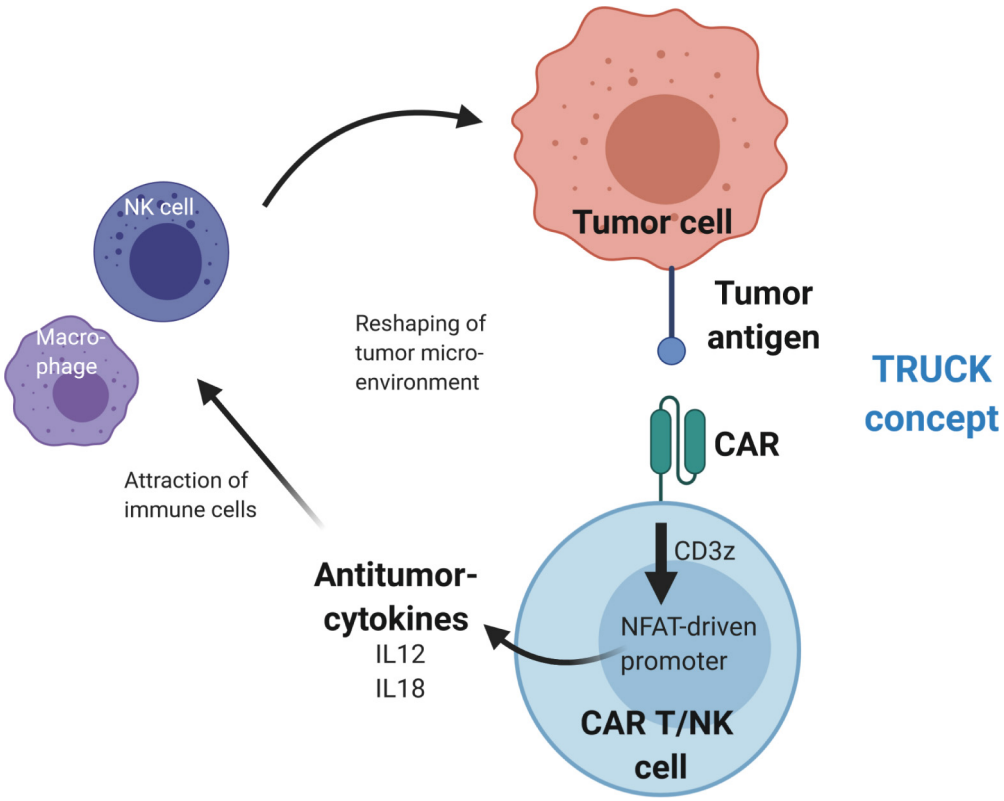

FIGURE 3 | Reshaping of the tumor microenvironment using T cells redirected for universal cytokine killing (TRUCK). Upon antigen binding, the CAR activates CD3z(eta) signaling, which leads to activation of an NFAT-driven promoter that controls expression of antitumor-cytokine cassette, e.g., IL12 or IL18. The cytokines are then secreted from the CAR T or CAR NK cells into the tumor microenvironment, where they recruit additional immune cells to enhance the antitumor activity.

rejection barriers, avoids time consuming *ex vivo* manipulation and cultivation of cells, and would directly reach the target effector cells of the individual. Of note, receptor-targeted vector particles can be as selective for their targeted cell type as antibodies for their antigen when applied systemically or locally in preclinical studies. In this regard, receptor targeting using viral vector nano-particles opens up the possibility for novel concepts in immunotherapy and cell type-specific delivery of CARs in *in vivo* settings (107). Similar delivery principles were shown for non-viral delivery of mRNA in lipid nanoparticles (108).

As a prerequisite for this approach, the natural tropism of the used viral vectors, e.g., gammaretro-/lentiviral and AAV vectors, needs to be blinded, so that the viral vectors no longer bind to their natural target receptors. In a second step, a defined and specified target selectivity has to be added by incorporation of a new selective target cell-binding principle, e.g., scFv antibody, peptide or DARPin (designed ankyrin repeat protein) (107, 109, 110).

Retroviral Vectors for "Off-the-Shelf" CAR Delivery

In the case of enveloped gammaretro- and lentiviral vectors, the envelope for pseudotyping is substituted for a "targeted" Measles (111) or Nipah virus-derived envelope (112) with a newly assigned target specificity. Proof-of-concept for targeted delivery was demonstrated for a variety of target cells, including various hematopoietic and endothelial cells. Of particular importance for CAR technology, surface-engineered lentiviral vectors were successfully applied to mediate selective gene transfer into various subtypes of lymphocytes, including T cells (110, 113) that, impressively, led to the *in vivo* generation of human CD19-CAR T cells with B-cell depletion and signs of cytokine release syndrome in a humanized mouse model (114).

AAV Vectors for "Off-the-Shelf" CAR Delivery

In the case of AAV vectors, which are derived from non-enveloped viruses, the capsid is the target of engineering. Prominent capsid structures are protrusions, which host the natural receptor binding motifs and pores used for loading of vector DNA. Genetic targeting approaches are the currently preferred strategy to modify vector tropism, and were used to insert receptor binding peptides [reviewed in (115)], immunoglobulin binding domains (116) or nanobodies (117) at the tip of the protrusions. Alternatively, the N-terminus of the non-essential capsid protein VP2 can be used as an insertion site. This is especially useful (I) to incorporate large peptides, (II) to target moieties that depend upon their 3D structure for

function or (III) to incorporate entire proteins (118–122). The respective fusion proteins become exposed on the capsid surface through the pore structures. Tropism can either be expanded or re-directed, depending on the specificity of the targeting moiety that is inserted, and whether or not the natural tropism has been ablated, for example by site-directed mutagenesis. The feasibility that off-target free, on-target delivery following intravenous administration of viral vector particles is possible was demonstrated by incorporating DARPins with antibody-like specificity via fusion to VP2 into AAV2 capsids blinded for binding to their primary receptor heparan sulfate proteoglycan (109). These AAV particles can efficiently discriminate between target and non-target cells *ex vivo* in mixed cell cultures as well as *in vivo*, e.g., as demonstrated by delivery of a suicide gene precisely into tumor tissue and specific targeting of $CD4^+$ lymphocytes *in vivo* (109, 122).

These systems will further enrich the portfolio of "off-the-shelf" applications for cancer immunotherapy.

DISCUSSION/OUTLOOK

Several factors impact the potency and successful translation of adoptive cell therapies like CAR T and CAR NK cells to treat cancer. As discussed above, selection of the cell source is a critical decision. The majority of CAR-based therapies use autologous T cells, which have been successfully administered in several clinical studies, with broader success in hematologic malignancies (especially of the lymphoid compartment) than in solid tumors thus far. Autologous CAR T cells have advantages such as no risk of GVHD and lower risk of rejection than allogeneic CAR T cells. However, autologous CAR T cells may have some immunologic defects and the patient must wait several weeks before the autologous CAR T cells are ready for application. As we seek to extend the clinical usefulness of CAR cell strategies, one obvious path forward is to commit more resources toward development of "off-the-shelf" CAR cell therapies, such as genetically modified "universal" allogeneic CAR T cells, NK cells, iPSC and progenitor-derived cells. Universal allogeneic CAR cell therapies are derived from healthy donors, so the immune cells should function properly, and the TCR and MHC are disrupted to avoid induction of GVHD or elimination by the host T cells. While such genome editing strategies to generate "off-the-shelf" CAR T cells are already in clinical practice (66, 71), these may have higher regulatory burden to demonstrate lack of off-target hits and translocations. Another advantage of allogeneic CAR cells is that they can be prepared in advance and stored until needed, thus reducing the time a patient must wait for treatment. As CRISPR-Cas9 genome modification procedures continue to become more efficient and precise, potential risks of genome modified cell therapies will decrease. For example, advances such as base editing make it possible to specifically edit the genome without the necessity to induce double strand DNA breaks, thus potentially increasing the safety of genome editing by reducing the risk of unwanted complications like chromosomal translocations in cell therapies. However, detection of off-target hits is even more difficult and will become even more challenging with advances like epigenome editing technologies.

Combinations of cell therapies may also be useful in this context, especially considering the complex interactions between different cell types during immune responses. For example, CAR T cell activity against colorectal cancer cells was recently shown to be improved by co-application of mesenchymal stem cells (MSC) genetically modified to release IL7 and IL12 (123). The authors exploited the natural capacity of MSC to home to tumor sites and thus support CAR T cells. Cross-talk between CAR T and MSC led to a greater persistence of CAR T, less activation-induced cell death and better anti-tumor activity as shown in *in vitro* and *in vivo* models. Therefore, generation of master cell banks of different types of universal allogeneic cells available as "off-the-shelf" living drugs may help increase the efficacy of immune cell therapies.

Therapeutic efficacy can be limited by loss of CAR T/CAR NK cell persistence due to rejection. As discussed above, humoral responses raised against murine-derived scFv may lead to loss of CAR-modified cells. In such cases, strategies to humanize the scFv can result in greater CAR cell persistence. Genetic ablation of MHC may also help to increase CAR cell persistence by evading the host T cell responses, but may also lead to increased detection by host NK cells.

Development of strategies to overcome tumor-induced immune suppression has been widely studied and use of immune checkpoint inhibitors or genetically engineering CAR T and CAR NK cells to be less responsive to checkpoint signaling are two main approaches to address this challenge. For example, CRISPR-Cas9-mediated elimination of the checkpoint receptor PD-1 from CAR T cells led to improved activity against the solid tumor glioblastoma in preclinical models (124). The possibility to simultaneously and efficiently modify multiple genes with CRISPR-Cas9 seems to be an advantage over ZFN and TALEN genome editing technologies.

Emergence of or selection for tumor cells that do not express the target antigen, a concept called "antigen loss," can also negatively impact CAR T and CAR NK cell antitumor activity. For example, relapsed/refractory B-cell acute lymphoblastic leukemia (B-ALL) patients who were previously administered blinatumomab, a bispecific antibody that targets CD3 on T cells and CD19 on B cells, were less likely to achieve minimal residual disease deep remission and were more likely to experience relapse due to antigen loss after treatment with anti-CD19 CAR T cells (125). Use of dual CAR concepts to target two tumor-associated antigens can lead to improved tumor control. However, the increased risk of on-target-off-tumor activity has to be taken into account.

In addition to antigen loss to avoid CAR T cell activity, decreased death receptor activity (FADD, BID, CASP8 and TNFRSF10B) was shown to be a mechanism of resistance to anti-CD19 CAR T cells (126). Importantly, pre-treatment leukemia-infiltrated bone marrow samples from patients who were treated with anti-CD19 CAR T cell therapy showed that lower death receptor gene expression associated with worse overall survival (126). Methods to restore or elevate death receptor expression and signal transduction activity in tumor cell target populations

could lead to improved tumor control. Identification of target molecules that are more specifically expressed on tumor cells and absent on healthy tissues will also increase the therapeutic efficacy of CAR T and CAR NK cell therapies. The recently described "Sequentially Tumor-selected Antibody and antigen Retrieval (STAR)" method led to isolation of nanobodies that preferentially bound acute myeloid leukemia (AML) cells, and identified CD13 as an AML-specific target (127). Generation of bi-specific CAR T cells that targeted CD13 and TIM3, a checkpoint inhibitor that was found to be upregulated in leukemic stem cells, led to improved elimination of AML. Furthermore, determination of general cancer-specific targets would alleviate the current need to target different antigens for different types of cancer. For example, designing CARs to target cancer-specific post-translational modifications such as Tn-glycosylated podoplanin (Tn-PDPN) (128) would be expected to result in fewer off-cancer effects as the 237Ab-derived 237 CAR T cells target Tn-PDPN, which is not present on normal tissue. As Tn glycosylation was present on all cancer cells evaluated, tumor escape is also less likely, which makes this a novel approach to improve CAR T cell efficacy (128). Along these lines, a genome-wide CRISPR-Cas9 screening method was recently used to discover a TCR that recognized the monomorphic MHC class I-related protein MR1 and T cells engineered to express this TCR killed several different types of human cancers without damaging healthy cells (129). Once verified, development of strategies such as these in allogeneic "off-the-shelf" cell sources could have great potential to exhibit anti-cancer activity against a broad spectrum of malignancies.

In summary, several possibilities to generate "off-the-shelf" anti-cancer immunotherapeutics are currently being explored. For example, control of TCR expression by genomic knockout or down-regulation via RNAi demonstrated the feasibility of generating "off-the-shelf" allogeneic CAR T cell products. However, also other allogeneic cell sources, such as NK cells and macrophages, appear to be suitable as "off-the-shelf" anti-cancer CAR cells. In addition to delivery of cell therapies, the possibility to apply viral vectors engineered for targeted *in vivo* modification of immune cells with CARs is another potent "off-the-shelf" strategy to generate CAR T and CAR NK cells. As research in these areas is rapidly progressing, we look forward to development of efficient "off-the-shelf" therapies that will be made broadly available to the many cancer patients world-wide.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

All authors contributed to conception and writing of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Automated Manufacture of Autologous CD19 CAR-T Cells for Treatment of Non-hodgkin Lymphoma

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Chimeric antigen receptor T cells (CAR-T cell) targeting CD19 are effective against several subtypes of CD19-expressing hematologic malignancies. Centralized manufacturing has allowed rapid expansion of this cellular therapy, but it may be associated with treatment delays due to the required logistics. We hypothesized that point of care manufacturing of CAR-T cells on the automated CliniMACS Prodigy® device allows reproducible and fast delivery of cells for the treatment of patients with non-Hodgkin lymphoma. Here we describe cell manufacturing results and characterize the phenotype and effector function of CAR-T cells used in a phase I/II study. We utilized a lentiviral vector delivering a second-generation CD19 CAR construct with 4-1BB costimulatory domain and TNFRSF19 transmembrane domain. Our data highlight the successful generation of CAR-T cells at numbers sufficient for all patients treated, a shortened duration of production from 12 to 8 days followed by fresh infusion into patients, and the detection of CAR-T cells in patient circulation up to 1-year post-infusion.

Keywords: automated, CAR-T, manufacturing, Prodigy, stem cell memory T

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INTRODUCTION

Genetically engineered T lymphocytes expressing a chimeric antigen receptor (CAR-T cells) are immune cells designed to lyse tumor cells upon CAR ligation with tumor-specific antigens. CD19 CAR-T cells are engineered to recognize CD19-expressing B cell malignancies and two CAR-T products are FDA approved for the treatment of relapsed/refractory patients with non-Hodgkin

lymphoma (NHL) (1). Approximately 50–70% of NHL patients receiving CD19 CAR-T cells achieve complete remission (2). Numerous preclinical studies and clinical trials are currently evaluating this therapy in additional contexts including solid tumors (3).

Current practice in the manufacture of CAR-T cells requires a series of hands-on steps that can often take 2 weeks to achieve a clinical cell dose. Upon collection of peripheral blood leukapheresis products from patients, T cells are isolated, expanded using anti-CD3/CD28 co-stimulation and cytokine supplementation, transduced with a CAR-encoding vector, and harvested for cryopreservation prior to infusion. Numerous quality control mechanisms are built in to ensure sample purity and tumor-induced effector activity. In attempts to optimize patient outcomes, several protocols have been designed to generate a mix of phenotypes in the final CAR-T product. In general, naive, memory, and stem-like phenotypes have shown the highest potential for achieving a long-lasting response, and CAR-T manufacture processes are designed to enrich for such phenotypes (4–8). Conditions that vary between protocols include the specific cytokines used, dose of cytokines, duration of expansion, and freeze-thaw conditions. The ultimate goal of CAR-T cell manufacturing is to deliver a highly cytotoxic product that exhibits high proliferative capacity, maintains long-term memory, is resistant to exhaustion, and does not produce cytokine release syndrome (CRS).

As the field rapidly progresses, there is a need for a standardized, sterile, reproducible and scalable protocol for clinical-grade manufacturing of CAR-T cells (9). While several methods are available, one platform, the CliniMACS Prodigy®, offers a closed automated system capable of manufacturing CAR-T cells outside of a cleanroom environment in an unclassified space while maintaining Good Manufacturing Practice (GMP) compliance (10). This technology not only increases the accessibility of CAR-T cell therapy to centers lacking sophisticated cell processing capabilities, but it is also adaptable to the needs of individual samples, offers sample monitoring, enables delivery of non-frozen cells, and rapidly yields a functional product within 2 weeks (11–13). Here, we demonstrate sufficient *ex vivo* expansion of CAR-T cells in a timespan as short as 8 days, a significant finding in consideration of a previous report that indicates there is improved anti-leukemic activity of CAR-T cells with shorter culture durations (14).

Also, we report the successful generation and infusion of autologous CD19 CAR-T cells in heavily pre-treated patients (15), and document enrichment of memory or stem-like qualities, which have been associated with improved patient response in other studies (16, 17). Specifically, we observed higher frequencies of T cell central memory (TCM) and stem cell memory (TSCM) phenotypes and cells expressing transcription factors TBET and GATA3. The CAR-T cells effectively killed $CD19^{+}$ cells *in vitro* and *in vivo* while maintaining polyfunctionality. We in addition observed a potential correlation between quality control potency assays and patient response rates. Moreover, by use of a commercially available, fluorescently labeled CD19 peptide we employed a method to measure circulating CD19 CAR T cells from patient peripheral blood by flow cytometry that correlates well with peripheral blood vector copy number.

MATERIALS AND METHODS

Cohort Description

Clinical leukapheresis products were obtained from NHL patients undergoing CAR-T cell therapy at University Hospitals Seidman Cancer Center under a phase I/II study (ClinicalTrials.gov Identifier: NCT03434769; IND 17932). Mononuclear peripheral blood cell apheresis products were processed within 24 h of receipt without cryopreservation. The study was approved by the institutional review board and all patients gave written informed consent.

CliniMACS Prodigy® Settings

CAR-T cells were manufactured within the CliniMACS Prodigy® (Miltenyi Biotec, Bergisch Gladbach, Germany) device using the TCT software program and TS520 tubing set (Miltenyi Biotec, Bergisch Gladbach, Germany). The main components of the Prodigy® and the instrument setup followed the protocol given by Miltenyi Biotec and were outlined in (13). All processing was performed at the Cellular Therapy Lab of University Hospitals Cleveland Medical Center Seidman Cancer Center/Case Western Reserve University Center for Regenerative Medicine.

Lentiviral Vector

All experiments described in this paper used a novel CD19 CAR vector, LTG1563, which was developed and provided by Lentigen, a Miltenyi Biotec company (Gaithersburg, MD, United States). The vector contains a scFv FMC63-based targeting domain, CD8-derived hinge region, TNFRSF19-derived transmembrane region, 4-1BB/CD137 costimulatory domain, and CD3-zeta chain intracellular signaling domain.

Cell Culture Reagents

Clinical-grade reagents used to manufacture the CAR-T cells included: CliniMACS Buffer, TexMACS Media, CliniMACS CD4 reagent, CliniMACS CD8 reagent, TransAct, and the cytokines IL-7 and IL-15 (Miltenyi Biotec, Bergisch Gladbach, Germany). Reagents were utilized according to manufacturer's instructions. One 25 μg vial each of IL-7 and IL-15 was added per 2L bag of media. A 25% stock solution of Human Serum Albumin (HSA) was used to supplement the CliniMACS Buffer to a concentration of 0.5%. Human AB serum was used to supplement the TexMACS Media to a concentration of 3% and was from Innovative Research (Novi, MI, United States). TexMACS media was supplemented with Human AB serum for 6 days of the cell culture and then replaced with media without Human AB serum for the duration of the culture.

Flow Cytometry

Prior to and post-CD4/CD8 enrichment, T cells were phenotyped with CD4 VioGreen, CD8 APC-Vio770, CD45 VioBlue, and 7AAD (reagents from Miltenyi Biotec, Bergisch Gladbach,

Germany). Final CAR-T products after harvest from the Miltenyi Prodigy® were phenotyped with CD19 Fc Chimera protein (R&D Systems, Minneapolis, MN, United States) followed by the anti-Fc-PE secondary antibody (Jackson ImmunoResearch, West Grove, PA, United States) as described previously (18), CD45 VioBlue, CD4 VioGreen, CD8 APC-Vio770, CD3 FITC, CD14 APC, CD20 PE-Vio770, CD56 PE, CD16 PE, and 7AAD (reagents from Miltenyi Biotec, Bergisch Gladbach, Germany) to establish the following populations: $CD45^+$ lymphocytes, $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+CD8^+$ T cells, $CD3^+$ T cells, $CD14^+$ monocytes, $CD20^+$ B cells, $CD56^+CD16^+$ NK cells, and $CD3^+CD56^+CD16^+$ NK T cells.

Flow analysis for circulating CAR-T cells was performed using a FITC-labeled CD19 peptide (amino acids 20–291) (ACRObiosystems, Newark, DE, United States), CD3 PE, CD8 APC-Vio770, CD45 VioBlue, and 7AAD (Miltenyi Biotec, Bergisch Gladbach, Germany). All samples from the panels above were analyzed on the CliniMACS MACSQuant Flow Cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). BD's 18-color Fortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ, United States) was used to assess CAR-T cell phenotype. The following antibodies were used for surface staining: Recombinant Human CD19 Fc Chimera Protein (R&D Systems, Minneapolis, MN, United States), Anti Fc-PE (Jackson ImmunoResearch, West Grove, PA, United States), CD3 AF700 (BD Biosciences, Franklin Lakes, NJ, United States), CD4 BV605 (Biolegend, San Diego, CA, United States), CD8 BUV737 (BD Biosciences, Franklin Lakes, NJ, United States), CD19 BV510 (BD Biosciences, Franklin Lakes, NJ, United States), CD45RO BUV395 (BD Biosciences, Franklin Lakes, NJ, United States), CD45RA BV650 (Biolegend, San Diego, CA, United States), CD45 BV786 (BD Biosciences, Franklin Lakes, NJ, United States), CD95 FITC (BD Biosciences, Franklin Lakes, NJ, United States), CD27 BV711 (BD Biosciences, Franklin Lakes, NJ, United States), CCR7 PE Cy7 (BD Biosciences, Franklin Lakes, NJ, United States); and Thermo Fisher's (Waltham, MA, United States) FOXP3 transcription factor buffer kit was used to intracellularly stain as per manufacturer's protocol for TCF7 AF647 (Biolegend, San Diego, CA, United States), Tbet PerCPCy5.5 (BD Biosciences, Franklin Lakes, NJ, United States), and GATA3 PECF594 (BD Biosciences, Franklin Lakes, NJ, United States). Data was analyzed by FlowJo 10.4.2 software (Ashland, OR, United States). In addition to manual gating for conventional subsets, high-dimensional clustering techniques were used to identify unique cell clusters within CAR-T products. Here, the Phenograph clustering algorithm (19) (Rphenograph package in R; default settings where k was set to 20) was used on pre-gated live CD3 + T cells. The phenograph clusters were projected on a 2-dimensional UMAP (20) (umap-learn in R; default settings) and statistical assessment of alteration in cluster frequencies within CAR-T cells was done using GraphPad Prism (GraphPad Software, San Diego, CA, United States).

Cytotoxicity Assay

Cytotoxicity assays were performed by co-culturing CAR-T cells with target cells at various effector to target ratios as previously reported (21). Briefly, target cells were pre-labeled with 1 μg/mL calcein-AM (Life Technologies, Carlsbad, CA, United States) for 30 min at 37°C. 10,000 target cells were plated per well of a 96-well plate and various ratios of CAR-T cells were added. After 4 h the cells were analyzed by flow cytometry to detect the loss of fluorescence in the target cells using the Attune NxT cytometer. Target cells used include Raji, Daudi, JVM2, and OCI-AML3 (all cell lines from ATCC, Manassas, VA, United States).

Animal Study

Six to eight week old NOD.*Cg-Prkdc*^{scid}*Il2rgt*$^{m1\,Wjl}$*/SzJ* mice (NSG, The Jackson Laboratory, Bar Harbor, NE, United States) were injected *i.v.* with 1.0×10^6 Raji-luciferase cells as previously described (18), with several modifications. Briefly, Raji-luciferase cells were injected into mice via tail vein. Mice were randomly split into 4 groups of 5 and received an *i.v.* injection of 2.5×10^6 CAR-T cells (normalized to the transduction efficiency; non-transduced T cell control group received 8×10^6 cells) 1 week after tumor cell injection. Disease progression was followed weekly by bioluminescence imaging using the IVIS Spectrum Imager (PerkinElmer, Waltham, MA, United States). Blood was collected at 2- and 4- weeks post CAR-T injection and analyzed by flow cytometry to track the circulating levels CAR-T cells. Mice were sacrificed at 5 or 6 weeks post CAR-T infusion. Blood, liver, kidney, spleen, bone marrow, and lymph nodes were isolated and cells were separated by homogenization with RBC lysis as needed, and then analyzed by flow cytometry.

Cytokine Assay

To evaluate the levels of plasma cytokines in tumor-bearing NSG mice receiving CAR-T therapy, we used the U-PLEX assay (Meso Scale MULTI-ARRAY Technology) commercially available by MSD (Meso Scale Discovery Rockville, MD, United States). Using this platform, a cytokine panel was screened: TGFβ1, TGFβ2, IFN-α2a, IFNβ, IFNγ, TNFα, TRAIL, VEGF, IL-6, IL-9, IL-15, IL-21, IL-22, and IL-29. 25 μL of plasma from each donor was used following the manufacturer's instructions. Electrochemiluminescence was detected using MESO QuickPlex SQ 120 (Meso Scale Discovery Rockville, MD, United States). The results were extrapolated from the standard curve from each specific analyte and plotted in pg/mL using the DISCOVERY WORKBENCH v4.0 software (Meso Scale Discovery, Rockville, MD, United States).

Proviral Detection

DNA was isolated from whole mononuclear cells and probed for rev-responsive element with a Taq-based qPCR assay (Lentigen Corporation, Gaithersburg, MD, United States). DNA was measured with a CFX Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States). Copy number per CAR-T cell estimated by the formula (copy#/ng) × (0.006ng/cell)/transduction rate.

Statistical Analysis

Unpaired two-tailed *t*-tests were used for comparison of two groups. One-way ANOVA was used when more than two groups were analyzed for statistical significance. All statistical analyses

were done using R in R Studio 1.1.456[1], GraphPad Prism 8.3.0 (GraphPad Software, San Diego, CA, United States) or Microsoft Excel 16.16.9[2]. Statistical significance was given as *, **, ***, **** by p-values less than <0.05, <0.01, <0.001, or <0.0001, respectively.

Data Sharing Statement

For original data, please contact dnw@case.edu.

RESULTS

Manufacturing Clinical Grade CD19 CAR-T Cells

Concerns exist on the ability to consistently expand patient-derived T cells, as these cells are often functionally impaired due to prior chemotherapy as well as the primary disease process (15). Here we present data on the manufacturing of CAR19 LTG1563 from autologous T cells. The T cell composition of patient apheresis samples were 19.06% ± 10.06 CD4 T cells and 27.9% ± 14.15 CD8 T cells (**Table 1**). Each apheresis collection was loaded onto the CliniMACS Prodigy® and column purified using the TS520 Tubing set and CliniMACS CD4 and CD8 reagents (microbeads). After column purification, CD4 and CD8 T cells were enriched to 37.85% ± 15.81 and 46.21% ± 15.88, respectively (**Table 1**). Interestingly, the expansion conditions led to a selective increase of CD4 T cells, increasing the average CD4:CD8 ratio from 1.03 post-CD4/CD8 enrichment to 2.48 by the end of culture (**Figure 1A**).

The CliniMACS Prodigy was loaded with ∼1 × 10^8 CD3 cells and the cells expanded with anti-CD3/CD28 co-stimulation and IL-7/IL-15 supplementation. After culture, we achieved a median 41.1-fold expansion of $CD3^+$ cells (range 30.0–78.6). To examine the transduction efficiency of manufactured CAR-T products, we performed flow cytometry using a peptide that binds the CD19 CAR (gating scheme depicted in **Figure 1B**). The median transduction efficiency of $CD3^+$ cells was 46.88% (range 29.02–61.09%). Median transduction of CD4 T cells was 57.80% (range 39.40–75.35%) compared to 46.79% (range 24.29–90.38%) for CD8 T cells. The increased transduction rate of CD4 T cells, combined with their greater expansion, led to an overall increase in CAR^+ CD4 T cells as compared to CAR^+ CD8 T cells (**Figure 1C**). All autologous samples were expanded and transduced at sufficient numbers for dosing in the clinical trial (**Table 2**). The multiplicity of infection of samples was approximately 20, leading to an average estimated vector copy number of 1.89 ± 0.71 per CAR-T cell.

Following the manufacturer's protocol, we harvested cells from the Prodigy® on day 12. However, to determine if the time to patient infusion could be shortened, we measured transduction efficiency and cell numbers at earlier time points. On days -1, 3, 6, 9, and 12, we assessed CD3, CD4, CD8, and the CD19 CAR expression by flow cytometry. As represented in **Figure 1D**, transduction efficiency reached a plateau on day 6. Accordingly, the transduction rate of $CD3^+$ cells after 8-day culture was comparable to that at day 12 at a median 44.3% (range 27.6–65.8%). Furthermore, cell counts at day 8 were found to be sufficient to meet the required cell numbers for infusion into patients in our clinical trial exceeding a dose of 1 × 10^6 transduced CD19 CAR-T cells/kg (**Figure 1C** and **Table 2**). We therefore reduced the duration of CAR-T production, and shortened the time from apheresis to treatment.

Product purity is a major concern for the safety and outcome of patients, particularly malignant B cell contamination since a single transduced B cell has been reported to lead to patient relapse (22). The purity of the CD19 CAR-T products was studied by flow cytometry. Samples were stained at harvest for T cells (CD3, CD4, CD8), B cells (CD20), monocytes (CD14), and NK/NKT cells (CD56, CD16). As shown in **Figure 1E**, there is little to no contamination of B cells (median 0.00%, range 0.00–0.04%), monocytes (median 0.18%, range 0.00–2.15%) and NK cells (median 0.13%, range 0.01–8.57%). Furthermore, the percent viability of CAR-T cells was measured by staining with 7-AAD and products showed a median 93.18% viability (range 86.58–96.25%) (**Figure 1F**). All products met acceptable standards of purity, viability for patient infusion and cell dose.

Phenotyping Manufactured CAR-T Product

As previous studies have emphasized, induction of quiescent TSCM and TCM phenotypes in CAR-T cells is desirable for improved self-renewal, effector differentiation, formation of memory, and lowered CRS (4–8, 23). Here, we expected the addition of IL-7/IL-15 in our manufacturing platform to result in a quiescent cellular profile despite extensive activation and 40-fold expansion. To confirm the abundance of quiescent subsets in the CAR-T product, we performed traditional T cell subset gating (**Table 3**) and observed an enrichment of CD4 and CD8 TSCM cells expressing high levels of CD95 (in addition to CD27 and CCR7) (**Supplementary Figures S1A–C**). To define unique phenotypes, we performed unbiased clustering using 41BB, CD8, CCR7, CD27, CD95, CD45RO, CD4, and CD127. The CAR-T products showed a distinct profile when compared to freshly isolated T cells while CAR expressing cells showed little variation from CAR^- cells within the CAR-T product (**Figure 2A**). Upon further analysis, we observed three predominant clusters overlap with CAR expression and identified these clusters as CD8 TSCM-like ($CD45RA^+CD27^+CCR7^-$), CD4 central memory ($CD45RA^-CD27^+CCR7^+$; TCM) and CD4 effector memory ($CD45RA^-CD27^-CCR7^-$; TEM) (**Figure 2A**). Each

[1]https://rstudio.com

[2]https://microsoft.com

TABLE 1 | Purity of apheresis and recovery post-enrichment of CD4 and CD8 cells.

	% Viable $CD45^+$ cells	
Parameter	**Pre-column purity**	**Post-column recovery**
CD4	19.06 ± 10.06	37.85 ± 15.81
CD8	27.9 ± 14.15	46.21 ± 15.88

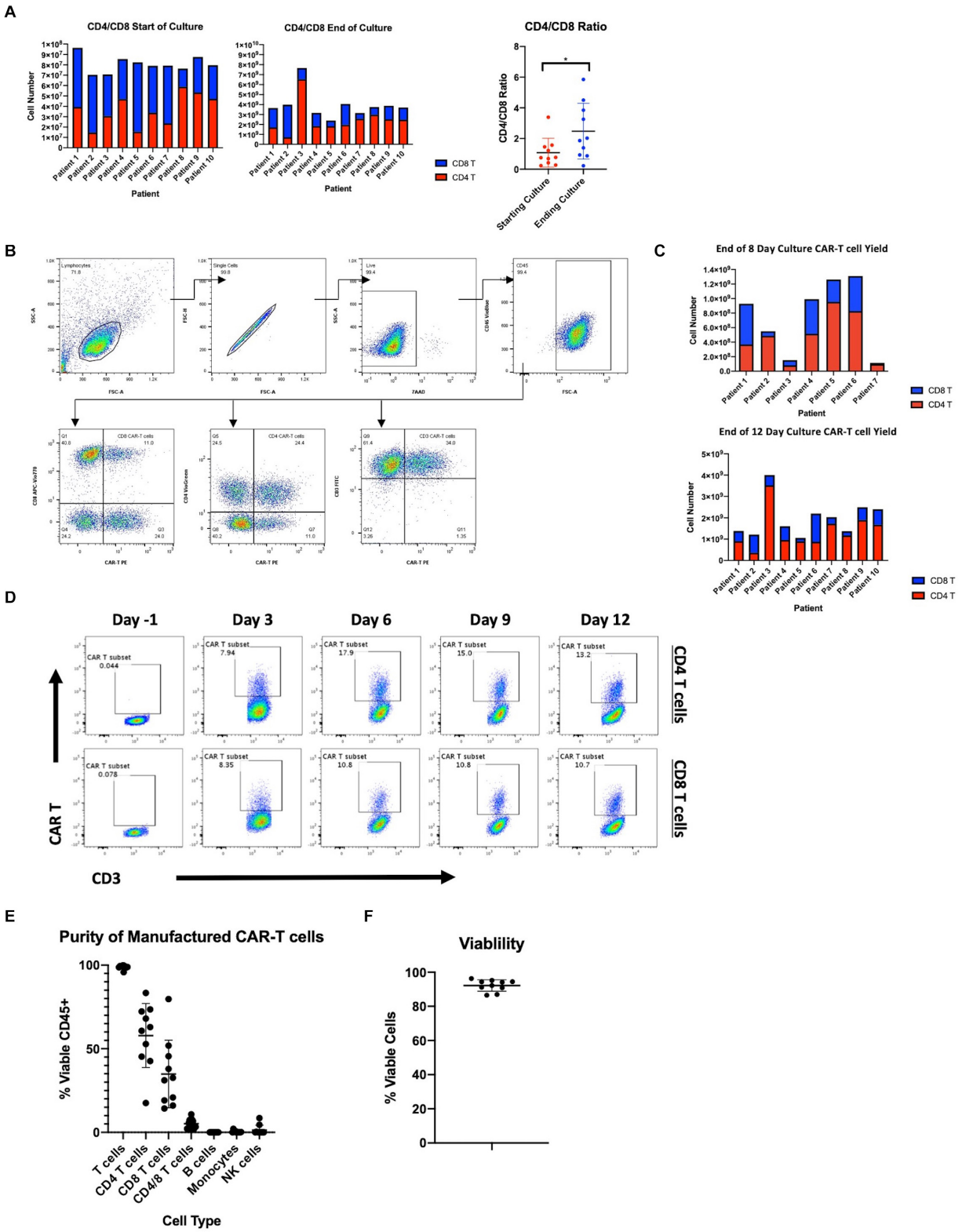

FIGURE 1 | Manufacturing clinical grade CD19 CAR-T cells using the CliniMACS Prodigy. **(A)** Total cell numbers and CD4 and CD8 T cell ratios at the start of culture after CD4/CD8 enrichment and after 12 days of culture. **(B)** Flow cytometric gating strategy for identification of CD19 CAR expressing T cells. **(C)** Number of CAR-T cells after 8 or 12 days of culture and CD4 and CD8 T cell composition. **(D)** Time course of CAR transduction rates of CD3 T cells for duration of culture (n = 1). **(E)** Purity of final product after expansion in the Prodigy (n = 10). **(F)** Percent viable T cells of CD45$^+$ cells after expansion in the Prodigy (n = 10). Statistical significance was given as *, **, ***, or **** by p-values less than < 0.05, < 0.01, < 0.001, or < 0.0001, respectively.

TABLE 2 | Culture yield, fold expansion, and transduction efficiency.

Parameter	12-day culture			8-day culture		
	Median culture yield ($\times 10^9$) (n = 10)	Median fold expansion (n = 10)	Median CAR^+ (% of $CD45^+$) (n = 10)	Median culture yield ($\times 10^9$) (n = 7)	Median fold expansion (n = 6)	Median CAR^+ (% of $CD45^+$) (n = 7)
CD4	2.22 (0.72–6.5)	51.5 (39.2–212.7)	31.18 (9.0–48.5)	1.0 (0.20–1.4)	24.0 (10.0–48.9)	35.5 (14.8–44.6)
CD8	1.27 (0.56–3.26)	36.0 (8.4–58.7)	13.46 (4.95–28.76)	0.59 (0.05–0.94)	10.9 (0.70–20.5)	14.4 (4.8–35.6)

TABLE 3 | T cell memory subset markers.

Marker	Naive	Stem cell memory	Central memory	Transitional memory	Effector memory	Terminal effector
CD45RA	+	+	−	−	−	+
CD45RO	−	−	+	+	+	−
CCR7	+	+	+	−	−	−
CD27	+	+	+	+	−	−
CD127	+	+	+	±	−	−
CD95	−	+	+	+	+	+

cluster expressed high levels of CD95 (likely resulting from prolonged exposure to IL-7/IL-15 during manufacturing) and was significantly enhanced among manufactured cells, regardless of transduction status (**Figure 2B**).

As current standard practice is for CAR-T cells to be cryopreserved at harvest and thawed before infusion into patients, we questioned whether freeze-thaw impacts the T cell memory phenotypes distribution observed in the product. The protocol for this clinical trial was to distribute the cells to each patient fresh for infusion, however, two patients received cryopreserved cells due to medical circumstances. While no change was observed in the frequencies of CD4, CD8, or CAR^+ T cells (**Figure 2C** and **Supplementary Figures S2D–F**), our data showed a favorable preservation of TSCM-like cells and loss of TEM (**Figure 2D**). These data suggest that although freeze-thaw does not alter CAR^+ cell frequencies or CD4/CD8 proportions, the reduced effector cells post-thawing could contribute to lower incidence of pathologies like CRS because of lower inflammatory response right after product infusion. Successful enrichment of a quiescent profile in the CAR-T cell product after freeze-thaw could promote clinical responses.

Next, to measure immune exhaustion vs. activation status of our product, we studied the levels of several immune checkpoint molecules such as PD1, CTLA4, BTLA, BLIMP1, and LAG3. We observed an increase in BLIMP1 and LAG3 within the product and a decrease of BTLA (**Figure 2E** and **Supplementary Figure S1G**). The increase in these markers (also up-regulated upon T cell activation) could be reflective of the highly activated state of the product. In order to further validate the lack of exhaustion, we assessed the relative expression of stemness markers OX40 and 41BB (24–26). As seen in **Figure 2F**, OX40 and 41BB were upregulated in the product with equivalent expression of 41BB in CAR^+ and CAR^- T cells, excluding the possibility of a vector-driven increase of 41BB. Given that our product showed an enrichment of both stemness and effector surface molecules, we sought to study the heterogeneity in CAR-T clusters using a high-dimensional flow cytometry panel that would show the distribution of transcription factors associated with effector function (TBET, GATA3), and stemness (TCF7) (27–29). To understand the expression of these markers relative to the memory phenotypes we identified earlier, clustering was performed with markers CD4, CD95, CD45RO, TBET, GATA3, TCF7, CD8, CCR7, CD27, and CD127. As seen in **Figures 2G,H**, expression of TBET, GATA3, and TCF7 colocalize with CAR expression, and GATA3 and TCF7 are specifically enriched among manufactured cells in the CD8 TSCM-like and CD4 TCM clusters. Manual gating on TBET, GATA3, and TCF7 further supports induced expression after culturing conditions and highlights a specific enrichment for CAR^+ cells among manufactured cells (**Figures 2I,J** and **Supplementary Figure S1H**). Altogether, despite higher levels of exhaustion markers, our results indicate the cells have a quiescent profile with the ability to self-renew and is suggestive of a polyfunctional CD4 and CD8 phenotype that is capable of differentiating into effector cells via the expression of stemness and effector transcription factors.

Manufactured CAR-T Cells Effectively Kill $CD19^+$ Cells *in vitro*

CAR-T cells show a dose-dependent cytotoxicity toward $CD19^+$ cancer cells *in vitro*. Effector cell function of the CAR-T samples was tested *in vitro* using a flow cytometry calcein-based cytotoxicity assay (21). Three $CD19^+$ B cell lymphoma lines were tested (Raji, Daudi, and JVM2) as well as an acute myeloid leukemia cell line (OCI-AML3) as a $CD19^-$ control. Results for cell lysis by three CAR-T samples are shown in **Figure 3A**. All samples tested showed significantly higher cell lysis of $CD19^+$ Raji, Daudi, and JVM2 cells compared to $CD19^-$ OCI-AML3 cells at a 10:1 effector to target ratio. Cell lysis decreases in a dose-dependent manner as the effector to target ratio is reduced. Furthermore, as shown in **Figure 3B**, CAR-T cell cytotoxic activity did not significantly differ when fresh or cryopreserved CAR-T cells were used at all ratios tested.

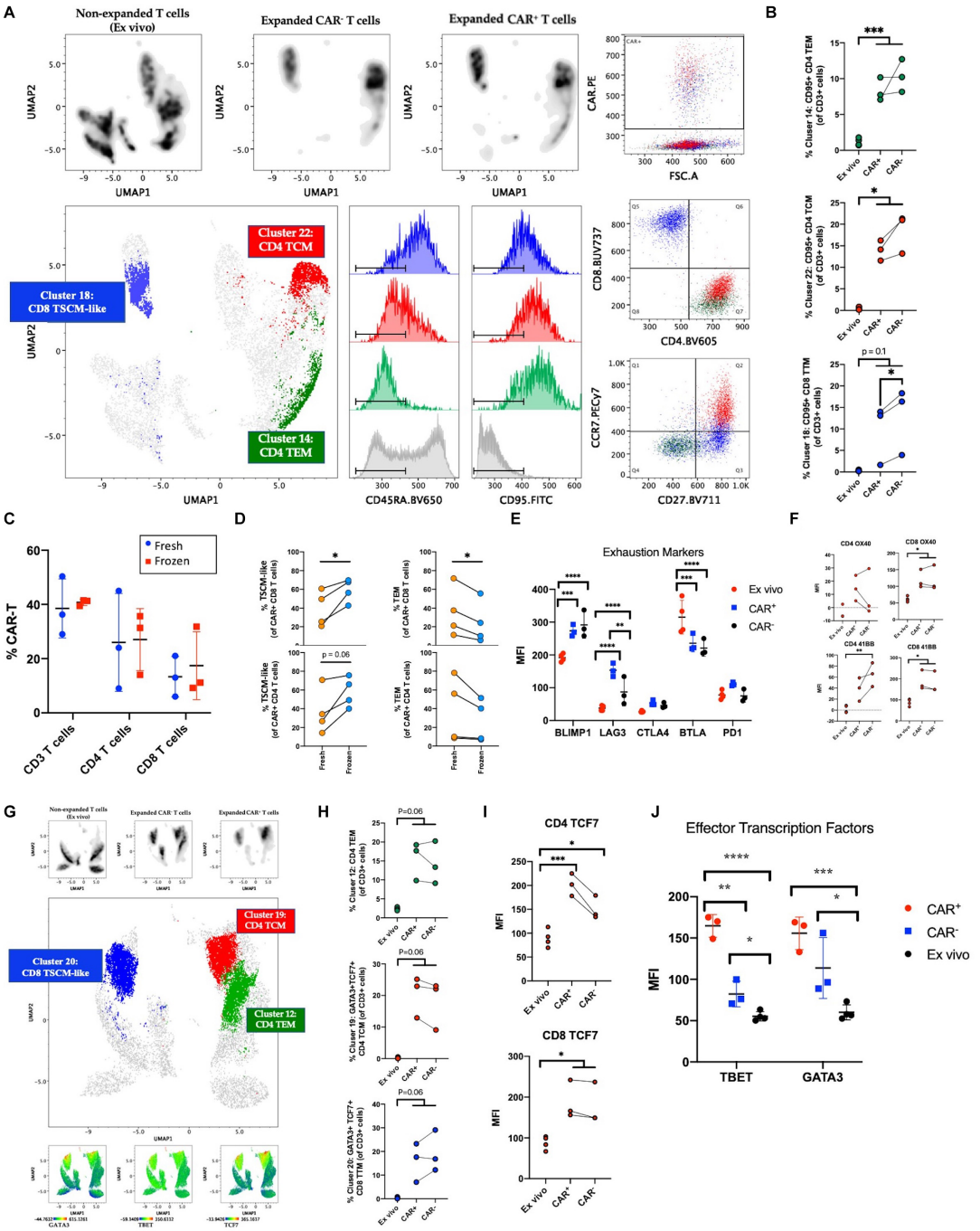

FIGURE 2 | Phenotyping manufactured CAR-T product. **(A)** Clustering analysis of CAR-T cell products (n = 3) and freshly isolated T cells (*ex vivo*; n = 4) using the markers 41BB, CD8, CCR7, CD27, CD95, CD45RO, CD4, and CD127. Cells were pre-gated on live CD3$^+$ singlets prior to analysis. Expanded CAR$^+$ and CAR$^-$ T cell populations separate by expression pattern from pre-culture T cells. CAR$^+$ cells predominantly express markers of stem cell memory (TSCM) among CD8 T cells and central memory (TCM) or effector memory (TEM) among CD4 T cells. **(B)** Percentage of CAR$^+$, CAR$^-$, and freshly isolated T cells among clusters from **(A)**. **(C)** Percentage of CAR expressing CD3, CD4, and CD8 T cells at harvest (fresh; n = 3) and after free-thaw (frozen; n = 3). **(D)** Changes in T cell memory subsets after freeze-thaw (n = 4). **(E)** Expression of inhibitory checkpoint molecules on *ex vivo* (n = 4) or expanded CAR$^+$ and CAR$^-$ T cells (n = 3). **(F)** Expression of OX40 and 41BB prior to culture (n = 4) and in post-culture CAR$^+$ and CAR$^-$ T cells (n = 3). **(G)** Clustering analysis of CAR-T cell products (n = 3) and freshly isolated T cells (n = 4) using the markers CD4, CD95, CD45RO, TBET, GATA3, TCF7, CD8, CCR7, CD27, and CD127. Cells were pre-gated on live CD3$^+$ singlets prior to analysis. Expanded CAR$^+$ and CAR$^-$ T cell populations separate by expression pattern from freshly isolated T cells. Expression of transcription factors GATA3, TBET, and TCF7 overlaps with CAR$^+$ cells. **(H)** Percentage of unique phenotypes associated with CAR$^+$ T cells of CD3$^+$ cells. **(I)** Expression of TCF7 prior to culture (n = 4) and in post-culture CAR$^+$ and CAR$^-$ T cells (n = 3). **(J)** Expression of TBET and GATA3 prior to culture (n = 4) and in post-culture CAR$^+$ and CAR$^-$ T cells (n = 3). Statistical significance was given as *, **, ***, or **** by p-values less than < 0.05, < 0.01, < 0.001, or < 0.0001, respectively.

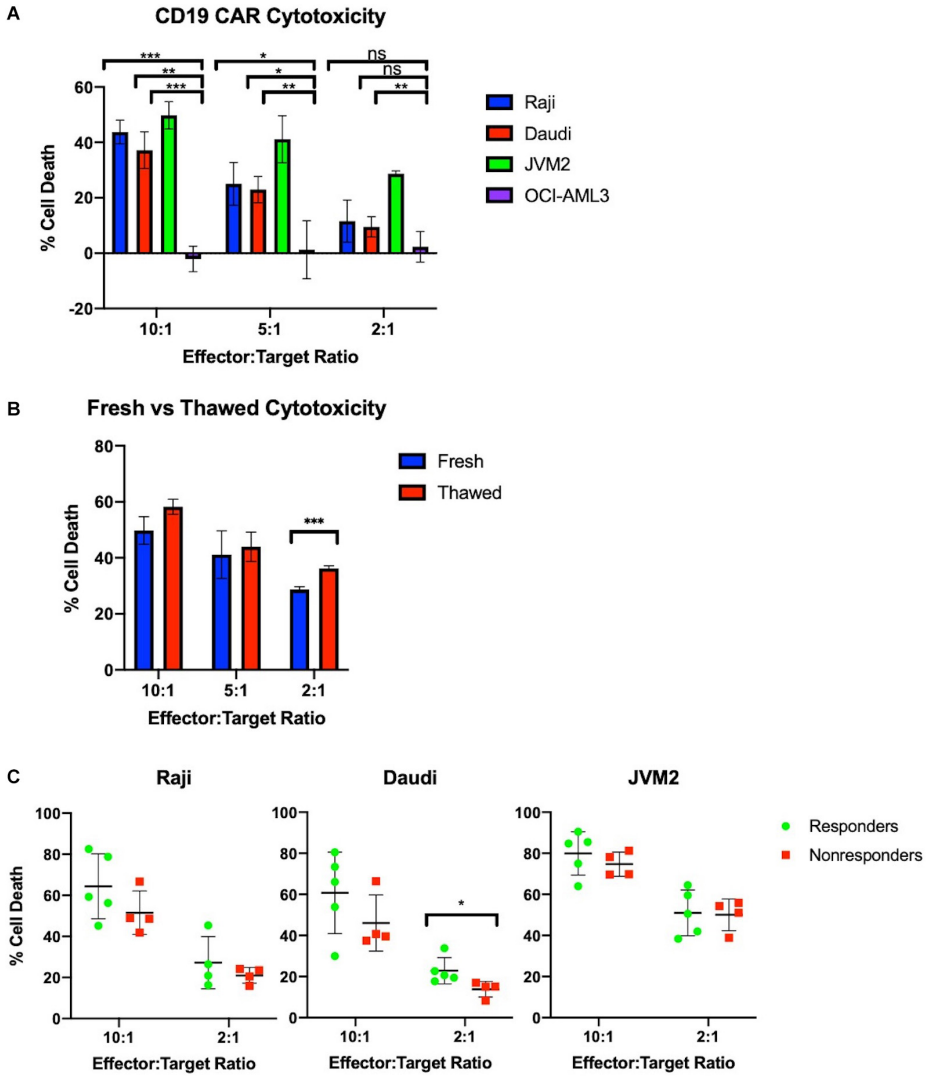

FIGURE 3 | Manufactured CAR-T cells effectively kill CD19$^+$ cells *in vitro*. **(A)** CAR-T cells selectively and effectively kill CD19$^+$ cells *in vitro* at 10:1 and 5:1 ratios as measured by number of calcein positive target cells compared to target cells alone (n = 3). **(B)** Freeze-thaw does not negatively impact JVM2 cell lysis at 10:1, 5:1, and 2:1 ratios (n = 3). **(C)** Cytotoxicity of autologous CAR-T cells against CD19$^+$ Raji, Daudi, and JVM2 cells stratified by patients with complete response (n = 5) or partial response/progressive disease (n = 4). Statistical significance was given as *, **, ***, or **** by p-values less than < 0.05, < 0.01, < 0.001, or < 0.0001, respectively.

To evaluate the potency of our products, we tested the CAR-T products of 9 patients prior to infusion against CD19$^+$ Raji, Daudi, and JVM2 cell lines at 10:1 and 2:1 ratios; CAR-T products were stratified by complete responders (CR) vs. non-responders (NR) (partial responders or patients with progressive disease). As shown in **Figure 3C**, CAR-T cells showed high potency across all three cell lines and a minor trend of enhanced cytotoxicity of CR is observed for all three cell lines.

Manufactured CAR-T Cells Effectively Clear CD19$^+$ Tumor Cells *in vivo*

Effector function of the CAR-T samples was tested *in vivo* in a lymphoma mouse model. NOD-SCID-IL-2 gamma-/- (NSG) mice were injected with luciferase-expressing Raji cells intravenously (*i.v.*) on day 0 and CAR-T cells were injected *i.v.* on day 7. The control mice were injected with comparable non-transduced T cell doses. Mice injected with CAR-T cells showed a significantly ($p < 0.01$) lower tumor burden compared to mice injected with un-transduced T cells as measured by bioluminescent imaging at all weeks tested (**Figure 4A**). The mice tolerated the CAR-T cells without any apparent toxicities or weight loss (**Supplementary Figure S2C**). At weeks 2 and 4 post-infusion, mice were bled for quantification of CD8 CAR-T cell expansion by flow cytometric analysis. Our results demonstrate robust expansion of circulating CD8 CAR-T cells by week 4 (**Figure 4B**). At weeks 5 and 6, mice were sacrificed and organs (spleen, liver, bone marrow, kidney, lymph

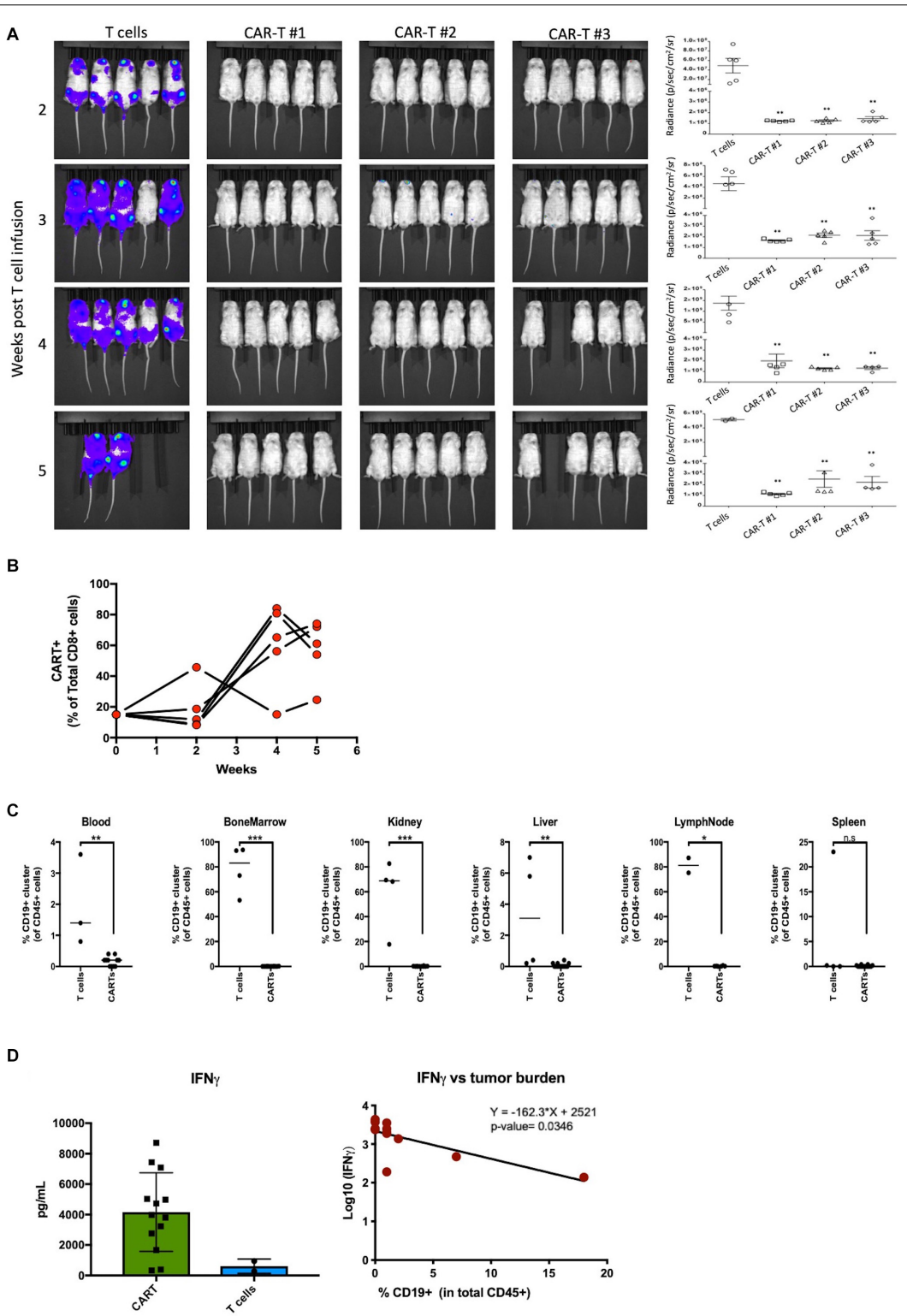

FIGURE 4 | Manufactured CAR-T cells effectively clear CD19^{+} tumor cells *in vivo*. **(A)** Left – Luciferase-expressing Raji cells injected into mice in groups of 5 were treated with CAR-T cells or non-transduced T cell control group and bioluminescence was measured at indicated weeks. Right – Quantification in radiance (p/sec/cm^2/sr) of tumor burden in mice groups. **(B)** Effector CD8 CAR-T cells expand in mice over 5 weeks in proportion to CAR negative human CD8 T cells. **(C)** Quantification of tumor burden between mice receiving *ex vivo* T cells (n = 2–4) or CAR-T cells (n = 14) in blood, bone marrow, kidney, liver, lymph nodes, and spleen. **(D)** Mice treated with CAR-T cells have higher levels of effector cytokine IFNγ which negatively correlate with tumor burden. Statistical significance was given as *, **, ***, or **** by p-values less than < 0.05, < 0.01, < 0.001, or < 0.0001, respectively.

nodes, and blood) were harvested and homogenized for flow cytometric analysis. As shown in **Figure 4C**, a Raji tumor cell ($CD19^+CD45^+$) cluster, demarked in an oval, was driven by similar expression of CD19. Quantification of Raji tumor cells demonstrates extensive tumor clearance in all CAR-T treated mouse blood and tissues, whereas tumor burden is present in all organs but the spleen of mice receiving non-transduced T cells (**Figure 4D**). To assess what cytokines were prevalent in the blood of mice receiving CAR-T cell treatment, we utilized the U-PLEX assay (**Supplementary Figure S2A**). As expected, we observed increased levels of the CD8 effector cytokine IFNγ in mice receiving CAR-T cells, which significantly correlated with decreased tumor burden (p-value = 0.036) (**Figure 4D**). Complementing the *in vitro* culture results (see **Figure 2**), our data also show that an increase in IFNγ production was associated with higher levels of TNFα, likely resulting from a polyfunctional CD8 CAR-T cell response (**Supplementary Figure S2A**). This increase went hand-in-hand with higher levels of innate immune cascades (i.e., higher IL-6 and IL-1b) and of IL-17A and IL-4 (likely a result of CD4 T cell differentiation). A likely counter to this inflammatory response is seen by the induction of TGF-β2 and a decrease in gamma-chain cytokines like IL-2 and IL-7 (**Supplementary Figure S2A**). Interestingly, of all cytokines measured (**Supplementary Figure S2A**), TNFα and TGF-β2 also correlated significantly with mouse tumor burden (**Supplementary Figure S2B**).

Detection of Manufactured CAR-T Cells in NHL Patients

An evaluation of the safety of anti-CD19 CAR-T cells manufactured using the CliniMACS Prodigy platform is currently underway in a phase I/II clinical trial enrolling patients with relapsed/refractory NHL. To date, patients have been treated with two dose levels at 0.5×10^6 and 1×10^6 CAR-T cells per kg. While a full assessment of patient responses is currently in progress, here we present the persistence of the manufactured CAR-T cells. Peripheral blood samples were obtained at 6, 14, 21, 30, 60, 90, 180, and 365 days after CAR-T cell infusion. Analyses were conducted by qPCR and flow cytometry to quantify CAR-T cell persistence. qPCR analysis was performed by probing for the pro-viral rev-responsive element and is represented by copy number per nanogram of DNA. Flow cytometric analysis of CAR-T cell persistence was performed by staining cells with a FITC-conjugated CD19 peptide. As shown in **Figure 5A**, patients generally experienced a peak in circulating CAR-T cell levels at day 14 through day 21, with gradual subsequent decreases. Peak expansion or persistence was not affected by cell dose. Flow cytometric quantification of CD8 and CD4 CAR-T cell subsets revealed that despite the predominance of CD4 CAR-T cells in the product, *in vivo* expansion is driven by CD8 CAR-T cells (**Figure 5B**). CD19 CAR-T cells were detectable by both methods up to 1 year after infusion, and CAR T cells were detected in all patients at all studied time points. Additionally, we demonstrate for the first time the utility of a FITC conjugated CD19 peptide to monitor CD19 CAR-T cells in patient samples and its strong agreement with existing qPCR data (**Figure 5C**).

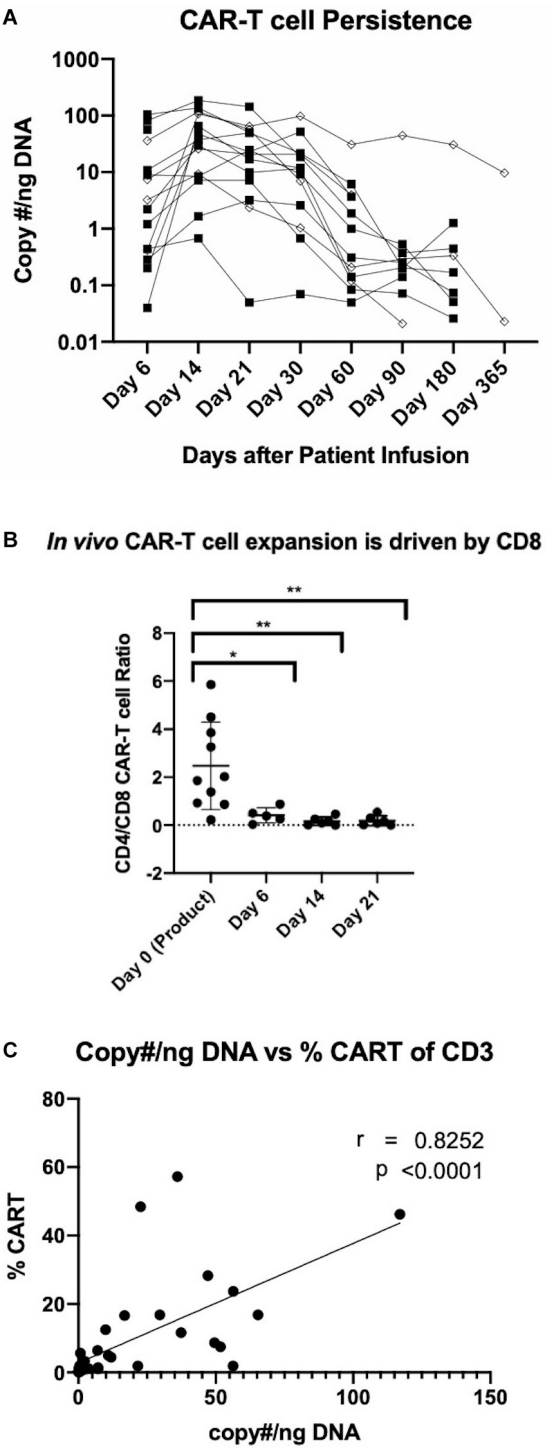

FIGURE 5 | Detection of manufactured CAR-T cells in NHL patients. **(A)** Detection of CAR in patient circulation by PCR for rev-responsive element up to 1 year post-treatment. Diamond and square shapes represent 0.5×10^6 (n = 4) and 1.0×10^6 (n = 11) CAR-T cells/kg doses, respectively. **(B)** CD4/CD8 CAR-T cell ratios in patients at day 0 (pre-infusion) (n = 15), day 6 (n = 5), day 14 (n = 6) and day 21 (n = 6) after treatment. **(C)** Correlation between flow cytometric and RT-qPCR detection of CAR-T cells in patient circulation (n = 36). Statistical significance was given as *, **, ***, or **** by p-values less than < 0.05, < 0.01, < 0.001, or < 0.0001, respectively.

DISCUSSION

Our findings demonstrate that it is possible to robustly manufacture CAR-T cells within a hospital setting using a GMP-compliant closed system. Furthermore, we were able to reduce the turn-around cell manufacturing time to 8 days, lowering the cost and expediting patient treatment. Such fast turn-around time is one of the main advantages of local manufacturing over centralized CAR-T manufacturing strategies. Interestingly, the conditions studied here appear to increase CAR-T cell potency by preventing differentiation and promoting a quiescent/memory profile (14). While our platform allowed patient treatment with fresh CAR-T products, the hypothesis that non-cryopreserved cells might provide better outcomes cannot be answered as we lack a comparison cohort in this study.

In addition to demonstrating the reproducibility of the closed cell manufacturing system, our study emphasizes the need for the standardization of several clinical grade reagents to ensure robust production of CAR-T cells. Unlike previous studies that have reported selective expansion of CD8 CAR-T cells (12, 13), we observed an increase in the frequency of CD4 T cells. This observed difference may be attributable to supplementation with IL-7/IL-15 rather than IL-2, although IL-7/IL-15 stimulation in a similar study led to no noticeable effect on T cell populations [Dominik (30)]. Notably, this affected the final product, with a predominance of CD4 CAR-T. Nonetheless, expansion of CD8 CAR-T cells dominated *in vivo*. In agreement with our findings, previous studies have reported enrichment of a TSCM-like subset from naive T cells upon IL-7/IL-15 supplementation, despite the use of differing gating criteria [(5); Dominik (30)]. In support of this result, we identified increased expression of TCF7, OX40, and 41BB on CAR^+ cells, indicative of the potential of CAR-T cells for self-renewal and effector differentiation. These CAR expressing cells that maintained a TCM/TSCM (CD27+) phenotype had higher levels of CD95, suggestive of an activated state. The activated state of these cells was further confirmed by an increase in the canonical Th1 and Th2 transcription factors TBET and GATA3, and an induction of activation-associated upregulation of LAG3, CTLA4, and PD1. The cell subset described here is compatible with both effector and stemness properties; together these qualities could be crucial to safe and long-lasting responses.

Another technical challenge addressed by our study is the development of a flow cytometric assay to monitor the persistence of circulating CAR-T cells in patients. While there are available reagents such as a monoclonal antibody detecting the scFv region of the FMC63 CD19 CAR (31), protein L (32), or fluorescent-tags, these assays are not commercially available, lack specificity, or are technically challenging in the clinical setting. Here, we employed a commercially available FITC-conjugated CD19 peptide with great success as demonstrated by a strong correlation between detection of circulating CAR-T cells in patient blood samples by viral qPCR and flow cytometry.

Since a major hurdle in the CAR-T cell therapy is the ability to predict the effectiveness of CAR-T products, we performed a potency assay (cytotoxicity assay) and correlated with clinical responses. Though a larger number of patients will be needed to appreciate its value, a subtle pattern was observed whereby the CAR-T products of patients achieving CR had greater cytotoxicity. This indicates the presence of intrinsic differences in CAR-T products which may affect response to CAR-T cell therapy. Recent studies have suggested various factors that may influence CAR-T cell response, such as age or the exhaustion phenotype of the starting sample (33, 34). Thus, our results warrant further investigation of the differences between CAR-T of responding and non-responding patients, particularly through functional assays or at the single cell level to understand product heterogeneity.

CONCLUSION

In conclusion, we report an efficient means to reproducibility manufacture functional autologous CD19 CAR-T cells for clinical application. We demonstrate co-stimulation of patient-derived T cells with IL-7 and IL-15 promotes a TSCM-like phenotype that is efficacious in the lysis of CD19 expressing cells *in vitro*, *in vivo*, and in the clinic. CAR-T cells persisted *in vivo* up to 6 weeks in our mouse tumor model and were detectable in circulation of patients up to 1 year after infusion. Lastly, we provide manufacturing benchmark numbers for inter-institutional comparisons of this approach in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available upon request by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University Hospitals Seidman Cancer Center Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

ZJ and AR wrote the manuscript. ZJ, AR, AS, and FL analyzed the data. ZJ, AR, AS, FL, and SM designed and performed the experiments. DS and RO invented the CAR construct utilized in this study. YX performed the experiments. DS and YX developed the CAR staining protocol and contributed to the development of CliniMACS Prodigy® procedures. AR and RL performed the animal study. SK-B and JR developed the Prodigy manufacture for this CAR-T product and the procedure for 8 day culture. SK-B, JR, and KZ developed the *in vivo* CAR-T staining. KZ and JS performed CAR-T detection studies. DW, ML, and R-PS

designed and supervised the study. DW and ML funded the experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01941/full#supplementary-material

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Conflict of Interest: YX, WK, AW, MK, DS, and BD are employed at Lentigen, a Miltenyi Biotec Company.

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A Novel Siglec-4 Derived Spacer Improves the Functionality of CAR T Cells Against Membrane-Proximal Epitopes

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A domain that is often neglected in the assessment of chimeric antigen receptor (CAR) functionality is the extracellular spacer module. However, several studies have elucidated that membrane proximal epitopes are best targeted through CARs comprising long spacers, while short spacer CARs exhibit highest activity on distal epitopes. This finding can be explained by the requirement to have an optimal distance between the effector T cell and target cell. Commonly used long spacer domains are the CH2-CH3 domains of IgG molecules. However, CARs containing these spacers generally show inferior *in vivo* efficacy in mouse models compared to their observed *in vitro* activity, which is linked to unspecific Fcγ-Receptor binding and can be abolished by mutating the respective regions. Here, we first assessed a CAR therapy targeting membrane proximal CD20 using such a modified long IgG1 spacer. However, despite these mutations, this construct failed to unfold its observed *in vitro* cytotoxic potential in an *in vivo* model, while a shorter but less structured CD8α spacer CAR showed complete tumor clearance. Given the shortage of well-described long spacer domains with a favorable functionality profile, we designed a novel class of CAR spacers with similar attributes to IgG spacers but without unspecific off-target binding, derived from the Sialic acid-binding immunoglobulin-type lectins (Siglecs). Of five constructs tested, a Siglec-4 derived spacer showed highest cytotoxic potential and similar performance to a CD8α spacer in a CD20 specific CAR setting. In a pancreatic ductal adenocarcinoma model, a Siglec-4 spacer CAR targeting a membrane proximal (TSPAN8) epitope was efficiently engaged *in vitro*, while a membrane distal (CD66c) epitope did not activate the T cell. Transfer of the TSPAN8 specific Siglec-4 spacer CAR to an *in vivo* setting maintained the excellent tumor killing characteristics being indistinguishable from a TSPAN8 CD8α spacer CAR while outperforming an IgG4 long spacer CAR and, at the same time, showing an advantageous central memory CAR T cell phenotype with lower release of inflammatory cytokines. In summary, we developed a novel spacer that combines cytotoxic potential with an advantageous T cell and cytokine release phenotype, which make this an interesting candidate for future clinical applications.

Keywords: chimeric antigen receptor, hinge, spacer, Siglec, CH2-CH3, IgG, CAR design

INTRODUCTION

The unprecedented therapeutic efficacy of CAR T cells in previously refractory blood cancers is considered to be one of the major breakthroughs in cancer immunotherapy, culminating in the recent market approvals by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for two CAR T cell products (1–7). While CAR therapies have now achieved public recognition, their development and the quest for optimal CAR design has been a multistep process stretching over several decades. Ever since their initial description in 1989 by Eshhar et al. (8), the receptors have evolved from a two-chimeric-TCR chain architecture to a one-protein design. This design commonly incorporates a single-chain variable fragment (scFv) of a given antibody as the antigen binding moiety, an extracellular spacer and a transmembrane region as structural features, as well as signal transduction units for T cell activation. Originally, the spacer domain was introduced into the CAR framework as an inert building block to allow the antigen binding moiety to extend beyond the T cell's glycocalyx and improve antigen accessibility (9). Following this assumption, a plethora of spacer regions were designed simultaneously ranging from the immunoglobulin (Ig) domains of the crystallizable fragments (Fc) of antibodies to extracellular domains of CD8α, CD28, the TCRβ chain or NKG2D (10–16) and were applied without comparative analyses. However, already very early on, Patel and colleagues provided the scientific proof that the spacer region can be of paramount importance for the receptor function and affects its expression, surface stability through the turnover rate, and signal transduction (17). More recent accumulating research has further been showing that in addition to the nature of the spacer, effective antigen recognition depends on the functional interplay between the spatial localization of the target epitope and the CAR spacer length (18–20). For instance, membrane-distal epitopes were shown to most efficiently trigger CARs with short spacers, while membrane-proximal epitopes required receptors with extended spacer domains to elicit accurate effector function, in this way emphasizing the biological requirement of optimal T cell-target cell distance (18–22). Thus, the design of CARs against novel antigens needs to consider both the epitope position within the target antigen as well as the nature and length of the spacer region and customize these variables accordingly.

The use of Ig-derived spacers is particularly attractive as it provides the opportunity to modulate the spacer length into long (CH2-CH3 domain), medium (CH3) and short (hinge only) structures, while retaining the nature of the parent protein. However, Ig-derived spacers have faced various complications during their development. In particular, off-target activation, CAR T cell sequestration in the lung, tonic signaling and activation-induced cell death (AICD) have been described leading to only a limited T cell persistence (23–26). Although these effects could be abrogated by mutating the amino acid sequence essential for FcR binding (23, 25, 27), it needs to be taken into consideration that these experiments were conducted in immunosuppressed NSG mice and whether FcR binding can be entirely eliminated in humans remains unclear. Of note, several clinical studies that used IgG-derived spacers described only limited anti-tumor efficacy and low CAR T cell persistence (28–31) while others are showing some promising clinical responses (32–34). Interestingly, the first commercially available CAR T cell-based therapies use CD28 (Yescarta) and CD8 (Kymriah) derived spacer domains.

Taking into account the shortage of well-described long spacer domains with a favorable functionality profile, we endeavored to develop a novel long spacer for membrane-proximal epitopes, which naturally lacks an FcR binding domain. Based on the postulated spatial requirements between CARs and their target antigens, we anticipated finding a CAR spacer construct whose functionality against membrane-proximal epitopes extends beyond that of a CD8α spacer CAR. Hence, we generated novel CAR spacers and analyzed their efficacy side-by-side to the cognate CD8α spacer counterpart – a comparison that has not been extensively undertaken thus far. The design of the novel spacers was based on the sialic acid binding Ig-like lectin (Siglec) receptor family, whose members are broadly expressed on various immune cells (35, 36). Structurally, each receptor member is composed of an N-terminal Ig-like V-set domain which is involved in sialic acid binding and a defined number of Ig-like C2-set domains that serve as a structural spacer and extend the binding moiety away from the plasma membrane. The selection of the Siglec family was inspired by the hypothesis that the incorporation of naturally occurring spacer domains into the CAR architecture will preserve the biological requirements of a spacer region and minimize unspecific interactions with other cells.

In this study, we confirm this strategy of using naturally occurring spacer domains by first demonstrating, that in a $CD20^+$ lymphoma model a long IgG1 spacer CAR is as functional as the CD8α spacer *in vitro*, but fails to translate its effectiveness *in vivo*, despite containing the earlier reported mutations to abrogate FcR binding (23). Subsequently, we evaluate novel spacers derived from the Siglec family of proteins and identify a long alternative spacer derived from Siglec-4 that performs with equal efficiency to the CD8α spacer *in vitro*. Finally, we demonstrate in a solid tumor model that the novel Siglec-4 spacer CAR does not exceed, but rather matches the CD8α spacer CAR cytotoxic activity *in vivo* on membrane-proximal targets, while maintaining a favorable cell phenotype profile and cytokine release pattern.

MATERIALS AND METHODS

CAR Gene Construction

Commercial gene synthesis in combination with an optimization algorithm for codon usage in humans (ATUM) was used to construct the *CAR* genes of interest. The CD20-specific scFv was derived from the murine monoclonal antibody Leu16 as originally described by Jensen and colleagues (37), while the CD66c- and TSPAN8-targeting scFv sequences were derived from the antibody clones REA414 (CD66c) and REA443 (TSPAN8) (Miltenyi Biotec). All antigen binding domains contained a $(G_4S)_3$-linker between the V_L and the V_H regions. To

facilitate receptor trafficking to the plasma membrane, a human CD8α leader signaling peptide was added N-terminally to the respective scFv sequence. The spacer region downstream of the scFv encompassed either the domain for IgG1 hinge-CH2CH3 (234 amino acids), IgG4 hinge-CH2CH3 (228 amino acids), or CD8α hinge (45 amino acids). To abrogate potential interactions of the Fc spacer CARs with FcR-expressing cells, the PELLGG and ISR motives in the IgG1 CH2 domain were replaced by the corresponding IgG2 amino acids (23). In the case of the IgG4 CH2 domain, the APEFLG sequence was replaced by APPVA from IgG2 and an N279Q mutation was introduced to remove glycosylation at this site (25). Spacers derived from the Siglec family were designed based on the protein sequences extracted from UniProt and the plasma membrane-proximal domains were incorporated into the CAR architecture. Thus, the Siglec-3 spacer comprised the amino acids 145–259 of the parent protein with a C169S mutation to abrogate unspecific disulfide-bond formation. The Siglec-4 spacer contained the amino acids 238–519, the Siglec-7.1 spacer the amino acids 150–353, the Siglec-7.2 spacer the amino acids 234–353, and the Siglec-8 spacer the amino acids 241–363 of the respective parent protein. All spacers were linked to the transmembrane domain of human CD8α, the intracellular domain of 4-1BB, and the CD3ζ signaling domain as derived from UniProt. The *CAR* genes were fused to a Furin-P2A sequence to include co-expression of the truncated low affinity nerve growth factor receptor (ΔLNGFR). Transgene expression was promoted by the PGK promoter located upstream of the *CAR* gene.

Lentiviral Vector Production

Second generation self-inactivating VSV-G-pseudotyped lentiviral vectors were produced by transient transfection of adherent HEK293T cells. One day before transfection, 1.6×10^7 HEK293T cells were seeded per T175 flask to reach a confluency of 70–90% on the following day. Each T175 flask was then transfected with a total of 35 μg plasmid DNA composed of pMDG2 (encoding VSV-G), pCMVdR8.74 (encoding gag/pol), and the respective transgene-encoding transfer vector using MACSfectin reagent (Miltenyi Biotec). All transfection reactions were performed with a DNA: MACSfectin ratio of 1:2. Following overnight incubation, sodium butyrate was supplied at a final concentration of 10 mM and at 48 h after transfection the medium was collected, cleared by centrifugation at 300 × g and 4°C for 5 min and filtered through 0.45 μm-pore-size PVDF filters. Concentration of the viral stock was performed by centrifugation at 4°C and 4,000 × g for 24 h. Pellets containing lentiviral vector were air-dried and resuspended at a 100-fold concentration with 4°C cold PBS. Lentiviral vector aliquots were stored at −80°C.

Generation of CAR T Cells

Automated CAR T Cell Generation

The CliniMACS Prodigy® TCT (T cell transduction) application was used for the automated manufacturing of large amounts of gene-modified T cells. Technical features and experimental procedures have previously been described in detail (38, 39). In brief, T cells were obtained from non-mobilized leukapheresis from healthy anonymous donors (University Hospital Cologne or the German Red Cross Ulm) and were typically processed 24–48 h after collection. Transduced and enriched CAR T cells were finally formulated and harvested in Composol® solution (Fresenius Kabi), supplemented with 2.5% human serum albumin (Grifols). For quality assurance, the transduction efficiency and T cell phenotype was determined using a MACSQuant Analyzer 10 (Miltenyi Biotec) after the TCT process. Transduction efficiency were determined by flow cytometry on days 5 and 12 of the TCT process using a flow cytometer.

Manual CAR T Cell Generation

Buffy coats from healthy anonymous donors were obtained from the German Red Cross Dortmund. Peripheral blood mononuclear cells (PBMCs) were then isolated from buffy coats by density gradient centrifugation. T cells were purified from PBMCs applying the Pan T Cell Isolation Kit, human (Miltenyi Biotec) and activated in TexMACS™ Medium (Miltenyi Biotec) supplemented with T Cell TransAct™, human (Miltenyi Biotec) and 100 IU/ml of recombinant Human IL-2 IS, research grade (Miltenyi Biotec). T cells were transduced 24 h after activation using VSV-G pseudotyped lentiviral particles. 3 days post activation, T Cell TransAct™, human and excess viral vector were removed and T cells were cultured in TexMACS™ Medium only supplemented with IL-2. T cells were expanded for 12 days and used directly for *in vitro* assays or frozen in TexMACS™ Medium containing 10% DMSO for later *in vivo* use. Frozen T cells that were used for *in vivo* testing were thawed 24 h before injection and cultivated at 37°C in TexMACS™ Medium without further supplements.

Target Cell Lines

HEK293T, JeKo-1, Raji and AsPC1 cells were obtained from ATCC and cultured as recommended. Raji cells were transduced with with a ffLuc cassette for *in vivo* detection and AsPC1 cells were transduced with with a eGFP/ffLuc cassette for *in vitro* and *in vivo* detection. To validate authenticity of the cell lines used, we used the Human STR Profiling Cell Authentication Service (ATCC).

Flow Cytometry

Antibodies specific for anti-human CD62L, CD45RO, CD95, CD271 (LNGFR), CD107a, TNF-α, CD223 (LAG3), CD279 (PD1), CD366 (TIM3), CD137 (4-1BB), CD4, CD8, CD3 were monoclonal recombinant antibodies (Miltenyi Biotec). For anti-CD20 CAR detection the CD20 CAR Detection Reagent (Miltenyi Biotec) was used. Staining of Miltenyi Biotec antibodies was performed according to the supplier's instructions. For direct CAR detection of CD66c and TSPAN8 specific CARs a sequential staining was used. First, samples were incubated with polyclonal Fab specific anti-mouse IgG antibodies produced in goat (Merck) at concentrations of 10 μg/ml for 30 min at 4°C. Samples were washed and then incubated with polyclonal anti-goat IgG antibodies produced in chicken (Thermo Fisher) at concentrations of 10 μg/ml for 30 min at 4°C. Stained samples were measured on a MACSQuant® Analyzer 8 or

MACSQuant Analyzer 10 (Miltenyi Biotec) and analyzed using the MACSQuantify™ Software.

In vitro Functional Assays

With JeKo-1 Target Cells

1×10^5 JeKo-1 and 1×10^5 CAR T cells were co-cultured in TexMACS™ Medium (Miltenyi Biotec) for 24 h in 96-well round bottom plates. Supernatants were collected at the endpoint and used to detect the cytokines released by anti-CD20 CAR T cells using the MACSPlex Cytokine 12 Kit (Miltenyi Biotec) with the four selected human cytokines IFN-γ, IL-2, TNF-α and GM-CSF, according to the manufacturer's instructions. The cytolytic activity of the engineered T cells was evaluated by using 1×10^4 $CD20^+$ JeKo-1 cells labeled with 1 μM CellTrace™ Violet (Life Technologies), as target cells. Effector and target cells were co-cultured for 24 h at the indicated ratios (E:T) in 96-well round bottom plates. Detection of the specific lysis was performed by quantitation of Violet dye labeled target cells using a MACSQuant Analyzer 8 (Miltenyi Biotec). Mock-transduced T cells were used as control at the same effector-to-target ratios.

With Raji Cells

2×10^5 CAR T cells were incubated with 2×10^5 $CD20^+$ Raji cells in 200 μl TexMACS™ Medium at 37°C. In addition, the medium was supplemented with 20 μl of a CD107a specific antibody. After 1 h of incubation the protein transport inhibitors Monensin and Brefeldin A (BD Biosiences) were added as recommended for 4 h. After this incubation period, cells were washed and first surface stained with LNGFR specific antibodies to label transduced T cells and subsequently intracellularly stained for TNF-α using the Inside Stain Kit and a TNF-α specific antibody (all Miltenyi Biotec). Cells were then measured by flow cytometry. For TIM3, LAG3 and PD1 detection 1×10^5 CAR T cells were inoculated with 2×10^5 $CD20^+$ Raji cells in 200 μl TexMACS™ Medium at 37°C for 24 h. Subsequently T cells were stained and analyzed by flow cytometry.

For functionality assays in the presence of NSG macrophages, 2×10^5 CAR T cells were incubated in a 1:1:1 ratio with Raji target cells and macrophages derived from a peritoneal lavage. The assay was performed in the presence or absence of murine FcR-blocking reagent. After 24 h of incubation, detection of the specific lysis was performed by quantitation of Violet dye labeled target cells via flow cytometry using a MACSQuant Analyzer 8 (Miltenyi Biotec).

With AsPC1 Cells

GFP^+/Luc^+ AsPC1 target cells were inoculated in 96-well plates at 2.5×10^4 cells per well in TexMACS™ Medium. CAR T cells or untransduced Mock T cells were added with at an E:T ratio of 2:1. The amount of T cells in the Mock control was adjusted to the number of total T cells in the CAR group with the highest total cell count. Cytotoxicity was measurement as the decrease of green surface area as assessed by the IncuCyte® S3 Live-Cell Analysis System (Sartorius). Measured values were normalized to the start of the experiment. After 24 h a supernatant sample was taken for cytokine measurements using the MACSPlex Cytokine 12 Kit. At the end of the experiment expression of LAG3, PD1, and 4-1BB were measured using a MACSQuant Analyzer 8 (Miltenyi Biotec). Specific endpoint killing was calculated from the green surface area values with the following formula:

$$specific\ killing\ [\%] = 100 - \left(100 * \frac{green\ area\ Mock}{green\ area\ CAR}\right).$$

In vivo Assays

Experiments involving animal handling were approved by the Governmental Review Committee on Animal Care in NRW, Germany and performed according to guidelines and regulations (Landesamt für Natur, Umwelt and Verbraucherschutz NRW, Approval number 84-02.04.2015.A168 and Approval number 84-02.04.2017.A021).

Raji lymphoma were established by tail vein injection of 5×10^5 Raji Luc^+ cells. After 7 days, 1×10^6 CAR T cells or Mock GFP-transduced T cells, adjusted to the total amount of T cells according to transduction efficiency of the CARs, were infused intravenously.

For AsPC1 GFP^+/Luc^+ cell line derived tumors 1×10^6 cells were injected subcutaneously in the right flank of NOD SCID gamma (NSG; NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ) mice (Jackson Laboratory, provided by Charles River). When tumors reached a size of 25 mm^2, 5×10^6 CAR T cells were injected into the tail vein. The amount of injected untransduced Mock T cells was adjusted to the number of total T cells in the CAR group with the highest total cell count.

Therapeutic response was measured longitudinally using the IVIS Lumina *in vivo* imaging system (PerkinElmer) after intraperitoneal injection of 100 μL (30 mg/mL) D-Luciferin (for Raji studies: XenoLight Rediject D-Luciferin Ultra (PerkinElmer). For AsPc1 studies: Potassium Salt, LUCK-2G, GoldBio) and additionally by manual caliper measurement for pancreatic tumors. All measures to secure the well-being of mice were executed following the relevant animal use guidelines and ethical regulations. Upon reaching the endpoint (weight loss of >19%, paralysis, stress score of >20 or end-point of the experiment, Day 20 for the lymphoma model and Day 29 for the pancreatic model), animals were euthanized according to guidelines and post-mortem analysis was performed in order to determine tumor burden, persistence and killing of the different CAR T cell constructs. In particular blood, bone marrow and spleen were subjected to flow cytometric analysis. Therefore, spleen was dissociated using the gentleMACS™ Octo Dissociator with Heaters according to the manufacturers protocol (Miltenyi Biotec) and bone marrow was extracted from the femurs and tibias of mice by cutting off the epiphyses of the bones and rinsing the inner fragments. The cell suspensions were filtered through a 70 μm pore size MACS SmartStrainer (Miltenyi Biotec) and following red blood cell lysis on blood, bone marrow and spleen single cell suspensions using Red Blood Cell Lysis Solution (Miltenyi Biotec), samples were stained and analysis was conducted on a MACSQuant Analyzer 8.

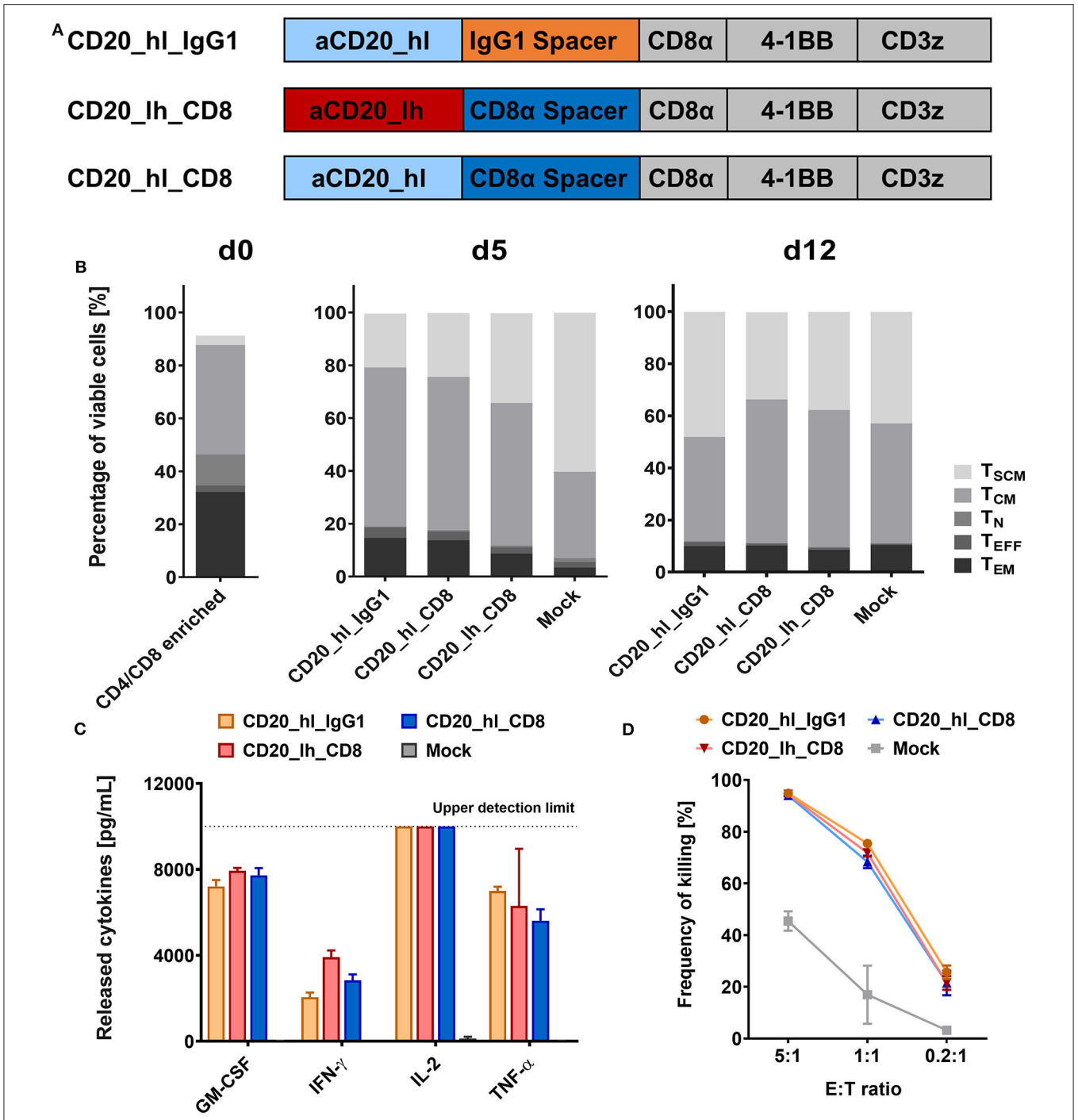

FIGURE 1 | CD20 specific CAR T cells with short CD8α and long IgG1 CH2-CH3 spacers show similar *in vitro* functionality. **(A)** Structure of the three CD20 CAR constructs. **(B)** T cell phenotypes in the CD4/CD8 enriched fraction on d0, d5, and d12 of the automated T cell transduction process by flow cytometry. **(C)** GM-CSF, IFN-γ, IL-2, IL-6, and TNF-α production after 24 h co-culture of CD20 CAR T cells with $CD20^+$ JeKo-1 target cells at 1:1 effector to target ratio analyzed by flow cytometry. $n = 3$. **(D)** Cytolytic activity of the engineered CAR T cells. Effector CAR T cells and target-positive JeKo-1 target cells were co-cultured for 24 h at the indicated ratios (E:T). Detection of the specific lysis was performed by flow cytometry. $n = 3$.

Statistics

Unless otherwise specified, all graphical error bars represent standard error of the mean. Statistical comparisons between more than two groups were conducted by One-way ANOVA with $p < 0.05$ using GraphPad Prism 7. To facilitate the statistical overview of the *in vivo* experiments, the significance analyses

were organized in a pairwise significance matrix (PSM) where each box represents a comparison between two groups, as shown by Al Rawashdeh et al. (40). The order, in which the groups were compared, is illustrated in **Figures S1, S4**. Significant differences between two comparing groups are defined by a green box, while insignificant differences by a red box.

RESULTS

CD20 Specific CD8α and IgG1 CH2-CH3 Spacer CARs Exhibit Comparable *in vitro* Activity

During pre-clinical development of a CD20 directed CAR candidate (39) we also evaluated a number of different CAR configurations (**Figure 1A**). We used an scFv derived from the Leu16 monoclonal antibody (30, 41), binding to the large extracellular loop of CD20 (42). This loop is only 47 amino acids long, which is why we hypothesized it would be more effectively targeted with a flexible CD8α or long IgG spacer. We generated two second generation CAR constructs, that comprised a CD8α transmembrane domain, a 4-1BB co-stimulatory domain and a CD3ζ main activator domain. Both bind CD20 via the Leu16 derived scFv in a V_H-V_L orientation and only differed in the spacer domain. The CD20_hl_IgG1 CAR comprises an IgG1 CH2-CH3 spacer while the CD20_hl_CD8 CAR possesses a CD8α spacer. The PELLGG and ISR motif of the IgG1 CH2-CH3 spacer were replaced by the corresponding IgG2 amino acids to reduce Fcγ-Receptor binding, as described previously (23). To assess whether the order of binding domains in the scFv also can play a role in CAR function, we constructed a CD8α spacer CAR with swapped scFv orientation (CD20_lh_CD8). We generated CD20 specific CAR T cells by genetically modifying CD3/CD28 polyclonally activated T cells with lentiviral vectors in a fully automated manner in a closed system using the CliniMACS Prodigy® as described previously (39). At the end of the manufacturing on day 12, similar T cell phenotypes were obtained for the samples modified with the different CAR constructs and the untransduced Mock control (**Figure 1B**). More than 80% of T cells had a memory phenotype (central memory T cell (T_{CM}) and stem cell memory T cell (T_{SCM}) as defined by their phenotypes being $CD62L^+/CD45RO^+/CD95^+$ and $CD62L^+/CD45RO^-/CD95^+$, respectively). Also, the three constructs demonstrated comparable functionality in terms of cytokine release (**Figure 1C**) and cytotoxicity (**Figure 1D**) upon co-culture with $CD20^+$ JeKo-1 target cells.

CD8α and IgG1 CH2-CH3 Spacer CARs Differ in Their *in vivo* Performance

Having assessed the *in vitro* activity, we next analyzed the same lentivirally modified T cells in a pre-clinical NSG mouse model. 5×10^5 $CD20^+$ Raji cells, which were modified to constitutively express luciferase, were injected into the tail vein of each mouse. Seven days later, 1×10^6 CD20 specific CAR T cells or GFP transduced Mock T cells (**Figure 2A**) were also applied intravenously. Tumor burden was monitored longitudinally over 3 weeks by non-invasive bioluminescent imaging (BLI) of tumor cells *in vivo*. Neither the Mock treated group nor mice treated with the IgG1 spacer CAR showed any control of tumor growth compared to the untreated group, and the animals in these groups were sacrificed according to the ethical code on day 17 and day 15, respectively (**Figure 2B**). On the other hand, significant therapeutic responses were achieved by the CD20_hl_CD8 and CD20_lh_CD8 CAR T cells (**Figures 2B,D**). Both groups exhibited a reduced tumor growth 6 days post T cell injection. While the CD20_hl_CD8 CAR T cells reached background fluorescence on day 13, CAR T cells equipped with the same CAR but with the scFv in the converse orientation needed longer to reduce tumor burden and did not reach background levels until the end of the experiment. This difference between the scFv variants could be attributed to a single mouse having remnants of tumor present in the jawbone, which in our experience is difficult to treat and possibly inaccessible to CAR T cells. We verified that the scFv orientation indeed had only a minor influence by repeating the experiment with the CD8α spacer CARs with the different scFv orientations using a different donor (**Figures 2C,E**). Again, both groups of CAR-modified T cells were effective in rapidly controlling the tumor growth, with no significant difference being observed between the different scFv orientations. *Ex* vivo analysis of spleen, bone marrow and blood at the end of the study showed no detectable IgG1 spacer CAR T cells in the treated mice while CAR T cells with the CD8α spacer could be readily detected, implying a reduced *in vivo* persistence or expansion of the T cells modified with the IgG1 spacer CAR (**Figure S2**).

These findings were in line with earlier results of other groups, showing reduced *in vivo* efficacy of full length IgG family spacers (25, 27). These groups mutated FcR binding sites or developed other solutions in order to decrease off-target binding of the T cell, which we were also able to confirm in an *in vitro* assay using mouse macrophages (**Figure S3**), but it is unclear whether all potential off-target binding has been abrogated as binding to other lower affinity FcγRs may be retained (25).

Construction and Characterization of a New Family of CAR Spacers

These findings motivated us to develop a new class of CAR spacer regions that naturally lack FcR binding sites. In this context we identified the Sialic acid-binding immunoglobulin-type lectin (Siglec) family whose members are expressed on various immune cells and incorporate Ig-like domains in their receptor architecture (43, 44). More specifically, while the membrane distal sialic acid binding Ig-like V-set domain is positioned N-terminally, the more C-terminally located Ig-like C2-set domains, which vary in number, serve as spacer regions. Based on previous reports describing that CAR T cell activation can be optimized according to the epitope location and spacer length, we selected one, two or three C2-set domains derived from Siglec-3, -4, -7, or -8 for spacer design (**Figure 3**).

To confirm correct translation and surface expression of the constructs, bicistronic lentiviral expression vectors were generated with a downstream ΔLNGFR gene linked to the CAR by a P2A sequence (**Figure 4A**). After transfection of the DNA

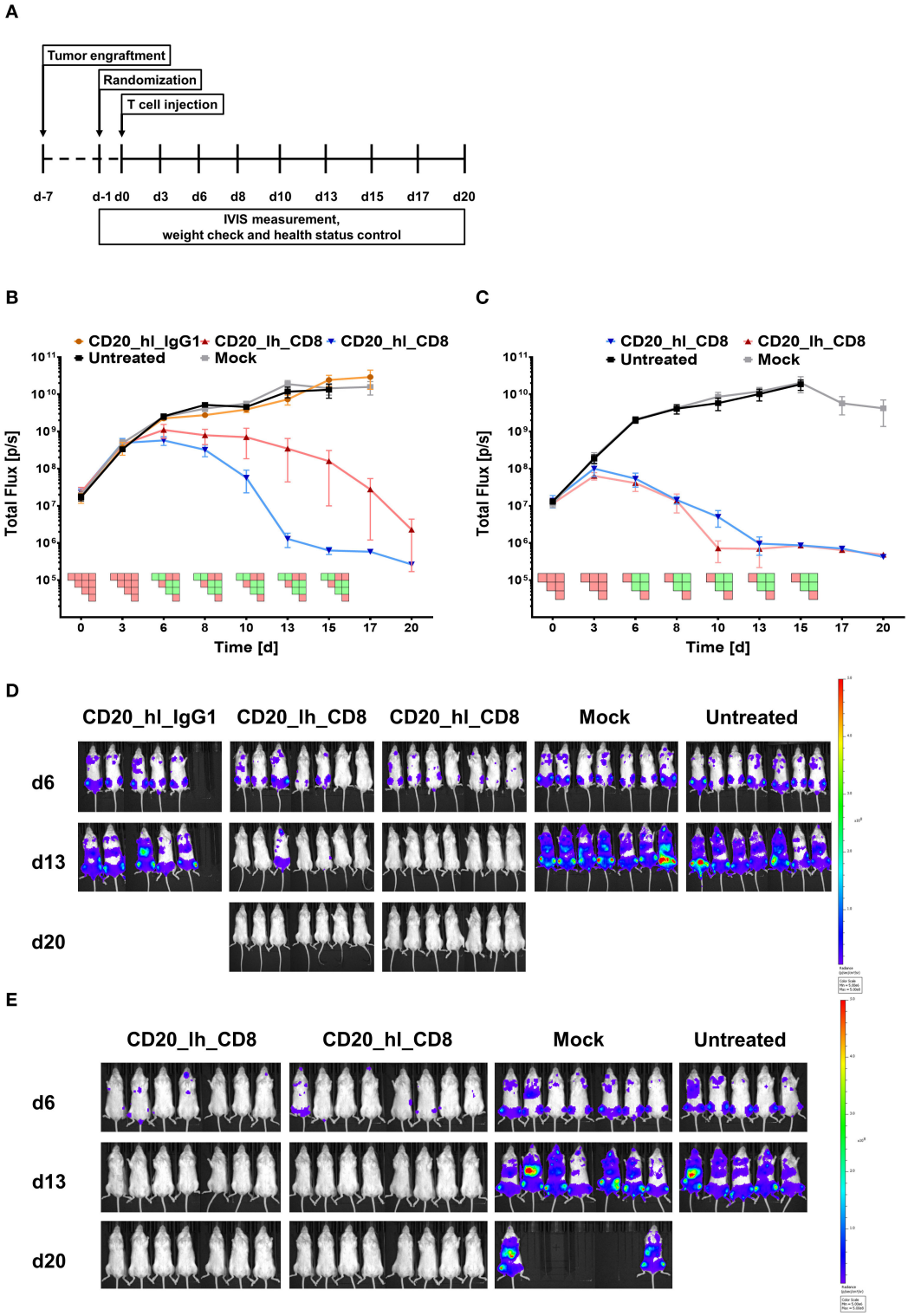

FIGURE 2 | CD20 specific CAR T cells with an IgG1 spacer domain fail to exhibit *in vivo* efficacy. **(A)** Overview of the study workflows. **(B)** Tumor burden change over time in mice treated with anti-CD20 IgG1 CH2-CH3 and CD8α spacer CAR T cells from one donor. $n = 5$/group. PSM $p < 0.05$ (green) [one-way ANOVA]. **(C)** Tumor burden change over time in presence of the two different CD8α CAR constructs with T cells from a second donor. $n = 6$/group. PSM $p < 0.05$ (green) [one-way ANOVA]. **(D)** Representative *in vivo* bioluminescence images of tumor bearing mice from **(B)**. Images are arranged according to the treatment group and time after CAR T cell injection. T cells were generated from one donor. Scale factor: min: 5×10^6, max: 5×10^8 p/s. **(E)** Representative *in vivo* bioluminescence images of tumor bearing mice from **(C)**. Images are arranged according to the treatment group and time after CAR T cell injection. T cells were generated from a second donor. Scale factor: min: 5×10^6, max: 5×10^8 p/s.

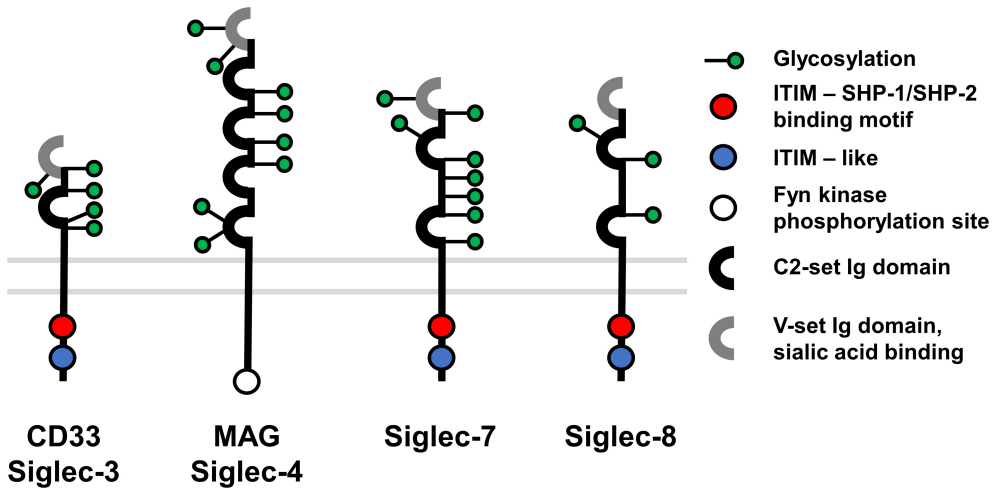

FIGURE 3 | Overview of Siglec membrane proteins used for CAR construction.

constructs into HEK293T cells, detection of the reporter protein ΔLNGFR confirmed successful transcription and translation of the CAR cassette, while direct staining of the CAR with a CD20 CAR detection reagent (PE) visualized surface expression of the CAR constructs. All constructs showed both ΔLNGFR and CAR expression in >80% of HEK293T cells (**Figure 4B**). Subsequently, we transduced primary T cells with lentiviral vectors and assessed the CAR expression 6 days post transduction (**Figure 4C**). The ΔLNGFR reporter protein was expressed in all cases demonstrating effective lentiviral transduction of the T cells and translation of the expression cassette (range 46–75% LNGFR$^+$ T cells). However, while three CAR constructs showed CAR expression levels comparable to the CD8α spacer CAR control, no CD20_hl_Sig7.1 CAR expression was detectable and the CD20_hl_Sig3 CAR was expressed on only 5% of the T cells. Based on these results, we excluded these constructs from further analysis.

Siglec-4 Spacer Shows Comparable *in vitro* Functionality To CD8α Spacer in a CD20 CAR Model

Next, we investigated the cytotoxic potential of the novel constructs. We co-cultured CAR T cells for 5 h with CD20$^+$ Raji cells or CD20$^-$ HEK293T cells at an E:T ratio of 1:1. Effector function was assessed by determining degranulation and intracellular detection of the cytokine TNF-α in the transduced cells (gated on ΔLNGFR expression). Only CAR T cells co-cultured with CD20$^+$ target cells showed significant degranulation (**Figure 4D**). Strongest degranulation could be observed for the CD8α and Siglec-4 spacer variants with around 35% of CD107α positive cells. The Siglec-7.2 spacer CAR produced an intermediate amount of CD107α at 20% positive cells and the Siglec-8 variant had the lowest degranulation with only 10% positive cells but still more than the negative controls (**Figure 4D**). Similar to the degranulation analysis, the proportion of ΔLNGFR$^+$/TNF-α$^+$ cells was also highest in CD8α spacer CAR T cells (**Figure 4E**; 31%) but the CD20_hl_Sig4 CARs only displayed 18% of TNF-α positive cells, followed by Siglec-7.2 and Siglec-8 spacer CARs. Again, no unspecific activation could be observed in the controls.

We also assessed the activation state of the modified T cells by analyzing TIM3, LAG3, and PD1 surface expression. CD20$^+$ Raji cells were co-cultured with CAR T cells for 24 h at an E:T ratio of 1:2. The CD8α and Siglec-4 spacer CAR modified T cells contained the largest fraction of TIM3/LAG3/PD1 triple positive cells (**Figure 4F**). As the Siglec-7.2 and Siglec-8 spacer CAR T cells displayed lower degranulation and upregulation of activation markers after antigen engagement throughout these *in vitro* experiments, we decided to investigate only the Siglec-4 spacer in more detail.

The Siglec-4 Spacer CAR Displays High Functional Potency Against Membrane-Proximal Targets

In our CD20$^+$ Raji lymphoma model the Siglec-4 spacer CAR demonstrated a comparable *in vitro* functionality to the CD8α spacer CAR. As described above the Leu16 epitope is very proximal to the target cell membrane, making it more susceptible for engagement with long spacer CARs. From the CAR variants that could be efficiently expressed in T cells, the Siglec-4 spacer was the only spacer with three C2-set Ig domains, agreeing with previous work that long spacers are excellent for targeting "short," membrane-proximal targets. To verify this hypothesis and to prove the robustness of the Siglec-4 spacer functionality, we assessed the Siglec-4 spacer CAR in an additional solid tumor model of pancreatic ductal adenocarcinoma (PDAC). We have recently identified CD66c and TSPAN8 as novel target candidates for cellular treatment of PDAC (Schäfer et al. manuscript under revision). These two target molecules are especially suitable for investigating our novel long spacer, as the scFv binding epitopes differ greatly in terms of membrane proximity.

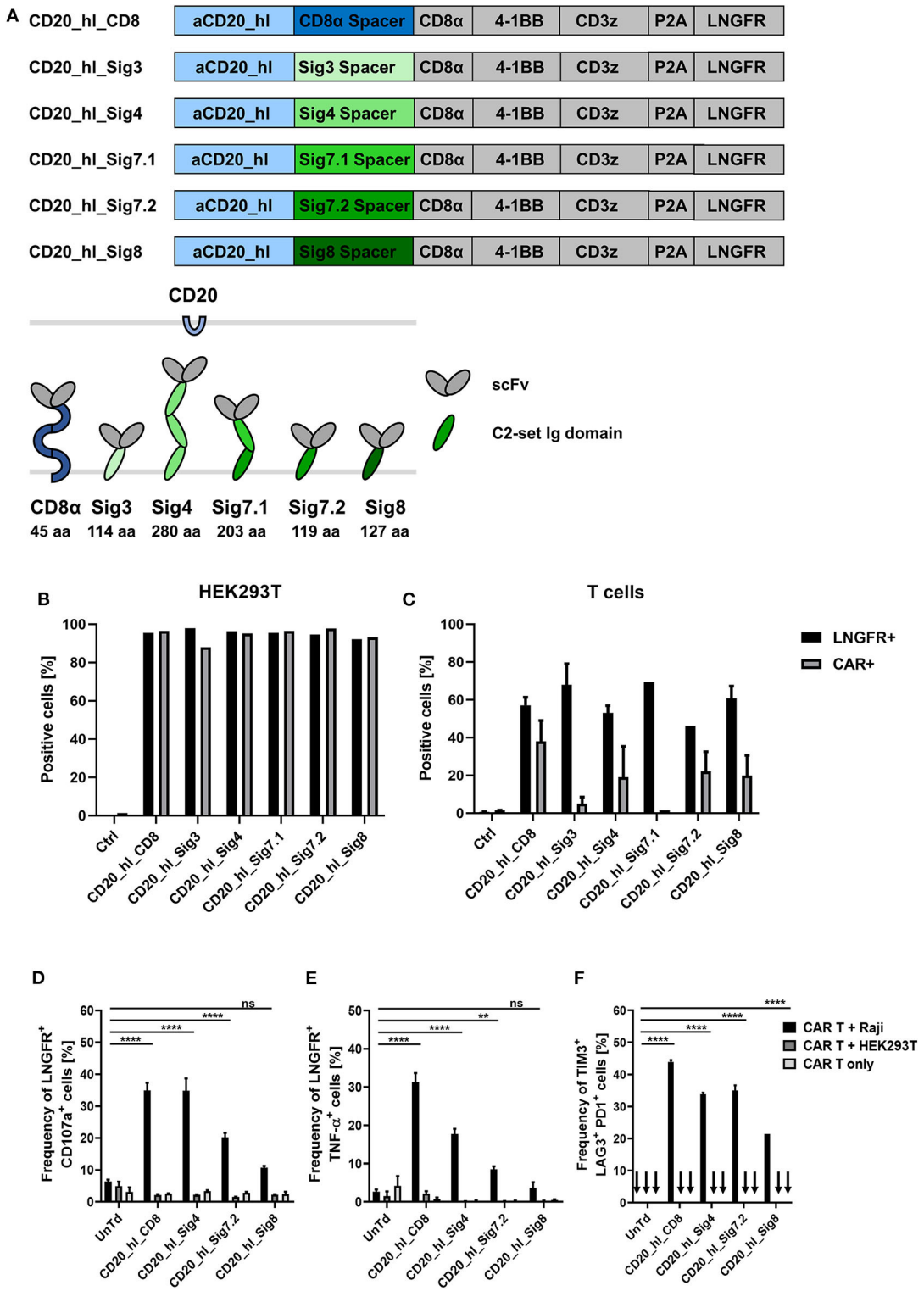

FIGURE 4 | *In vitro* evaluation of novel CD20 specific Siglec spacer CAR T cells. **(A)** Modular structure of the CD20 CAR constructs with the Siglec spacers and extracellular domain comparison of the CAR constructs. **(B)** Expression analysis of the CAR constructs in transiently transfected HEK293T cells 24 h post transfection and **(C)** in transduced T cells from two donors 6 days post transduction. LNGFR and CAR expression were evaluated by flow cytometry. **(D,E)** Siglec spacer CAR T cells were cocultured with Raji or HEK293T cells for 5 h at a ratio of 1:1 and T cell expression of CD107a **(D)** and intracellular TNF-α **(E)** were analyzed by flow cytometry. **(F)** The frequency of TIM3, LAG3, and PD1 triple positive CAR T cells was analyzed after 24 h co-culture at a 1:2 ratio of CAR T cells to Raji or HEK293T cells by flow cytometry. CAR T cells alone were also cultured in order to exclude unspecific activation. $n = 3$. Error bars, mean ± SD. *ns* > 0.05, $^{**}p < 0.01$, and $^{****}p < 0.0001$ [one-way ANOVA, CAR T + Raji compared to Untreated (UnTd)].

TSPAN8 has two extracellular loops extending from the membrane that span 24 and 96 amino acids, respectively, the larger having two interconnecting disulfide bonds. Thus, the whole protein is very membrane proximal. On the other hand, CD66c is a glycophosphatidylinositol anchored protein and consists of two C2-set domains and one V-set domain. In consequence it extends further into the extracellular space compared to TSPAN8. In addition, the epitope of the aCD66c scFv is localized on the outer N terminal V-set domain. In summary, TSPAN8 can be considered a membrane proximal target, while CD66c is a membrane distal target.

We exchanged the Leu16 scFv from our CD20_hl_Sig4 CAR with the CD66c and TSPAN8 specific scFvs that were previously identified (**Figure 5A**) (Schäfer et al. manuscript under revision). Additionally, we incorporated in our experiments CD66c and TSPAN8 specific CD8α spacer CARs and a TSPAN8 specific IgG4 CH2-CH3 spacer CAR, which contained a 4/2 NQ mutation in the CH2 domain as well as a S→P substitution which has been reported to reduce FcR binding also *in vivo* (25), which was not the case for our IgG1 construct (25).

$CD66c^+$/$TSPAN8^+$ AsPC1 PDAC cells that were additionally modified to express GFP and luciferase were co-cultivated with CAR T cells specific for CD66c and TSPAN8 at an E:T ratio of 2:1 and analyzed using a fluorescent live cell analysis system. We assessed cytotoxicity as a decrease in green fluorescence surface area normalized to 2 h after co-inoculation. After 48 h, a supernatant sample was taken for cytokine quantitation while activation markers were measured at the end of the experiment (132 h).

Both, the CD66c_lh_Siglec-4 CAR T cells, as well as the untransduced control T cells showed no specific killing of target cells, while the CD66c_lh_CD8 CAR showed a specific endpoint killing of 42%, (**Figure 5B**). On the other hand, when targeting the membrane proximal TSPAN8, the Siglec-4 spacer CAR T cells showed a similar killing to that of the TSPAN8_hl_CD8α CAR T cells approaching 60% endpoint killing. In contrast, CAR T cells modified with a TSPAN8 CAR with the alternative long IgG4 CH2-CH3 spacer exhibited only 40% killing at the end of the experiment, showing the weakest cytotoxicity of all tested TSPAN8 CAR T cells. The CD66c_lh_Sig4 CAR T cells, which showed no cytotoxicity, also expressed no activation markers (**Figure 5C**). The strongest upregulation of activation markers 4-1BB, LAG3 and PD-1 was observed in TSPAN8_hl_CD8α CAR T cells. Interestingly, the TSPAN8 specific Siglec-4 CAR T cells displayed a lower expression of activation markers, even though the cytotoxicity equalled that of the CD8α spacer CAR T cells. This difference between the CD8α and the Siglec-4 spacer CAR T cells was even more striking at the cytokine level (**Figure 5D**). The TSPAN8_hl_CD8 CAR T cells released markedly higher levels of cytokines than the other CAR T cells. The TSPAN8_hl_Sig4 CAR T cells secreted cytokines at levels more similar to CD66c_lh_CD8 and TSPAN8_hl_IgG4 CAR T cells, which was very surprising, with regard to the same observed cytotoxicity as the TSPAN8 CD8α CAR T cells.

The Siglec-4 Spacer Is Highly Efficacious in an *in vivo* PDAC Model

Finally, we investigated the functionality of the three TSPAN8 specific CAR constructs *in vivo* in a pre-clinical PDAC tumor model. 1×10^6 AsPC1 $eGFP^+/Luc^+$ cells were injected subcutaneously in NSG mice. Tumor growth was measured non-invasively by BLI imaging and furthermore assessed by physical caliper measurement. When the first tumors reached a diameter of 25 mm^2, treatment groups were randomized according to the BLI signal and tumor size, and treatment was started by i.v. injection of 5×10^6 CAR T or untransduced Mock T cells (**Figure 6A**). Untransduced T cells did not display a therapeutic benefit over the untreated group (**Figure 6B**). All mice from these two groups had to be sacrificed before the end of the experiment as tumor ulcerations began to become established. The therapeutic effect for the CD8α and Siglec-4 CARs became apparent in BLI measurements from day 6 onwards. The tumor burden within the groups treated with the CD8α and Siglec-4 spacer CARs decreased in a comparable manner and reached baseline by the end of the experiment 29 days after T cell injection. At the same time, tumor growth was controlled by the IgG4 CH2-CH3 spacer group, but there was no tumor clearance as seen with the other groups. Persistence of CAR T cells could be demonstrated in the spleens of all CAR T cell treated groups with the highest amounts found in the CD8α spacer CAR and Siglec-4 spacer CAR groups (**Figure 6C**). A markedly lower amount of CAR T cells could be recovered from the IgG4 spacer CAR group. Interestingly, when the phenotype of the human T cells was examined the proportion of T_{CM} was twice as high in CD4 and CD8 CAR T cells of the Siglec-4 spacer CAR group as compared to the CD8α spacer CAR T cells (**Figure 6D**).

DISCUSSION

Despite the largely empirical design of CARs based on the functional principles of an antibody and the T cell receptor (TCR), CAR T cell therapies have demonstrated remarkable efficacy in the hematological tumor setting. Although a direct comparison of results across CAR T cell-based clinical trials is difficult due to the various differences in protocols, target antigens, co-stimulatory signaling, treatment regimen, patient groups and disease burden, the rough trend can be observed that those receptors that incorporate a CD8α or CD28 spacer region in their architecture display better therapeutical efficacy than those that utilize IgG-based Fc domains (1–7, 28–31). Non-clinical studies investigating this effect suggest that the inferiority of IgG spacers is due to the engagement with FcγR-expressing myeloid cells (23) resulting in off-target activation of both gene-modified T cells and the respective $Fc\gamma R^+$ cells. In parallel, additional work has been demonstrating that the exemplary performance of CD8α or CD28 spacer CARs is partially also attributed to the epitope location on the targeted antigen CD19 and a number of studies have affirmed the postulate that membrane-proximal epitopes are best targeted by

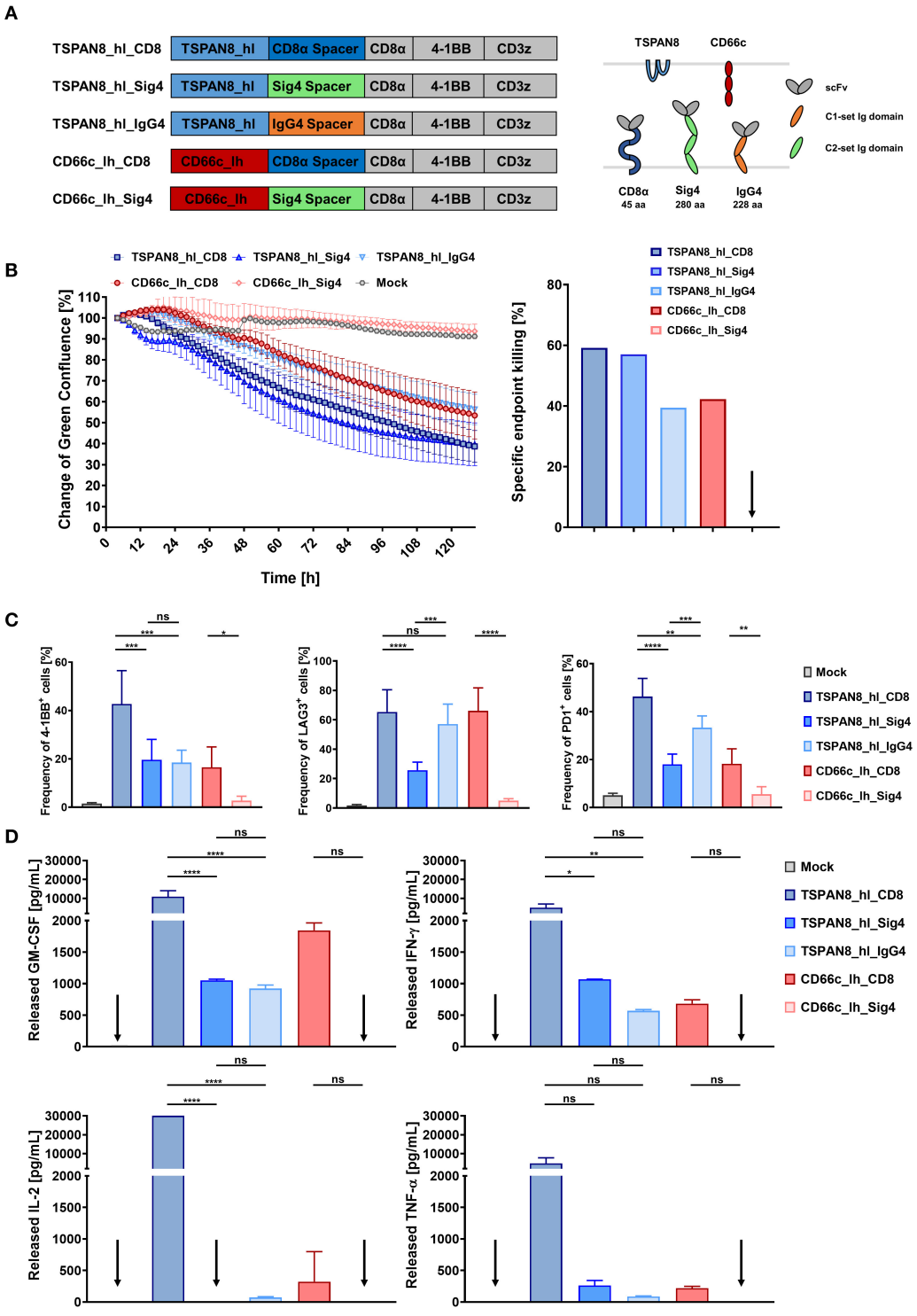

FIGURE 5 | *In vitro* comparison of T cells transduced with TSPAN8 and CD66c CAR constructs, incorporating different spacer domains. **(A)** Structure of the TSPAN8 and CD66c CAR constructs with the Siglec spacers and extracellular domain comparison of the CAR constructs and target molecules. **(B)** Cytolytic kinetics and specific endpoint killing of AsPC1 target cells incubated with CAR T cells and Mock T cells from three different donors in effector to target ratios of 2:1. $n = 6$. **(C)** Frequency of 4-1BB, LAG3 and PD1 positive CAR T cells was analyzed at the end of the cytolytic evaluation with AsPC1 cells by flow cytometry. **(D)** GM-CSF, IFN-γ, IL-2, IL-6, and TNF-α production after 24 h of co-culture of TSPAN8 or CD66c CAR T cells with AsPC1 cells from one donor assessed by flow cytometry. $n = 2$. Data from **(B–D)** were taken from the same experiment. Shown is the mean ± SD. *ns* > 0.05, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ [one-way ANOVA, multiple comparisons].

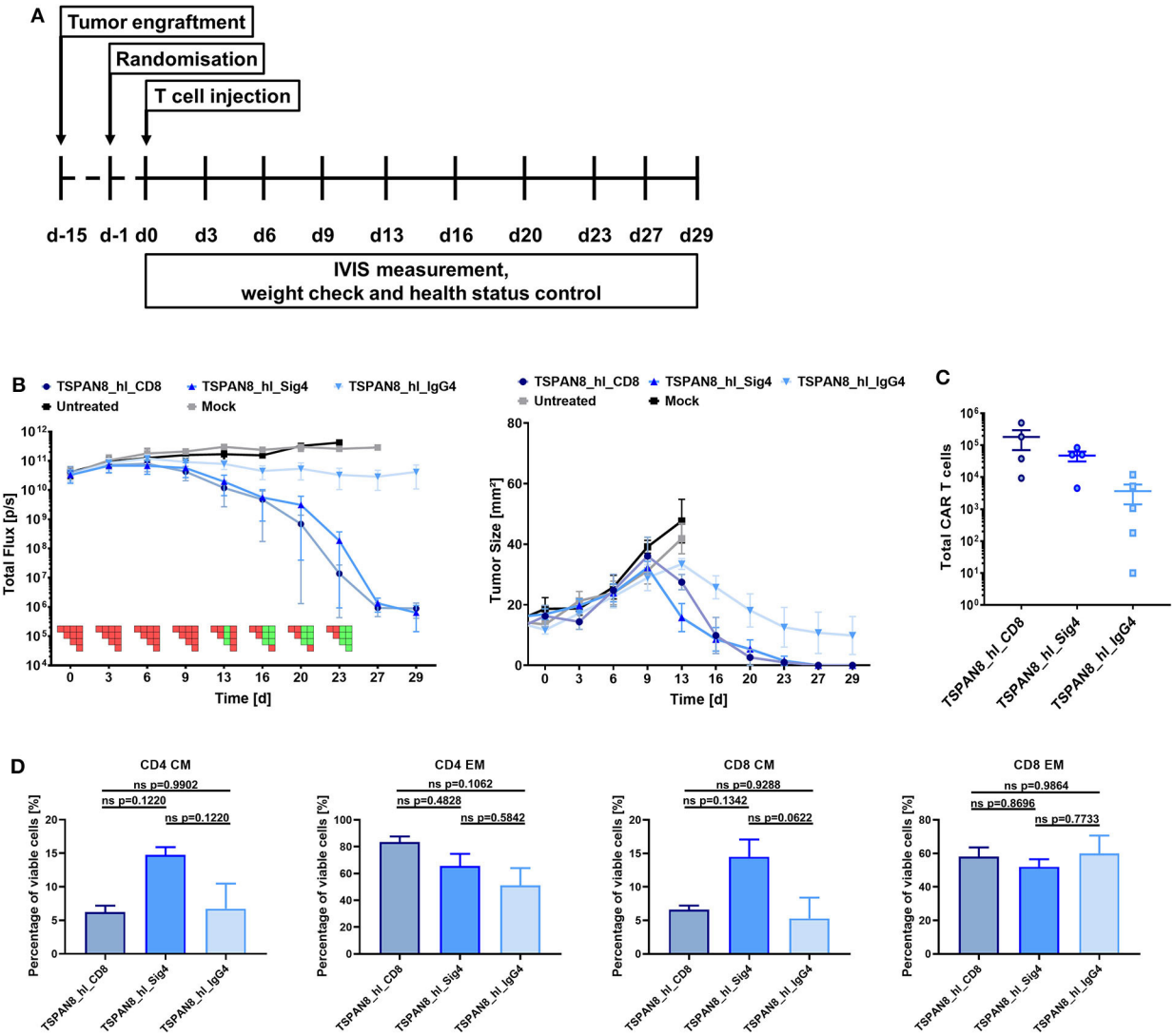

FIGURE 6 | The TSPAN8 specific Siglec-4 spacer CAR T cells exhibit the same anti-tumor efficacy as the CD8α spacer CAR T cells, while retaining a more memory-like phenotype. **(A)** Overview of the study workflow. **(B)** Tumor burden and change in tumor size over time after TSPAN8 CAR T cell infusion. Untreated and Mock T cell treated animals served as controls, T cells from one donor were used. IgG4: $n = 5$; Sig4 and CD8α: $n = 4$. PSM $p < 0.05$ (green) [one-way ANOVA, multiple comparisons]. **(C)** Total number of CAR positive T cells recovered from spleens of TSPAN8 CAR-treated animals at the end of the experiment calculated after flow cytometric analysis. IgG4: $n = 5$; Sig4 and CD8α: $n = 4$. **(D)** CD4 and CD8 CAR+ T cell phenotypes in the spleens of TSPAN8 CAR-treated animals analyzed at the end of the experiment by flow cytometry. $n = 4$.

long spacer modules while membrane-distal epitopes are effectively recognized by CARs incorporating short spacer elements (18–22, 45).

In light of these developments, we identified a shortage of functional CAR spacer modules for membrane-proximal epitopes. Taking advantage of the well-described CD20 antigen and the membrane-proximal binding epitope of Leu16-derived anti-CD20 scFv (42), we sought to characterize the properties of CD8α- vs. IgG-based spacer CARs against CD20 *in vivo*. To avoid unintended cross-activation of CAR- and FcγR-expressing cells in the context of the IgG spacer, the amino acid sequence for IgG1-FcγR interactions in the IgG1-CH2 extracellular domain of the CAR was replaced by the corresponding IgG2 amino acids as described previously (23). However, contrary to reports describing increased anti-tumor activity and CAR T cell persistence following modifications in the IgG4 spacer to abrogate FcγR-binding in the CAR spacer domain (25, 27), we did not observe any *in vivo* therapeutic efficacy of IgG1 CAR T cells after similar modifications in our study. More specifically, the lack of efficacy was accompanied with an inefficient persistence of the gene-modified T cells. These results were in stark contrast to the functional capacity of the CD8α CAR T cells which – according to current understanding – display a less favorable receptor architecture due to the short spacer

region. Although it is reasonable to conclude that the introduced mutations into the IgG spacer domain may not entirely abrogate FcγR binding, it cannot be ruled out that additional mechanisms are in play that sacrifice the therapeutic efficacy. For example, it has already been described that murine scFvs and other non-self gene products can elicit HLA-restricted T cell-mediated immune responses (3, 46, 47). Thus, the possibility exists that the introduced mutations into the Fc region can create immunogenic peptides by the T cell's antigen processing machinery which are then presented on the T cell's HLA and render the gene-modified lymphocytes susceptible to TCR-triggered fratricidal activity. Therefore, it is to be appreciated that the interplay of CAR T cells with their cognate counterparts and the immune system is complex and further work is required to understand the full immunogenic potential of CAR molecules.

To exclude the possibility of potential immunological barriers elicited by the spacer region, we switched our test system to the IgG4 backbone which was previously described to show *in vivo* performance (25, 27) and which has also shown successful translation to the clinic (34). In addition, a new set of spacer domains was designed based on the Siglec family whose members are expressed throughout the immune system and display evolutionary structural similarities to the constant region of immunoglobulins, but lack the inherent ability to interact with FcγRs (35, 36). To determine systematically the optimal spacer length for the membrane-proximal CD20 epitope, five Siglec spacer CAR variants were generated incorporating either one, two or three Ig domains. Of note, different parent proteins were selected, as different Siglec molecules encompass distinct glycosylation patterns which are likely involved in modulating the protein's stability, flexibility, spatial architecture etc. and thus may have different effects on the CAR molecule. Moreover, in an attempt to maintain the original architecture of the molecule, the domains closest to the plasma membrane were selected. Consequently, the Siglec spacer regions within the otherwise identical CAR framework encompassed either a 114 amino acid (aa) Siglec-3, 119 aa Siglec-7, 127 aa Siglec-8, 203 aa Siglec-7, and 280 aa Siglec-4 spacer domain as opposed to the control 45 aa CD8α spacer domain.

Subsequent expression profiling revealed that not all Siglec spacer-based CARs were efficiently expressed on the T cell surface. In particular, Siglec-7.1 and Siglec-3 spacer CARs showed the lowest expression efficiency emphasizing the importance of the spacer region not only on the receptor's functionality but also on its optimal expression. In fact, Patel and colleagues have already described that the CAR spacer domain can affect the receptor's stability and modify its turnover rate (17). It is plausible that the glycosylation patterns present in Siglec-7.1 and Siglec-3 spacer CARs render the receptors less stable, in this way increasing the turnover kinetics and a decreased CAR detectability on the cell surface. Another potential reason for the inefficient expression of the Siglec-3 spacer CAR may lie in the C169S mutation which was introduced in order to abrogate unspecific disulfide bond formation as C169 is involved in an interdomain disulfide bond within the parent protein. Moreover, it is possible that the Siglec-3 C2-set domain *per-se* is instable when isolated from the membrane-distal V-set domain. Although a splice variant of CD33 has been described, which lacks the N-terminal domain ($CD33^{\Delta E2}$), these reports rely on mRNA analyses (48, 49). Protein-based detection using antibodies remains controversial, as it is still not clear whether a clone exists that can specifically recognize the Siglec-3 C2-set domain (49, 50). Importantly, using lentiviral transduction of His-tagged $CD33^{\Delta E2}$, Laszlo and colleagues have shown that the expression of the splice variant is also dependent on the cell type (49). In this context, HEK293T exhibited highest transgene expression while hematopoietic cells displayed only low level expression of the truncated immune receptor which is in line with our observations on the expression of the Siglec-3 spacer CAR (**Figures 4B,C**).

In the next series of experiments, the three best expressed Siglec spacer CAR candidates were analyzed for their ability to induce T cell effector function upon antigen engagement. Consistent with previous reports (17–19, 25, 26, 51), our study provides evidence that the CAR spacer region can modulate the effector function of transgenic T cells. Intriguingly, however, we find that depending on the effector function analyzed, the functional hierarchy may vary. In particular with regard to cytotoxicity, no significant differences between the CD8α spacer (45 aa in length, no Ig domain) and Siglec-4 spacer (280 aa in length; three Ig domains) CAR can be observed while in terms of cytokine secretion the CD8α spacer CAR displays a significant dominance over other CAR constructs. Importantly, in addition to the CD20 system, this observation was further confirmed in the setting of another membrane-proximal antigen, TSPAN8, indicating a common functional feature for membrane-adjacent epitopes.

It has already been demonstrated in the TCR-context that distinct thresholds exist for the cytolytic machinery, the proliferative induction as well as the cytokine production system (52–56) and emerging work suggests similar principles for CAR-triggered T cells (26). The current study further supports this finding and the data obtained indicate that the nature of the spacer region can modulate the nature and degree of effector function. An alternative strategy has been described by Liu and colleagues (57) and Caruso and colleagues (58) in two independent studies, in which they demonstrate the ability of effector function fine-tuning through scFv affinity modulation. The clinical impact of such modifications was impressively demonstrated by Ghorashian and colleagues, who reported a better overall therapeutic profile of CD19 CAR T cell therapies in patients who received lower affinity CARs compared to the commonly used FMC63-scFv-based CARs (59). In particular, while the antileukemic activity was retained, the CAR T cells displayed an enhanced proliferative capacity and reduced severity of cytokine release syndrome (CRS). Though this clearly reveals the effectiveness of such an approach, scFv affinity modulation is a laborious undertaking and bears the risk to result in unwanted modifications to the target specificity. Therefore, fine tuning the chimeric receptor's spacer region provides a time-profitable option with a lower risk profile. More importantly, it further allows to create a variety of receptors with a range of signal transduction intensities independent of the binding domain.

Besides, based on the efficacy data obtained with the CD8α spacer (45 aa) vs. Siglec-4 spacer (280 aa) CARs targeting CD20 and TSPAN8, we find that the receptors' cytotoxic efficacy is not dominated by the spatial constraints of the CAR and its target epitope. This is significant as previous studies reporting such a trend were performed primarily in the context of IgG-derived sequences (25, 26, 51) and have not been compared extensively to spacers derived from other parental proteins. Thus, our work demonstrates that not only structural and spatial elements in CAR T cell:target cell interaction influence a receptor's bioactivity, but also additional factors are in play that are not entirely understood or fully considered yet. It is likely that e.g., CAR flexibility/rigidity and surface stability may have a greater relevance than previously assumed. For instance, Patel and colleagues have shown that the spacer domain can diminish a CAR's functionality by increasing its turnover rate (17). Thus, it is important to take into consideration that Ig domains as they are present in IgG and Siglec spacer domains display a distinct structural folding while the CD8α spacer is derived from a stalk connecting an Ig-like domain with the membrane. Attempts to resolve the structure of the CD8α hinge domain were of limited success so far, indicating the relative flexibility of this region (60). The Siglec-4 or the IgG spacers are missing this flexibility and in this way reduce targetable epitopes to the ones located in membrane proximity.

Another important aspect to be taken into consideration is the tendency of the CD8α stalk region to heterodimerize with CD8β, the subunit that contains raft-localizing determinants (61). As lipid rafts contain an accumulation of accessory molecules decisive for signal transduction and the intracellular CD8β domain has been described to promote association with the two crucial players Lck and LAT (62), it is likely that – in the context of cytotoxic T cells – the CD8α spacer region is capable of attenuating the effector function threshold by fostering interaction with downstream signaling molecules. These effects are absent in IgG- and Siglec-based spacers, so that the overall induction of T cell function is likely primarily guided by the number of triggered CAR molecules (**Figure S5**).

In support of the *in vitro* data, the Siglec-4 spacer CAR displayed a similar anti-tumor efficacy *in vivo* as the cognate CD8α spacer CAR against TSPAN8 and both therapies were superior to the IgG4-based spacer CAR treatment. Taking into account the length of the spacer regions (CD8α: 45aa; Siglec-4: 280 aa; IgG4: 228aa), we could not observe any obvious correlation with CAR potency and rather identified an intrinsic inferiority of the IgG4 spacer *in vivo*. However, in the context of the TSPAN8 targeting, the modified IgG4 spacer CAR showed a much better relative *in vivo* performance to the CD8α spacer compared to the IgG1 spacer performance in the CD20 study. Indeed, the modified IgG4 spacer (25) has now demonstrated good efficacy in ongoing clinical studies (34) indicating that other factors in CAR design such as the scFv binding domain, transmembrane domain or the drug product formulation may also play a role in *in vivo* function and T cell persistence.

Strikingly, however, while the cytotoxic activity was comparable between the CD8α and Siglec-4 spacer CARs, we observed a reduced secretion of pro-inflammatory cytokines and an attenuated upregulation of activation/exhaustion markers such as 4-1BB, LAG3, and PD1 in the Siglec-4 spacer CAR T cells. Moreover, while the proliferative capacity of Siglec-4 CAR T cells was slightly lower compared to CD8α spacer CAR T cells, the Siglec-4 CAR-treated mice featured a trend toward a higher fraction of T_{CM} phenotype within the CAR T cell cohort which is associated with better overall remission and decreased likelihood of relapse in a clinical context.

It is currently widely established that CAR efficacy correlates closely with the development and severity of CRS in the clinic, an adverse event whose management has proven challenging in the clinical setting. Although tocilizumab and glucocorticoids have been described as effective intervention options, finding the right timing for their application represents a big hurdle (1, 63). In fact, too early intervention may jeopardize the therapeutic efficacy and increase the risk of relapse, while too late intervention bears the risk of CRS-induced multi-organ failure and irreversible neurotoxicities resulting in a patient's death (1, 3, 5, 7, 64–68). Thus, a treatment modality that retains the cytotoxic ability of currently approved CAR T cell therapies but attenuates the levels of secreted cytokines may turn engineered T cells not only into a reliable and effective, but also a safer platform. Moreover, a concomitant increase of the memory phenotype in the CAR T cell cohort of the patient holds promise to further increase the therapeutic efficacy while reducing life-threatening side effects.

Although the phenotype of Siglec-4 CAR modified T cells bodes well for future clinical application, what is the potential toxicity profile of this novel spacer structure? The parent protein Siglec-4, also known as myelin-associated glycoprotein (MAG), has been reported to be exclusively produced by myelinating glial cells such as oligodendrocytes in the central nervous system (CNS; 1% of total protein mass) and Schwann cells in the peripheral nervous system (PNS; 0.1% of total protein mass) (69, 70). Its specific expression on the innermost layer of myelin directly opposite to the axon surface supports its crucial role in the stabilization of axon-myelin interactions, the regulation of myelination, and the inhibition of axon regeneration after injury (71–73). These effects have been first described to be mediated by the N-terminal V-set domain of the receptor, as determined by ligand specificity analyses, site-directed mutagenesis and analogy to the crystal structure of Siglec-1 (74–77). In our evaluation of homology studies, we found the protein sequence to be the best conserved among the Siglecs and within mammalian species. Indeed, the highest sequence homology was identified to lie within the first two N-terminal domains of Siglec-4 (78). Consequently, in order to abrogate these interactions, both N-terminal domains were excluded from our CAR spacer design.

More recent studies, however, suggest that an alternative binding domain exists that interacts with the Nogo receptor 1 and 2 (NgR1, NgR2), but not NgR3 (79–82). Deletion analysis demonstrated that while the first three Ig-like C2-set domains (amino acids 17-325) of Siglec-4 are involved in these interactions, C2-set domains 3-5 (amino acids 234-506) as they are present in our CAR architecture fail to associate with NgR1 or NgR2 (83) indicating that domains 1 and 2 are the major interaction partners. Interestingly, both a soluble and membrane-bound receptor construct comprising the C2-set domains 3-5 of

Siglec-4 (amino acids 234-506) are still able to inhibit neurite outgrowth in the CNS, suggesting the existence of an as of yet unidentified ligand partner (83, 84). This observation may indicate the potential risk of unwanted interactions of the Siglec-4 spacer-based CAR T cells with this unknown binding partner. Although the CNS is an immune-privileged organ an intensive infiltration by CAR T cells has been shown to occur as a result of blood-brain-barrier (BBB) damage due to strong CRS. However, as Siglec-4 spacer CAR T cells appear to produce lower levels of cytokines, BBB disruption is expected to be mitigated, in this way minimizing CNS accessibility for CAR T cells.

In support of this hypothesis, despite the high homology between human and rodent Siglec-4 of 95% at the amino acid level over the entire extracellular domain (85, 86), we did not observe any toxicities in the mouse cohort receiving Siglec-4 spacer CAR therapy in our *in vivo* studies. Nevertheless, since - to the best of our knowledge - human-mouse cross-reactivity of Siglec-4 and its interaction partners has not been determined, these data need to be handled with care and further analysis is required to investigate the extent of potential side-effects of Siglec-4 spacer-based CAR T cells.

In summary, this study introduces the new class of Siglec CAR spacers, which structurally resemble IgG class spacers without their FcγR binding features. The Siglec-4 spacer proved to be as efficient as a conventional CD8α spacer in both *in vitro* and *in vivo* CAR function, but exhibited advantageous traits in terms of the T cell phenotype and CAR T cell cytokine release, which make it an interesting candidate CAR structure to translate into future clinical applications.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by German Red Cross Dortmund, University Hospital Cologne and German Red Cross Ulm. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Landesamt für Natur, Umwelt, and Verbraucherschutz NRW, Approval numbers 84-02.04.2015.A168 and 84-02.04.2017.A021.

AUTHOR CONTRIBUTIONS

RP designed the Siglec based spacers. DS and RP designed the Siglec spacer-based CARs. DS, JH, RP, WA, IJ, and OH designed the study. DS, JH, AS, JB, CB, DG, and WA conducted experiments. DS, JH, and RP wrote the manuscript with input from all authors. NM-T, RP, WA, IJ, and OH supervised the project. All authors discussed the data and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01704/full#supplementary-material

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chimeric Antigen Receptor T Cell Therapy for Pediatric B-ALL: Narrowing the Gap Between Early and Long-Term Outcomes

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Chimeric Antigen Receptor (CAR) T cell therapy targeting CD19 has introduced a paradigmatic shift in our treatment approach for advanced B cell malignancies. A major advance has been in the field of pediatric B-ALL where complete responses have been achieved across clinical trials with rates of 65–90% in the relapsed/refractory setting. These striking early response rates led to FDA approval of Tisagenlecleucel, CD19-specific CAR T cells, in August 2017. With broadened access and available longitudinal follow up, it is imperative to study the true durability of CAR-mediated responses and establish long-term relapse free and survival outcomes following CAR therapy. Phase I and II clinical trials have reported event-free survival rates of 50% at 1 year following CD19-CAR infusion in children and young adults with B-ALL. Here, we review some of the major challenges accounting for the discrepancy between early response rates and long term outcomes. In specific, relapse with $CD19^{+}$ or $CD19^{-}$ disease has emerged as a major challenge following CD19-CAR T cell therapy. Related, is the issue of CAR persistence which has been shown to correlate with long-term outcomes. We highlight select efforts to optimize clinical strategies and CAR design to promote enhanced persistence. To date, we do not have robust predictors of response durability and relapse following CAR therapy. The ability to identify patients at risk of relapse in an *a priori* manner may introduce an interventional window to consolidate CAR-mediated remissions and enhance response durability. This review highlights the need to bridge the gap between the remarkable early complete responses achieved with CD19-CAR T cell therapy and the long-term survival outcomes.

Keywords: chimeric antigen receptor, B cell-malignancies, acute lymphoblastic leukemia, pediatrics, CD19 antigen, adoptive immunotherapy

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INTRODUCTION

Relapsed and refractory B cell Acute Lymphoblastic Leukemia (B-ALL) is accompanied by a dismal prognosis and accounts for a significant amount of cancer-related mortality, specifically in the pediatric population, where ALL remains the most common cancer subtype (1). The development of chimeric antigen receptor (CAR) T cell therapy has introduced a new therapeutic option for this patient population and has demonstrated remarkable clinical outcomes. In specific, CAR T cells targeting the B-cell associated antigen, CD19, has achieved complete response (CR) rates of

65–90% across clinical trials spanning institutions in patients with B-cell leukemias (2–6). These early results led to FDA approval of CD19-specific CAR T cells, Tisagennlecleucel (kymriah), in August 2017, and prompted mass efforts to permit scalability and access. The approved indication is for the treatment of pediatric and young adult patients with refractory B-ALL or B-ALL in second or greater relapse. With FDA approval, CAR T cell therapy has become available for commercial use. With broader access to CAR T cell therapies and increased experience and longitudinal follow up, it is now pertinent to understand the long-term outcomes using CAR T cell therapy (**Figure 1**). Here we review a limited background on CAR T cell therapy, highlight successes using CD19-CAR T cells in achieving early responses in Pediatric B-cell ALL, address the importance of identifying predictors of CAR responses and resistance and highlight factors challenging long-term CAR responses.

BACKGROUND: THE CAR CONSTRUCT

CARs are artificial antigen receptors engineered to fuse an antibody-binding domain targeting a tumor-specific antigen to T cell derived-signaling domain/s (7). T cells can be engineered to express CARs targeting tumor-specific antigens, endowing T cells with tumor-specific cytotoxicity. The majority of CARs designed for clinical development have targeted the B-cell associated CD19 surface antigen and have been used in B cell malignancies, including B-cell leukemias and lymphomas across ages (2–6, 8–13). To date, the majority of CARs developed for clinical trials have included a primary CD3-ζ signaling domain and a CD28 or 4-1BB secondary co-stimulatory domain, based on early pre-clinical work demonstrating independent properties of CD28 and 4-1BB in enhancing cytotoxicity beyond first-generation CARs housing a singular CD3-ζ signaling domain (7, 14, 15). Physiologic T cells rely on antigen presentation within the major histocompatibility complex (MHC) and are dependent on secondary costimulatory signals to permit effective cytotoxicity. In contrast, antibodies can bind to surface antigens in an MHC-independent manner. Tumors often downregulate MHC and can lack costimulatory ligands, rendering them immune to T-cell mediated cytotoxicity. The design of a CAR permits MHC-independent antigen-binding by the inclusion of an antibody-derived binding domain. Additionally, by including both primary T cell signaling and endogenous costimulatory signals, CARs are not reliant on tumor expression of costimulatory ligands and can effectively induce T-cell mediated cytotoxicity by inducing multiple signals with the binding of a single tumor-specific antigen (7).

SUCCESSES USING CD19-SPECIFIC CAR T CELLS IN ACHIEVING EARLY COMPLETE RESPONSES

Efficacy of CD19-specific CAR T cell therapy was first reported in small case series in adults with indolent lymphoma and chronic leukemia (8, 9, 12). Shortly after, acute B-cell lymphoblastic leukemias were found to be exquisitely sensitive to CD19-specific CAR T cell therapy (2, 6). Phase I studies in pediatric B-ALL using independent CAR constructs across institutions demonstrated conserved efficacy ranging from 65 to 90% (2–4). The challenge of scalability required organized collaboration, merging pharmaceutical and academic efforts. A phase II global, single-cohort study using Tisagenlecleucel demonstrated that the single-institution phase I outcomes were paralleled in this multi-institutional study with a CR rate of 81% following a single infusion of CAR T cells (5). This landmark study importantly demonstrated feasibility of centralized CAR manufacturing for scaled, global use and led to FDA approval of Tisagenlecleucel for children and young adults with B-ALL.

RELAPSE PATTERNS POST-CAR AND DURABILITY OF CAR-MEDIATED RESPONSES

Post-CAR Relapse

As described, CD19-CAR T cell therapy has proven effective at achieving early complete response rates. With increasing follow up, the emergence of relapse post-CAR T cell therapy, however, poses a significant clinical challenge to the durability of CAR-mediated remissions (16, 17). The two most prominent patterns of relapse include relapse with conserved $CD19^{+}$ expression, most often in context of CAR loss, and relapse with CD19-negative or downregulated disease in context of ongoing CD19-targeted pressure. Studies using CD19-targeting CARs in adults and children with B cell malignancies across institutions demonstrate relapse rates of 30–60% with both $CD19^{+}$ and $CD19^{-}$ disease accounting for relapses (16). The largest phase II pediatric study described above however, identified $CD19^{-}$ relapse to be a predominant pattern of relapse in their cohort of 75 infused patients with 15 of 16 evaluable relapses having CD19-downregulation (5). A phase I single-institution study evaluating 43 pediatric patients infused with a similar 4-1BB-based CD19-specific CAR construct demonstrated a lower rate of $CD19^{-}$ relapse, possibly explained by decreased persistence and less ongoing targeted-pressure, with 7 of 18 relapses demonstrating CD19 negativity and 11 relapses with conserved CD19 expression (4). Mechanistic studies describe mutated CD19 RNA isoforms with alternatively spliced CD19 and underlying genetic mutations in CD19 driving truncated protein production or dysfunctional or absent transmembrane domains, that account for absence of surface CD19 in context of $CD19^{-}$ relapse (18, 19). Studies are ongoing to identify subpopulations of B-ALL that predispose patients to $CD19^{-}$ relapse. Patients with KMT2A (mixed lineage leukemia, MLL) rearrangement have been shown to have increased risk of $CD19^{-}$ relapse in context of myeloid-transformation post-CAR (20, 21). To date, however, we lack extensive established predictors identifying patients at risk of

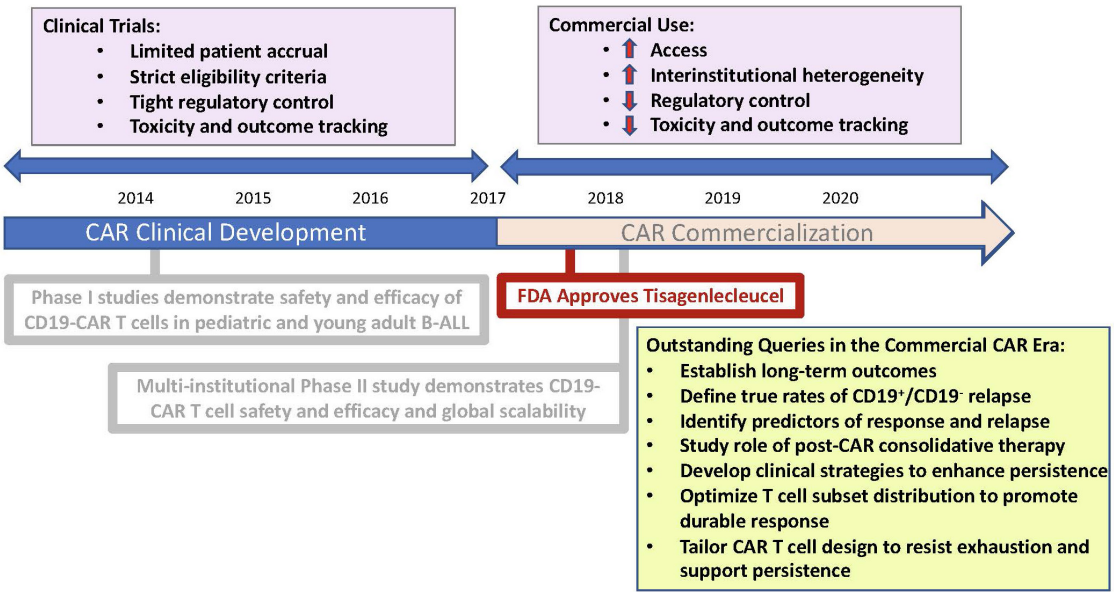

FIGURE 1 | Schematic representation mapping the chronology of CAR commercialization. Illustration highlights practice changes characterizing the era of CAR commercialization and pertinent queries requiring further study.

relapse or at risk of specific relapse patterns. With CAR commercialization, specific relapse patterns are not captured in the form of clinical trial monitoring and tracking of CAR-mediated outcomes occurs at institutional discretion. With commercialization and extended CAR access comes a responsibility to continue to study specific relapse patterns in efforts to establish the true rate of $CD19^+$ and $CD19^-$ relapse in the pediatric CAR setting. Additionally, it is vital to study predictors of both $CD19^+$ and $CD19^-$ relapse so we can better differentiate patients likely to achieve durable responses and patients at high risk for post-CAR relapse in an *a priori* manner, permitting pre-emptive intervention to prevent relapse.

CAR T Cell Persistence and B Cell Aplasia

Duration of B cell aplasia, as a representation of ongoing CAR T cell persistence, has been associated with durability of CAR-mediated remission. Direct testing for CAR T cell persistence can be done using PCR or flow cytometry, however, these tests remain research tests at this time and the standard for clinical CAR T cell persistence monitoring remains indirect testing of B cell apalasia. In pediatric ALL, it has been demonstrated that a longer duration of CD19-CAR persistence correlates with the durability of remission (4, 22). CD28 ad 4-1BB costimulation demonstrate distinct properties *in vivo*, with 4-1BB mediating protection against T cell exhaustion and facilitating long-term persistence and immune memory (23). Tisagenlecleucel, the CD19-specific CAR commercially approved for pediatric B-ALL, houses the 4-1BB costimulatory domain.

OPTIMIZATION STRATEGIES TO ENHANCE CAR T CELL PERSISTENCE

B cell and disease factors may impact CAR T cell persistence. In specific, low CD19 antigen load prior to lymphodepletion has been identified in one case series as a risk factor for early CAR T cell loss (4). A phase II study investigating the role of upfront CAR T cells in pediatric patients with refractory disease as detected by flow cytometry minimal residual disease (MRD) following consolidation (NCT03876769) is ongoing and will predominantly treat patients with low disease burden and may yield insight into the expansion and persistence potential of CAR T cells in a low disease burden setting. A novel strategy to promote *in vivo* CAR expansion uses T cells engineered to express the CD19 antigen (T antigen-presenting cells, T-APCs) as booster cells post-initial CAR infusion. Analysis of pediatric CD19-CAR recipients with low CD19-antigen burden or rapid CAR T cell loss or contraction treated with T-APCs demonstrated early evidence of secondary CAR T cell expansion, supporting *in vivo* antigen delivery as a potential approach toward reinvigorating CAR expansion and enhancing CAR persistence (24). Additionally, in efforts to augment CAR T cell persistence and reverse exhaustion, checkpoint inhibition is being clinically explored following CD19-CAR therapy, in event of incomplete-response or early CAR-T cell loss. Early data demonstrates safety of this strategy and preliminary promise, specifically in patients with early CAR-loss and bulky extramedullary disease (25).

Selecting for T Cell Subsets

T cell factors likely contribute to CAR T cell persistence. Patients treated with CAR T cell therapy are often multiply

relapsed and have had significant prior exposure to leukemia therapies including cytotoxic chemotherapies and allogeneic hematopoietic stem cell transplantation. These factors likely account for significant variation in the composition and representation of naive, memory subsets and stem central memory T cells and contribute to heterogeneity of T cell fitness across patients. Pre-clinical data demonstrate that T cell subsets of early lineage, including naive and stem central memory T cells, confer improved expansion during *ex vivo* CAR manufacturing. Specific chemotherapy agents such as cyclophosphamide and cytarabine associate with depletion of early lineage T cells in pediatric leukemia patients, supporting the impact of prior therapy on CAR T cell subset distribution and expansion potential. Culture methods including select use of IL7 and IL15 cytokines to enrich expansion of early lineage T cells are being explored (26). To date, with the exception of a single-institutional effort to evaluate CAR products of defined CD8 and CD4 T cell formulations (4, 13), the majority of clinical CAR-T cell products for B-ALL are made up of heterogeneous T cell subset distributions, reflecting the individual patient's circulating T cell pool. Although pre-clinical data support use of naive and stem central memory T cell subsets, optimization of clinical CAR products enriched for select T cell lineage subsets to promote CAR persistence remains an area under study. It is vital to pursue measured study of T cell subset distribution of apheresis and CAR products across patients as they relate to CAR T cell persistence and CAR-mediated outcomes.

Optimizing CAR T Cell Engineering

There is active preclinical effort to identify methods to enhance CAR signaling while mitigating CAR T cell exhaustion. CAR T cell engineering for clinical trials is primarily achieved using viral-mediated transduction with random insertion. Targeted CAR insertion to the T-cell receptor α constant (TRAC) locus using CRISPR/Cas9 has been explored as a method to avert tonic signaling and defer effector-T cell differentiation and exhaustion (27). Efforts to characterize properties driving T cell exhaustion using a tonically active CAR model identified deficiency of the AP-1 factor, c-Jun, as a driver of T cell-exhaustion. Engineering CAR-T cells to over-express c-Jun is an alternative approach to rendering CAR T cells resistant to exhaustion that has demonstrated pre-clinical promise (28).

Although, as described, costimulatory domains such as CD28 and 4-1BB are known to have variable properties driving expansion and persistence, the CAR structure is modular, housing additional transmembrane domains and immunoreceptor tyrosine-based activation motifs (ITAMs) that can be engineered to titrate function. Manipulation of quantity and position of functional ITAMs demonstrates that CD1928ζ CARs expressing a single, proximal ITAM maintain cytotoxicity while protecting against T cell differentiation and exhaustion as compared to CD1928ζ CARs with additional or distal ITAM signaling (29). Precise modulation of a 4-1BB CAR to express a CD28-hinge/transmembrane domain and inclusion of additional ITAM domains has been shown to permit CAR activity in response to lower-antigen expression while preserving 4-1BB-mediated persistence (30). Further understanding of T cell factors driving activation, persistence and exhaustion underpin our ability to engineer CAR constructs optimized for low-threshold activation and enhanced persistence.

CAR-MEDIATED TOXICITIES

Early CAR translation identified CAR-mediated cytokine release (CRS) syndrome and immune effector cell-associated neurotoxicity syndrome (ICANs) to be the two most-common post-CAR toxicity syndromes, with CRS having increased frequency and generally preceding ICANs (11, 31–33). CRS is characterized by fever but can be associated with constitutional symptoms, vital sign instability including hypotension and hypoxia and in severe cases may warrant vasopressor-use, intubation, PICU level care and rarely result in fatalities. ICANs also spans severity with symptoms ranging from mild confusion, aphasia, impairment of cognitive or motor skills to seizures, loss of consciousness and in the rare case cerebral edema and death. Treatment for both these syndromes may be limited to supportive care or may include agents such as tocilizumab, an anti-IL-6 receptor antibody that abrogates IL-6 signaling and disrupts toxicity symptoms without compromise of efficacy, or steroids (32, 34). Initial concerns that disrupting CAR-mediated toxicity will in parallel disrupt efficacy have been dispelled and agents such as tocilizumab and steroids are currently used more liberally in response to toxicity. Additional agents are under investigation for treatment of CAR related toxicities including Siltuximab, a direct IL-6-targeting antibody, and Anakinra, an anti-IL-1 receptor antagonist that has been shown to have efficacy in CAR-mediated Hemophagocytic Lymphohistiocytosis (HLH), a post-CAR toxicity on the spectrum of severe CRS seen following CD19-CAR T cell therapy (35) and recently reported to have greater frequency following CD22-CAR T cell therapy (36). Increased disease burden has been shown to be a predictor of CAR-toxicity and studies are ongoing to establish further predictors of toxicity and understand toxicity as it relates to efficacy (11). CAR-mediated toxicities such as CRS and ICANs generally occur concurrently with CAR T cell expansion and commonly manifest within the first 28 day window post-CAR therapy. Long-term neurocognitive effects in patients experiencing neurotoxicity have yet to be established and remains an area under study. Aplasia of physiologic B cells is an additional expected on-target, off-tumor side effect of CD19-CAR T cell therapy that is effectively managed with IVIG replacement. Durability of B cell aplasia is desirable, as it represents ongoing CAR persistence (37). In the pediatric registration trial of Tisagenlecleucel, all patients responding to CAR therapy developed B cell aplasia with a probability of ongoing B cell aplasia at 6 months post-CAR of 83% (5). Related hypogammaglobulinemia has been shown to be higher in pediatric patients as compared to adult CD19-CAR recipients, likely due to decreased established antibody-producing-$CD19^-$plasma cell clones in children (38). Understanding specifics of adaptive T cell immune responses and reconstitution following combined lymphodepletion and CD19-CAR T cell therapy remains an area under study.

ROLE OF CONSOLIDATIVE HEMATOPOIETIC STEM CELL TRANSPLANTATION FOLLOWING CAR THERAPY

One additional outstanding question in the field is the role for consolidative allogeneic hematopoietic stem cell transplantation (HSCT) following CAR T cell therapy. In context of data demonstrating that despite achievement of early responses post-CAR, many patients will go on to relapse with $CD19^+$ or $CD19^-$ disease, HSCT has been used at many centers to consolidate CAR-mediated remissions (39). Pediatric data following use of short-lived CD19-CARs, harboring the CD28 costimulatory domain, demonstrate decreased relapse in patients consolidated with HSCT. Of 28 patients achieving minimal residual disease (MRD) negative responses by flow cytometry across pediatric CD19-CAR T cell trials at the National Cancer Institute (NCI), 2 of 21 patients consolidated with HSCT relapsed as compared to 6 relapses of 7 patients who did not undergo post-CAR HSCT (40). Long-term Phase I data using CD28-based CAR T cells in adults with ALL did not show a benefit in event-free-survival (EFS) or overall survival (OS) in patients receiving consolidative HSCT post-CAR (11), however, Phase I/II data studying 4-1BB CD19-CAR T cells in adult ALL, showed prolonged EFS in patients undergoing HSCT post-CAR (41). Data analyzing pediatric ALL patients receiving 4-1BB-harboring CD19-CAR T cells, support benefit in patients receiving consolidative HSCT post-CAR. Of 38 patients achieving remission following CD19-4-1BB CAR T cells, 3 of 13 consolidated with HSCT relapsed as compared to 20 relapses of 25 patients who did not undergo post-CAR HSCT (22). Patients who are HSCT-naive prior to CAR or who experience early CAR-loss (within 63 days) post-CAR have been identified as cohorts who may specifically benefit from consolidative HSCT (22, 42).

Due to HSCT-related toxicity risks, many centers and patients/families will opt to undergo surveillance and monitor for minimal residual disease (MRD) and B cell aplasia and only proceed with HSCT in context of loss of CAR persistence or evidence of detectable MRD. In some patients, loss of B cell aplasia will may precede disease recurrence introducing a window to proceed with HSCT while the patient is in continued remission, however, some patients will relapse concurrently with CAR loss and may lose their therapeutic window for HSCT. Additionally, many patients enter CAR after prior-HSCT, and toxicity and survival outcomes of a second HSCT are significantly inferior to outcomes following initial HSCT. To date, the decision whether to pursue a consolidative HSCT has not yet been standardized and differs across institutions and depends upon individualized factors including status of prior HSCT, CAR persistence, comorbidities, donor availability and patient/family preferences. Prospective trials on the role of first or second HSCT post-CAR remissions are essential to understanding the role of HSCT in the CAR era.

DISCUSSION

We have learned that CD19-specific CAR T cells are effective at achieving early remissions in relapsed/refractory pediatric and young adult B-ALL, but the rate of lasting relapse-free curative outcomes lags behind with longer follow up. A phase I intent-to-treat study of CD19-CAR T cells in pediatric and young adult patients demonstrated an event-free survival (EFS) of 50.8% at 12 months in a cohort of 45 patients. Similarly, the long-term follow up of the phase II global, multi-institutional study analyzing Tisagenlecleucel in pediatric and young adult patients demonstrated an EFS rate of 50% at 12 months in a sample size of 75 infused patients. With CAR commercialization in 2017, longitudinal data is only recently becoming available and it is imperative we understand when and if the overall and event-free survival curves flatten post-CAR T cell therapy. The promising early post-CAR responses have introduced high expectations on behalf of CD19-CAR T cell recipients. Establishing the true curative potential and limitations of CAR-T cell therapy is vital to managing clinical expectations of this therapy and identifying interventional windows to further enhance the durability of outcomes. To date, we do not have robust predictors of response durability and relapse. It is a priority to pursue further interrogation of such predictors so we can identify and differentiate patients expected to have lasting responses following CAR and patients who may achieve early remission but require further consolidative therapy to maintain responses. Additionally, extensive pre-clinical efforts are ongoing to further tailor CAR design to promote CAR-persistence and long-term immune memory.

Despite major advances with CAR clinical development and commercialization, the field remains in its infancy with many outstanding queries and areas for long-term outcome optimization. Ongoing effort is in order to narrow the gap between early response rates and long-term outcomes and further harness the power of this potent therapeutic to achieve reliable, durable CAR-mediated cures.

AUTHOR CONTRIBUTIONS

LS wrote the manuscript in entirety and is accountable for the content of the work.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Engineering Immune Cells for *in vivo* Secretion of Tumor-Specific T Cell-Redirecting Bispecific Antibodies

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Immunotherapeutic approaches based on the redirection of T cell activity toward tumor cells are actively being investigated. The impressive clinical success of the continuously intravenously infused T cell-redirecting bispecific antibody (T-bsAb) blinatumomab (anti-CD19 x anti-CD3), and of engineered T cells expressing anti-CD19 chimeric antigen receptors (CAR-T cells) in hematological malignancies, has led to renewed interest in a novel cancer immunotherapy strategy that combines features of antibody- and cell-based therapies. This emerging approach is based on the endogenous secretion of T-bsAbs by engineered T cells (STAb-T cells). Adoptive transfer of genetically modified STAb-T cells has demonstrated potent anti-tumor activity in both solid tumor and hematologic preclinical xenograft models. We review here the potential benefits of the STAb-T strategy over similar approaches currently being used in clinic, and we discuss the potential combination of this promising strategy with the well-established CAR-T cell approach.

Keywords: cancer immunotherapy, T cell-redirection, bispecific antibodies, chimeric antigen receptors, *in situ* secretion

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INTRODUCTION

The immune system plays an important role in shaping the immunogenicity of tumors (1). The T cell receptor (TCR)-mediated recognition of processed tumor-associated antigens (TAAs) drives the elimination or sculpting of developing cancer cells, which can generate immune-resistant cell variants (1, 2). Due to this selective immune pressure, these variant cells display a multitude of evasion mechanisms from immune recognition and destruction, such as abnormalities in the antigen presentation machinery (2), and the generation of an immunosuppressive environment that promotes tumor growth (3). In the past few decades extensive research has been made to develop cancer immunotherapy approaches aimed at stimulating anti-tumor T cell responses (4, 5). Most notably the emergence of immune checkpoint inhibitors blocking negative regulators of T cell immunity (6), the systemic administration of bispecific antibodies (bsAbs) (7), and the adoptive transfer of genetically engineered T cells expressing chimeric antigen receptors (CARs) (8). However, only a limited proportion of patients benefit from these strategies. Therefore, intense efforts are being made to improve the currently available immunotherapies and to design new strategies to strengthen anti-tumor immune responses.

CURRENT T CELL-REDIRECTING STRATEGIES

T cell-redirecting immunotherapies are intended to specifically eliminate tumor cells by physically joining lymphocytes and cancer cells using tumor-targeted cell-cell bridging (CCB) molecules (9). CCBs can be generated using engineering approaches to manipulate the membrane of immune cells (cell surface engineering), to create artificial soluble molecules (antibody engineering) or a combination thereof (4, 5). In fact, some of these CCB-based strategies, such as membrane-anchored CARs or soluble T cell-redirecting bsAbs (T-bsAbs), are revolutionizing the treatment of B cell malignancies (10).

CAR-Engineered T (CAR-T) Cells

CARs are synthetic receptors consisting of three domains: an antigen-binding ectodomain, the transmembrane domain, and the signaling endodomain (5). The ectodomain is usually a single-chain fragment variable (scFv) antibody, that allows the synthetic receptor to specifically recognize a user-defined cell surface TAA in an major histocompatibility complex (MHC)-independent manner, and is tethered to the transmembrane domain through the spacer or hinge region (8) (**Figure 1**). The third component is the endodomain, most often the CD3ζ intracellular signaling domain linked to one or more co-stimulatory domains (5, 11). First-generation CARs contain solely the intracellular signaling region of CD3ζ (12). Second-generation CARs generated by adding a co-stimulatory domain (from CD28 or CD137) in tandem with the CD3ζ chain (13) have been a major advance in CAR-T cell therapy because co-stimulation is a necessary component of physiological T cell activation, thereby improving proliferation, survival, cytokine secretion and cytotoxicity. Third-generation CARs further expanded on the second-generation by adding an additional co-stimulatory domain (14, 15).

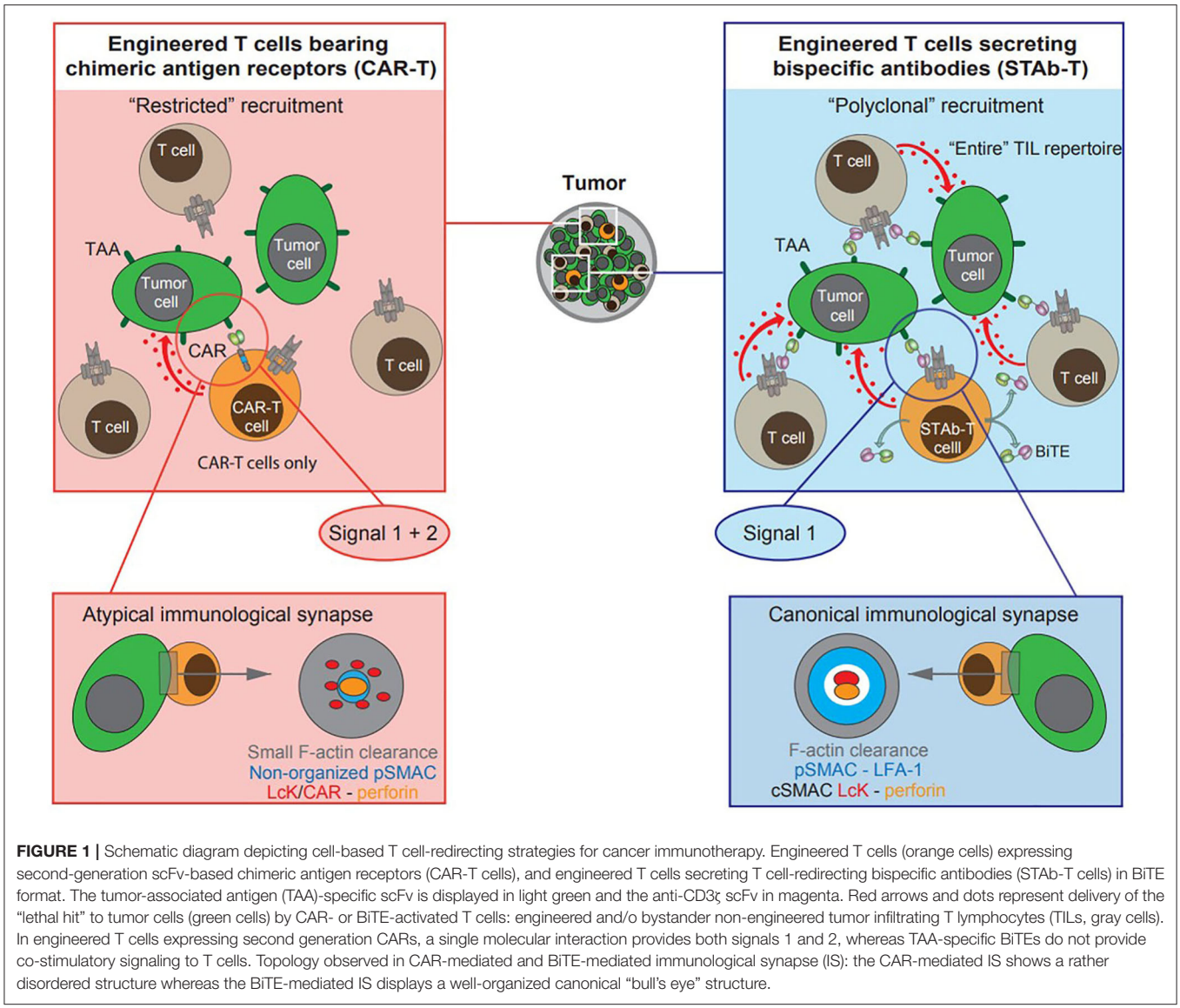

FIGURE 1 | Schematic diagram depicting cell-based T cell-redirecting strategies for cancer immunotherapy. Engineered T cells (orange cells) expressing second-generation scFv-based chimeric antigen receptors (CAR-T cells), and engineered T cells secreting T cell-redirecting bispecific antibodies (STAb-T cells) in BiTE format. The tumor-associated antigen (TAA)-specific scFv is displayed in light green and the anti-CD3ζ scFv in magenta. Red arrows and dots represent delivery of the "lethal hit" to tumor cells (green cells) by CAR- or BiTE-activated T cells: engineered and/o bystander non-engineered tumor infiltrating T lymphocytes (TILs, gray cells). In engineered T cells expressing second generation CARs, a single molecular interaction provides both signals 1 and 2, whereas TAA-specific BiTEs do not provide co-stimulatory signaling to T cells. Topology observed in CAR-mediated and BiTE-mediated immunological synapse (IS): the CAR-mediated IS shows a rather disordered structure whereas the BiTE-mediated IS displays a well-organized canonical "bull's eye" structure.

TABLE 1 | Pros and cons of current T cell-redirecting strategies.

	Adoptive cell therapies		Protein-based therapies
	CAR-T cells	STAb-T cells	Systemic administration T-bsAbs
Active trafficking to tumor sites	✓	✓	×
Co-stimulatory signal/s	✓	×[a]/✓[b]	×
Long lifespan	✓	✓	×[c]/✓[d]
"Off-the-shelf" therapy	×[e]/✓[f]	×[g]/✓[h]	✓
Polyclonal recruitment of T cells	×	✓	✓
Canonical immunological synapse	×	✓	✓

[a] *Monocystronic approach.*
[b] *Bicystronic approach.*
[c] *Bolus therapy with small Fc-less T-bsAbs (e.g., BiTE).*
[d] *Continuous intravenous infusion (CIV)/Half-extension technologies or Fc-engineered Ig "silent" T-bsAbs.*
[e] *Autologous CAR-T cells.*
[f] *"Universal" CAR-T cells.*
[g] *On-tumor strategy.*
[h] *Off-tumor strategy/"Universal" STAb-T cells.*

This structure endows CAR-T cells with several valuable attributes for a T cell-redirecting strategy. As CARs are not MHC-restricted, they can be used to treat patients without regard to MHC haplotypes, and circumvent MHC down-regulation, one of the most important mechanisms of immune evasion (11). In addition, CARs provide both activating and co-stimulatory signals which are required to achieve full T cell activation (**Figure 1** and **Table 1**) (16). The success of anti-CD19 CAR-T cells in clinical trials prompted the approval of two second generation CAR-T cells products, tisagenlecleucel (2017) and axicabtagene ciloleucel (2018), by the US FDA for the treatment of pediatric and young adult patients with relapsed or refractory B cell acute lymphoblastic leukemia (B-ALL) (17) and adult patients with relapsed or refractory large B cell lymphomas (18), respectively.

Nevertheless, the use of CAR-T cells presents some limitations (19), mainly severe toxicities related to a massive release of pro-inflammatory cytokines (cytokine release syndrome, CRS) and neurotoxicity (20). In addition, the majority of TAAs are also expressed on normal tissues, leading to on-target/off-tumor toxicity (19, 21). Solid tumors present additional challenges, due to the highly immunosuppressive tumor environment. Additionally, 30–60% of patients that achieve complete response, relapse after anti-CD19 CAR-T cell therapy (22).

Bispecific Antibodies

BsAbs are artificial molecules recognizing two different epitopes either on the same or on different antigens, and by simultaneously recognizing a cell surface TAA and an activating receptor on the T cell surface (CD3e), are able to activate and redirect T effector cells to kill cancer cells in a MHC-independent manner (5, 23). In recent years a considerable number of new bsAb formats have been designed, many of which are small-sized Fc-less molecules, built by connecting scFv and/or single-variable domain/heavy chain-only (V_{HH}) antibodies (23, 24). These antibodies are specifically designed to promote an efficient T cell/tumor cell synapse formation, and avoiding Fc-induced off-target toxicities (24). Among them, diabodies consist of two polypeptidic chains containing counterpaired V_H and V_L domains, connected by a short linker that prevents intramolecular pairings, resulting in the formation of dimeric molecules (55 kDa) (25). Tandem scFvs (ta-scFvs), consist of two scFvs connected by a flexible linker on a single polypeptide chain (57 kDa) (26). Those bispecific ta-scFv antibodies recognizing a tumor cell surface TAA and CD3e on T cells are so-called **bi**specific **T** cell-**e**ngagers (BiTEs) (26). The bispecific **li**ght **T**-cell **e**ngager (LiTE), consisting of a TAA-specific V_{HH} antibody fused to an anti-CD3scFv, is a recent evolution of this concept (27). The smaller size (43 kDa) and quicker diffusion of LiTE antibodies could allow them to reach tumor areas, which are inaccessible for larger bsAbs (27).

More than 30 T cell-redirecting bsAbs (T-bsAbs) have entered clinical development (28), but only one is presently in clinical use: blinatumomab, an anti-CD19 BiTE, for the treatment of relapsed/refractory B-ALL (29) and minimal residual disease-positive B-ALL (30). Despite the impressive responses observed with blinatumomab (31, 32), significant challenges still hamper the clinical application of BiTEs and similar bsAb formats. Off-target toxicities (mainly CRS and neurotoxicity), due to the expression of the targeted TAA on non-tumor cells, is a major concern for patients treated with systemically administered BiTEs (33). In addition, the short serum half-life of small-sized T-bsAbs requires continuous intravenous administration at a constant flow rate using infusion pumps (34). Another concern regarding the use of T-bsAbs is the lack of co-stimulatory signaling capacity. However, the ability of BiTEs to induce potent T cell cytotoxicity in the absence of co-stimulation has been well-documented (35). Although the reasons for this "co-stimulation independence" are not clear, it may result from the ability of Fc-less T-bsAbs to induce the formation of conventional mature immunological synapses (ISs) between T cells and tumor cells (36, 37).

NEXT-GENERATION T CELL-REDIRECTING STRATEGIES

As previously described, both CAR-T cells and systemically infused T-bsAbs have shown encouraging clinical responses but still must overcome important hurdles. In an attempt to combine the strengths of both therapies a novel strategy based on the endogenous **s**ecretion of **T**-bs**Ab**s (STAb) is being developed. We have previously classified STAb strategies as "*on-tumor*" and "*off-tumor*" depending on whether the T-bsAbs are secreted in the tumoral or peritumoral environment or from tumor-distant locations, respectively (10). The *in vivo* production of small-sized T-bsAbs by genetically modified T cells could result in effective and persistent concentrations of antibodies, compensating for their short-serum half-life (10). Moreover, this approach might

circumvent problems of tumor penetration and systemic toxicity, due to tumor trafficking of adoptively transferred T cells and subsequent intratumoral secretion of T-bsAbs (**Table 1**) (10). In addition, *in vivo* secretion avoids potential concerns regarding the formulation and long-term storage of bsAb therapeutics, preventing aggregation and deterioration (10, 38). Finally, in the STAb-T strategy, and in contrast with CAR-T therapy, T cell recruitment is not restricted to engineered T cells, as T-bsAbs secreted in the tumor may redirect bystander non-engineered infiltrating T cells to tumor cells, leading to a significant boost in anti-tumor T cell responses (**Figure 1** and **Table 1**) (4).

The STAb concept is now attracting attention but is not new. In 2003, a study demonstrated that human cells could be engineered to secrete a functionally active anti-CEA x anti-CD3 diabody, with ability to redirect T cell-mediated cytotoxicity against CEA-expressing tumor cells *in vitro*, and recruit bystander T cells *in vivo* to delay tumor growth (39). Moreover, anti-CEA x anti-CD3 diabody-secreting primary T cells were generated by lentiviral transduction and such STAb-T cells significantly reduced *in vivo* tumor growth in human colon cancer xenografts (40). More recently, the ability of an anti-EphA2 BiTE secreted by retrovirally transduced primary T cells demonstrated the ability of STAb-T cells to redirect the cytotoxic activity of non-transduced T cells specifically to $EphA2^+$ cancer cells *in vitro* and showed potent anti-tumor activity *in vivo* (41). Likewise, systemic infusion of retrovirally transduced T cells secreting an anti-CD19 BiTE induced tumor regression of leukemia and lymphoma in preclinical models (42). Another study reported that STAb-T cells secreting an anti-CD123 BiTE redirected bystander T cell cytotoxicity against $CD123^+$ acute myelod leukemia (AML) cells and induced regression of AML in xenograft models (43). Interestingly, efficient STAb-T cells have been generated not only using viral vectors, but also by RNA-transfection. In this regard, anti-CD19 STAb-T cells generated by electroporation of a messenger RNA encoding an anti-CD19 BiTE showed superior anti-tumor activities compared with RNA anti-CD19 CAR-T cells, achieving complete remission in a leukemia mouse model (44). It has been demonstrated that *in situ* secreted anti-CD19 BiTEs are loaded onto the T cell surface (42, 44). Therefore, it is tempting to speculate that the "arming of the CD3 complex" by *in vivo* secreted BiTEs in the peritumoral environment, could provide a significant therapeutic advantage over systemically administered BiTEs (e.g., blinatumomab).

Other cell types, such as mesenchymal stem cells (MSCs), and endothelial cells are suitable candidates to be engineered for "off-tumor" STAb strategies, based on the endogenous secretion of T-bsAbs from tumor-distant sites (45–47). The feasibility of *in vivo* secretion of T-bsAbs after systemic or local delivery of several types of nucleic acids or viruses has also been demonstrated (10, 48). Systemic administration of engineered mRNA (49) or minicircle DNA encoding T-bsAbs (50) induced sustained antibody secretion in mice and elimination of established human carcinoma xenografts. In another study, a single intramuscular injection of plasmid DNA induced secretion of functional T-bsAbs for 4 months and delayed cancer progression in mice (51). In addition, several types of oncolytic viruses have been armed with expression cassettes encoding T-bsAbs, to combine both direct oncolysis and T cell-mediated killing (52–55).

Nevertheless, T cells represent ideal vehicles for STAb therapy due to their capacity to migrate to tumor sites and their ability to act simultaneously as antibody factories and effectors (10). In addition, T-bsAb-mediated activation has been shown to induce an increase in transgene expression (41), which may favor the secretion of the T-bsAbs primarily at the tumor site and, consequently, reducing systemic toxicity.

OPEN QUESTIONS AND FUTURE PROSPECTS

Co-stimulatory and Co-inhibitory Receptors

Although only extensive research and clinical trials will determine the ultimate therapeutic potential of next-generation T cell-redirecting strategies, the STAb strategy may have important conceptual advantages over the CAR strategy (10), such as the polyclonal recruitment of the entire pool of tumor infiltrating T cells, and the reduction of systemic on-target/off-tumor toxicity due to the local secretion of the T-bsAbs (**Figure 1** and **Table 1**) (4, 5). In fact, Liu et al. have shown greater anti-leukemia activities of anti-CD19 BiTE-RNA electroporated T cells, compared to anti-CD19 CAR RNA-electroporated T cells in a Nalm6 tumor model (44). The authors highlighted the potential of anti-CD19 STAb-T cells to cure $CD19^+$ neoplasia with controlled toxicities (44). By contrast, Choi et al. have reported differences between CAR-T and STAb-T cells in terms of persistence and exhaustion, supporting the notion that CAR-T cells might be superior (56). In the experimental system used, T cell activation mediated by a locally secreted anti-EGFR BiTE resulted in a progeny of phenotypically exhausted cells, with reduced proliferative capacity and persistence, compared to anti-EGFRvIII CAR-activated T cells (56). The authors suggest that these differences may be attributable to the 4-1BB co-stimulatory domain used in the CAR construct (56), although the influence of other factors, such as the different TAA targeted, their cell density, as well as the location of the epitope recognized by both anti-EGFR and anti-EGFRvIII scFvs has not been considered. The positive effects of 4-1BB-mediated co-stimulation on reducing T cell exhaustion have also been demonstrated on engineered T cells expressing a second-generation anti-CD19 CAR (BBζ) (57).

STAb-T cells have demonstrated significant anti-tumor activity in different preclinical models, without additional co-stimulation (40–42, 44, 58). However, the provision of co-stimulatory signals may be instrumental to enhance anti-tumor efficacy especially in the context of solid tumors. In fact, we have demonstrated that simultaneous secretion of an anti-CEA x anti-CD3 diabody and a tumor-specific co-stimulatory ligand comprising the extracellular portion of CD80 fused to an anti-CEA antibody (59) increased anti-tumor activity in human colon carcinoma xenografts (4). Recent studies have shown that the expression of 4-1BB and CD80 ligands on the surface of engineered T cells secreting and anti-CD19 BiTE significantly increased the antileukemia activity *in vivo* (60). Collectively, these studies showed that STAb-T cells could be easily equipped

with physiological or tumor-specific co-stimulation systems using cell surface or antibody engineering strategies.

On the other hand, blockade of the PD-1/PD-L1 interaction can induce durable anti-tumor responses in a wide range of solid and hematological tumors (61). Several blocking antibodies against PD-1/PD-L1 have been approved for clinical use in humans (6), and preclinical studies have demonstrated that combining PD-1/PD-L1 axis blockade with CAR-T cells or systemically administered T-bsAb can improve anti-tumor activity (62–64). Importantly, several studies have demonstrated the therapeutic potential of engineered CAR-T cells secreting either anti-PD-1 or anti-PD-L1 blocking antibodies, and are currently being evaluated in clinical trials (65). In addition, CAR-T cells that express PD-1 dominant-negative receptors (66) or chimeric PD-1:CD28 switch-receptors (67) have been reported to increase anti-tumor effects and reduce susceptibility to tumor-induced T cell dysfunction. Finally, the rapid advancements in precision genome editing techniques, such as CRISPR-Cas9 system, has enabled to disrupt PD-1 function in CAR-T cells/T cells for cancer therapy (68, 69). All these "protective strategies" could also be easily implemented in a STAb-T cell context to improve their therapeutic potential.

Tumor Antigen Escape

Another relevant issue in a tumor-specific T cell-redirecting context is the loss of the targeted TAA. Here, it is important to highlight that among relapsing patients treated with anti-CD19 CAR-T cells, 10–20% are CD19-negative (22), while CD19 loss is infrequent following blinatumomab therapy (7). Several mechanisms have been proposed to explain antigen loss, such as accumulation of genetic and epigenetic mutations during tumor progression and selection of antigen-negative variants due to immune pressure (22). Interestingly, trogocytosis, a process whereby lymphocytes capture fragments of the plasma membrane from antigen-presenting cells and express them on their own surface (70), has been reported to occur following CAR-T cell interaction with CD19 (71). Trogocytosis leads to reversible antigen loss that reduces both TAA density on tumor cells and CAR expression on the T cell surface, presumably as a consequence of CAR internalization. Moreover, the transfer of CD19 protein from leukemia cells to T cells promotes fratricidal T cell killing and T cell exhaustion (71). Trogocytic target acquisition seems to be a general feature of CAR-T cells, as this phenomenon has been observed with CARs targeting different antigens (71). Regarding BiTE-stimulated T cells, trogocytic mechanisms have not been reported so far, although additional studies are needed to further clarify this issue.

Immunological Synapse

An important unresolved issue refers to structure of the IS formed by the CCB molecules in T cell redirecting strategies (**Figure 1**) (36). Although CAR-T cell stimulation induces an efficient microtubule organizing center and lytic granule secretion, even faster than in the canonical TCR-initiated IS, the actin cytoskeleton is not completely depleted from the center of the synapse, that exhibited a disorganized multifocal signaling cluster structure, with major differences relative to the typical TCR-initiated IS (36, 72–74). Unlike CARs, small-sized T-bsAbs are able to induce the formation of a canonical "bull's eye" IS between T lymphocytes and tumor cells (35). Indeed, BiTE-initiated IS has been found to be identical in structure and molecular composition to TCR-induced IS (37). Further studies are needed to more precisely define the impact of the topology of the IS on the functional capacity and cytotoxic potential of CAR-T and STAb-T cells (36).

Development of Off-the-Shelf Universal Adoptive Cell Therapies

The use of allogeneic cells from healthy donors has significant advantages over autologous approaches, such as the immediate availability of cryopreserved batches and reduced cost. We have demonstrated that engineered MSCs might be incorporated into biocompatible scaffolds to secrete T-bsAbs that can act distantly at the tumor site, and can be retrieved a after a given period of time when the intended therapeutic effect has been achieved (45). Therefore, off-the-shelf stocks of gene-modified human allogeneic STAb MSCs might be easily generated and microencapsulated and implanted subcutaneously according to clinical need (75). The development of universal allogeneic CAR-T cells is an active area of research, and different strategies are being investigated to reduce the risk of graft-vs.-host disease and make cells less visible to the host immune system (76). Similar approaches could easily be implemented to the generation of universal STAb-T cells.

FINAL CONSIDERATIONS

STAb-T cell-based strategies have demonstrated encouraging anti-tumor effects in preclinical models, but their safety needs to be further explored in controlled clinical trials. Nevertheless, the administration of CAR-T or STAb-T therapies may not necessarily be mutually exclusive and both approaches might be used sequentially or simultaneously (56). Moreover, the use of CAR-T cells and STAb-T cells targeting different TAA could be relevant to overcome antigen loss, in a fashion similar to dual-antigen CAR-T cell targeting strategies (77–80). Such a strategy might consist of the simultaneous administration of CAR and STAb-T cells or the generation of a single cell product expressing both CCBs.

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BB and LA-V contributed to the conception and design of this review. BB and ÁR-F wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Highly Efficient Generation of Transgenically Augmented CAR NK Cells Overexpressing CXCR4

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Natural killer (NK) cells are a noteworthy lymphocyte subset in cancer adoptive cell therapy. NK cells initiate innate immune responses against infections and malignancies with natural cytotoxicity, which is independent of foreign antigen recognition. Based on these substantive features, genetically modifying NK cells is among the prime goals in immunotherapy but is currently difficult to achieve. Recently, we reported a fully human CAR19 construct (huCAR19) with remarkable function in gene-modified T-cells. Here, we show efficient and stable gene delivery of huCAR19 to primary human NK cells using lentiviral vectors with transduction efficiencies comparable to those achieved with NK cell lines. These huCAR19 NK cells display specific and potent cytotoxic activity against target cells. To improve homing of NK cells to the bone marrow, we augmented huCAR19 NK cells with the human CXCR4 gene, resulting in transgenically augmented CAR NK cells (TRACKs). Compared to conventional CAR NK cells, TRACKs exhibit enhanced migration capacity in response to recombinant SDF-1 or bone marrow stromal cells while retaining functional and cytolytic activity against target cells. Based on these promising findings, TRACKs may become a novel candidate for immunotherapeutic strategies in clinical applications.

Keywords: natural killer cell, chimeric antigen receptor, immunotherapy, fully human CAR, CD19, huCAR19, transgenically augmented CAR NK cell, chemokine receptor 4

INTRODUCTION

Cancer immunotherapy using CD19-specific chimeric antigen receptor (CAR) modified T-cells (CAR T-cells) has shown remarkable clinical benefit in patients with relapsed or refractory B-cell malignancies. The first CAR T-cell products, tisagenlecleucel (Kymriah, Novartis) and axicabtagene ciloleucel (Yescarta, Gilead), were approved in the United States in 2017 and Europe in 2018. However, so far, this success has not been translated to other hematological indications, such as

acute myeloid leukemia (AML) or even solid tumors. In addition, CAR T-cell therapies can be associated with severe, sometimes even life-threatening adverse events, such as cytokine release syndrome (CRS), neurotoxicity, B-cell aplasia, and/or graft-versus-host disease (GvHD) (1).

Another immune cell type that can recognize and destroy cancer cells is natural killer (NK) cells. Matching their name, this small subset of lymphocytes initiates the innate immune response against infections and malignancies with its natural cytotoxicity behavior in hand. NK cells rely on germline encoded inhibitory and activating receptors sensing a misbalance of the corresponding ligands on the target cell surface and are, therefore, independent of foreign antigen recognition. In recent years, NK cell–based immunotherapy approaches have been developed for the treatment of various malignancies in both autologous and allogeneic settings. The transfer of *ex vivo* expanded autologous and allogeneic NK cells has been found to be safe and well tolerated in a range of clinical trials with no signs of GvHD, CRS, or neurotoxicity, but the effect on tumor suppression appears to be low for autologous NK cell infusions or highly dependent on the type of cancer for allogeneic NK cell infusions (2).

Similar to T-cells, NK cells can be genetically modified with CAR genes to improve their antitumor potential. CAR expression on the surface of NK cells mimics an activating receptor providing a strong activation signal upon contact with its respective ligand on the tumor cell, resulting in CAR NK cell activation and target cell lysis. Considering the beneficial safety profile of NK cells and the possibility to use them as an "off-the-shelf" product in an allogeneic setting make CAR NK cells an attractive alternative to CAR T-cells. However, compared to T-cells, NK cells are harder to modify with viral vectors due to their intrinsic antiviral defense mechanisms (3). Transduction efficiencies of NK cells vary tremendously depending on the cell source, the viral vector system, and the transduction enhancer used (4). Lentiviral vectors (LVs) pseudotyped with the glycoprotein of the vesicular stomatitis virus (VSV-G) are classically used to generate CAR T-cells but are less efficient for NK cells with transduction efficiencies of 20–40% (5, 6). Therefore, optimization of gene transfer protocols for VSV-G pseudotyped LV for the generation of CAR NK cells is urgently required.

Today, fewer than 15 clinical trials using CAR NK cells are performed worldwide, which is far below the amount of ongoing CAR T-cell trials (>200) (1, 7). Similar to CAR T-cell trials, most CAR NK cell trials use a CD19-specific CAR molecule derived from the murine antibody FMC63 (8, 9). Due to the potential of murine-derived targeting domains causing anaphylaxis, more and more CAR T-cell studies are investigating humanized or fully human CAR constructs (1). In this respect, recently, fully human CD19-specific and fully human mesothelin-specific CAR constructs were reported showing efficient expression and strong antitumor activity in T-cells (10–13). Because murine-derived CAR molecules possess the same risk of causing anaphylaxis in engineered CAR NK cells, CAR NK cell approaches should evaluate the feasibility of humanized or fully human CAR constructs as well.

Persistent tumor cells in the bone marrow are a major cause of cancer relapse in several indications including AML (14–16). Trafficking of NK cells to and from the bone marrow essentially depends on chemotaxis. NK cells basically complete their maturation steps in the bone marrow, are retained there, or egress to and emerge in the blood circulation (17). Retention of NK cells in the bone marrow is primarily driven by the interaction of the CXCR4 chemokine receptor expressed on NK cells and its ligands SIP5 (sphingosine-1 phosphate receptor 5) and CXCL12, also known as SDF-1 (stromal cell–derived factor 1), abundantly represented by bone marrow stromal cells. During NK cell maturation, CXCR4 expression decreases, promoting NK cell release from the bone marrow (18). It has been shown that NK cell chemotaxis to the bone marrow is induced by SDF-1 and can be prevented utilizing AMD3100, a CXCR4 antagonist (Plerixafor), resulting in an increase of NK cells in the spleen and other peripheral organs (17, 18). In this respect, it was recently demonstrated that the expression of CXCR4 in NK cells can improve their homing to the bone marrow (19). Combined expression of CAR and CXCR4 might, therefore, enable enhanced chemotaxis of CAR NK cells to the bone marrow allowing more efficient targeting of persistent tumor cells in the tumor niche environment.

To our knowledge, we report here for the first time the highly efficient transduction of primary NK cells with VSV-G pseudotyped LVs delivering a fully human, CD19-specific CAR construct (huCAR19). Generated huCAR19 NK cells are shown to exhibit strong and specific antitumor activity. To augment homing to the bone marrow, NK cells were modified to express human CXCR4 in addition to the huCAR19 construct. These *transgenically augmented CAR NK cells* (TRACKs) are proven to overexpress CXCR4, which leads to enhanced chemotaxis toward SDF-1 and bone marrow stromal cells without any loss in their CD19-specific cytotoxic activity.

MATERIALS AND METHODS

Ethics Statement

This research was approved by the Ethics Committee of the University Hospital Frankfurt, Germany. Written informed consent was obtained from each donor.

Cell Lines and Primary Cells

HEK-293T (ATCC CRL-11268) and HT1080 (ATCC CCL-121) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biowest, Nuaillé, France) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany) and 2 mM L-glutamine (Sigma-Aldrich, Munich, Germany). Nalm-6 [peripheral blood derived B cell precursor leukemia cells (ALL), $CD19^+$], JeKo [peripheral blood derived B cell lymphoma cells (non-Hodgkins lymphoma), $CD19^+$], CD19-negative JeKo ($CD19^{neg}$JeKo), SUP-B15 [bone marrow derived B cell precursor leukemia cells (ALL), $CD19^+$], and BV-173 [peripheral blood derived B cell precursor leukemia cells (chronic myeloid leukemia), $CD19^+$]

cell lines were grown in RPMI 1640 (Biowest, Nuaillé, France) supplemented with 10% FCS and 2 mM L-glutamine. NK92, a malignant non-Hodgkins lymphoma cell line (ATCC® CRL-2407), and NKL, a large granulocyte leukemia cell line (20), were cultivated in X-Vivo 10 medium (Lonza, Belgium) supplemented with 5% human serum (Sigma, United States) and 100 IU/ml of IL-2 (Miltenyi Biotec, Bergisch-Gladbach, Germany). All cell lines were cultivated at 37°C, 5% CO_2, and 95% humidity for up to 1 month. Cells were split every 2 to 3 days and did not undergo more than 20 passages. Regular testing for Mycoplasma was performed for all cell lines using a PCR Mycoplasma Test Kit (PanReacApplichem, Germany).

Human PBMCs were isolated from the fresh blood of healthy anonymous donors or buffy coats purchased from the German blood donation center (DRK-Blutspendedienst Hessen, Frankfurt, Germany). Primary NK cells were purified by negative selection using the NK cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). After NK cell isolation, 2×10^6 cells were cultivated per well in a 24-well plate in 400 µl of X-Vivo 10 (Lonza, Belgium) medium supplemented with 5% human serum (Sigma, United States) and 100 IU/ml of IL-2 (Miltenyi Biotec, Bergisch-Gladbach, Germany) for at least 72 h at 37°C. The purity of NK cells after isolation was determined by flow cytometry (**Supplementary Figures S1B,C**).

Transgene Constructs

The fully human anti-CD19-CAR construct (kindly provided by Dr. Brian G. Till, Fred Hutchinson Cancer Research Center, Seattle, WA, United States) was previously described (11). In brief, the huCAR19 construct consists of the encoding sequences of the VL and VH region of the human 21 Da antibody linked by a 16 amino acid peptides followed by a hinge domain derived from human CD8a, the transmembrane and costimulatory domain of human 4-1BB and the signaling domain of CD3ζ. For the generation of the huCAR19.CXCR4 construct, the human CXCR4 coding sequence was cloned downstream to the huCAR19 sequence linked via self-cleaving peptide P2A. Both constructs reside in the clinical grade lentiviral expression vector SINpWPT (21) and are under the control of a human elongation factor-1 alpha (EF1α) promoter.

Lentiviral Vector Generation, Concentration, and Titration

Vector particles were generated by transient transfection of HEK-293T cells using polyethylenimine (PEI; Sigma-Aldrich, Munich, Germany) and third-generation packaging plasmids as described before (22–24). In brief, 1 day before transfection, 1.5-2×10^7 cells were seeded into a T175 flask. In total, 35 µg DNA was added to 2.3 ml of DMEM without additives and combined with 2.2 ml DMEM containing 140 µl of 18 mM PEI solution. The transfection solution was mixed and incubated for 20 min at room temperature. The culture medium was exchanged to 10 ml DMEM supplemented with 15% FCS and 2 mM L-glutamine before the transfection solution was added to HEK-293T cells. 4–6 h later, the medium was replaced by DMEM supplemented with 10% FCS and 2 mM L-glutamine. 2 days after transfection, the cell culture supernatant was collected, filtered via a 0.45 µm filter, and released vector particles were concentrated by a 20% sucrose cushion at 4500 × *g* for 24 h. The supernatant was discarded, and pellets were resuspended in 60 µl Dulbecco's Phosphate Buffered Saline (PBS; Lonza, Cologne, Germany) per T175 flask. A third-generation lentiviral packaging system was used to produce CAR-carrying lentiviral particles. Plasmid ratios for the generation of lentiviral particles were described previously (22) and can be found in the **Supplementary Table S1**.

All produced vector particles were titrated by transducing HT1080 cells with five serial dilutions of vector particles. The expression of huCAR19 transgene was detected 4 days post-transduction by flow cytometry. Titers were calculated as described before (25). Particle numbers were determined using Nano sight NS300 (Malvern Ltd., United Kingdom).

NK Cell Transduction

For transduction of NK cell lines, 3×10^4 of NK-92 or NKL cells were seeded into a single well of a 96-well flat-bottom plate in 100 µl X-Vivio10 medium supplemented with 5% human serum and 100 IU/ml IL-2 (complete X-Vivo medium). The transduction enhancer polybrene (Sigma) was added to the cells at a concentration of 8 µg/ml before adding 100 µl complete X-Vivo 10 medium containing 2 µl of concentrated vector stock.

For transduction of primary NK cells, 4×10^4 purified NK cells, if not otherwise specified, were seeded into a single well of a 96-well flat-bottom plate in 100 µl complete X-Vivo 10 medium 72 h post NK cell isolation. For each transduction experiment, 2 µl of concentrated vector stock was used. The transduction enhancer polybrene (Sigma-Aldrich, Germany) was utilized at a concentration of 8 µg/ml and added directly to the cells prior to transduction. Vectofusin-1 (Miltenyi Biotec, Germany) was used according to the manufacturer's instructions and, as described before (26), at a final concentration of 10 µg/ml. In brief, Vectofusin-1 and vector particles were diluted in X-Vivo10 medium without additives in a total volume of 50 µl before both solutions were mixed, incubated for 5–10 min at room temperature, and finally added to the NK cells. Retronectin (Takara, United States) was precoated in a 96-well flat-bottom plate at a concentration of 20 µg/cm^2 and incubated overnight at 37°C 1 day before transduction. Afterward, Retronectin solution was removed, the plate was blocked with 2% BSA in PBS for 30 min and washed with PBS before seeding NK cells and adding vector particles. If not otherwise specified, polybrene was used as a transduction enhancer.

To increase transduction efficiency, spinfection was performed at 850 × *g* at 32°C for 90 min. Afterward, NK cells were gently resuspended by pipetting. Fresh complete medium was added every other day after transduction until cell analysis. Transgene expression was determined by flow cytometry 3 days post-transduction. The functional capability of transduced cells, including migration assay, cytotoxic activity, cytokine production, and degranulation, was analyzed 4 days after transduction. An overview of the experimental setup is provided in **Supplementary Figure S1A**.

Cytotoxicity Assay

Cytotoxic activity of huCAR19-LV or huCAR19.CXC4-LV transduced NK cells was determined using $CD19^+$ Nalm-6 cells or other tumor cell lines. CAR expression was detected 72 h post-transduction by flow cytometry, and cytotoxic activity of NK cells was evaluated on the following day. 5×10^4, 2.5×10^4, or 1×10^4 CAR-positive NK cells or untransduced NK cells were cocultured with 1×10^4 Nalm-6 cells that were previously labeled with CellTrace™ CFSE (Invitrogen, United States) according to the manufacturer's instructions. To compensate for variations of transduction efficiency, the effector cell population was normalized to an absolute NK cell number by the addition of untransduced NK cells. Nalm-6 cells without effector cells were used as control. Coculture was performed for 4 h at 37°C and 5% CO_2 in a total volume of 200 µl RPMI medium supplemented with 10% FCS and 2 mM L-glutamine. Afterward, the cell mixture was stained for dead cells using the fixable viability dye eFluor 780 (Thermo Fisher Scientific, United States) according to the manufacturer's instructions and analyzed by flow cytometry. The percentage of dead target cells was analyzed as the CFSE positive, viability dye positive cell population.

Degranulation and IFNγ Secretion Assay

Four days after transduction, 2×10^4 CAR NK cells were seeded in to a single well of 96-well V-bottom plates together with 2×10^4 Nalm-6 cells in a total volume of 200 µl RPMI medium supplemented with 10% FCS and 2 mM L-glutamine and incubated at 37°C and 5% CO_2. Untransduced NK cells were used as a control. The anti-CD107a antibody (Biolegend, United States) was added directly after seeding the cells, and a mixture of protein transport inhibitors containing brefeldin A (BD Biosciences, United States), and monensim (BD Biosciences) were added after 1 h of coculture according to the manufacturer's instruction, and cells were incubated for 3 additional hours. At the end of the coculture, supernatant and cells were separately collected. To determine CD107a expression, NK cells were stained for CD3, CD14, CD19, and CD56 markers as well as for dead cells using fixable viability dye eFluor 506. NK cell degranulation was determined by analyzing CD107a expression as an NK cell degranulation marker on live, $CD3^-$, $CD14^-$, $CD19^-$, and $CD56^+$ cells by flow cytometry. Collected supernatants were stored at −80°C and used to detect secreted IFNγ by a Duoset ELISA kit (R&D systems, United States) according to the manufacturer's protocol.

Migration Assay

A migration assay utilizing polycarbonate transwell plates with a 6.5 mm diameter and 5 µm pore size (Corning Costar, United States) was used to assess the migration potential of NK cells. 4 days after transduction, 1×10^5 NK cells were loaded in the upper chamber in a total volume of 200 µl X-Vivo10 medium supplemented with 5% human serum and appropriate cytokines. Untransduced NK cells cultured in parallel were used as control. Next, 100 ng/ml of SDF-1 (CXCL12; SinoBiological, United States) diluted in 200 µl X-Vivo10 medium supplemented with 5% human serum (Sigma, United States) and 100 IU/ml of IL-2 were added in the lower well. After 2 h of incubation at 37°C and 5% CO_2, cells and medium of the lower chamber were harvested, and the number of migrated cells was quantified by flow cytometry analysis. To inhibit chemotaxis, 25 µg/ml AMD3100 (CXCL12 antagonist; Sigma Aldrich, Germany) was added to NK cells for 2 h at 37°C prior to the experiment onset.

To evaluate the cytotoxic functionality of migrated cells, a coculture assay of harvested NK cells with $CD19^+$ Nalm-6 cells was performed. For this purpose, migrated cells were collected, counted, and cocultured with Nalm-6 cells at a 1:1 ratio for 4 h at 37°C and 5% CO_2, followed by washing and staining for viability and CD19. The cytotoxic capacity of migrated NK cells was determined by the decrease in the percentage of living CD19 positive cells analyzed by flow cytometry.

Furthermore, to simulate the migration of NK cells toward the bone marrow niche, a migration assay with primary culture-expanded bone marrow–derived mesenchymal stromal cells (BMSCs) was performed. BMSCs were generated from pooled bone marrow mononuclear cells and expanded to the end of passage 3 as described (27). After thawing, cells were cultivated for 5 days in basal medium containing 10% platelet lysate. 1 day before starting the migration assay, 0.5×10^5 BMSCs were seeded in the lower chamber of a transwell plate. On the next day, the medium was exchanged to 200 µl X-Vivo10 medium without any supplement. Transduced and untransduced NK cells were labeled with CellTrace™ CFSE (Invitrogen, United States) according to the manufacturer's instructions, and 1×10^5 CSFE-labeled NK cells with or without AMD3100 treatment were loaded into the upper chambers of the transwell. After 2 h of incubation, microscopic pictures from the lower chamber were acquired (Zeiss AxioVert 200 Inverted Fluorescent Microscope, Germany; power x10 magnification), and the number of migrated cells was quantified by flow cytometry analysis.

Flow Cytometry

Flow cytometry analysis was performed using the MACSQuant® Analyzer 10 (Miltenyi Biotec, Bergisch-Gladbach, Germany), and data were analyzed by FCS Express 6 (*de novo* Software, Glendale, CA, United States). To determine the purity of NK cell purification on the day of isolation, 72 h after culture, and during the final analysis, cells were stained with fixable viability dye eFluor506 (e.bioscience), anti-CD3 (clone BW264/56), anti-CD14 (clone TÜK4), and anti-CD19 (clone LT19) antibodies all conjugated with VioGreen as well as APC-labeled anti-CD56 antibody (all Miltenyi Biotec, Germany). Viable cells that were $CD3^-$, $CD14^-$, and CD19 negative cells but positive for CD56 were considered to be NK cells according to the gating strategy in **Supplementary Figure S1B**. CXCR4 was detected using anti-CD184 (clone 12G5; BD Horizon, United States) conjugated to BV421. CAR expression was determined by cell labeling with biotinylated human recombinant CD19 protein (CD19-Fc, Acro Biosystemms, United States) followed by secondary labeling with PE-conjugated Streptavidin (Miltenyi Biotec, Germany). FITC conjugated anti-CD107a (LAMP-1) antibody (Biolegend, United States) was used to evaluate degranulation on NK cells after coculture with target cells. After staining, cells were washed

twice with washing buffer (PBS, 2% FCS, 0.1% NaN_3) and fixed with PBS containing 1% formaldehyde prior to analysis.

Statistical Analysis

Statistical analyses were performed with Prism 7 software (GraphPad, San Diego, CA, United States). Statistical significance was determined by applying the unpaired two-tailed Student's *t* test as indicated; *p* values less than 0.05 were considered significant.

RESULTS

Efficient huCAR19 Gene Delivery to Primary NK Cells

A fully human, second-generation CD19-specific CAR construct (huCAR19) as well as the huCAR19 construct linked to the human CXCR4 sequence via a P2A site (huCAR19.CXCR4) were used to produce CAR NK cells by lentiviral transduction. The huCAR19 gene cassette contains the scFv regions derived from the human 21D antibody as a binding domain followed by a CD8a hinge domain, the transmembrane, and the costimulatory domain from 4-1BB as well as the intracellular signaling domain of CD3ζ (**Figure 1A**). LVs encoding both CAR transgenes were produced in several batches. Characteristics of produced LV batches, including titer, particle count, and average size as well as corresponding multiplicity of infection (MOI), are listed in **Supplementary Table S2**.

First, CAR gene delivery to the NK cell lines NKL and NK-92 was analyzed by applying polybrene as a transduction enhancer, revealing very efficient gene transfer (**Figures 1B,C**). 3 days after transduction, nearly 100% of the NKL and NK-92 cells were transduced by huCAR19-LV. Next, generated huCAR19-LV particles were applied to transduce purified, primary NK cells in parallel to NKL and NK-92 cells in the presence of polybrene. Surprisingly, the transduction of primary NK cells was as efficient as those of NK cell lines determined by CD19-CAR detection 3 days after vector treatment (**Figure 1C**). On average 94.4% of NKL, 99.1% of NK-92, and 96.2% of primary NK cells were transduced. An overview of the experimental timeline and gating strategy for primary NK cells is provided in **Supplementary Figure S1**.

To investigate the effect of different transduction enhancers on gene delivery to primary NK cells, transduction of NK cells was performed in the presence or absence of polybrene, Vectofusin-1, and Retronectin. The most pronounced gene transfer was achieved in the presence of polybrene (nearly 90%) compared to Vectofusin-1 with 70.1% and Retronectin with 30.4% (**Figure 1D**). Samples without any enhancer showed a moderate transduction rate (5.4%). Therefore, all further experiments were performed using polybrene as an enhancer.

In the next step, we investigated whether adding the human CXCR4 gene sequence to the huCAR19 construct has any adverse effect on huCAR19 expression. Nearly equivalent transduction rates were achieved for both CAR constructs with a mean transduction efficiency of 87.11% for huCAR19-LV and 85.25% for huCAR19.CXCR4-LV (**Figures 1E,F**). Prolonged cultivation up to 2 weeks post transduction revealed that the CAR constructs were stably expressed on primary NK cells as determined by flow cytometry (**Supplementary Figure S2A**). These results demonstrate the feasibility of highly efficient CAR gene transfer to primary NK cells by applying LV vectors.

CXCR4 Expression Can Be Increased on Primary NK Cells Using huCAR19.CXCR4-LV

To enhance the migration capability of CAR NK cells toward tumor cells residing in the bone marrow, we generated huCAR19.CXCR4-LV particles delivering CXCR4 in addition to the CAR construct. To evaluate the ability of the vector to induce CXCR4 overexpression, primary NK cells were isolated from healthy donors and cultivated with IL-2 for 2 days before transduction with huCAR19.CXCR4-LV or huCAR19-LV particles. Prior to transduction, highly purified primary NK cells were less than 17% CXCR4 positive (**Supplementary Figure S1C**), and 72 h post-transduction, significant CXCR4 expression was detected on NK cells treated with LV particles harboring the huCAR19.CXCR4 construct (on average, 81.5% in the $CD56^+$ cell population), and co-expression of both inserts was observed (**Figure 2**). On average, more than 84% of the cells co-expressed CAR and CXCR4 upon huCAR19.CXCR4 gene delivery (**Figure 2B**). In contrast, CXCR4 was only expressed on 25.7% of $CD56^+$ or 27.1% of $huCAR19^+$ NK cells treated with huCAR19-LV particles (**Figure 2**). Notably, the level of CXCR4 on NK cells transduced with the huCAR19 construct alone was not significantly enhanced and was similar to the levels of untransduced NK cells cultivated in parallel (**Figure 2A**). Expression of CXCR4 was stable for at least 2 weeks post-transduction as determined by flow cytometry (**Supplementary Figures S2B,C**). Notably, cell viability, proliferation capacity, and phenotype of the generated CAR NK cells were comparable to untransduced primary NK cells cultured in parallel, demonstrating that the transduction process or transgene expression did not negatively affect NK cell cultivation (**Supplementary Figure S3**).

Functional Evaluation of huCAR19 Modified NK Cells

In the next step, the specific cytolytic activity of huCAR19 NK cells augmented with or without CXCR4 was tested. For this purpose, CFSE-labeled $CD19^+$ Nalm-6 cells were cocultured with the genetically modified NK cells at a different effector to target ratios for 4 h. In all experiments, at least 63% of the NK cells were CAR positive (**Supplementary Figure S4A**). Both types of huCAR19 NK cells efficiently killed the tumor cells in all applied ratios (**Figure 3A**). Already at an effector-to-target ratio of 2.5 to 1, nearly 80% of all tumor cells were lysed by the genetically modified NK cells. In contrast, untransduced NK cells showed only moderate cytotoxic activity against the $CD19^+$ Nalm-6 cells (less than 20% dead cells for all tested effector-to-target ratios; **Figure 3A**). To further demonstrate the specificity of the huCAR19 NK cells, CD19 positive (SUP-B15, BV-173, and JeKo) and negative ($CD19^{neg}$JeKo) tumor cell lines were

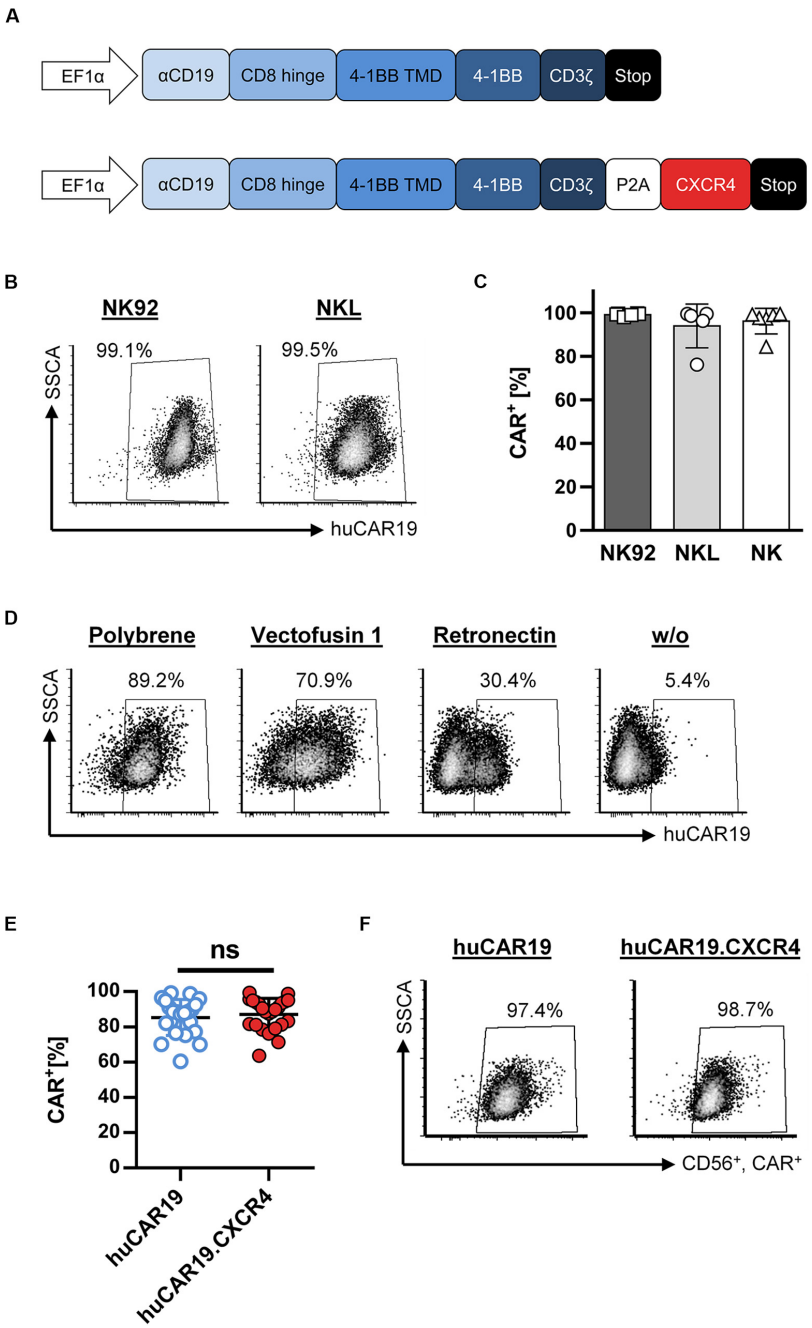

FIGURE 1 | Fully human CAR gene delivery to NK cells. **(A)** Schematic illustration of huCAR19 (top) and huCAR19.CXCR4 (bottom) constructs used to generate CAR NK cells. The second-generation fully human CAR consists of the anti-CD19 scFv derived from the human monoclonal antibody 21 Da (αCD19) linked to the hinge domain of human CD8a (CD8 Hinge), the transmembrane and costimulatory domains of human 4-1BB (4-1BB TMD and 4-1BB), and the signaling domain of CD3ζ. The coding sequence of human CXCR4 is connected to the CAR gene by a P2A self-cleaving peptide. Transgene expression is under control of the human elongation factor 1-α promoter (EFl-α). **(B,C)** NK-92 and NKL cells as well as primary NK cells were transduced with huCAR19-LV particles in the presence of polybrene and analyzed for CAR expression 3 days post-transduction by flow cytometry. The percentage of CD19-CAR-positive cells is indicated. Each transduction experiment was performed at least five times. Representative dot plots for NK-92 and NKL-transduced cells are shown in B. Individual results of transduced NK-92, NKL, and primary NK cells are shown in C as a bar diagram with mean value and SD. **(D)** Effect of different transduction enhancers on huCAR19 gene delivery to primary NK cells; 3×10^4 primary NK cells were transduced with huCAR19-LV particles in the presence or absence of polybrene, Vectofusin-1, and Retronectin. 3 days post-transduction, huCAR19 transgene expression was detected by flow cytometry. Representative dot plots are shown ($n = 2$, with technical replicates). The percentage of huCAR19-positive NK cells is indicated. **(E,F)** Addition of human CXCR4 sequence to the huCAR19 transgene did not impact huCAR19 expression. Primary NK cells of six different donors were transduced with huCAR19-LV or huCAR19.CXCR4-LV particles in the presence of polybrene. The percentage of CD19-CAR-positive cells was analyzed by flow cytometry 3 days post-transduction. Each transduction experiment was performed at least 10 times. Three to four individually produced vector stocks were applied. Individual results as well as mean value, SD, and significance are shown in E. Representative dot plots are shown in F. ns, not significant by unpaired *t* test ($p = 0.5245$).

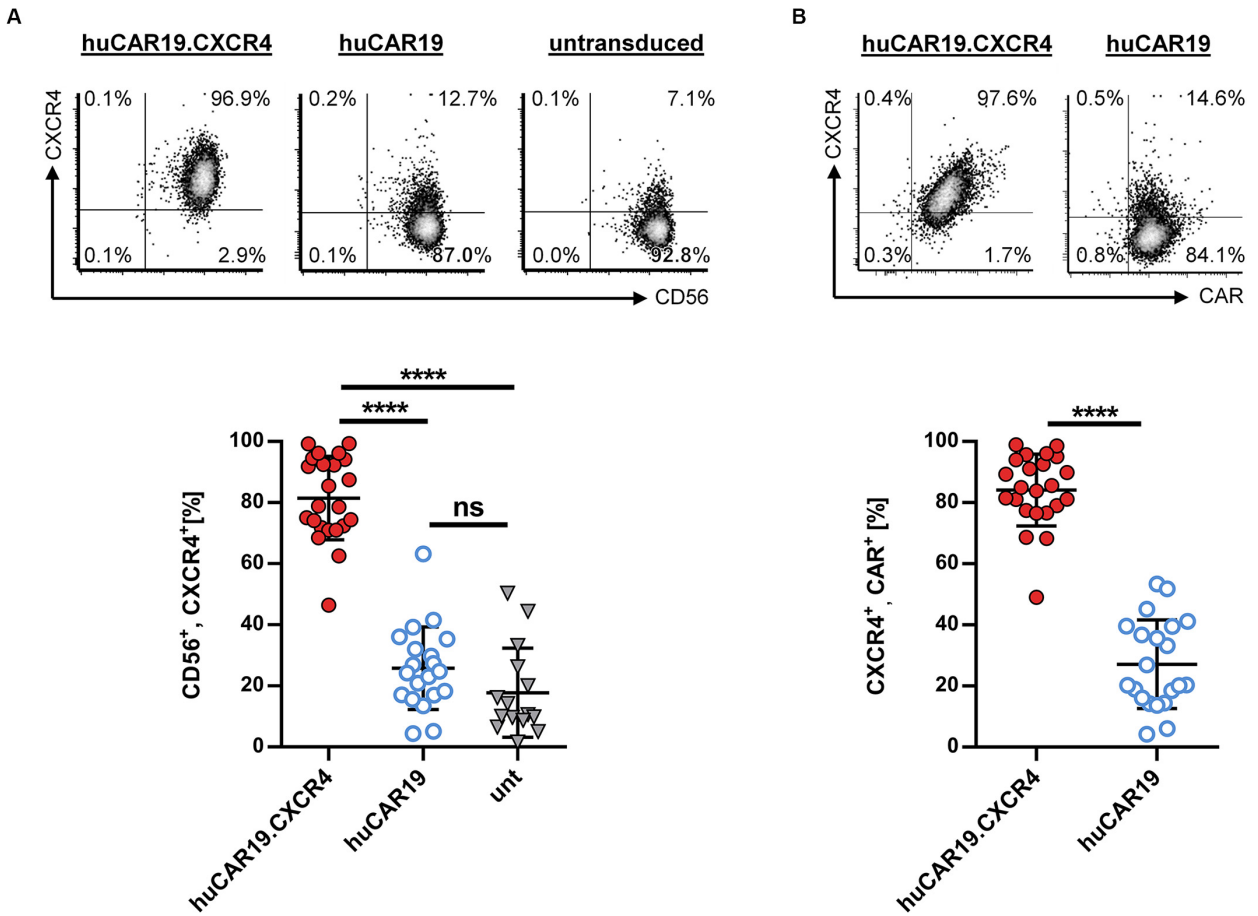

FIGURE 2 | CXCR4 overexpression on NK cells. Primary NK cells were transduced with huCAR19.CXCR4-LV or huCAR19-LV particles and analyzed for CXCR4 expression by flow cytometry 3 days post-transduction. Untransduced (unt) cells were cultured and analyzed in parallel. Each transduction experiment was performed at least 10 times with technical replicates. Three to four individually produced vector stocks were used. Mean value, SD, and significance are indicated. **(A)** Surface expression of CXCR4 on CD56-positive NK cells. Representative dot plots (upper panel) indicate the percentage of cells in each quadrant. Individual results of each experiment for CD56, CXCR4-double positive cells are shown in the lower panel. **(B)** Surface expression of CXCR4 on CD19-CAR-positive NK cells. Representative dot plots (upper panel) indicate the percentage of cells in each quadrant. Individual results of each experiment for CXCR4, CAR-double positive cells are shown in the lower panel. ****$p < 0.0001$; ns, not significant by unpaired t test. Characteristics of isolated, primary NK cells prior to transduction are shown in **Supplementary Figure S1**.

evaluated in a killing assay. SUP-B15, BV-173, and JeKo cells were efficiently lysed by both types of the huCAR19 NK cells, and only background killing was present for $CD19^{neg}$ JeKo cells or untransduced NK cells (**Supplementary Figures S4B–E**).

Subsequently, we investigated the activation-induced degranulation and interferon gamma (IFNγ) secretion by the TRACKs upon coincubation with $CD19^{+}$ Nalm-6 cells at an effector-to-target ratio of 1:1. Degranulation was measured by surface detection of the degranulation marker CD107a by flow cytometry and IFNγ secretion by ELISA of the coculture supernatants. The average percentage of degranulation marker after specific target stimulation was comparable for huCAR19 and huCAR19.CXCR4 NK cells (on average, 51.5 and 49.9%, respectively) and significantly higher than CD107a level in untransduced control cells (26.4%; **Figure 3B** and **Supplementary Figure S5**). Similarly, IFNγ secreted by huCAR19 and huCAR19.CXCR4 NK cells was significantly higher upon coculture with tumor cells compared to unmodified NK cells although no difference in IFNγ secretion was detected between both CAR NK cell products (**Figure 3C**). Altogether, these data show that both CAR NK cell products are functional with regard to CAR-dependent specific tumor lysis.

huCAR19.CXCR4 NK Cells Display Enhanced Chemotaxis Activity

Next, the ability of CXCR4 gene delivery to enhance NK cell chemotaxis toward CXCL12 was addressed. Migration of huCAR19.CXCR4 NK cells as well as of huCAR19 or untransduced NK cells was determined in a transwell migration assay. Chemotaxis of NK cells from the upper well toward the chemotactic factor SDF-1 in the lower chamber was investigated after 2 h by counting the amount of migrated NK cells. For huCAR19.CXCR4 gene-modified NK cells, the number

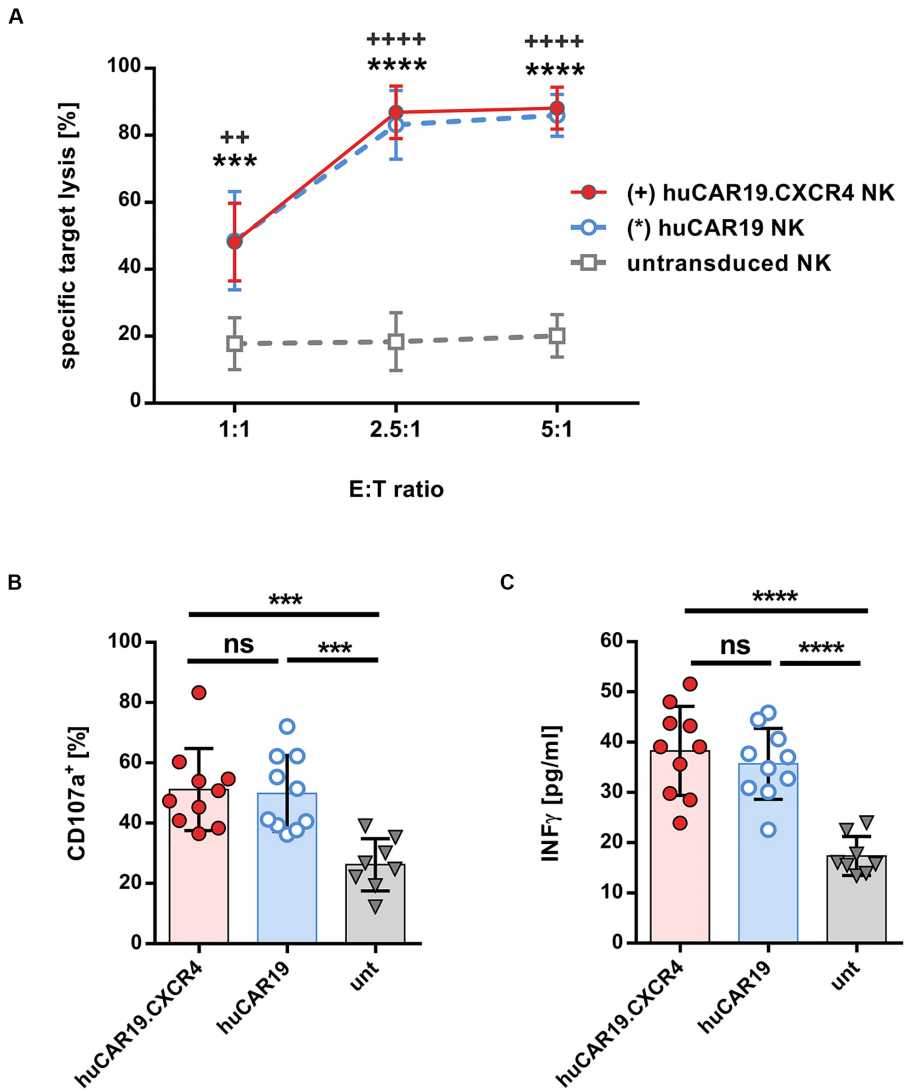

FIGURE 3 | Functional characteristics of gene-modified CAR NK cells. Analysis of cytotoxic function, degranulation, and IFN**γ** secretion of huCAR19 or huCAR19.CXCR4 gene-modified primary NK cells upon cocultivation with CD19-positive Nalm-6 cells. Untransduced primary NK cells cultured in parallel were used as a control. CAR NK cells were generated by transduction of primary NK cells with huCAR19-LV or huCAR19.CXCR4-LV particles at an MOI of 1–5. **(A)** Flow cytometry-based killing assay. After determination of huCAR19 expression, CAR NK cells as well as untransduced primary NK cells were mixed with CFSE-labeled Nalm-6 cells in different effector-to-target (E:T) ratios as indicated. The percentage of dead target cells were identified as double positive cells for CFSE and viability dye by flow cytometry. Mean values, SDs, and significance are shown. $n = 7$ with technical replicates. Unpaired t test was performed to determine significance comparing CAR NK cells (*for huCAR19.NK cells; $^{+}$for huCAR19.CXCR4 NK cells) versus untransduced samples. $^{++}p < 0.005$; $^{***}p < 0.001$; and ****, $^{++++}p < 0.0001$. **(B,C)** Degranulation **(B)** and IFNγ secretion assay **(C)**. Transduced as well as untransduced primary NK cells were mixed with Nalm-6 cells at an E:T ratio of 1. In the first hour of coculture FITC conjugated anti-CD107a antibody was added. After 4 h, cells and supernatant were collected. Harvested cells were used to determine CD107a expression by flow cytometry **(B)**. IFNγ secretion was determined in the supernatant by ELISA **(C)**. Bar diagrams show individual results as well as mean values, SDs, and significance. $n = 5$ with technical replicates. $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, non-significant by unpaired t test.

of migrated cells was nearly twofold enhanced compared to the huCAR19 NK cells and even threefold compared to the untransduced cells (**Figure 4A**). Untransduced NK cells exhibited a slight tendency to migrate through the transwell even in the absence of SDF-1 (**Figure 4B**).

To prove that the enhanced migration ability of huCAR19.CXCR4 NK cells is triggered by CXCR4 overexpression, chemotaxis toward SDF-1 was blocked by treating the NK cells with the antagonist AMD3100 for 2 h prior to the migration assay. The number of migrated huCAR19.CXCR4 NK cells after treatment with AMD3100 decreased dramatically from ∼13,100 to ∼2300 cells (roughly a sixfold decline), which is close to the baseline level defined by the amount of untransduced NK cells migrated in the absence of SDF-1 (∼1500; **Figure 4B**). Notably, the moderate migration ability of huCAR19-LV transduced or untransduced cells was

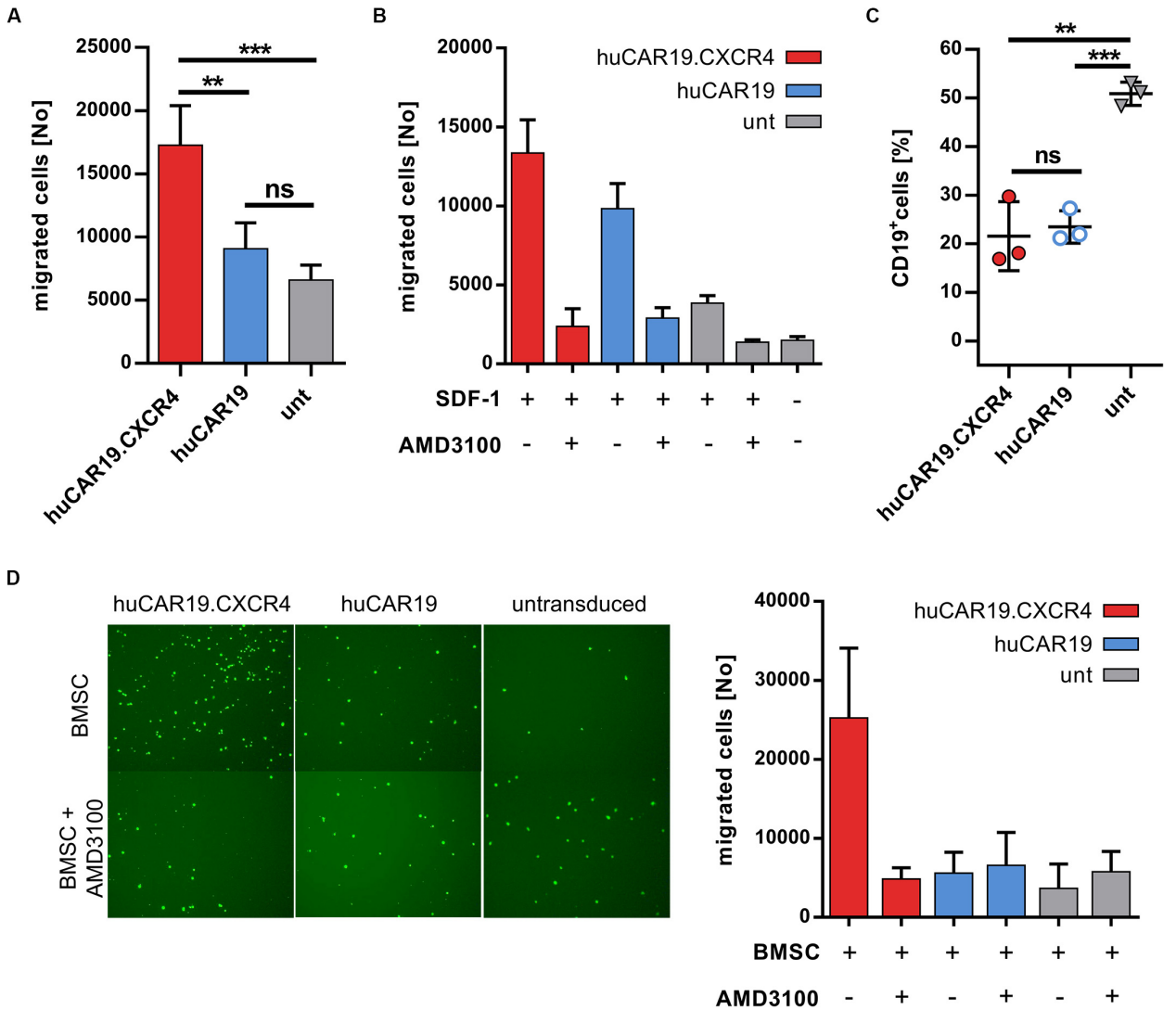

FIGURE 4 | Chemotaxis of NK cells is enhanced by CXCR4 overexpression. The migratory potential of genetically modified NK cells was evaluated in a migration assay using transwell plates. CAR NK cells were generated by transduction of primary NK cells with huCAR19-LV or huCAR19.CXCR4-LV particles. Untransduced primary NK cells cultured in parallel were used as a control. **(A)** Genetically modified or untransduced NK cells were placed in the upper chamber of the transwell plate and analyzed for their potential to migrate toward a lower chamber containing SDF-1 (CXCL12). The number of migrated NK cells was counted by flow cytometry. $n = 4$ with technical replicates. **(B)** Migration potential of NK cells was blocked by the CXCR4 antagonist AMD3100. Untreated or AMD3100 preincubated NK cells were placed in the upper chamber of the transwell plate and analyzed for their potential to migrate in the presence or absence of SDF-1. The number of migrated NK cells was counted by flow cytometry. The experiment was done in technical replicates. **(C)** A coculture assay was performed (in a 1:1 ratio) with migrated NK cells post chemotaxis to determine their cytotoxic potential. Specific target cell lysis was determined by decrease in the percentage of living CD19-positive cells analyzed by flow cytometry. $n = 3$. The experiment was done in technical replicates. Bar diagram show mean value, SD, and significance. $^{**}p < 0.005$; $^{***}p < 0.001$; ns, non-significant by unpaired t test. **(D)** The migration potential of CSFE-labeled NK cells with or without AMD3100 pretreatment toward BMSCs plated in the lower wells. Representative fluorescent microscopic pictures of migrated, CFSE-labeled NK cells (right) as well as quantitative data by flow cytometry (left) is shown. Bar diagram shows individual results as well as mean values and standard deviation. The experiment was carried out once with purified NK cells from two different donors.

reduced near to baseline levels in the presence of AMD3100 as well (**Figure 4B**).

To investigate the target-specific lysis potency of transduced cells after migration, a killing assay was performed following an independent transwell migration assay using SDF-1 as the chemotactic factor. In comparison to untransduced cells, significantly higher target cell lysis was observed in coculture with $CD19^+$ Nalm-6 cells in a 1:1 ratio by both types of huCAR19 NK cells (**Figure 4C**).

To further mimic the bone marrow environment and NK cell homing, another migration assay was performed, seeding BMSCs in the lower wells of the transwell plate instead of SDF-1. To discriminate migrated NK cells from BMSCs, NK cells were labeled with CFSE before loading into the upper

chamber of the transwell. Fluorescent microscopic imaging and quantification by flow cytometry of migrated cells revealed that the presence of BMSCs is sufficient to induce enhanced chemotaxis of CXCR4-augmented NK cells (more than fourfold compared to huCAR19 NK cells and untransduced cells), which can be abrogated by AMD3100 (**Figure 4D** and **Supplementary Figure S6A**). Notably, the addition of recombinant SDF-1 to BMSCs in the lower wells increased the total number of migrated huCAR19.CXCR4 NK cells for at least one donor, but also elevated the levels of migrated huCAR19 NK and untransduced NK cells, which were all close to the background upon AMD3100 treatment (**Supplementary Figures S6B,C**).

Taken together, these observations demonstrate that huCAR19 NK cells augmented with CXCR4 are able to eradicate $CD19^+$ tumor cells in a CAR-dependent manner, have significantly enhanced migration ability, and retain their functional activity post-migration.

DISCUSSION

Translation of CAR NK cell therapy into clinics is ongoing at a slow pace behind adoptive CAR T-cell therapy owing to some drawbacks regarding not only purification and expansion, but also efficient delivery of transgenes into NK cells and migration capacity (28, 29).

Natural killer cell lines can overcome these limitations, but they have other obstacles with regard to clinical applicability. Although, for basic research, several NK cell lines exist, the only clinically applicable cell line is NK-92. CAR-expressing NK-92 cells are currently under clinical evaluation, but clinical efficacy has to be awaited (30, 31). One major disadvantage of CAR NK-92 cells is their lymphoma origin, requiring irradiation before infusion, limiting their persistence, proliferation, and cytotoxic potential (7). This presents a challenge, especially when attempting to treat rapidly progressing cancers, such as AML. Therefore, CAR NK cells of primary origin are favored over CAR NK-92 cells for certain indications. In the current study, we demonstrate for the first time a highly efficient generation of CAR NK cells derived from peripheral blood encoding fully human CD19-specific CAR augmented by human CXCR4 with potent antitumor activity and migration capability. Genetic modification of primary NK cells was achieved by transduction with third-generation LV particles pseudotyped with VSV-G using polybrene as an enhancer. Interestingly, the transduction of primary NK cells was as efficient as that of the NK cell lines. This quite surprising CAR gene delivery to primary NK cells was not only reproducible in repeated experiments, but it was also stable for at least 2 weeks post-transduction. In addition, cell viability, proliferation capacity, and phenotype were not affected by polybrene or the transgene. This is especially important for the generation of a sufficient number of CAR NK cells for immunotherapy. Further, a high NK cell purity was achieved upon isolation, which is important to avoid T-cell mediated GvHD upon infusion of allogeneic NK cell products (2).

The choice of transduction enhancer and viral envelope protein can tremendously influence transduction efficiencies (26). Direct comparison of Retronectin, Vectofusin-1, and polybrene revealed that Vectofusin-1 and polybrene are beneficial for the transduction of primary NK cells using VSV-G peusdotyped LV particles and clearly outperform Retronectin. Polybrene is also a well-known transduction enhancer in the field of NK cells. High transduction efficiencies can be reached for NK cell lines, but for primary NK cells, gene transfer rates of only 8–55% were reported depending on the viral vector and cell source used (32–34). In our study, on average, more than 80% of gene modified NK cells could be reached. Notably, in contrast to Boissel and colleagues (33), we did not observe a negative effect of polybrene on NK cell viability or proliferation. Similar high gene transfer rates to primary NK cells were reached using Vecotfusin-1 as an enhancer, which might even be increased when using a different viral envelope. For Vectofusin-1, it was recently demonstrated that transgene delivery to primary NK cells can be improved by pseudotyping LVs with the envelope protein from endogenous feline virus (RD114) or baboon endogenous retrovirus (BaEV) instead of VSV-G (5, 6). In the field of CAR T-cells gamma-retroviral vectors or VSV-G pseudotyped LVs are most commonly used for their generation. Therefore, clinical translation of CAR NK cells will benefit from the use of an already approved viral vector, highlighting the value of the demonstrated high transduction efficiency of primary NK cells in the current study with VSV-G pseudotyped LVs (33).

A clinical grade lentiviral expression backbone harboring a fully human CD19-specific CAR construct with or without additional human CXCR4 gene was used to generate huCAR19 and huCAR19.CXCR4 NK cells, respectively. Both CAR NK cells showed potent and specific antitumor activity on CD19-positive patient-derived primary tumor cell lines of various origin, including peripheral blood– or bone marrow–derived B-cell precursor leukemia cells from ALL or chronic myeloid leukemia patients and peripheral blood–derived B-cell lymphoma cells from a patient with non-Hodgkins lymphoma. In addition, upon antigen stimulation, robust INF-γ secretion and upregulation of the degranulation marker CD107a were induced. These results demonstrate that the fully human CD19 CAR construct not only shows strong antitumor activity in T-cells (11), but is also very effective upon introduction to NK cells in terms of cytokine secretion and tumor cell lysis. Notably, the types of proinflammatory cytokines secreted by NK cells differ from those produced by T cells. *Ex vivo* expanded primary human NK cells are known to mainly secrete IFN-γ and GM-CSF upon activation, which are relatively safer than the proinflammatory cytokines produced by T-cells, including TNF-α and IL-6 (35, 36). Secretion of IL-6, IL-1, and TNF-α by CAR T-cells is associated with the development of CRS, a common and severe side effect of CAR T cell therapy. In contrast to CAR T-cells, activated CD19-specific primary CAR NK cells, CAR NK92 cells, or CAR CIK cells were shown by us and others to secrete high levels of IFN-γ and/or GM-CSF but only moderate levels of TNF-α, and production of IL-6 was not observed (37–39). A recent report of a clinical trial did reveal that, among 11 patients with relapsed or refractory CD19-positive malignancies, the majority had a clinical response to treatment with primary CD19-specific CAR NK cells without the development of major toxic effects, including CRS (40),

supporting the hypothesis that CAR NK cells might be a safer alternative to CAR T-cells.

Concerning the limited migratory potency of NK cells to malignant cells residing in bone marrow niches or other tumor sites, promoting NK cell chemotaxis via chemokine receptor upregulation has been considered before. Primary NK cells engineered to express the chemokine receptor CCR7 or CXCR2 were demonstrated to have an improved migratory ability toward their respective ligands (41, 42). In an NK cell line, even the combination of CAR and CXCR4 were evaluated, showing that YT2 cells expressing EGFRvIII-CAR and CXCR4 migrate to and specifically kill SDF-1-secreting glioblastoma cells (43). For T-cells, CXCR4 overexpression enhanced their bone marrow migration resulting in local engraftment (44). For NK cells, the power of CXCR4 expression was recently demonstrated by exploring the CXCR4 gain of function mutation present in WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome, resulting in an increased NK cell migration toward bone marrow (19, 45). In our study, we could show efficient overexpression of native human CXCR4 on primary CAR NK cells upon genetic engineering. These TRACKs displayed not only strong and specific antitumor activity, but also enhanced chemotaxis toward recombinant SDF-1 and BMSCs. This boosted migratory potential might facilitate an improved clearing of CD19 positive tumor cells residing in the bone marrow *in vivo*, which has to be explored in future work. In conclusion, this work demonstrates that TRACKs could be an important player for NK cell–based adoptive cell therapy and should be further investigated for their translation to clinics.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University Hospital Frankfurt. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AJ designed and performed the experiments. AJ and JeH evaluated the data. FT contributed to the experimental design and data evaluation. SA helped with data evaluation. HM contributed to the construct design. EU, HB, FT, and SA contributed protocols and reagents. ZM, JaH, and JeH conceived and designed the study. JeH and EU supervised the work. AJ and JHar prepared the figures and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02028/full#supplementary-material

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REVIEW
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Immunoregulatory Roles of Extracellular Vesicles and Associated Therapeutic Applications in Lung Cancer

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Lung cancer represents a fatal condition that has the highest morbidity and mortality among malignancies. The currently available treatments fall short of improving the survival and quality of life of late-stage lung cancer patients. Extracellular vesicles (EVs) secreted by tumors or immune cells transport proteins, lipids, and nucleic acids to other cells, thereby mediating immune regulation in the tumor microenvironment. The cargo carried by EVs vary by cellular state or extracellular milieu. So far, multiple studies have suggested that EVs from lung tumor cells (TEVs) or immune cells promote tumor progression mainly through suppressing antitumor immunity. However, modified or engineered EVs can be used as vaccines to elicit antitumor immunity. In addition, blocking the function of immunosuppressive EVs and using EVs carrying immunogenic medicine or EVs from certain immune cells also shows great potential in lung cancer treatment. To provide information for future studies on the role of EVs in lung cancer immunity, this review focus on the immunoregulatory role of EVs and associated treatment applications in lung cancer.

Keywords: extracellular vesicles, lung cancer, immunosuppression, immunostimulation, therapeutic application

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Abbreviations: AICD, activation-induced cell death; APCs, antigen-presenting cells; APO2L, Apoptosis ligand 2; ATMP-MTX, ATMPs loaded with methotrexate; ATMPs, autologous tumor-derived microparticles; BAG6, BCL2-associated athanogene 6; CAR, chimeric antigen receptor; CCL, chemokine (C-C motif) ligand; CCR, C-C motif chemokine receptor; CD40L, CD40 ligand; CDKIs, cyclin-dependent kinase inhibitors; CEA, carcinoembryonic antigen; cGAS, Cyclic GMP-AMP synthase; CTLA4, cytotoxic lymphocyte antigen 4; DCs, dendritic cells; DEXs, exosomes from dendritic cells; DMA, Dimethyl amiloride; ESCs, embryonic stem cells; ESEVs, EVs from embryonic stem cells; ESEXs, exosomes from embryonic stem cells; EV-PTX, PTX encapsulated EVs; EVs, extracellular vesicles; FasL, Fas ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; Her2/neu, human epidermal growth factor receptor 2; HSP70/72, Heat Shock Protein 70/72; HS-TEXs, Heat-stressed lung tumor cell-derived exosomes; IDO, indoleamine 2,3-dioxygenase; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; ISEV, The International Society for Extracellular Vesicles; LEVs, EVs from T lymphocytes; LLC, Lewis lung cancer; LMPs, T lymphocytes-derived microparticles; MAGE, melanoma antigen gene; MDSCs, myeloid-derived suppressor cells; MEXs, exosomes derived from macrophages; MHC I/II, major histocompatibility complex I/II; MMP9, matrix metalloproteinase 9; MTX, methotrexate; MVBs, multivesicular bodies; MyD88, myeloid differentiation factor 88; NKp30, natural killer (NK) cell receptor; NSCLC, non-small cell lung cancer; OVs, Oncolytic virus; PBMCs, peripheral blood mononuclear cells; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; pMHC II, peptide-MHC II; SOCS3, suppressors of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; STING, stimulator of interferon gene; TABs: lung tumor-derived apoptotic bodies; TEVs, EVs from lung tumor cells; TEXs, Lung cancer-derived exosomes; TGF-β, transforming growth factor-β; TIDC, tumor-infiltrating DCs; TIME, tumor immune-microenvironment; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg cells, regulatory T-cells; UV, ultraviolet; UV-TEXs, UV-exposed tumor-derived exosomes; UV-TMPs, UV-exposed tumor-derived microparticles; VEGF, vascular endothelial growth factor; WT1, Wilms' tumor gene 1.

INTRODUCTION

Lung cancer remains the most frequently diagnosed cancer, accounting for 11.6% of total cases, and a leading cause of cancer-related mortality, responsible for 18.4% of total cancer deaths around the globe (1) with its 5-year survival being 19% (2). Most lung cancer patients, especially late-stage patients, have unfavorable prognoses due to delayed diagnosis and unresponsiveness to conventional therapies (surgery, chemotherapy, and radiotherapy) (3). Recently, mounting attention has been paid to immunotherapy for its good curative effects. For instance, chimeric antigen receptor (CAR) T-cells can successfully eradicate hematologic malignancies, but their effect on solid tumors, including lung cancer, has not been satisfactory (4). Treatments involving immune checkpoint inhibitors (such as programmed death-1 (PD-1) inhibitor, programmed death-ligand 1 (PD-L1) inhibitor, and cytotoxic lymphocyte antigen 4 (CTLA4) inhibitor) are remarkably effective in many malignancies, including lung cancer. Nonetheless, only about 20% of lung cancer patients respond to these therapies, and the efficacy largely depends on the adequate expression of PD-1 or PD-L1 on the immune or cancer cells (3, 5). These advances indicate that immunotherapy has great potential in the treatment of lung cancer, and further breakthroughs are needed. We recently found that autologous lung tumor–derived microparticles (ATMPs) loaded with methotrexate (ATMP-MTX) had a good safety profile and were effective for the treatment of advanced lung cancer with malignant pleural effusion (6). EV-associated therapy for lung cancer has become a research hot spot and may result in a major breakthrough in the treatment of lung cancer in the future (7, 8).

Extracellular vesicles, a collective term for various membrane structures released by virtually all cells principally involve exosomes, microparticles, and apoptotic bodies (9). EVs contain a variety of cargo from donor cells, including proteins, lipids, and genetic substances (10, 11). The cargo can be transferred by EVs to recipient cells, resulting in phenotypic changes in the latter and vice versa (12). Cargo possessing distinct properties are selectively enriched within different subtypes of EVs from various donor cells under specific ambient conditions (13, 14). As a medium that mediates cell-to-cell communication, EVs are implicated in a wide array of biological activities in malignancies, including lung cancer (12, 15). These activities include metastasis (16), angiogenesis (17), and regulation of host immune function (15, 18). Currently, immune regulation of EVs is a hot spot of related research. Multiple studies have reported that EVs from both cancer and immune cells are involved in the immune regulation of lung cancer (11, 18). More specifically, natural EVs mainly play an immunosuppressive role in various cancers, including lung cancer (19–23) although, on the other hand, modified EVs may serve as activators of antitumor immunity (6, 24–26).

Accordingly, EV-associated therapies have been tried in the treatment of lung tumors (6, 11, 18, 19, 21, 24–35). EVs involved in these studies range from modified or engineered TEVs and DEVs to EVs from embryonic stem cells (ESEVs) and T-lymphocytes (LEVs). Discrepancies in efficacy among these trains of research can mainly be ascribed to differences in study design, such as the different subtypes of EVs used. Therefore, there is urgent need to comprehensively review and characterize the immunological features of EVs and to make full use of previous research findings concerning EVs to develop effective treatment methods for lung cancer. In this paper, we principally review the roles of EVs as mediators of intercellular communication between tumor and immune cells in the modulation of antitumor immunity and relevant therapeutic applications in lung cancer.

BIOGENESIS OF EVs

The existence of vesicles in the extracellular milieu in mammalian tissues or fluids was first described in the late 1960s, and since then, mounting attention has been paid to their roles (36, 37), and remarkable results have been accomplished regarding EVs over the past decades. "Extracellular vesicles," as a generic term, was recommended by the International Society for Extracellular Vesicles (ISEV) to refer to the "particle released from the cells that are delimited by a lipid bilayer and cannot replicate." It is so defined mainly because, up to now, no consensus has been reached among researchers concerning the specific markers for various subtypes, mainly including exosomes, microparticles/microvesicles/ectosomes, oncosomes, and apoptotic bodies (9, 38). In terms of the assembling and releasing process, it has now been generally accepted that "ectosomes" (microparticles/microvesicles) are formed by outward budding of the plasma membrane, exosomes derive from fusion of multivesicular bodies (MVBs) with the plasma membrane (39, 40), and apoptotic bodies are generated by cells undergoing apoptosis (40, 41). In different biogenetic processes, certain substances are selected and enriched within specific subtypes of EVs, and constitution of cargo in one type of EV might change as the microenvironment of donor cells changes (13, 14, 40). Accordingly, particular cargo dictates the properties of various subtypes of EVs, which indicates that different EV subtypes play variant roles in tumor immunomodulation (13, 14).

IMMUNE CARGO OF EVs

The proportion of EVs secreted by a cell varies depending on the donor cell type and its state (42). The production of a certain subtype of EV also changes with the transformation of donor cells (43, 44). The bioactive molecules in EVs are derived from cell membranes and endosomes (12, 39), and the uniqueness of these molecular characteristics is related to the donor cell (45). EVs produced by lung tumor cells or immune cells contain plenty of bioactive substances, such as proteins, lipids, and genetic DNA/mRNA/non-coding RNA, which are transported between cells and can deliver information about immune processes (11). In most cases, EVs from lung tumor cells contain certain cargo that induces immune escape (19, 21, 46, 47). Nonetheless, after modification, immunogenic components (e.g., tumor-associated antigens) on EVs derived from tumor cells gradually function and can activate antitumor immunity (24–26, 48, 49). Similarly,

TABLE 1 | Immunosuppressive cargo of EVs in lung cancer.

Cargo	EV types	Modifications or treatments	Mechanisms	Functions in immunity	References
NA	TEXs	\	IL-6/STAT3^	Inhibiting the maturation of DCs	(19)
NA	TEXs	\	NA	Inducing Treg cells	(19)
NA	TEXs	\	NA	Decreasing c-c/c-x-c chemokine receptor on DCs	(19)
NA	TEXs	\	NA	Upregulating immunosuppressive molecules on DCs	(19)
NA	TEXs	\	NA	Downregulating immunostimulatory molecules on DCs	(19)
PD-L1	TEXs	\	PD-L1/PD-1	Inducing anergy or apoptosis of T cells	(20)
HSP72/HSP70	TEXs	\	TLR2/MyD88/ IL-6/STAT3	Activating immunosuppressive function of MDSCs	(21)
Non-coding RNAs	TMPs	UV-irradiation	TLR3/IL-1β	Increasing secretion of IL-1β from M2 type macrophages and promoting tumor progression	(47)
DNAs^	TMPs	UV-irradiation	cGAS/STING/ TBK1/STAT6	Inducing M2 polarization of macrophages	(78)
U1snRNA	TMPs	\	TLR3	Inducing tumor-promoting inflammation	(22)
FasL	LMPs	Activation	APO2L/TRAIL; Fas/FasL	Inducing death of T cell; Inducing apoptosis of DCs	(81, 82) (23)

NA, not available; ^, uncertain; \, none; EVs, extracellular vesicles; TEXs, Lung cancer-derived exosomes; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; DCs, dendritic cells; Treg cells, regulatory T-cells; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; HSP72/HSP70, Heat Shock Proteins 70/72; TLR2, toll-like receptor 2; MyD88, myeloid differentiation factor 88; MDSCs, Myeloid-derived suppressor cells; TMPs, lung tumor-derived microparticles; UV, ultraviolet; TLR3, toll-like receptors 3; IL-1β, interleukin-1β; cGAS, Cyclic GMP-AMP synthase; STING, stimulator of interferon gene; FasL, Fas ligand; LMPs, T lymphocytes-derived microparticles; APO2L, Apoptosis ligand 2; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

EVs derived from immune cells contain many functional molecules and mediate communication among immune cells (43, 50–54). Additionally, EVs from embryonic stem cells also possess some antigens similar to those from cancer cells (31). All immunosuppressive and immunostimulatory cargo in EVs discussed in this review are listed in **Tables 1**, **2**, respectively.

LUNG TUMOR IMMUNE-MICROENVIRONMENT (TIME)

The TIME of lung cancer has to be mentioned before we discuss the immunoregulatory roles of EVs because EVs are just one of the mediators of immune regulation in the TIME.

Tumors are considered to be caused by genetic mutations that may generate neoantigens and trigger immune surveillance to clear or suppress non-self tumor cells. However, this self-protection mechanism frequently fails as cancer develops (55). The cancer immunoediting hypothesis, designed to explain this phenomenon, assumes that there exist three phases during the process of tumorigenesis and tumor progression, namely "elimination," "equilibrium," and "escape" (56). This complicated process is actually a battle between cancer cells and the host immune system. In the "elimination" phase, the host immune system prevails, whereas during the "escape" phase, cancer cells defeat the host immune system. "Equilibrium" refers to a standoff between the two sides. The entry into the "escape" phase involves an interaction among various players, including tumor cells, tumor stroma, and the host immune system (56). Briefly, the negative results of this "struggle" include impaired immune recognition (such as loss of tumor antigens), increased resistance to the cytotoxic effects of immunity, or especially, establishment of an immunosuppressive state within the tumor microenvironment (56). The formation of the immunosuppressive microenvironment involves immunosuppressive cytokines (such as vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), galectin, or indoleamine 2,3-dioxygenase (IDO)] released by cancer cells; these cytokines block the maturation of immune cells and promote the recruitment of immunosuppressive cells (57).

Research looking into the maturation of dendritic cells (DCs) from lung cancer biopsies shows that DCs consist of three types, i.e., $CD11c^{high}$ mDCs, $CD11c^{-}$ pDCs and $CD11c^{int}$ mDCs, in terms of their expression levels of CD11c, and most tumor-infiltrating DCs (TIDC) are "semi-mature" or even immature (58). Moreover, those TIDCs isolated freshly from non-small cell lung cancer (NSCLC) underwent only slight phenotypic maturation and showed poor antigen-presenting ability after toll-like receptor (TLR) activation *in vitro* (58). Increased proportions of $CD4^{+}CD25^{+}$ Treg cells secreting TGF-β were found in tumors and peripheral blood from patients with lung cancer, and tumor-infiltrating lymphocytes showed only marginal production of Th1 or Th2 cytokines (59). Macrophages in tumor tissue can be stimulated by tumor-derived cytokines and polarized into the M2 type. The latter could subvert adaptive immunity and promote tumor progression (60). Secretion of EVs with suppressors of cytokine signaling 3 (SOCS3) from alveolar macrophages were inhibited in patients with non-small cell lung cancer and in a lung cancer mouse model, which promoted the development of lung tumors (61). Myeloid-derived suppressor cells (MDSCs) are a group of phenotypically heterogeneous immature cells of bone

TABLE 2 | Immunostimulatory cargo of EVs in lung cancer.

Cargo	EV types	Modifications or treatments	Mechanisms	Functions in immunity	References
CCL2-5, CCL20	TEXs	Heat treatment	CCL/CCR	Recruiting and stimulating $CD11c^+$DC and $CD4^+/CD8^+$ T-cells	(25)
CD54, CD86	TEXs	Heat treatment	NA	Adhering to DCs	(25)
MHCI/II	TEXs	Heat treatment	NA	Antigen presentation	(25)
NA	TEXs	Rab27a overexpression	NA	Inducing maturation of DCs	(26)
CD40L	TEXs	CD40L-engineering	CD40/CD40L	Inducing maturation of DCs	(49)
Her2/neu, CEA, WT1, MAGE2, and survivin peptides	Lung tumor-derived apoptotic bodies	MAGE-engineering and UV-irradiation	NA	Presenting antigens to DCs and stimulating DCs maturation	(28)
MAGE peptides	DEXs	DEX incubated with MAGE peptides	pMHCII costimulatory molecules/TLR	Antigens cross-presentation among DCs, NK cell activation	(29)
Certain carcinoembryonic antigens	ESEXs	GM-CSF engineering	NA	Inducing antigen-specific antitumor immune response	(31)
MTX	LMPs	MTX-incorporation and UV-irradiation	NA	Inducing immunogenic death of lung cancer cells	(6)

NA, not available; CCL, chemokine (C-C motif) ligand; CCR, C-C motif chemokine receptor; MHCI/II, major histocompatibility complex I/II; Her2/neu, human epidermal growth factor receptor 2; CEA, carcinoembryonic antigen; WT1, Wilms' tumor gene 1; MAGE, melanoma antigen gene; pMHCII, peptide-MHCII; BAG6, BCL2-associated athanogene 6; NKp30, natural killer (NK) cell receptor; ESEXs, exosomes from embryonic stem cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; MTX, methotrexate.

marrow origin and have a remarkable ability to suppress T-cell activation (62). These cells were found to be more suppressive and apparently increased in peripheral blood, tumor tissues, spleen, and lymph nodes in tumor-bearing mice or humans compared to normal controls (62).

In summary, lung cancer cells will do all in their power to escape from host antitumor immunity for their survival. Understanding this essential concept can help us decipher the roles of EVs in lung cancer immunity.

ROLES OF EVs IN IMMUNOREGULATION IN LUNG CANCER

As an efficient medium conveying information between cells, EVs contain specific antigens or immune molecules from tumor or immune cells, and they play a vital role in cancer immunoediting (11). EVs produced by tumor cells can be internalized by immune cells, thereby altering the function of immune cells and vice versa.

In almost all TIMEs, EVs act as an immunosuppressor (19–23) (**Figure 1**). More precisely, in the process of immunoediting, EVs may serve as an immune stimulator at the germination of cancer cells (which may not go through an immunoediting process) and then convert to an immunosuppressor during the progression of cancer. A classical research, though not studying lung cancer cells, demonstrates that exosomes from poorly metastatic melanoma cells can potentially inhibit cancer metastasis to the lung by stimulating an innate immune response and triggering cancer cell clearance at the pre-metastatic niche (63) while exosomes from advanced and highly metastatic melanoma help create pre-metastatic niches in remote microenvironments to favor metastasis (64).

It is worth noting that TEV-mediated tumor immunoregulation is closely related to TEV-mediated tumor metastasis; the latter is a complicated process dubbed the infiltration-metastasis cascade (65). On the one hand, TEVs activate epithelial-mesenchymal transition (EMT) in neoplastic, mesothelial, and vascular endothelial cells through various signaling pathways (such as TGF-β, Wnt5b, or caveolin-1 signaling pathways), thereby enhancing the migration ability of cancer cells and increasing the permeability of blood vessels in the peritumoral matrix (65–69). Particularly, in addition to metastasis promotion, TGF-β also plays an important role in immune regulation (70) as TGF-β could induce fibroblasts to release the immunomodulatory protein PD-L1 into extracellular vesicles, resulting in inhibition of T-cell proliferation. At the same time, PD-L1 knockdown could reduce the induction of TGFβ-dependent extracellular matrix protein production and, thus, suppress cell migration (71). On the other hand, TEVs can be systemically transported to distant locations, thereby fostering a pre-metastatic niche via activating a reactive, myofibroblast-rich stroma and promoting immune evasion (65, 68, 72). Therefore, the immunosuppressive action and metastasis-promoting effect of EVs complement each other to promote tumor progression.

Nevertheless, other researchers found that, under certain stresses (such as exposure to radiation, heat, or ultraviolet light) or when engineered with a specific aptamer, reconstructive tumor and immune cells can release corresponding EVs with a stronger immune-stimulating property but reduced immunosuppressive ability (6, 24–26) (**Figure 2**). Anticancer immunotherapy using EVs is precisely premised on these findings.

In this section, we mainly discuss the immunosuppressive roles of natural TEVs and T-lymphocyte–derived MPs (LMPs) in lung cancer and the immunostimulatory evidence of processed or engineered TEVs and DEVs. Knowing these regulatory mechanisms is pivotal for developing optimal protocols to use EVs to effectively elicit antitumor immunity.

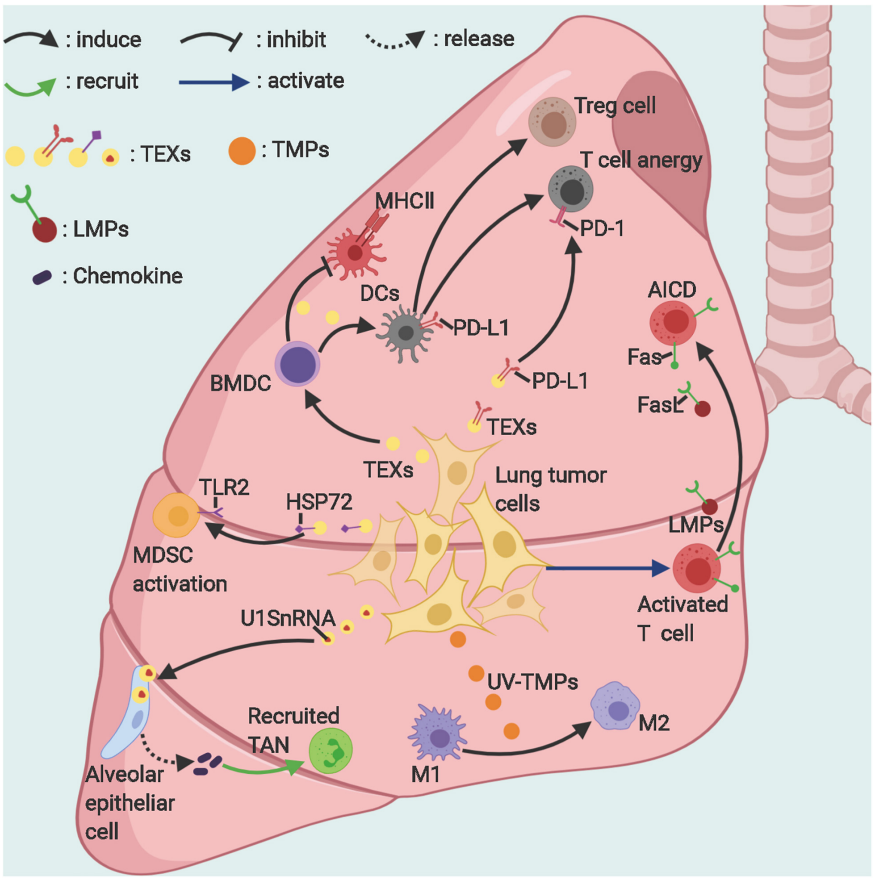

FIGURE 1 | The suppressive roles of EVs in lung cancer immunity. Lung tumor–derived exosomes and microparticles can suppress antitumor immunity in various ways. Activated T-cells release microparticles, which induce their own death via Fas/FasL signaling. *TEXs, Lung tumor-derived exosomes; TMPs, tumor-derived microparticles; LMPs, T-lymphocyte-derived microparticles; TAN, tumor-associated neutrophil; AICD, activation-induced cell death; MDSCs, Myeloid-derived suppressor cells; M1/2, macrophages subtype 1/2*. Figure created with BioRender.com.

Immunosuppressive Roles

Natural EVs From Lung Cancer Cells

Inhabiting function of dendritic cells

It has been demonstrated that exosomes derived from Lewis lung cancer (LLC) cells could block the differentiation and maturation of myeloid precursors into DCs and induce apoptosis of myeloid precursors in the presence of FLT-3L *in vitro* (19) as indicated by decreased $CD11c^+$ DCs and downregulated maturation markers of CD80/CD86/MHCII on DCs (19). The underlying mechanism has not been specifically explored by any studies, but it can be speculated on the basis of findings of other similar studies concerning other cancers. For instance, research shows that the exosomes from murine or human breast cancer cells could block the differentiation of murine myeloid precursor cells into immature $CD11c^+$ DCs by inducing expression of interleukin-6 (IL-6) and activating the signal transducer and activator of transcription 3 (STAT3) (46).

LLC exosomes can induce the expression of immunosuppressive molecules, including PD-L1, CD11b, and Arginase I, and downregulate the expression of immune activating/stimulatory molecules, such as CD80, CD86, and MHC-II on dendritic cells (19). These treated DCs also decrease the mRNA level of certain immunocompetent molecules, such as tumor necrosis factor-α (TNF-α), IL-6, and inducible nitric oxide synthase (iNOS), and the aforementioned changes in DCs eventually lead to T-cell anergy (19). A PD-L1-blocking antibody can partially eliminate the inhibitory effect of DCs treated by LLC exosomes rather than 4T1 exosomes, indicating that other molecules, rather than PD-L1 on DCs treated by the 4T1 exosome, mediate the immunosuppressive effects (19).

Lung cancer (LLC)-derived exosomes (TEXs) inhibit migration of DCs to lymph nodes by decreasing most C-C/C-X-C chemokine receptors, especially CCR6, CCR7, and CXCR3 on DCs (19). These TEXs inhibit the migration of DCs to draining lymph nodes and block the interaction between DCs and T-lymphocytes. However, little is known about what substances on TEXs mediate this effect and how they work.

Induction of apoptosis of T-cells

PD-L1 was found on exosomes as well as donor lung cancer cells, and these PD-L1-expressing exosomes can suppress cytokine secretion and induce anergy or apoptosis of PD-1-expressing

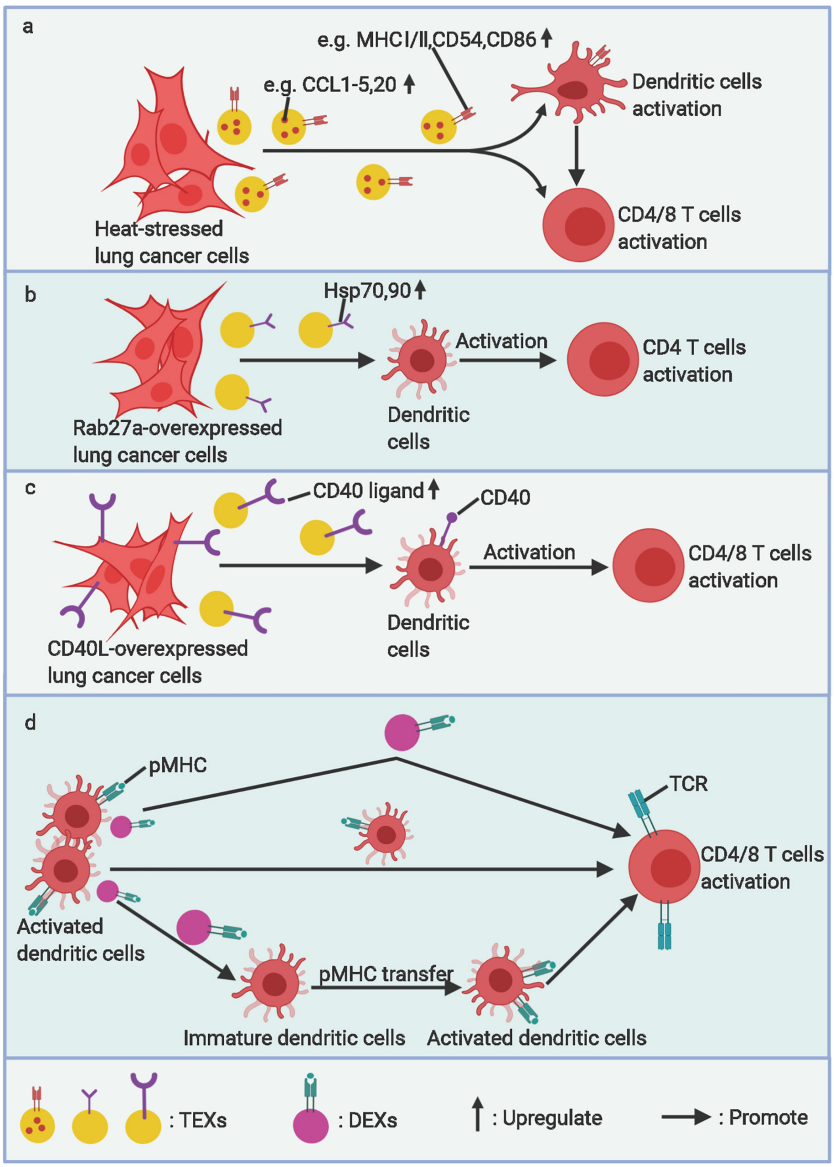

FIGURE 2 | The stimulatory roles of EVs in lung cancer immunity. **(a)** Heat stressed, **(b)** Rab27a overexpressed, and **(c)** CD40L overexpressed lung cancer cell–derived exosomes (TEXs) can activate DC maturation and arouse specific antitumor immunity. **(d)** Activated dendritic cell–derived exosomes (DEXs) can present antigens to T-cells directly or by transferring pMHC to other immature dendritic cells and amplifing this antigen-presentation effect. *TCR, T-cell receptor*. Figure created with BioRender.com.

activated T-cells (20). The mRNA level of PD-1 in circulating exosomes of patients with NSCLC was found to be significantly associated with the effect of anti-PD-1 therapy (73). Indeed, it was demonstrated that a high level of PD-L1-expressing TEXs in blood, as a variant of secreted PD-L1, could neutralize the administrated anti-PD-1 antibodies (74) before they reached tumor tissues, thereby resulting in poor response and outcomes (75).

Induction of immunosuppressive immune cells

FOXP3-expressing regulatory T (Treg) cells, as a significant component maintaining immune homeostasis, also suppress antitumor immunity. The infiltration of $FOXP3^{+}$Treg cells into a tumor was found to be highly related to poor prognosis of cancer patients. TEX-treated DCs were shown to induce the differentiation of $CD4^{+}FOXP3^{+}$Treg cells while suppressing the differentiation of $CD4^{+}IFN\text{-}\gamma^{+}$ Th1 cells (19).

Various factors in a tumor could induce the expansion or activation of MDSCs through multiple pathways, including STAT3 or IL-4Rα–STAT6 pathways, resulting in the suppression of T-cell function (62). EVs have been increasingly shown to take part in the communication between tumor cells and MDSCs. Research in mice and humans shows that TEXs (including lung cancer exosomes) can activate immunosuppressive function

of MDSCs (21). Specifically, heat shock protein 72 (Hsp72) expressed on TEXs interacts with the TLR2 on MDSCs, which triggers the TLR2/MyD88 signaling pathway, induces the autocrine production of IL-6, and causes Stat3 phosphorylation, which ultimately activates the suppressive function of MDSCs (21). Of note, TEXs induce the activation of Stat3 without promoting MDSC expansion while the tumor-derived soluble factors (TDSFs) trigger the expansion of MDSCs by activating the Erk signaling pathway (21). On the whole, these results are supplementary to the findings that regulation exists between tumor cell and MDSCs and are conducive to the further understanding of the development of MDSCs against a tumor background.

Additionally, TEXs were also found to induce tumor-promoting inflammation in sites far from the primary tumor and to mediate tumor metastasis (22). U1 snRNAs in exosomes derived from LLC or B16/F10 melanoma cells can be transferred to alveolar epithelial cells and could be sensed by TLR3. Activated TLR3 signals increase the production of chemokines, which promotes the recruitment of neutrophils to the lung. These infiltrating neutrophils would be polarized into tumor-promoting subtypes (tumor-associated neutrophils, TANs) (76, 77) and eventually enhance the formation of a pre-metastatic niche in the lung (22). The mechanism is akin to that by which the non-coding RNA in lung tumor–derived microparticles (TMPs) stimulates TAM to secrete IL-1β as described below (47).

MPs From Ultraviolet (UV)-Treated Lung Cancer Cells

MPs from UV-treated lung cancer cells could induce polarization of M2 macrophages to suppress antitumor immunity and promote tumor progression *in vitro* and *in vivo* (47, 78). Specifically, macrophages treated with MPs from UV-treated lung cancer cells upregulate the expression of M2-type surface markers CD163, CD206, VEGF, IL-10, and arginase 1 and downregulate the level of M1-type surface markers IL-12, iNOS, and TNF-α (47, 78). The mechanism of MPs stimulating M2 type macrophages and promoting tumor progression involves the activation of cGAS/STING/TBK1/STAT6 pathways in macrophages (78). Furthermore, whether DNAs in these TMPs activate the cGAS/STING pathway in M2 macrophages needs further investigation (78). Meanwhile, the tumor-promoting effects of M2 macrophages induced by lung cancer microparticles are associated with increased IL-1β secretion after macrophages sense the non-coding RNA in TMPs through the TLR3 signaling pathway (47). IL-1β has been proven to promote the angiogenesis (79) and stemness of tumor cells (80).

EVs From Activated T-Lymphocytes

Activated T-cells could release microvesicles carrying Fas ligand (FasL) and apoptosis ligand 2 (APO2L), which mediates the activation-induced cell death (AICD) of mature T-cells (81, 82). Additionally, exosomes from mature T-cells can be taken up or internalized by DCs via exosomal LFA-1, thereby downregulating the expression of peptide/MHC I on DCs and inducing the apoptosis of DCs via the Fas/FasL signaling pathway (23).

Immunostimulatory Roles

Modified EVs From Lung Cancer Cells

Although natural TEVs mainly mediate immunosuppressive roles in lung cancer as aforementioned, TEVs do share similar antigens with donor tumor cells as indicated by their ability to induce a tumor-specific immune response (25). Mounting evidence suggests that EVs from stress-treated (25) or modified (49) lung cancer cells with stronger immunogenicity can activate DC maturation and specific T-cell immune response (**Figures 2a–c**). Some artificially immunogenicity-enhanced TEVs used for stimulating immunity are detailed in following paragraphs.

Heat-stressed 3LL lung tumor cell–derived exosomes (HS-TEXs) could more efficiently induce DC activation and an antigen-specific T-cell immune response than unprocessed TEXs (25) (**Figure 2a**). This is attributed to the increased level of chemokines, including CCL2, CCL3, CCL4, CCL5, and CCL20 in HS-TEXs, which contribute to the recruitment and stimulation of $CD11c^+$ DC and $CD4^+/CD8^+$ T-cells both *in vitro* and *in vivo* (25). Actually, heat stress promotes these chemokines to assemble into lipid rafts, which are then enriched in EVs (25). Nonetheless, the exact mechanism of enhanced immunogenicity of the EVs from stress-treated tumor cells remains unclear.

Wenhai and colleagues (26) found that exosomes released by Rab27a-overexpressed A549 cells had more typical exosomal proteins, including Hsp70 and Hsp90. A mouse model study showed that these EVs induced more BMDCs into mature DCs and then promoted $CD4^+$ T-cell proliferation and, thus, gained a stronger antitumor effect (26) (**Figure 2b**). The authors postulated that the mechanism might be associated with more immunogenic molecules, such as Hsp70 and Hsp90, on modified exosomes (26). Because the postulation is not consistent with the prior (21) and following (27) studies, further research is needed to determine this issue.

Furthermore, TEXs directly decorated with costimulatory molecules also show enhanced immunogenicity. The CD40 ligand (CD40L) can ligate with CD40 expressed on the unique antigen-presenting cells (APCs), including DCs, resulting in enhanced maturation of DCs as well as stronger antitumor CD4 + /CD8 + T-cell immunity (83, 84) (**Figure 2c**). But, under normal circumstances, CD40L is predominantly expressed on primed T-cells rather than naive T-cells, which may restrict the activation of DCs (85). Researchers successfully designed CD40L-carrying exosomes that possess a stronger ability to trigger maturation of DCs by integrating the features of tumor antigens in TEX, CD40/CD40L targeting DCs, and CD40 signaling (49).

Artifactitious EVs From DCs

Dendritic cells are some of the most important antigen-presenting cells. EVs from tumor antigen–stimulated DCs contain pMHC complex, costimulatory molecules, and adhesion molecules and have been proven to be able to elicit MHC-restricted T-cell immunity (50, 52, 86) (**Figure 2d**). In fact, studies show that the *in vitro* effect of activating T-cells by EVs from APCs is conspicuously weaker as compared to that by corresponding APCs, and the presence of unactivated APCs could apparently enhance EVs' ability to stimulate T-cells. In other words, *in vivo* activation of T-cells by EVs from APCs

entails the assistance of APCs (50, 86, 87). Further studies have revealed that these MHC antigen–bearing exosomes from DCs (DEXs) could be transferred to other DCs, resulting in activation of antigen-specific naïve $CD4^+$T-cells (53, 88). Even MHC class II-deficient DCs with costimulatory molecules CD80 and CD86 can adopt DEXs and activate $CD4^+$T-cells. In this way, activated DCs may enhance their ability to stimulate T-cells by generating large amounts of DEXs with pMHC complexes (88). The mechanisms of the antigen-presenting roles of EVs have been well reviewed previously (7, 11).

Therefore, we are led to assume an effective adaptive immune activation process in which foreign TEVs can be recognized, processed, and presented by DCs to T-cells. Meanwhile, stimulated DCs release EVs, which causes other unstimulated DCs to engage in antigen presentation and finally enhance their immunostimulatory effects.

ASSOCIATED THERAPEUTIC APPLICATIONS OF EVs IN LUNG CANCER

Because the mechanisms by which the EVs from tumor or immune cells regulate immunity in lung cancer have been partially, if not fully, understood, we may work out protocols that specifically target relevant mechanisms to minimize EVs' immunosuppressive effects or maximize their antitumor immunity by modifying EVs or using EVs as immunogenic drug carriers (**Figure 3**). In the following sections, we discuss existing or ongoing research exploring EV-related applications in the treatment of lung cancer.

Blocking the Function of EVs

Directly Blocking the Production of EVs

Because EVs play significant roles in suppressing anticancer immunity and tumor progression through various pathways (21), blocking the generation or release of EVs is believed to be a feasible way to eliminate their immunosuppressive function. As described above, Hsp72 of TEXs from various tumor cells, including lung cancer, could restrain tumor immune surveillance by activating the MDSCs' immunosuppressive activity by triggering the TLR2 signaling pathway (21). Dimethyl amiloride (DMA), an inhibitor of the H^+/Na^+ and $Na^+/Ca2^+$ channels, and omeprazole, a K^+/H^+ ATPase inhibitor, can inhibit the secretion of exosomes (89, 90) (**Figure 3B**). Therefore, authors have tried to learn if the two drugs can decrease exosome secretion and reverse the activation of MDSCs (21). Chalmin F et al. proved that DMA alone exerted little or no effect although combined therapy (cyclophosphamide plus DMA) could apparently enhance the tumor-inhibitory ability of cyclophosphamide (stimulating T-cell immunity by eliminating regulatory T-cells) by blocking the immunosuppressive function of MDSCs in three non-lung cancer models (21). DMA in combination with CpG could also achieve a synergic effect. Moreover, a human study that used its analog, amiloride (currently used for the treatment of edema or high blood pressure and shown to be able to decrease secretion of exosomes), in 11 patients with colorectal metastatic cancer and high blood pressure showed that MDSCs in the blood of patients had lower phosphorylation of STAT3 and suppressive function (21). Overall, this research proves that blocking the secretion of TEXs could restore certain immune function and enhance the effectiveness of other treatments. Moreover, multiple chemicals that block generation or secretion of EVs were also tried as therapeutic agents for lung cancer (to reverse tolerance to chemotherapies) or other cancers (91). Their potential roles in restoring the immune function of patients with lung cancer need to be further confirmed.

Blocking the Interaction Between EVs and Targeting Cells

Apart from decreasing the production of EVs, blocking the contact of EVs with recipient cells might produce similar or better effects. Using an A8 peptide that competitively binds to the domain of membrane HSP70 on tumor-derived exosomes can block the combination of HSP70 with TLR2 and restore the anticancer immune response (27) (**Figure 3B**). Elevated levels of PD-L1 on DCs induced by exosomes derived from the LLC may decrease the proliferation of $CD4^+$T-cells and their differentiation into $CD4^+IFN\text{-}\gamma^+$ Th1 cells but increase the differentiation of Treg cells (19). Anti-PD-L1 antibodies have been shown to significantly reverse this immunosuppressive effect (19). It is also worth noting that the 4T1 exosomes (from breast cancer cells) had a weaker suppressive effect on the $CD4^+$T-cells than LLC exosomes. In line with that, treatment with an anti-PD-L1 antibody exerted little restoring effect on the differentiation of $CD4^+$T-cells into $CD4^+IFN\text{-}\gamma^+$ Th1 cells (19). These findings suggest that exosomes from different cancer cell types work differently in suppressing immunity. Identification of new molecules/ligands mediating immunosuppression on TEVs will allow us to find novel and more effective therapeutic targets.

Engineering TEVs Into Immunity-Inducing Vaccines

Many studies find that UV exposure (6, 24), heat treatment (25), or other stresses strengthens the immunogenicity of EVs from tumor cells and, meanwhile, reduces their tumor-promoting properties (**Figure 3A**). Moreover, gene-modified ligands on EVs can strengthen their ability to target tumor and immune cells and finally enhance tumor-specific immunity (26, 49) (**Figure 3A**).

UV Irradiation

Previous studies proved that UV-exposed tumor-derived microparticles (UV-TMPs) (from melanoma, hepatocellular, colon, and lung carcinoma), rather than naturally secreted TMPs, had the ability to stimulate DC maturation and induce T-cell–dependent antitumor immunity (6, 24). The mechanism underlying the maturation of DCs might involve innate DNA in TMPs implicated in the cGAS/STING pathway in DCs. The activation of the pathway could induce the production of type I IFN (24). With regard to antitumor effects, this UV-TMP vaccine has been shown to be effective only as a prophylactic measure and not as a treatment alternative for preexisting tumors (6, 24). Of note, UV-TMPs were more immunogenic than UV-exposed

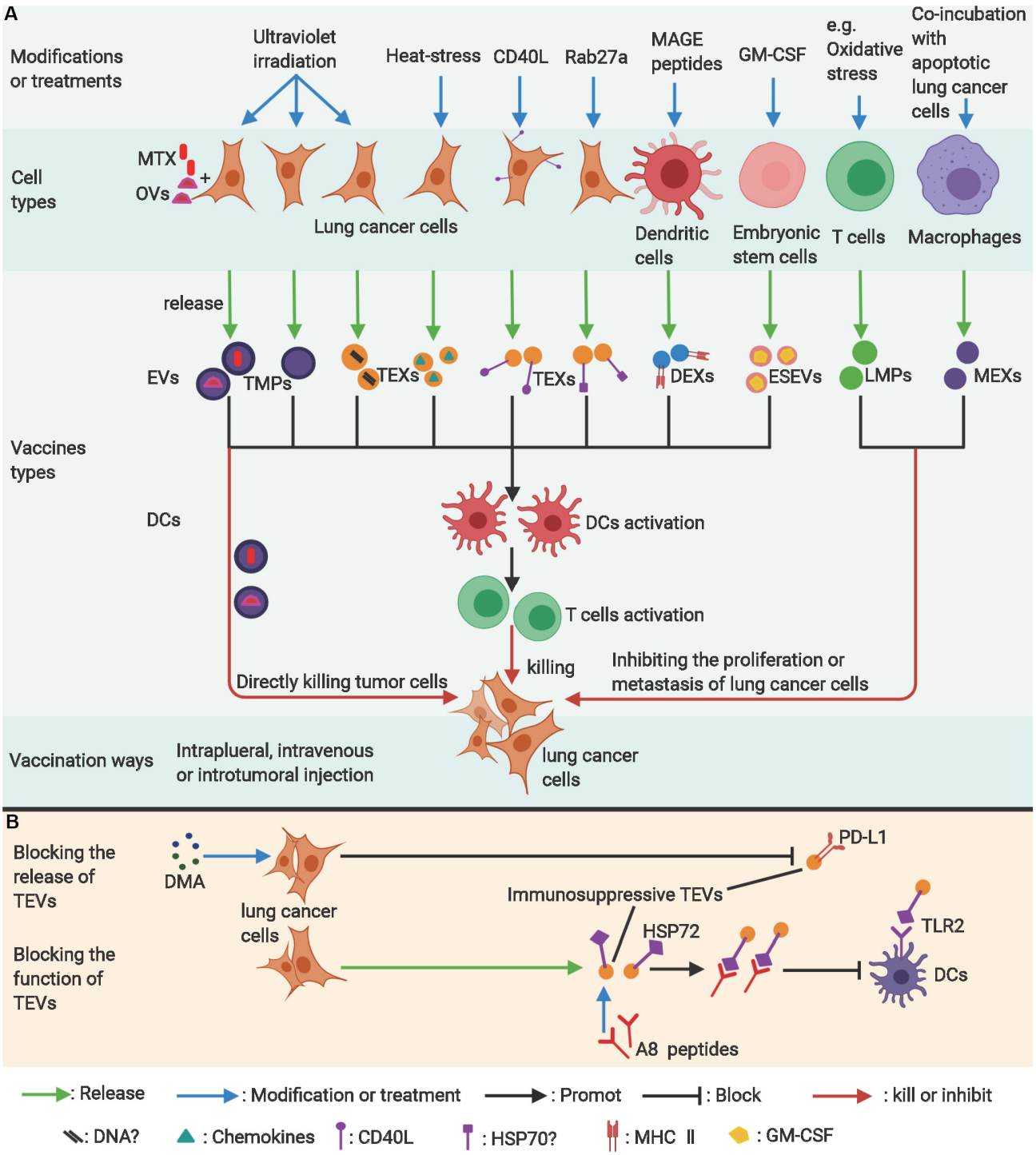

FIGURE 3 | The applications of EVs in lung cancer therapy. **(A)** Modified lung cancer cells, dendritic cells, or embryonic stem cell–derived extracellular vesicles can be used to stimulate antitumor immunity as vaccines. Modified T-cells or macrophage-derived extracellular vesicles can potentially inhibit proliferation of lung cancer cells. **(B)** Blocking the release of exosomes from lung cancer cells (TEXs) or neutralizing the immunosuppressive molecules on TEXs are potentially effective antitumor ways. *?, uncertain; MAGE, melanoma antigen gene; GM-CSF, granulocyte-macrophage colony-stimulating factor; MTX, methotrexate; OVs, Oncolytic virus; TMPs, lung tumor-derived microparticles; TEVs, EVs from lung tumor cells; TEXs, Lung cancer-derived exosomes; DEXs, exosomes from dendritic cells; ESEVs, EVs from embryonic stem cells; LMPs, T-lymphocyte-derived microparticles; MEXs, exosomes derived from macrophages; DMA, Dimethyl amiloride; HSP72, Heat Shock Protein 72; TLR2, toll-like receptor 2*. Figure created with BioRender.com.

tumor-derived exosomes (UV-TEXs) and tumor-cell lysates, suggesting that different types of EVs may possess various immunogenicity (24). Understanding this difference and the underlying mechanisms can help us select EVs that work best as the most effective cell-free vaccines against tumors. However, other studies show that UV-processed TMPs are not invariably immunostimulatory (47, 78) as discussed in previous sections.

Heat Treatment

In addition, other researchers find that heat-stressed 3LL lung tumor cell–derived exosomes (HS-TEXs) induce more efficient DC activation and antigen-specific T-cell immune response than their unprocessed counterparts (25). This might be ascribed to increased content of various inflammatory chemokine ligands in HS-TEXs, which attract and activate $CD11c^+$ DCs and $CD4^+/CD8^+$ T-cells both *in vitro* and *in vivo* (25). Consequently, intratumoral injection of HS-TEXs could more effectively activate specific antitumor immune response than untreated tumor-derived exosomes, thus inhibiting tumor growth and significantly prolonging survival of tumor-bearing mice (25).

Rab27a Overexpression

Rab27a is generally seen as a key regulator for exosome secretion from donor cells (92, 93). Johnson and colleagues proved that Rab27a regulates the azurophilic granule exocytosis of neutrophils, which was intimately linked to its microbicidal function (94). Rab27a deficiencies in mice impaired the secretion of myeloperoxidase stimulated by lipopolysaccharides (LPSs) *in vivo* (95). Wenhai et al. (26) find that exosomes from Rab27a-overexpressed A549 cells induce more BMDCs into mature DCs than normal exosomes and, subsequently, promote $CD4^+$ T-cell proliferation and exhibit a strong antitumor effect in a mouse model. This mechanism of immune activation might be explained by an increased amount of immunogenic molecules (such as Hsp70 and Hsp90) on the modified exosomes, but this hypothesis needs further verification (26). Intriguingly, other research shows that Rab27a deficiencies decrease the secretion of exosomes and inhibit primary tumor growth and pulmonary dissemination of a metastatic carcinoma (4T1) (93). Actually, exosome secretion does not depend exclusively on Rab27a or Rab27b (12) and may vary with different cells (93). Moreover, Rab27a also participates in the secretion of some non-exosome-associated proteins, which include the metastasis-promoting matrix metalloproteinase 9 (MMP9) (93). The contradictions among these studies involving Rab27 proteins in tumors will not be resolved after fully understanding the role of the Rab27 family.

CD40L Modification

It is feasible to directly engineer costimulatory molecules on TEXs to enhance their immunogenicity (49). Researchers attempted to use CD40L-carrying exosomes as a stronger signal to trigger maturation of DCs by combining the features of tumor antigens in TEX and CD40L on exosomes targeting CD40 on DCs (49). As expected, results show that exosomes from CD40L gene-modified 3LL lung cancer cells had stronger ability than normal TEXs in activating the maturation of DCs and then inducing tumor-specific T-cell activation and protracting the survival of mice inoculated with 3LL cells (49).

The foregoing studies show that tumor-derived extracellular vesicles (TEVs) have the potential to induce specific anticancer immunity in either their natural states or artificially engineered forms. On the basis of these findings, we are led to conclude that the centerpieces of all these studies are the tumor-associated antigens on TEVs, and manipulations of TEVs only serve to enhance their interaction with immune cells while reducing their "immunosuppressive components." However, in fact, antitumor effects of engineered TEVs have been found to be limited in animal research. Moreover, the potential risks of immunosuppression and promoting tumor growth and metastasis may also restrict further application of TEVs as vaccines in clinical practice. On the other hand, even though we can't guarantee that engineered TEVs are adverse reaction–free, we believe that DCs activated by TEVs and DEXs have no undesirable properties (e.g., favoring tumor growth, angiogenesis, or metastasis) and possess similar or even stronger abilities to stimulate adaptive immunity (52–54, 87). In fact, DC vaccines pretreated with TEVs (28) or DEXs (29, 30, 96) for clinical use are being studied actively.

DC Vaccines Pretreated With Modified TEVs

A clinical trial employed autologous DCs as multivalent vaccine in 16 patients with stage IA to IIIB NSCLC who had previously received treatment (28). This DC vaccine was stimulated by apoptotic bodies secreted from an irradiated allogeneic NSCLC cell line that overexpressed Her2/neu, CEA, WT1, Mage2, and surviving cells (28) (**Figure 3A**). Results show that the vaccine was well tolerated although no apparent benefits in clinical outcomes were achieved except in two individuals (28). In addition, though specific and non-specific immunologic responses to vaccines could be found in some patients, there was no significant association between immune responses (as measured by IFN-γ ELISPOT) and clinical outcomes. The result might be attributed to the use of an improper indicator for monitoring immune activation (28). Anyway, this research proves that the DC vaccine is feasible and does have certain biological activities. The study provides some useful information for improving the design of future studies. Indeed, use of multivalent antigens from modified allogeneic tumors and the heterogenicity of patients might be two major causes responsible for the limited efficacy (28).

Engineering DEXs Vaccines

The feasibility of producing autologous DEXs loaded with specific MAGE peptides and the tolerance and safety of the vaccine in $MAGE^+$ NSCLC patients has been proven by a phase I clinical trial (29) (**Figure 3A**). Only grade 1–2 adverse events were monitored, and a few patients with advanced NSCLC achieved a long PFS after immunization (29). Activation of NK cells could be observed in some patients, but no significant increase was found in antigen-specific T-cell activity, which might be ascribed to increased $CD4^+CD25^+$ T-regulatory T-cells (29).

Meanwhile, another phase I study using MAGE-loaded DEXs in patients with MAGE3$^+$ advanced melanoma yielded similar or more optimistic results (96). Encouraged by these successes, the researchers initiated a phase II clinical trial with second-generation DEXs (IFN-γ-DEXs, derived from IFN-γ-stimulated mature dendritic cells) loaded with MHC class I- and class II-restricted MAGE antigens as vaccines (30). This vaccine was designed to enhance both NK and T-cell immune functions and to explore whether it could improve the clinical outcomes of chemotherapy-stabilized/responding NSCLC patients (30). Results suggest that IFN-γ-DEXs could enhance the functions of NKp30-dependent NK cells but failed to significantly induce the activation of antigen-specific T-cells (30). Further studies indicate that the functional enhancement of NK cells was correlated with prolonged PFS of the patients, and this enhancement depended on the reaction of NKp30 on NK cells with its ligand BAG6 on the IFN-γ-DEXs (30). It is noteworthy that previous melanoma studies showed that NK activation, induced by DEX from immature DCs, relied on NKG2DL and the IL-15Ralpha signaling pathway (96, 97). Questions remain to be answered regarding the limited effects of DEX vaccines: whether other antigens could be engineered on DEXs to arouse stronger antitumor immunity and whether combined treatment of DEX vaccines and immune-checkpoint blockers generate stronger immune synergy (30).

Engineering Embryonic Stem Cell (ESC)–Derived Exosomes as Vaccines

Common antigens between tumor and embryonic cells are the immunological basis for using embryonic cells as antitumor vaccines (98). An interesting attempt was conducted to stimulate antitumor immunity by using exosomes from ESCs, which may express similar carcinoembryonic antigens as some tumor cell types (31) (**Figure 3A**). Vaccination with exosomes from granulocyte-macrophage colony-stimulating factor (GM-CSF)–expressing murine embryonic stem cells could prevent the growth of implanted LLC lung adenocarcinoma, B16-F10 melanoma, MC-38 colon adenocarcinoma, and 4T1 mammary carcinoma but not E0771 medullary breast adenocarcinoma in allogeneic mice (31). This vaccine resulted in increased tumor cell-specific CD8$^+$ T-cells and a decreased percentage of MDSCs in the spleen and raised the ratio of CD8$^+$ T-effector cells to Tregs in the tumor (31). This ESEV vaccine could avoid the risk of embryomas/teratomas caused by the whole-ESC vaccine. More studies are needed to ascertain the common antigens between the ESCs and lung cancer cells for the future application of this vaccine.

Functioning as an Immunogenic Drug–Delivery System for Its Specific Tumor Tropism

Tumor-derived EVs have a specific tumor tissue/cell tropism (99). A *in vivo* study using fluoresce DiIC18 to label the EVs showed that paclitaxel (PTX)-encapsulated EVs (EV-PTX) could transform PTX-induced systemic inflammation to peritumoral inflammation (32). Some reviews (100–102) mention that EVs can be made into an effective drug-delivery system for cancer therapy by modifying their tropism.

Human EVs from lung cancer cells have been shown to serve as vehicles for delivering oncolytic virus (OVs) and PTX to reduce tumor growth in nude mice with compromised immune systems (33) (**Figure 3A**). Compared to OVs alone or OVs + PTX, EVs encapsulation could apparently increase infectious titer or transduction ratio of OVs in lung cancer cells and showed a stronger tumor-suppressing effect (33). Further studies proved that the murine lung cancer cell–derived EVs containing OVs and PTX, but not EVs alone, could induce immunogenic death of cancer cells *in vitro* as indicated by the increased expression of calreticulin on the cell surface and the extracellular release of ATP (32). Treatment with a virus, EV-Virus and EV-Virus-PTX, could selectively induce peritumoral inflammation, although not systemic inflammation, as indicated by increased infiltration of TILs (32). The EV-virus could induce stronger cytotoxic immunity than the virus alone, which might be because EV encapsulation may protect the virus from immune surveillance (32). Notably, a systemic inflammatory reaction would take place upon treatment with PTX alone although EV encapsulation could significantly prevent the systemic reaction, suggesting that EVs derived from tumor cells do have strong specific cancer tissue tropism (32).

Recently, in a phase I clinical trial, we intrapleurally administered ATMPs-MTX to advanced lung cancer patients with malignant pleural effusion and produced encouraging results (6). TMPs could be intrapleurally injected into mice quickly, and most of TMPs stayed or assembled in the lungs and tumor tissues. TMPs-MTX show apparent tumor tropism and exert cytotoxicity on tumor cells and tumor-associated macrophages but not on T-cells, and apoptotic tumor cells treated by TMPs-MTX could activate DCs both *in vitro* and *in vivo* (6). Intrapleural infusion of ATMPs-MTX into patients has been shown to be safe, well tolerated, and clinically beneficial without grade 3 or higher toxic effects. In particular, it could create an immune-activated intrapleural microenvironment as indicated by increased effector immune cells and cytokines and decreased suppressive immune cells (6).

Collectively, certain molecules contained in TEVs could make them more stable in blood and help them target tumor issues more accurately, thereby making TEVs an excellent delivery carrier. Enhanced tropism conferred by OVs (32) and modified ligands or use of proper local administration routes (e.g., intrapleural injection) in combination with encapsulated chemotherapeutic agents can effectively enhance the antitumor efficacy of TEVs, partially by activating antitumor immunity.

Modified EVs From T-Cells and Macrophages Directly Inhibit Lung Cancer

Intriguingly, microparticles derived from three types of T-lymphocytes (LMPs), including hominal peripheral T-lymphocytes stimulated by various stimuli (apoptosis, cell division, or oxidative stress), could inhibit the proliferation

of various tumors, including lung cancer *in vitro* and *in vivo* (35) (**Figure 3A**). The mechanism of these non-species-specific antitumor effects is associated with arrest of the cell cycle at G_0/G_1 for upregulated expression of cyclin-dependent kinase inhibitors (CDKIs) in lung cancer cells, consistent with which these microparticles were ineffective for quiescent cancer cells (35). Additionally, researchers have not reached a consensus on the effect of the LMPs on angiogenesis under different stimuli (34, 103, 104). Moreover, researchers also found that exosomes from activated T-cells could enhance the invasiveness of 3LL cancer cells by upregulating the expression of MMP9 through the Fas/FasL signaling pathway (105). In summary, better understanding of how EVs from T-cells under various conditions work differently on lung cancer cells in the future is a prerequisite for developing them as an effective treatment for lung cancer.

A recent study shows that exosomes derived from macrophages (MEXs), stimulated with UV-induced apoptotic lung cancer cells, could inhibit lung metastasis (106) (**Figure 3A**). The mechanism involves the transportation of PTEN protein from MEXs to cancer cells, thereby inhibiting epithelial-mesenchymal transition (106).

Reviewing these related studies can give us a holistic view of the roles of EVs from immune cells on cancer cells and helps us further study the mechanism by which EVs from vaccine-stimulated T-cells (or other immune cells) kill or inhibit tumors.

CONCLUSION AND FUTURE DIRECTIONS

In summary, EVs, as a communication medium between cancer and immune cells, plays significant roles in lung cancer immunity. Modification of tumor or immune cells can alter the immunoregulatory function of EVs even from immunosuppressive roles to immunostimulatory ones. Treatment of lung cancer can be approached by targeting the mechanisms by which EVs mediate lung cancer immunity.

Further exploration of immunoregulatory mechanisms and therapeutic applications of EVs in lung cancer is essential, and existing studies have shown good prospects. On the basis of the aforementioned literature focusing on EVs' roles in immunoregulation and therapy of lung cancer, the authors believe future research efforts should be directed to the following fields.

First, so far, there is still no definitive markers and effective isolation methods for different EV subtypes (9, 107). This means that certain subtypes of EVs in existing studies are most likely to be a mixture of different EV subsets in various proportions. Identifying them was and will be the greatest challenge for the study of EVs. Second, most of the studies focus on the immunoregulatory roles of exosomes and microparticles rather than apoptotic bodies. Generally speaking, they all play an immunosuppressive role during the development of lung cancer, and all of them can be modified in various ways to induce antitumor immunity. Nonetheless, few studies compared the immunoregulatory ability of these EV subsets in cancer. For instance, H22 hepatocarcinoma cell–derived microparticles (H22-MPs) are shown to be more immunogenic than tumor cell lysates and tumor cell–derived exosomes in inducing T-cell-dependent antitumor immunity (41). Of note, though the microparticles, exosomes, and tumor cell lysates in that study were collected from the same number of H22 cells, the pretreatment of donor H22 cells was different, which might impact the immunogenicity of these EV subsets. Therefore, more efforts are needed to compare the immunogenicity of these EV subsets to figure out which type of EV subsets works best as an antitumor vaccine. Third, previous research focuses principally on the phenotypic changes of recipient cells of EVs and not on the underlying mechanisms (19, 26, 78). Further studies should identify potential substances on EVs and associated signaling pathways underlying those functions. Fourth, the accurate assessment of potential risks associated with the application of TEX vaccines, such as metastasis (16) and/or angiogenesis (17), will also determine whether they will be safely put into clinical use. Fifth, Some researchers proved that UV-TMPs could induce differentiation of suppressive M2 macrophages and inhibit antitumor immunity (47, 78), and others proved that UV-TMPs could activate DCs and stimulate antitumor immunity (6, 24). Further studies should look into the functional changes of both DCs and macrophages in the same subject to ascertain the final immunoregulatory outcomes of UV-TMPs. Sixth, effective immune activation entails three prerequisites, including antigen presentation, activation of CD4 + /CD8 + T-cells, and persistent stimulation of cytokines. The immunoregulatory network is so complicated that EV vaccines used for human lung cancer therapy are far from satisfactory (29, 30). ATMPs in combination with MTX have shown encouraging clinical benefits in advanced lung cancer patients with malignant pleural effusion (6). Therefore, EVs in combination with other therapies, such as PD-1/PD-L1 blockade or radiotherapy and so on, should be tried to explore optimal therapeutic regimens for lung cancer.

AUTHOR CONTRIBUTIONS

ZY, JF, and YJ participated in the study design and the manuscript preparation. ZY and JF wrote the manuscript. JX, YL, and MZ contributed to figure preparation. LD and TL made the tables. SW, WG, and YJ were involved in manuscript revision. All authors read and approved the final manuscript.

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REVIEW
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TCR Redirected T Cells for Cancer Treatment: Achievements, Hurdles, and Goals

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Adoptive T cell therapy (ACT) is a rapidly evolving therapeutic approach designed to harness T cell specificity and function to fight diseases. Based on the evidence that T lymphocytes can mediate a potent anti-tumor response, initially ACT solely relied on the isolation, *in vitro* expansion, and infusion of tumor-infiltrating or circulating tumor-specific T cells. Although effective in a subset of cases, in the first ACT clinical trials several patients experienced disease progression, in some cases after temporary disease control. This evidence prompted researchers to improve ACT products by taking advantage of the continuously evolving gene engineering field and by improving manufacturing protocols, to enable the generation of effective and long-term persisting tumor-specific T cell products. Despite recent advances, several challenges, including prioritization of antigen targets, identification, and optimization of tumor-specific T cell receptors, in the development of tools enabling T cells to counteract the immunosuppressive tumor microenvironment, still need to be faced. This review aims at summarizing the major achievements, hurdles and possible solutions designed to improve the ACT efficacy and safety profile in the context of liquid and solid tumors.

Keywords: TCR - T cell receptor, genetic engineering, cancer immunotherapy, adoptive T cell immunotherapy, cancer immunoediting

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INTRODUCTION

Adoptive T cell therapy for cancer (ACT) is a branch of cancer immunotherapy that relies on the ability to redirect T cell specificity to selectively target tumor antigens. ACT stemmed from two remarkable clinical observations: (i) The magnitude of T cells infiltrating tumor masses often correlates with response to treatment (1) and (ii) Allogeneic donor T cells infused in the context of hematopoietic stem cell transplantation promote clinical response in hematological malignancies (2). Initially, ACT solely relied on tumor-specific T cells isolated from the tumor masses and expanded *in vitro* (3). This approach was limited to resectable tumors from which enough T cells could be harvested and expanded. The development of gene engineering technologies dramatically changed the landscape of the ACT field, rapidly making this treatment accessible to an unprecedented number of patients and tumor types. By inserting an exogeneous T cell receptor (TCR) into cells, T cells specificity could be precisely redirected toward selected tumor

antigens (**Figure 1**). This new opportunity shifted the research focus and raised some novel questions: the main issue was no more how to harvest a sufficient number of tumor-specific T cells from each single patient, but how to isolate and harness high-avidity tumor-specific TCRs, and how to proficiently generate and expand the most fit engineered T cells. The flexibility of the genetic modification tools offered the chance to insert and/or remove different genes in T cells and to permanently express, in the therapeutic products, entirely synthetic molecules. A striking deliverable produced by these efforts is represented by T cells expressing Chimeric Antigen Receptors (CARs), that generated astonishing clinical results against blood malignancies (4–9).

The outcomes of the first ACT clinical trials contributed to further elucidate the complex interplay between the immunosuppressive tumor microenvironment and the cellular players of immunity. Results suggested that modulation of *ex vivo* T cell expansion protocols and additional engineering of the T cell genome could be used to tweak T cell qualities, improving persistence and functionality of the therapeutic products. Several strategies were proposed to improve engineered T cell persistence, homing ability to the tumor site, capacity to recognize and eliminate tumor cells, and represent today's intense research lines. The possibility to modulate TCR affinity and T cell costimulatory and inhibitory signal pathways opens up novel therapeutic scenarios. The following review has the scope to summarize the cornerstones and the most relevant hurdles and efforts currently pursued to improve ACT.

FROM ALLOGENEIC STEM CELL TRANSPLANTATION TO ADOPTIVE T CELL THERAPY

Allogeneic Hematopoietic Stem cell transplantation (Allo-HSCT) is a therapeutic modality relying on the infusion of hematopoietic stem and progenitor cells, harvested from a healthy donor, to a patient previously conditioned with high-doses chemo-radiotherapy. Although initially developed to regenerate the bone marrow of patients with genetic diseases or with hematological malignancies requiring strong myeloablative chemotherapy (10, 11), Allo-HSCT proved able to control malignant cells largely through an immunological mechanism, as delineated by two major observations. Firstly, T-lymphocyte depleted grafts had a decreased efficacy in eradicating malignant diseases, suggesting that the donor to host immune response, and in particular the activity of allogeneic T cells, had *per se* an effect in abating the risk of relapse after transplant (2, 12, 13). Secondly, the infusion of circulating mature lymphocytes harvested from the donor (donor lymphocyte infusion, DLI) (14) correlated with the anti-leukemic effect (graft vs. leukemia, GvL) in a dose-dependent fashion (15). The efficacy of Allo-HSCT and DLI in restoring a state of disease remission represents one

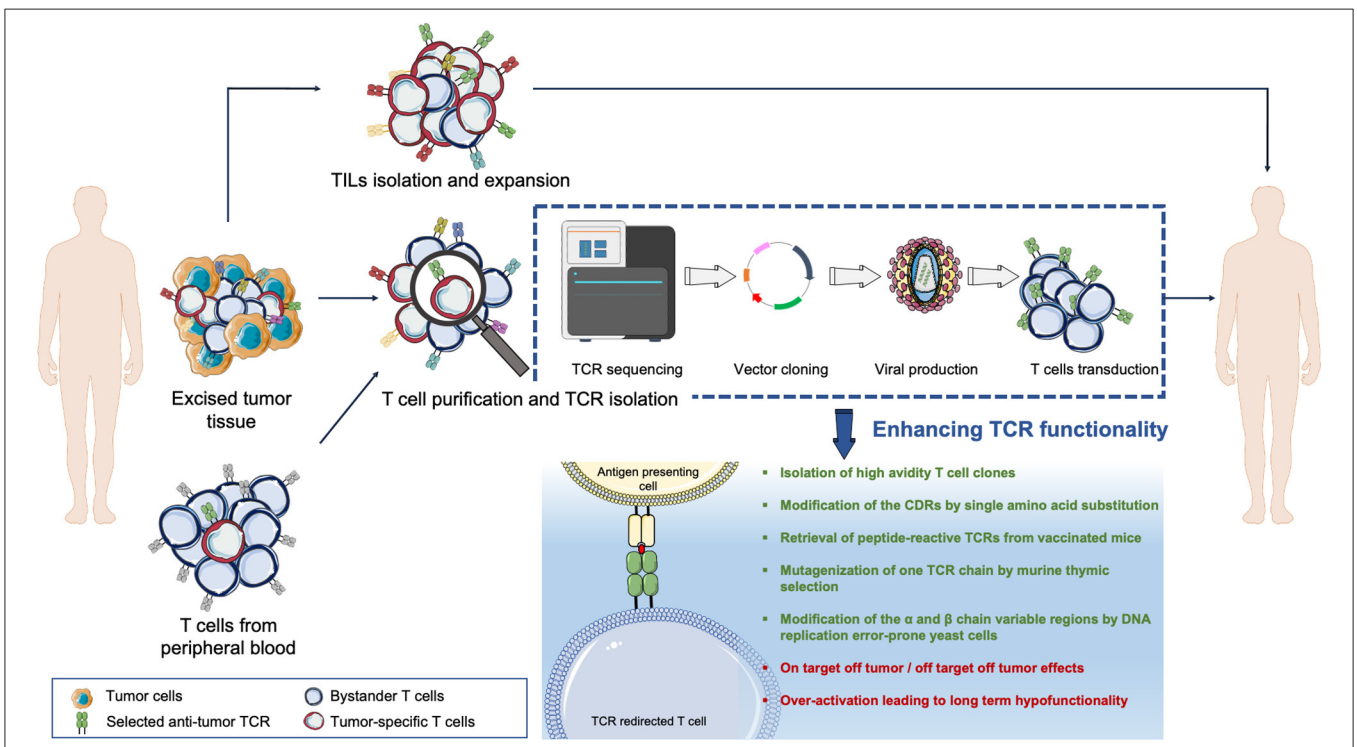

FIGURE 1 | Overview of the TCR adoptive T cell therapy. Tumor-reactive lymphocytes can be isolated from either the tumor mass (tumor-infiltrating lymphocytes, TILs) or from the T cell pool circulating in patients' peripheral blood. T cells can be expanded *in vitro* and then re-infused back into the patient such as in TILs therapy. Else, the tumor-reactive T cell Receptor (TCR) genes can be isolated, sequenced, and transferred into acceptor T cells via vectors to redirect T cell specificities against tumor epitopes.

of the first compelling evidences of the potential of adoptive T cell therapy. Unfortunately, the benefits of allogeneic transplant and DLI against cancer are counterbalanced by toxicities, mainly due to the presence of a heterogeneous TCR repertoire with unknown specificities in the infused T cell population. Indeed, it's been calculated that ∼10% of the T cell repertoire circulating in healthy donors is alloreactive (16). The most common manifestation of such toxicities is graft vs. host disease (GvHD), an immune reaction against the host's healthy tissues, occurring with varying degrees of severity but potentially fatal. The efforts to reduce toxicity while preserving the efficacy of DLI, and to export this therapeutic opportunity beyond the HSCT context, were the driving forces in promoting innovative ACT approaches.

The first ACT strategies tested with autologous T lymphocytes were based on the isolation of T cells infiltrating primary lesions resected from patients with melanoma (tumor-infiltrating lymphocytes, TILs), followed by their *in vitro* expansion with high-doses of interleukin-2 (IL-2) (17). The infusion of these cellular products, composed of an oligoclonal T cell repertoire incorporating $CD4^+$ and $CD8^+$ T cells, mediated potent anti-tumor responses with no toxicities in cell types other than melanocytes (3, 18, 19). The Objective Response Rate (ORR) observed was 41% across various clinical trials for patients with metastatic melanoma (20). Based on these encouraging results, the approach was widened and offered to patients affected by other solid tumors with variable outcomes, promising in some settings [e.g., sarcoma (21), cervical and ovarian cancer (22, 23)] but rather modest in others [e.g., renal (24), metastatic renal (25) and, colorectal (26, 27) cancer, **Table 1**]. The inconsistent efficacy of TILs may be linked to various causes: (i) the technical difficulties in isolating T cells from immune-cold tumors (44); (ii) the poor reactivity of the screened T cells, especially in tumors characterized by a low mutational burden (45, 46), and (iii) the overall low frequency of tumor-specific T cells infiltrating cancer lesions when compared to bystander T cells (47, 48). The high success rate of TILs therapy in melanoma can be in fact explained by the melanoma cells high tumor mutational burden, resulting in a heightened immunogenicity and a consequent enrichment of tumor-specific T lymphocytes (49).

To expand the beneficial effect of TILs while overcoming the hurdles intrinsically associated with this therapy, the use of circulating T cells, harvested from patients and stimulated *in vitro* with immunogenic cancer epitopes, was proposed. This approach promotes the selective expansion of the tumor-specific T cell fraction, in numbers sufficient to enable their re-infusion to patients, resulting in clinical benefits (50, 51). Nonetheless, the use of TILs and circulating T cells lead to the generation of a T cell population for which the affinity and functionality of the TCR could not be predicted *a priori* and whose ability to effectively induce clinical responses was tightly linked to the expansion potential of harvested cells.

Gene editing and gene transfer technologies greatly boosted the ACT field, allowing modification of the T cell genome and redirection of T lymphocytes specificities by inserting highly functional, tumor-specific TCRs (52) into patients' T cells, that could be subsequently expanded *in vitro*. In the 90s the discovery that the Fab region of an antibody could be efficiently fused to the CD3 zeta chain and to other costimulatory intracellular domains to create Chimeric Antigen Receptors (CARs), further revolutionized the T cell-based immunotherapy field. CARs are able to activate T cells upon binding to a surface receptor expressed by the target tumor cell (53) and their use proved instrumental in widening the therapeutic window of blood tumor treatment in otherwise poor survivors (4–9), thus confirming TCR-engineered T cells as a new therapeutic.

MAKING TUMOR SPECIFIC T CELLS: FROM TCR GENE TRANSFER TO TCR GENE EDITING

The ability of T cells to respond to a wide spectrum of foreign antigens relies on the high variety of TCRs, heterodimeric glycoproteins composed of one α and one β chain associated to the CD3 complex (54), able to specifically interact with antigenic peptides bound to human leukocyte antigen (HLA) restriction elements (55). A series of genetic rearrangements in the α and β chain genes occur slightly differently in every single cell, thus creating a heterogeneous TCR repertoire that can recognize a vast epitope array. Hence, to fully characterize the T cell specificity it is necessary to determine the rearranged α and β chain sequences.

In the 80s, the progression of genomics allowed the isolation of TCR genes (56, 57) and the study in detail of their sequences. The advent of next generation sequencing technologies rendered feasible a comprehensive identification of tumor specific TCR sequences that are today used to genetically engineer T lymphocytes in adoptive T cell therapy studies.

Gene Transfer at the Service of ACT

Most TCR-based gene therapy approaches rely on the *ex-vivo* transduction of T cells with viral vectors. The first vectors used in gene therapy were adenoviruses (58), vectors endowed with high cargo capacity (up to 30 kb) but unable to foster transgene integration in the host genome. This feature reduced the adenoviruses utility for ACT: since T cells robustly proliferate upon antigen encounters, integration of the transferred TCR genes in their genome is critical to the preservation of transgenic specificity in T cell progeny. Furthermore, the immunogenicity of adenoviral proteins, highlighted by the high incidence of adenovirus-specific neutralizing antibodies in humans, potentially leads to viral inactivation (59) or to life-threatening inflammatory responses (60), thus limiting their exploitation. Retroviral vectors (RV), instead, have been broadly used because of their wide cell tropism (61, 62), good integration capacity, and for the high and stable gene expression they convey. Cell division is required for RV transduction, but this limitation does not impact their use since T cells are highly proliferating *in vitro*. RV have been widely used to deliver a variety of molecules, including suicide genes (63–65), TCRs (28, 66), and CARs (53) in T lymphocytes. Lentiviral vectors (LV) gained interest more recently, particularly for their efficiency profile and their capacity for transducing dividing as well as non-dividing cells, a feature particularly relevant for the genetic

TABLE 1 | Overview of TCR-engineered T cell-based clinical trials.

Disease	Epitope	Antigen	Antigen type	HLA restriction	Vector (nuclease)	Number of treated patients	ORR (%)	Infusion toxicities	References
Melanoma	AAGIGILTV	MART-1	TAA (tissue restricted)	HLA-A*0201	Retrovirus	17	2 (12%)	none	(28)
Melanoma	AAGIGILTV	MART-1	TAA (tissue restricted)	HLA-A*0201	Retrovirus	20	6 (30%)	14 (skin rash), 11 (uveitis), 10 (hearing loss)	(29)
Melanoma	KTWGQYWQV	gp100	TAA (tissue restricted)	HLA-A*0201	Retrovirus	16	3 (19%)	15 (skin rash), 4 (uveitis), 5 (hearing loss)	(29)
Melanoma and synovial sarcoma	SLLMWITQC	NY-ESO-1	TAA (cancer/testis antigen)	HLA-A*0201	Retrovirus	11 and 6	5 (45%) and 4 (67%)	none	(30)
CRC metastatic and synovial sarcoma	IMIGVLVGV	CEA	TAA (tissue-restricted)	HLA-A*0201	Retrovirus	3	1 (33%)	3 (severe colitis)	(31)
Melanoma	EVDPIGHLY	MAGE-A3	TAA (Cancer/testis antigen)	HLA-A*01	Lentivirus	2	n.a.	2 (death due to cardiac toxicity)	(32)
Metastatic melanoma, sinovial sarcoma and esophageal cancer	KVAELVHF	MAGE-A3	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus	7, 1 and 1	5 (56%)	2 (death), 2 (CNS symptoms)	(33)
Metastatic melanoma	EAAGIGILTV	MART-1	TAA (tissue restricted)	HLA-A*0201	Retrovirus	13	9 (69%)	2 (skin rash), 2 (CRS)	(34)
Esophageal cancer	KVAELVHF	MAGE-A4	TAA (cancer/testis antigen)	HLA-A*2402	Retrovirus	10	0 (0%)	None	(35)
Multiple Myeloma	SLLMWITQC	NY-ESO-1	TAA (cancer/testis antigen)	HLA-A*0201	Lentivirus	20	16 (80%)	None	(36)
Sarcoma plus myeloma	SLLMWITQC	NY-ESO-1	TAA (cancer/testis antigen)	HLA-A*0201	Retrovirus	18 and 20	11 (61%) and 11 (55%)	None	(37)
Leukemia	CMTWNQMNL	WT1	TAA (Transcription Factor)	HLA-A*2402	Retrovirus	8	2 (25%)	None	(38)
Metastatic synovial sarcoma	NY-ESO-1^{c259}	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus	12	6 (50%)	11 (BM suppression)	(39)
Leukemia	RMFPNAPYL	WT1	TAA (Transcription Factor)	HLA-A*0201	Lentivirus	12	12 (100%)	9 (GvHD)	(40)
Synovial sarcoma, osteosarcoma, liposarcoma, peripheral malignant nerve sheet tumor	SLLMWITQC	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Retrovirus	10	2 (20%)	1 (CRS)	(41)
Myeloma/liposarcoma	SLLMWITQC	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus (CRISPR-Cas9)	3	0 (0%)	None	(42)
Synovial sarcoma	SLLMWITQC	NY-ESO-1	TAA (Cancer/testis antigen)	HLA-A*0201	Lentivirus	30	9 (30%)	n.a.	(43)

BM, Bone Marrow; CEA, Carcino Embrionic Antigen; CNS, central nervous system; CRS, Cytokine Release Syndrome; gp100, glpycoprotein 100L; MAGE-A3/A4, Melanoma-Associated Antigen A3/A4; MART-1, Melanoma Antigen Recognized by T cells 1; NY-ESO-1, New York Esophageal Squamous Cell Carcinoma-1; ORR, Overall Response Rate; TAA, Tumor-Associated Antigen; GvHD, Graft vs. Host Disease; TCR, T cell Receptor; WT1, Wilms Tumor 1.

manipulation of stem cells (67). The safety profile of RV and LV is guaranteed by a vector design ensuring replication incompetence (68, 69) and has been proven in human trials (70–72). Adeno-Associate Viruses (AAV) (73) have been widely used in cancer gene therapy and proven to be well-tolerated and safe. Still, the need to synthesize the complementarity strand to promote transgene integration represents a limitation. To circumvent the process, both strands can be packaged as a single molecule to pair and form a dsDNA as a self-complementary AAV vector (scAAV). While this technological advancement allowed AAV to be independent from host cell complementary strand synthesis (74), it almost halved the vector packaging capacity. Nonetheless, scAAV outperformed conventional AAVs in terms of efficacy in preclinical models (75, 76).

Integrating viral vectors insert the genetic cassette semi-randomly into the host genome, thus potentially leading to unwanted insertions in exons, that leads to disruption of the gene hit, or in enhancer regions, potentially altering gene regulation. Theoretically, viral integrations in oncogene regulatory elements or in tumor suppressor genes may contribute to oncogenic transformation (77), a rare event that, most importantly, has never been reported in engineered T cells. Nevertheless, several strategies have been implemented to increase the safety profile of integrating vectors. These include the elimination of viral genes responsible for virulence (78), splitting packaging genes into different plasmids (79), and the introduction of inactivation switches in the vectors constructs (80). In addition, chromatin insulator elements can be added to the flanking regions of the insertion cassette, acting as physical barriers to hamper the interactions between viral enhancers and other regulatory elements (81). To increase the safety profile of viral-mediated gene delivery, it is nowadays possible to instruct viral vectors to integrate into specific "safe harbors," genomic regions distant from transcribed genes, enhancers, regulatory RNA, or microRNA regions to minimize the risk of perturbing gene expressions (82, 83). Identified safe harbors are either housekeeping genes, e.g., AAVS1 (84, 85) and ROSA26 (86), or specific loci not affecting gene expression and identified by mapping the viral vectors integration sites (87).

In addition to viral vectors, a variety of non-viral gene transfer methods have been explored to transfer transgenes into T cells (**Figure 2**). Transposons are mobile elements composed

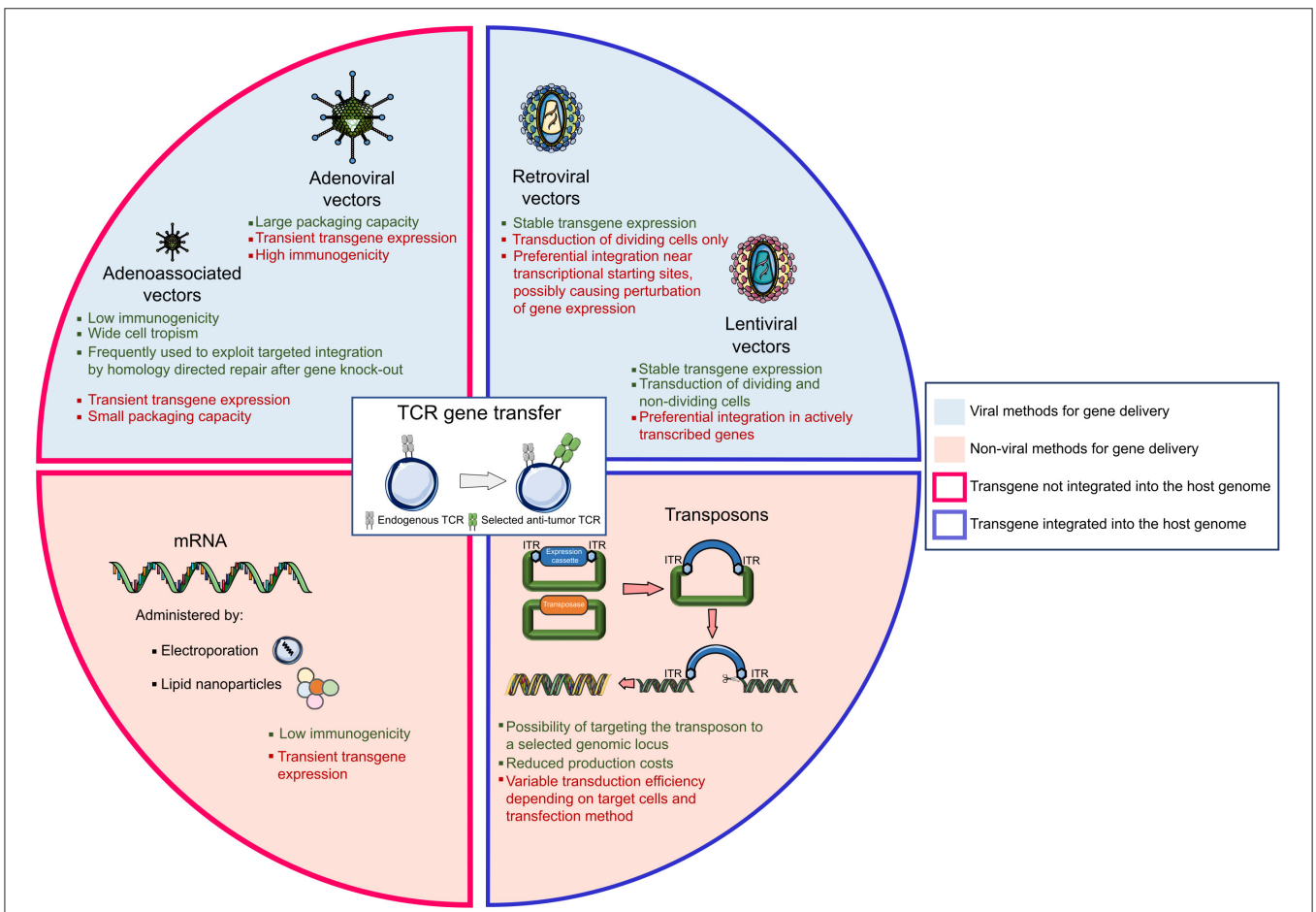

FIGURE 2 | The landscape of gene delivery methods. The genetic transfer of an exogeneous T cell receptor (TCR) into a donor T cell can be obtained with different vectors, the most widely used being viral vectors, mRNA, and transposons systems. Strengths and weaknesses are listed for each technology.

of a transposase gene flanked by inverted terminal repeats (ITRs) (88). For the purpose of gene therapy, two plasmids are transfected together, one encoding for the transposase and the other one containing the expression cassette flanked by ITRs; upon entry, the transposase integrates the gene of interest in the genome. The so-called "Sleeping Beauty" transposon system gained the widest application, being able to transfer up to 6 kilobases into mammalian cells (89). This system may be considered as efficient as viral gene transfer, at least *in vitro*, if the transposon/transposase ratio is tightly controlled (90) to avoid the formation of functionally inactive transposase oligomers (91). In general, transient expression of the transposase is usually preferred, and ensured by mRNA electroporation (92). Transposons have an acceptable production cost and a low immunogenicity potential. Nonetheless, gene transfer efficiency varies according to the target cell and is sensitive to the size of the expression cassette. In the ACT field, transposons have been efficiently utilized to express functional CARs (9, 93–95) and TCRs (96–98) but their exploitation in clinical practice is still limited.

Messenger RNA-based gene transfer, usually achieved by mRNA electroporation or by enclosing the mRNA into lipid nanoparticles (99), is devoid of insertional mutagenesis risk. However, mRNA can convey only transient transgene expression and *in vitro* transcribed mRNAs could trigger cellular inflammatory reactions, whose incidence can be mitigated by introducing base modifications in the synthetic RNA (100). In ACT studies, CAR-T cells have been generated using mRNA gene transfer; still, multiple administrations of the engineered T cells were necessary to mediate tumor regression. Of interest, in a phase I clinical trial, CAR-T cells targeting mesothelin generated upon mRNA electroporation and administered to patients with advanced cancers proved safe and mediated anti-tumor activity despite transient persistence (101).

TCR Gene Transfer

In 1986, a murine exogenous α and β TCR gene pair was successfully transferred into another cytotoxic T cell, endowing the recipient cell with a new TCR specificity (52). The efficacy of the TCR gene transfer was tested in immortalized T cells, where the cDNA of a MART-1-specific TCR isolated from melanoma TILs was stably expressed (102) and, shortly after, on primary human T cells, granting recipient T cells cytolytic activities specifically toward their target epitope. The encouraging safety and efficacy pre-clinical results observed by targeting MART-1 (103, 104), the murine MDM2 oncoprotein (105) and the EBV-associated LMP protein (106), prompted the approval of TCR gene transfer in human clinical trials. In the context of metastatic melanoma, TCR-transferred T cells successfully induced tumor regression in two out of 15 patients and persisted *in vivo* for at least 2 months after infusion (28). The safety profile of TCR-transferred T cells specific for MART-1 or gp100 was similar to that of TILs, with on-target off-tumor toxicities toward skin and eye melanocytes (107). Thanks to these seminal results, the TCR transfer clinical application was widened to other relevant targets, such as the New York esophageal squamous cell carcinoma (NY-ESO)-1, expressed in Melanoma and Synovial Sarcoma (30, 43) and the carcinoembryonic antigen (CEA), expressed in colorectal cancer (31).

The broader use of TCR transfer, however, underlined some of the limitations of this new technology. Firstly, endogenous and exogenous TCRs competed for assembly with the CD3 subunits (108), thus resulting in suboptimal surface expression of the transferred receptor. Secondly, α and β chains from exogenous and endogenous TCRs could mis-pair, further diluting the expression of the correctly paired tumor-specific receptor and introducing new specificities, potentially leading to unwanted toxic reactivities (109). TCR mispairing has been described *in vitro* by using human cells (109) and was associated to immune-mediated toxicities in murine models (110). So far, no events potentially associated to TCR mispairings have been reported in clinical trials. Nonetheless, to address this safety issue and to increase the expression level of the exogenous TCR, several strategies have been proposed: (i) the replacement of the human TCR constant region, essential for pairing, with a murine-derived sequence (111), (ii) the introduction of cysteine residues to stabilize proper pairing of the TCR chains via disulphide bonds (112, 113), (iii) the generation of a human TCR incorporating the CD3ζ chain (114), (iv) the swapping of TCR constant domains between the α and β chains (115), and (v) the incorporation in the vector cassette of small interfering RNA sequences able to reduce the expression of the endogenous TCR genes (116). To overcome the limitations of TCR gene transfer, nascent genome editing technology has been exploited to develop the TCR gene editing approach (117).

Genome Editing in the Service of ACT

The use of artificially modified nucleases enables the disruption of the genes encoding α and β chains of the endogenous TCR, thus completely and permanently avoiding the risk of TCR mispairing and the mutual dilution effect resulting from the expression of four TCR chains in a single cell. Artificial nucleases bind DNA in selected genomic regions, in which they mediate a DNA double-stranded break (DSB), either repaired by the high-fidelity homologous direct repair (HDR) system or by the mutagenic non-homologous end joining repair machinery (NHEJ). HDR uses a DNA template, usually the sister allele, to correct the break and restore gene function, while NHEJ introduces or erases a variable number of nucleotides upon repair, with the chance of creating premature stop codons and frameshift mutations. Both repair mechanisms can be exploited for gene therapy purposes, with different aims: HDR is suitable for gene correction when an exogenous donor DNA template is delivered with the nuclease (118), while NHEJ is preferred if a gene has to be disrupted (119).

The zinc fingers nucleases (ZFNs), among the first efficient gene editing tools developed, are large multimeric molecules, each monomer targeting a 3-4 DNA base pair sequence, linked to the FokI endonuclease (120). While the multimers confer ZFNs specificity, that can be increased even by elongating the length of the multimers, the endonuclease mediates DNA cleavage. This gene editing tool supported the first genome editing clinical applications (121) and the first TCR gene editing approach. In fact, ZFN-mediated disruption of the endogenous TCR has been combined with LV gene transfer to efficiently generate

WT1-specific TCR-edited T cells that outperformed TCR gene transferred T cells in safety, specificity, and efficacy *in vitro* and *in vivo* (117). Despite these encouraging results, the first protocol reported required 40 days of manufacturing to be completed and multiple manipulation steps. To improve feasibility, a single-editing strategy, based on the sole disruption of the TCR α chain gene was proposed resulting in optimal expression of a NY-ESO-1-specific TCR and efficient tumor rejection in animal models, in the absence of adverse events (122).

An alternative to the ZFNs system is represented by transcription activator-like effector nucleases (TALENs), small (33–35 amino acids) transcription factors fused with an endonuclease domain (123). TALENs specificity is modified by mutating the two hyper-variable residues that bind the DNA helix. The nucleotide sequence recognized by TALENs is fairly short, increasing the likelihood of off-target binding sites throughout the genome and potentially leading to unwanted DNA breaks. To overcome this limitation, the DNA binding regions can be elongated by multiplying the hyper-variable residues, hence increasing TALENs specificity at the expense of a more complicated protein design. In the ACT context, TALENs have been proficiently used to disrupt endogenous TCR genes in preclinical models and in clinical trials (124, 125).

Meganucleases represent alternative genome editing tools originating from naturally occurring endonucleases that directly bind DNA (126). Meganucleases present some advantages, such as the generation of a 3′overhang at the cleavage site that favors HDR when compared with 5′overhang, and their overall small size, suitable for several delivery methods (127). Still, the difficulty in separating the endonuclease cleavage domains from the DNA binding site limits the number of DNA sequences that can be targeted. To circumvent this obstacle, chimeric proteins have been generated by fusing meganucleases with ZFNs and/or TALENs DNA binding domains, at the expense of increased manufacturing complexity (128, 129).

The introduction of the CRISPR/Cas9 nucleases, bacterial proteins adapted to excise phage DNA fragments (130), completely revolutionized the genome editing field. While ZFNs and TALENs recognize the target DNA sequence via protein-DNA interaction, the CRISPR/Cas9 system relies on a short RNA sequence (single guide RNA, sgRNA). The RNA interacts with the Cas9, conferring the binding specificity and guiding the nuclease activity (131). The CRISPR/Cas9 platform is highly efficient and versatile, since the specific DNA binding is entirely mediated by the sgRNA, short enough to be easily synthetized *in vitro* but long enough to ensure high specificity. Compared to the previously developed nucleases, the CRISPR/Cas9 system provides three major advantages: (i) rapid and relatively inexpensive manufacturing, (ii) the possibility of multiplex genome engineering obtained by simultaneously targeting several genes, and (iii) compliance with several delivery systems adapted to different cell types (132).

Multiplex genome engineering is a remarkable feature of CRISPR/Cas9, not easily achieved with other nucleases. The possibility of disrupting genes in a single step streamlined different editing procedures (133) and had a direct impact on ACT manufacturing processes, where the synchronous disruption of the α and β TCR chains (42, 134) can sensibly decrease the *in vitro* manipulation time. In addition, a template strand can be delivered together with the CRISPR/Cas9 system, allowing the integration of the genetic material exactly at the cleavage site (135).

Different tools can be employed to deliver CRISPR/Cas9 complexes into cells. Plasmid delivery has been used (136), but with suboptimal efficiency and with an increased risk of plasmid integration in the host genome. Furthermore, the expression of the Cas9 protein is retained for a fairly long amount of time, increasing the likelihood of adverse immune responses or off-target gene editing. An alternative approach depends on delivering the sgRNA together with the *in vitro* transcribed Cas9 mRNA (137), ensuring transient Cas9 expression but posing the risk of decreased cleavage efficiency. Lastly, the native Cas9 protein can be pre-assembled *in vitro* with sgRNA in a ribonucleoprotein complex and then electroporated into the target cells (138). This transfer method overcomes the need for transcription/translation and the risk of intracellular degradation of the free sgRNA (139), thus improving safety and reducing off-target mutagenesis risks.

As for ZFNs (140–142) and TALENs (143, 144), a side effect of the CRISPR/Cas9 system is the risk of editing off-target genes. The nuclease activity can potentially cause DNA strand breaks in other genomic regions, knocking-down unwanted genes or promoting genome translocations (145, 146). Off-target editing can also affect the RNA transcriptome, with toxic consequences for the cell (147). Three methods have been employed to minimize off-targets while increasing on-target activity when using CRISPR/Cas9: (i) the use of modified sgRNAs with higher specificity for the target site, (ii) the titration of the sgRNA and Cas9 ratio (148), and (iii) the introduction of a single point mutation in the Cas9 (149). Furthermore, additional enzymes have been incorporated in the system to mediate base editing without affecting the transcriptome (150).

Several techniques have recently been optimized to map off-target cleavage sites. Mutation detection assays using T7 endonuclease followed by deep sequencing of the resulting amplicons have been initially used, but their sensitivity is limited, especially when dealing with large deletions (151). The tendency of integrase-defective lentiviral vectors to incorporate into DSBs can be exploited to barcode regions of Cas9 activity (152). BLESS [direct *in situ* Breaks Labeling, Enrichment on Streptavidin and next-generation Sequencing (153)] can map double-strand breaks by using biotinylated linkers that are incorporated at the DSB site; the biotinylated DNA regions are then purified and the captured DNA fragments sequenced. One of the most sensitive off-target detection assay is GUIDE-seq (genome-wide, unbiased identification of DSBs enabled by sequencing), where short phosphorylated double-stranded oligo-deoxynucleotides are incorporated into DSBs to detect Cas9 cleavage sites (154). More rarely, nucleases can cause chromosomal translocation that can be detected using high-throughput, genome-wide translocation sequencing (HTGTS) methods (155), chromatin immunoprecipitation sequencing (ChIP-seq) (156), and digenome-seq or the recently proposed CIRCLE-Seq (157).

All described genome editing technologies have been employed for the modification of either hematopoietic stem cells or T cells. As summarized in **Figure 3**, genome editing has been exploited with several purposes in the ACT field: to completely redirect T cell specificity (42, 117, 124, 158–160), to avoid the risk of GvHD or fratricide effects mediated by CAR-T cells (161–165), to make adoptively transferred T cells resistant to the immunosuppressive environment (42, 166–173) or to lymphodepleting drugs (174, 175). The high efficiency of gene editing and the overall flexibility of the CRISPR/Cas9 system makes it the most relevant tool to precisely and rapidly edit high numbers of T cells to be used in ACT. Nonetheless, issues remain to be addressed regarding the manufacturing, the delivery, and the broad accessibility of genome editing products to patients (176).

ANTIGEN IDENTIFICATION AND TCR GENE HUNTING

Different Classes of Target Antigens

One of the major questions in today's adoptive T cell gene therapy is the choice of the target antigen. Theoretically, the ideal candidate should be (i) expressed on tumor cells and not on healthy tissues (to avoid toxicities), (ii) expressed on cancer stem cells (to promote tumor eradication), (iii) associated with the oncogenic process (to reduce the risks of tumor immune evasion), (iv) able to elicit an immune response, and (v) efficiently processed and presented in the context of a common HLA allele (177). Unlike CAR-T cells, TCR-redirected T cells can target antigens independently of their intracellular localization, as soon as they are processed and presented by HLA molecules. For the majority of cancer types, the ideal antigen is yet to be identified, and the search is proving more difficult than expected, particularly for those tumors with undefined clonal evolution and not fully understood in terms of molecular pathogenesis.

The two major classes of antigens in the context of ACT are tumor-associated antigens (TAAs) and neoantigens. TAAs are epitopes originated from endogenous wild-type proteins whose expression is increased in tumors and limited in magnitude or in spatial expression in healthy tissues; neoantigens are instead epitopes derived from somatic DNA alterations.

Different TAAs have been investigated for their potential therapeutic relevance (178): cancer/testis antigens such as melanoma-associated antigen (MAGE)-A3 (179, 180), MAGE-A4 (35, 181), and New York esophageal squamous cell carcinoma (NY-ESO)-1 (182); oncogenes/oncosuppressors such as WT1 (50) and p53 (183); tissue-restricted/differentiation antigens such as MART-1 (28, 34), gp100 (29), or CEA (31). The toxicity and safety profile of T cell therapies targeting TAAs seem to be heterogeneous, depending on the chosen epitope. Several TAA-specific TCRs showed important side effects: cardiovascular and neurological toxicities [with MAGE-3 specific T cells (32, 33)], undesired recognition of melanocytes (with MART-1 specific T cells) (28), and severe transient colitis (with CEA specific T cells) (31). TCR targeting NY-ESO-1, instead, conveyed no toxicities but limited clinical response in a small cohort of patients affected by melanoma and synovial sarcoma (30) and of sarcomas/nerve sheet tumors (41). Its application appeared promising in various pre-clinical tumor models, both in terms of efficacy and safety [bladder (184), ovarian (185), esophageal (186) and prostate cancers (187), multiple myeloma (188), medulloblastoma (189), non-small cell lung carcinoma (190) mesenchymal tumors (191), and breast cancer (192)] but clinical studies are needed to validate these results. The expected good toxicity profile may reside in the limited NY-ESO-1 expression in healthy tissues, essentially restricted to the gonads, an immune-privileged site.

Focusing on neoantigens appears to be another efficient choice for cancer immunotherapy (193). Neoantigens represent the main target of autologous T cell responses in patients treated with TILs (194). Since neoantigens peptides are not presented to thymocytes, T cells specific for those epitopes are not deleted by central tolerance mechanisms, thus the chance of retrieving high affinity TCRs is enhanced. However, the clinical exploitation of neoantigens in TCR-mediated ACT is hindered by their intrinsic qualities: (i) neoantigen-forming mutations tend to differ among patients, making difficult the development of a widely applicable immunotherapeutic product, and (ii) neoantigen expression might be heterogeneous across the tumor tissue. This is particularly true for passenger mutations, random alterations caused by genome instability and not homogeneously spread in the tumor mass (45). In addition, the mutational rate is highly variable in different tumors. In fact, the likelihood of identifying neoantigens from tumors with a low mutational load is poor and their relevance as therapeutic targets is limited (195). Despite these limitations, initial reports highlighting the occurrence of immunogenic neoantigens widely shared in tumor cells and among patients are emerging (196). Additionally, thanks to their broad expression in cancer cells and to their involvement in oncogenesis, founder mutations may be considered promising ACT candidate targets (197). However, epitopes arising from founder mutations may be poorly immunogenic or differ among patients, thus reducing their appeal.

Overall, the interest in neoantigens has increased in recent years, and more than 100 clinical trials exploiting these candidates are currently in progress. However, the ongoing trials largely rely on *in vivo* peptide vaccination rather than on engineered T cell infusions, further underlining the difficulties in validating a proper target for adoptive T cell therapy (198).

A third possible choice of targets is represented by Minor Histocompatibility antigens (MiHA), peptides derived by polymorphic intracellular proteins, potentially overexpressed by tumor cells. The most known examples are HA-1 and HB-1 (199–201), expressed selectively by hematopoietic cells and by different liquid cancers, thus constituting highly relevant targets (202, 203). Since MiHA derive from coding regions of polymorphic genomic sites, coupling HA-1-directed T cell therapy with Allo-HSCT from a recipient not harboring the same single nucleotide polymorphism (SNP) could provide a valuable therapeutic strategy, able to spare the donor hematopoietic stem cell population while eradicating cancer. So far, HA-1-specific engineered T cells have been tested *in vitro* and in preclinical models, showing an optimal efficacy and safety profile (204). However, MiHA targeting requires specific combination of SNPs

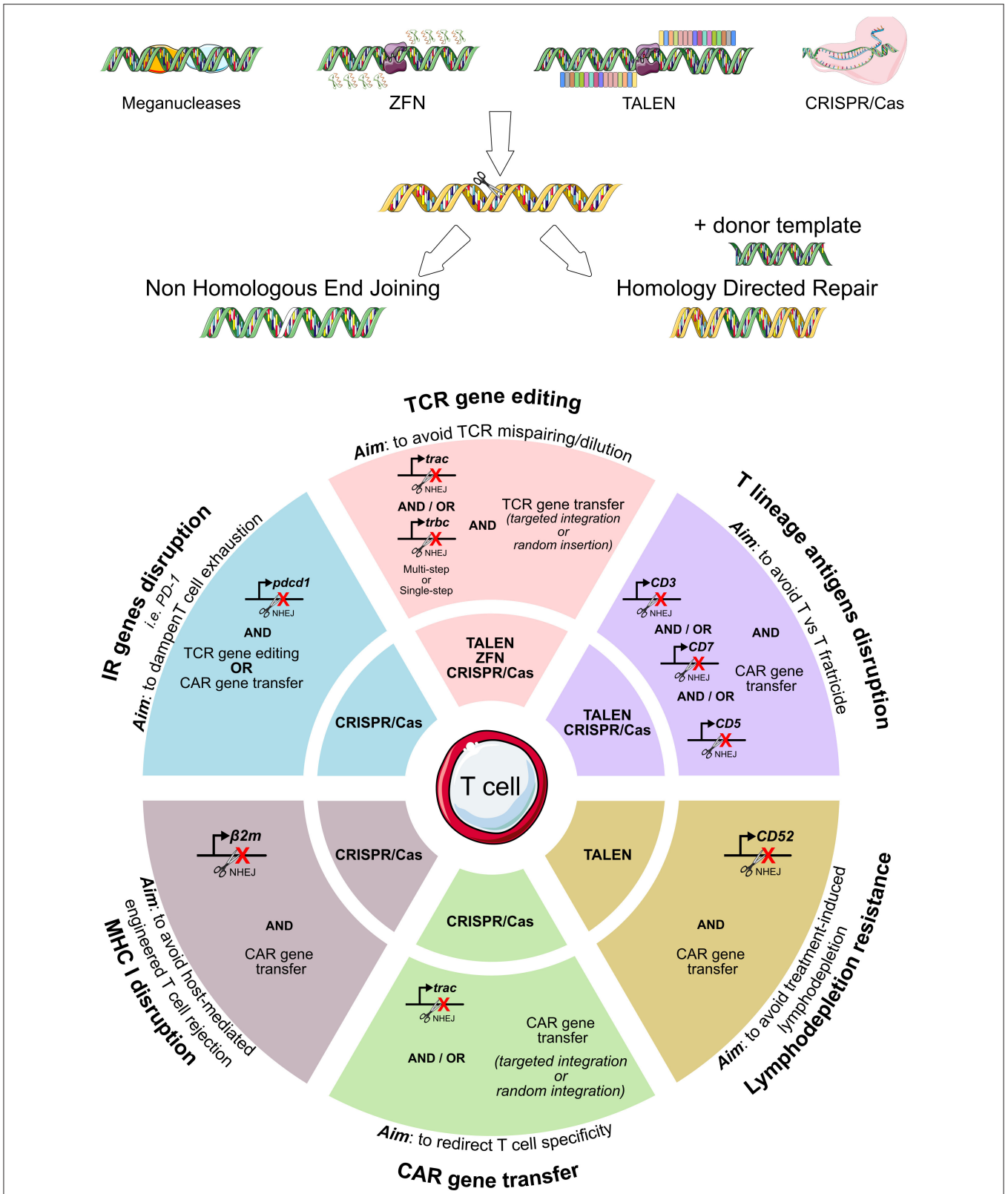

FIGURE 3 | Genome editing exploitation for adoptive T cell therapy. To eliminate the expression of T cell genes, meganucleases, transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs), the CRISPR/Cas9 system can be employed. A summary of the genes edited in the context of adoptive T cell therapy (TCR- or CAR-T cell immunotherapies) is reported, together with the specific nuclease system used. CAR, chimeric antigen receptor; TCR, T cell receptor.

and HLA allele between the donor and recipients, which reduces the broad applicability of this approach.

A fourth source of antigens are proteins encoded by oncogenic viruses. Being involved in tumorigenesis, viral epitopes are shared uniformly by the tumor mass and, due to their nature of foreign molecules, they're potentially highly immunogenic. Hereby, the isolation of high-avidity viral-specific T cells is easier than with other antigen classes, making these targets particularly appealing for ACT. Unfortunately, few tumors have a clear viral pathogenesis, thus this antigen source is currently mostly limited to some HPV and EBV-associated malignancies (205, 206).

The Challenges in Selecting Tumor Antigens and Tumor-Specific TCRs

Apart from choosing the antigen class of interest, tumor screening for immunologically relevant epitopes is a particularly laborious process. The most used and standardized strategy relies on paired whole-exome sequencing or, alternatively, the comparative RNA sequencing analysis of tumor and of healthy tissues to determine differently expressed genes. Once the most promising hits are identified, *in silico* assessment of HLA presentation and binding (207) is required. In this regard, prediction algorithms are still suboptimal (208), not always accurate and they very often lead to false positive results (209). Thus, extensive validation of the results is always required. The analysis of the tumor ligandome can now be considered a good alternative approach. This technology is based on mass spectrometry typing of all the peptides eluted from the HLAs of a specific tumor type or tumor cell line (210). Here too, the amino-acidic sequences retrieved need to be validated for their relevance in the tumor setting. Despite the great potential of this methodology, some limitations still remain, namely the high number of tumor cells needed, an endpoint is not always attainable when using primary tumor samples, and the low number of epitopes retrieved upon *in silico* and *in vitro* screening (211, 212).

Finding the perfect immunogenic epitope is only half of the issue, since the typing of epitope reactive TCRs may prove challenging as well. To isolate a tumor-reactive T cell, a proper source must be selected, the clone of interest must ideally harbor a high avidity TCR and reach a significant level of frequency and purity. These prerequisites are fundamental for the successful retrieval of functional TCR αβ nucleotide sequences.

T cell isolation is particularly challenging especially for poorly immunogenic tumors and for those in which the T cell infiltrate is scant. Recent works showed that, in different cancers, tumor-reactive TILs represent only a minimal fraction of the total T cell subpopulation infiltrating the tumor (47) and that reinvigoration of tumor immunity is associated with recruitment of new T cell clones (213). These observation lead to the hypothesis that selecting T cell clones on the basis of their abundance in the tumor may be misleading. In addition, the poor efficiency of the T cell *ex vivo* expansion procedures may be taken in consideration as a limiting factor in the retrieval of tumor-specific T cells, especially when studying lymphocytes originated from tumors characterized by a microenvironment known to blunt T cell proliferation (214).

When tumor specific T cells are retrieved, the greatest challenge of the TCR sequencing step is the correct pairing of the cancer-specific α and β TCR chains for each T cell clone. The initial approach for the identification of the TCR repertoire was based on single cell cloning coupled with Sanger sequencing (215, 216), which makes it difficult to estimate the overall repertoire diversity. The field greatly benefited from the introduction of high-throughput sequencing technologies which enabled researchers to profile the diversity of millions of TCR molecules in the analyzed samples. With this approach a complete overview of the TCR sequences constituting the repertoire of the sample is obtained (217–219). However, to successfully characterize and select the TCR αβ pair of interest among the numerous sequences retrieved, the specimen needs to be enriched in anti-tumor specificities in order to obtain an oligoclonal population. To this aim sequencing can be preceded by enriching steps, such as co-culturing TILs with tumor cells harvested *ex vivo* or performing serial stimulations with professional antigen-presenting cells pulsed with the peptide (or peptide library) of interest. In the latter case, an entire protein sequence can be screened by epitope scanning (220).

An interesting approach that avoids the *ex vivo* enrichment step is PAIRseq: the sample is split into parallel PCR runs, each one tagged with a specific barcode, and then the results are deconvoluted to identify proper TCR pairs (221). An evolution of this laborious setting is perhaps single cell RNA sequencing, where the use of a cell-specific oligo-DNA barcode allows researchers to retrieve TCRs at single cell resolution (222). The typical output of these systems is generally hundreds or thousands of TCR pairs, questioning whether or not faster sequencing translates to a more laborious validation phase. Alternatively, recent advances in the proteogenomic field may speed up the selection of tumor-specific TCRs. In fact, it is now possible to combine single-cell resolution TCR sequencing with barcoded multimers loaded with a specific HLA molecule and with a selected tumor epitope (222). This approach is extremely helpful in characterizing, in a single step, both the tumor specific TCR sequence and its epitope specificity. This new technology may greatly speed up the isolation of TCRs directly from human samples, avoiding any enriching steps and jumping directly to the functional validation of newly retrieved TCR sequences *in vitro*.

Another point of discussion is the choice of the ideal specimen to be used for the isolation of tumor-reactive T cell clones. Hunting for tumor-specific T cell receptors directly from the tumor site in patients has historically been the most straightforward choice (17) since anti-tumor reactivities can be intuitively more abundant in the tumor mass. However, the development of a tumor implies an escape from immune surveillance, suggesting either that the infiltrating T cells present at the tumor site were not efficient enough to eradicate the disease, thus questioning their use, or that these highly specific cells were blunted in their activity by the tumor microenvironment (223). In the latter case, the anti-tumor efficacy of the tumor-specific T lymphocytes assessed by *in vitro* functional assays upon isolation may not be informative, but

the TCR is worth isolating and employing in TCR transfer approaches. Recent reports (224, 225) also demonstrated that the exhaustion signature could be exploited, defining a T cell subset enriched with neoantigen-specific T cells.

Since tumor-reactive T cells also circulate throughout the body, patients' peripheral blood and lymph nodes from tumor patients are suitable sites to harvest these cells, and often the only available sites for tumors that cannot be excised. Tumor-specific T cells have been found in tumor-draining lymph nodes, and efficiently used for ACT (226). Melanoma-specific T cells have been enriched and expanded *ex vivo* starting from peripheral blood (227). However, at least in some tumor types, the low frequency of circulating tumor-reactive T cell clones (228) might impair their retrieval.

Trading Toxicity With Efficacy

In the process of hunting for new TCR specificities, the aim is to define the TCR sequences most efficiently mediating tumor lytic functions. These highly promising TCRs can be collected and used for off-the-shelf immunotherapeutic approaches readily accessible for each candidate patient. The leading TCRs are the ones with the highest binding affinity and avidity (229) toward the HLA-peptide complex, the fastest association rate and the slowest dissociation speed (230, 231). According to this concept, isolated TCRs were screened for strong and fast killing efficacy and the most suitable ones were further developed. In addition, TCRs were modified in their complementary-determining regions (CDRs) *in vitro* to artificially increase their affinity for the target, thus overcoming the barrier of thymic selection, that deletes thymocytes harboring autoreactive high avidity TCRs. Several approaches have been recently exploited with the ultimate aim of generating high affinity TCRs: (i) mutations in complementarity determining regions by sequential single amino acid substitutions (232–234), (ii) vaccination of mice and consequent retrieval of peptide-reactive TCRs (235), (iii) murine thymic selection to mutagenize one of the TCR chains (236), (iv) transfer of the entire human TCR αβ gene loci into mice to educate T cells against human self-antigens (237, 238), and (v) DNA replication error-prone yeast cells to modify the α and β chain variable regions (239). These techniques have been particularly useful when dealing with TAAs, since highly avid T cell clones recognizing these antigens in a specific and efficient manner have been difficult to isolate.

The advantages of TCR affinity enhancement have been demonstrated *in vitro* (240–242) and in clinical trials (30, 36, 37, 39). The opposite side of the coin, though, is the risk of enabling engineered T cells to respond to tissues displaying low antigen expression, fostering on- or off-target off-tumor toxicities (243). A clinical trial with an artificially enhanced TCR directed against MAGE-A3 proved highly efficient in eradicating tumor cells but was also endowed with a remarkable off-target off-tumor cardiac toxicity (32, 244), leading to the suspension of the trial. On the same line, an artificially-enhanced TCR directed against CEA was associated with the occurrence of on-target off-tumor reactions and strong systemic inflammation, underlining the limitation of procedures aimed at enhancing TCR avidity when targeting TAAs (31). As a matter of fact, it proved very challenging to predict any possible cross-reactivity of engineered T cells against human tissues. The most commonly used techniques, *in vitro* testing and epitope alanine scanning, have been further refined in recent years (241, 244) and extended to scan all the possible amino acid substitutions in the target epitope (245). Nevertheless, concerns about affinity enhancing techniques still persist. In this context it might prove safe to introduce a kill switch in engineered T cells, enabling their ablation if necessary (63, 246). Otherwise, the conditioning regimen prior to T cell infusion can be modulated, reducing therapy-induced tissue damage and antigen spreading, two phenomena potentially fostering off-target reactions.

In addition to the adverse events observed in clinical trials, the modification of TCR affinity may also convey excessive activation signals to T cells, that could lead to hypo-functionality and/or premature T cell death. An extensive and continuous activation is indeed detrimental for T cell function (247–249). Reports in the context of TCR engineering are still scant (40) but new insights from the field of CAR-T cell therapy have highlighted this issue (250). Since TCRs is even more sensitive to antigen density variations than CARs (251), it's reasonable to suppose that this mechanism could be relevant in the context of TCR engineering. The picture might even be more complex with TCRs, since costimulatory signals are more tightly involved in the immunological synapse (252) than in CAR-T cells, a feature that provides more flexibility but that requires greater attention to signal tuning.

PERSISTENCE OF ADOPTIVELY TRANSFERRED T CELLS AND CLINICAL RESPONSES

Nowadays, it's still unclear which variables impact long-term persistence in adoptively transferred T cells the most. This scientific question is particularly relevant because T cell persistence is a fundamental requisite for durable immunosurveillance. Whether immunosurveillance is required for the maintenance of clinical remission is still a matter of debate. However, reports indicate that the sustained and prolonged *in vivo* expansion of engineered T cells correlates with relapse-free survival and tumor control (39, 40). Several measures have been implemented to foster ACT persistence, including the use of preconditioning regimens and the choice of manufacturing protocols able to enrich memory cells. The use of a lymphodepleting conditioning regimen prior to ACT inhibits host immune cells, including regulatory T cells (T_{regs}), and favors the accumulation of homeostatic cytokines (253), critical in sustaining engineered T cell engraftment and expansion (254, 255).

The administration of low-doses IL-2 can also sustain adoptively transferred T cell proliferation *in vivo* (17, 19) and has been included in several ACT protocols. The use of IL-2, however, conveys the risk of toxic reactions related to the activation of bystander host cells. Furthermore, prolonged administration of this cytokine was shown to preferentially expand T_{regs} (256).

The cellular composition, in terms of subsets and differentiation of the therapeutic product, has a direct impact on efficacy. Long telomeres (257), CD27, and CD28 co-expression (258) on TILs were associated to clinical responses in initial ACT trials. With engineered T cell products, the co-infusion of CD4 and CD8 cells fosters T cell persistence (8). In some ACT applications, manufacturing includes a selection step to enrich the product in CD8 central memory (T_{CM}) lymphocytes (259). The polyfunctionality of adoptively transferred engineered T cells correlated with clinical responses in several clinical trials, targeting NY-ESO-1 (36), MART-1 (29), and WT1 (38). The relevance of the intrinsic qualities of the infused T cells was further highlighted by the observation that even low numbers (10^5) of highly fit engineered T cells were sufficient to mediate anti-tumor responses (260, 261). Based on these observations and with the final aim of improving the fitness of the infused T cell products, different T cell expansion protocols have been developed and compared. T cell activation with phytohaemagglutinin (PHA) was shown to promote T cell expansion, but also T cell terminal differentiation (262), whereas stimulation with an anti-CD3 monoclonal antibody coupled with high doses of IL-2 reduced the TCR repertoire diversity and enhanced apoptosis (263). The combination of TCR triggering with co-stimulation, obtained thanks to the use of anti-CD3 and anti-CD28 antibodies, followed by the culture of the T cells in the presence of high-doses of IL-2, improved the fitness of the cellular products (264).

Despite showing potent tumor killing abilities *in vitro*, effector T cells were paradoxically less effective than early differentiated T cells when transferred in tumor-bearing mice (265). These results can be explained by the progressive model of mature T lymphocyte differentiation (266). Upon antigen encounter naïve T cells differentiate into stem cells memory T cells (T_{SCM}), T_{CM} and subsequently in effector memory and terminally differentiated cells, progressively losing proliferating and persistence ability. Recent studies demonstrated that T_{SCM}, originating directly from naïve T cells (267, 268), are endowed with stem cell-like properties and with the ability to persist for decades *in vivo* (269, 270). The persistence capacity of T_{SCM} was confirmed in patients treated with genetically engineered T cells, in the context of both malignant (271) and non-malignant (272) diseases. In the attempt to preserve this early-differentiated T cell subset, the *in vitro* protocol used for TILs expansion was shortened (273) and the anti-CD3/anti-CD28 antibodies were conjugated to cell-size beads (274) or nanomatrixes (275). The cytokine cocktail used to sustain T cell expansion *in vitro* also plays a major role in determining the fitness of cellular products. The introduction of Interleukin-21 in the culture medium promotes a T_{CM} phenotype (276) while Interleukin-7 and Interleukin-15 supplementation, in the absence of IL-2, expands the T_{SCM} pool (277, 278).

As already mentioned, the intrinsic T cell fitness has an impact on the persistence and thus on the efficacy of the T cells used in ACT. In CAR-T cell therapy trials, CAR-T cells isolated from poor responders expressed genes associated with effector memory differentiation and apoptosis, a glycolytic metabolism, and hypo-functionality. Conversely, efficient anti-tumor activity was associated with an early-memory differentiation signature, expression of CD27, and absence of the exhaustion marker PD-1 (259, 279, 280). Similar observations were reported in TCR-based studies. The transfer of a WT1-specific TCR into Epstein-Barr virus-specific donor CD8 T cells has been exploited to generate functional, memory-like cellular products (281). Using this manufacturing procedure, high levels of engraftment and long-term persistence were observed in humans (40). Furthermore, in an ACT trial with TCR engineered T cells, the extent of cytokine release was associated with anti-tumor activity (107).

Once adoptively transferred, T cells interact with the host immune system. Competition of infused and unmodified T cells for proliferative signal accessibility may decrease cell survival. Furthermore, engineered T cells may be recognized and rejected by the host immune system, thus abrogating ACT efficacy. In the autologous setting, rejection could be due to an immune response against the transgene products, as observed in preclinical models (282). An immune response against the murine-derived CD19 CAR was described before and after cell therapy. In the JULIET study (283), the majority of treated patients showed detectable levels of pre-existing anti-murine CD19-specific antibodies, that further increased upon CAR-T cells infusion. Nonetheless, the kinetics of engraftment was unmodified, and rejection barely detected, questioning the relevance of these markers in predicting CAR-T cells persistence. T cell mediated immune responses against Herpes Simplex Virus-derived Thymidine Kinase (TK) epitopes were described in patients treated with TK-DLI, often leading to the elimination of genetically engineered T cells (284). The immunogenicity of TK could be overcome in the HSCT context by infusing transduced T cells during the immunosuppressive phase that follows transplantation (285). For ACT applications that do not involve HSCT, the minimization of transgene immunogenicity remains a desirable and relevant goal.

OVERCOMING BARRIERS TO T CELL HOMING AT TUMOR SITE

The efficacy of ACT is strictly dependent on the ability of the infused product to infiltrate neoplastic lesions. This is particularly difficult in solid tumors, often characterized by a dense stromal architecture, an abnormal vessel structure, and by alteration of chemo-attractants that impinge T cell homing (286–288). In recent years, different strategies have been developed to counteract these factors and hence, increase the ability of T cells to migrate inside neoplastic lesions, where they can properly exert their anti-tumor activity.

Interfering With Cancer Metabolism and Chemokines to Increase ACT Infiltration

The connection between metabolism and oncogenesis is well-documented. Metabolic reprogramming, a hallmark of cancer, does not only impact on cancer cell survival and proliferation, but also on the immunological microenvironment. The presence of reactive nitrogen species (RNS), produced by several human tumors, can induce nitration of different proteins present in

the tumor microenvironment (TME) with consequences on T cell functions (289). As an example, the nitration of the CCL2 chemokine decreases its binding affinity for CCR2, thus reducing T cell recruitment. In mouse models, preconditioning of the tumor microenvironment with small molecules blocking RNS production increased the CCL2-mediated recruitment of adoptively transferred tumor-specific CD8 T cells (290), making it an interesting target for further therapeutic development.

Tumors can alter the fucosylation of T cell surface glycoproteins (291), again impinging T cell homing at tumor sites. The *ex vivo* glycoprotein fucosylation increases *in vivo* migration and cytotoxic abilities of tumor-specific T cells in leukemia, breast cancer, and melanoma models (292).

The CXCL12/CXCR4 is an additional relevant axis activated by neoplastic cells and cancer-associated fibroblasts in several human tumors. CXCR4 expression correlates with desmoplasia, metastases formation, and immunosuppression (293–298). In murine models of leukemia, melanoma and ovarian cancer, CXCR4 inhibition, obtained with blocking antibodies or with the CXCR4 antagonist AMD3100, increased the effector to T_{regs} ratio at the tumor site and reduced tumor growth (298–301). Stemming from these observations, several clinical trials are now exploring the efficacy of CXCR4 blockade in solid tumors (302).

Exploiting Cancer Vasculature to Foster ACT Infiltration

Tumor neo-angiogenesis involves the formation of a disorganized network of irregular and leaky vessels, inefficient in delivering oxygen, drugs, and immune cells to the neoplastic microenvironment. This process is largely orchestrated by the vascular-endothelial growth factor (VEGF) and results in tumor growth promotion and altered inflammation (303). VEGF inhibitors are currently used in the treatment of several cancers (304) owing, in particular, to their ability to increase T cell tumor homing (305–309). By promoting vessel maturation, VEGF inhibitors positively impact on immunotherapy and ACT (310). Vascular-targeting peptides represent additional effective tools for the precise delivery of small molecules capable of inducing tumor vessels normalization and increased T cells infiltration. In mouse models, tumor-necrosis factor-targeted (TFN) delivery to the tumor vasculature by linking the TNF protein with the CNGRCG angiogenic vessel-homing peptide (NGR-TNF fusion protein) enhanced the local production of immunomodulatory cytokines, favoring the extravasation of immune cells and improving ACT therapeutic activity (311). The fusion of the TNF superfamily member LIGHT with a vascular targeting peptide (LIGHT-VTP) (312, 313) and the specific delivery of IFN-γ and TNF-α by the homing peptide TCP-1 (314) enhanced endothelial permeability and T cell infiltration in mouse models. The cyclic peptide iRGD (315) and the targeting of the vascular integrity regulator VE-cadherin by CD5-2, a specific inhibitor, facilitated T cell homing to the TME in tumor-bearing mice treated with ACT (316).

Although only some of these therapies have been tested in association with ACT, their effect in increasing T cell migration into tumors strongly suggests that strategies aimed at normalizing the neoplastic vasculature could potentially increase ACT efficacy.

Enforcing Chemokine Receptor Expression in T Cells

The interaction of specific chemokines and cytokines with their receptors is a key determinant of immune cell migration, and a mismatch between the chemokines secreted by neoplastic or stromal cells and the receptors expressed by T lymphocytes strongly limits T cell homing in tumors (317–320).

In recent years, attempts have been made to correct this mismatch by engineering T cells with receptors for chemokines or cytokines abundant in the TME, showing preliminary encouraging results. In TRAMP mice with metastatic prostate adenocarcinoma expressing high levels of CCL2, the expression of CCR2 in SV40 Tag-specific CD8 lymphocytes increased T cell homing to the tumor (321). In xenograft models, T cell transduction with a RV encoding CX3CR1 enhanced migration toward human cell lines expressing Fractalkine, the CX3CL1 ligand, and inhibited tumor growth (322). In a lymphoma murine model, adoptively transferred CD8 T cells, overexpressing CXCR4, were preferentially recruited by CXCL12 expressing cells in the bone marrow, to promote tumor control (323). Anchoring IL-4 receptors to the membrane of adoptively transferred T cells increased *in vivo* tumor homing, cytokine secretion, and the killing of melanoma (324). Lentiviral T cell transduction to express CXCR2 enhanced *in vivo* homing toward human melanoma in xenograft models, by exploiting the high IL-8/CXCL8 secretion levels (325).

Classical chemo-radiotherapy is also able to modulate T cell recruitment at the tumor site, suggesting that its use with ACT can have additive effects. In murine models, chemo-radiotherapy has been shown to increase the local release of CCL5, CXCL9, and CXCL11 in the TME, improving ACT-induced tumor growth control (326). Treatment with doxorubicin in mouse models bearing either murine or human melanoma induced CXCL9 and CXCL10 expression by neoplastic cells and increased the infiltration of adoptively transferred T lymphocytes. The effect was further enhanced if ACT followed a combined treatment with doxorubicin and IL-2 (327). Unfortunately, the involved chemokine receptor pattern was not deeply investigated, leaving undemonstrated a causal relation between the increased chemokine secretion by tumor cells and the increased T cell homing.

Overall, these studies prompt further investigation and possible exploitation of chemokine receptors in the context of ACT. Interestingly, a large analysis of 142 patients enrolled in ACT trials revealed the association of genetically determined alterations in chemokine receptors expression with response to therapy (328), underlining the knowledge gap on the impact of chemokines in ACT.

Generating Genetically Engineered T Resident-Memory Cells

Among TILs, T resident-memory (T_{RM}) cells are permanent tissue-resident T cells able to mount an immune reaction; they've

been proposed to be key determinants in the magnitude of anti-tumor immune responses, and their presence in different human cancers correlates with survival (329–336). It is tempting to assume that the induction and/or manipulation of this T cell subset might improve anti-tumor immunity and disease control. Indeed, in murine models, the administration of anti-cancer vaccines through routes that enhance the induction of T_{RM} is associated to tumor growth inhibition and, importantly, provides protection also at distant sites (330, 337–339). In a melanoma mouse model, ACT with T cells lacking RUNX3, a transcription factor essential for T_{RM} development, strongly reduced TILs accumulation and treatment efficacy (340). These results suggest that the association of ACT with strategies directed at promoting tissue residency of the infused cells might be beneficial to improve tumor control.

SURVIVING THE TUMOR MICROENVIRONMENT (TME)

As already demonstrated in several disease contexts, a long-lasting protective memory is a critical requirement for a successful ACT. Despite improvement in manufacturing protocols and fitness of the final cellular products, adoptively transferred T cells have to face a harsh environment while interacting with cancer cells (**Figure 4**). Different immunosuppressive mechanisms act at the tumor site, with the potential to reduce or even dampen the curative action of ACT. If a T cell encounters the cognate antigen in an immune active environment with plenty of additional co-stimuli, the resultant immune reaction is most likely to be efficient in clearing antigen-bearing cells. However, at the tumor site a variety of signals make T cells chronically exposed to antigenic stimulation in the absence of appropriate co-stimuli. Different TME resident cells and tumor cells are responsible for this, either by directly interacting with T cells or by releasing soluble factors. As a consequence, T cell depletion, anergy, exhaustion, and accumulation of T_{regs} (341–345) promote tumor immune escape (214).

Checkpoint Blockade and ACT

Tumor-specific T cells infused to patients and chronically exposed to tumor antigens often enter a dysfunctional state defined as T cell exhaustion that hampers the anti-tumor response and fosters tumor escape (346).

T cell exhaustion is a process of progressive and hierarchical loss of effector functions (e.g., chemokines production and cytolytic activities), resistance to activation through TCR engagement, metabolic deregulation, and failure to acquire an antigen-independent memory state (347). The sustained co-expression of multiple inhibitory receptors (IRs) such as PD-1, CTLA-4, LAG-3, Tim-3, 2B4, CD39, CD160, BTLA, and TIGIT was identified as a hallmark of exhausted T cells (T_{EX}) (249) in solid and hematological tumors (e.g., melanoma, leukemia, breast, prostate, ovarian, renal, lung, and hepatocellular carcinoma (348–358). Interestingly, the pattern of inhibitory receptors expressed by T_{EX} significantly varies among different tumor types. This indicates that exhaustion mechanisms are differentially shaped by various tumor microenvironments (248) and suggests that ACT approaches need to be tailored according to the specific features of each tumor.

Blocking the interactions between the IRs expressed by tumor-reactive T cells and their cognate ligands leads to the reversal of T cell exhaustion (359, 360). Monoclonal antibodies impeding the PD-1/PD-L1 binding (361) and affecting the CTLA-4 axis (362, 363) are able to restore tumor T cell recognition and tumor regression in a relevant subset of terminally ill patients. Due to their efficacy, the use of monoclonal antibodies in association with ACT could greatly benefit T cell resistance toward the tumor microenvironment. Although still exploratory, this combinatory approach showed encouraging preclinical results in the context of CAR-T cells (364).

The use of an immune checkpoint blockade is associated with significant toxicity, indicating that the indiscriminate blockage of inhibitory receptors on the entire T cell repertoire may be deleterious. Indeed, immune-related adverse events (irAEs) occurred in up to 80% of treated patients, and were life threatening in a significant fraction of cases (365). irAEs mainly occurred because potential autoreactive T cells were unleashed and T_{regs} functionality was dampened (366). In order to maintain the benefit of inhibitory receptor blockade while reducing toxicities, it may be beneficial to counteract inhibitory axes selectively on tumor-specific T cells. This is currently one of the focuses in the T cell-based immunotherapy field. The increased anti-tumor activity of CAR- and TCR-engineered T cells in which an inhibitory receptor gene has been disrupted was shown in different preclinical models (161, 166, 171, 367). Recently, the results of the first-in-human phase I trial with PD-1 disrupted TCR-edited autologous T cells in patients with refractory tumors have been published, highlighting the feasibility and safety of multiplex gene-editing in tumor-specific T cells (42).

T cell exhaustion can also be exploited for tumor-reactive T cell isolation purposes. In fact, in the context of melanoma, it was shown that PD-1 expressing TILs are enriched in melanoma-specific T cells. These cells, despite being exhausted, could be isolated and their function restored (224). In addition, circulating PD-1 positive T cells showed to be enriched in neoantigen-specificities (368) when compared to the PD-1 negative counterparts.

Counteracting Immune Suppressive Cells Accumulating in TME

Besides T cell exhaustion, the presence of immunosuppressive cell subpopulations or soluble cytokines may also dampen anti-tumor T cells responses. In tumors, monocytes have been described to preferentially polarize into M2-tumor associated macrophages (M2-TAM) (369) or Tie-2-expressing monocytes (TEM) (370). M2-TAM and TEM sustain tumor survival and blunt immune reactions. Myeloid-derived suppressor cells (371) and different components of the stroma have been implicated as well in tumor progression, through different mechanisms: (i) the expression of inhibitory receptor ligands (372), (ii) the production of metabolites or soluble factors [e.g., indoleamine

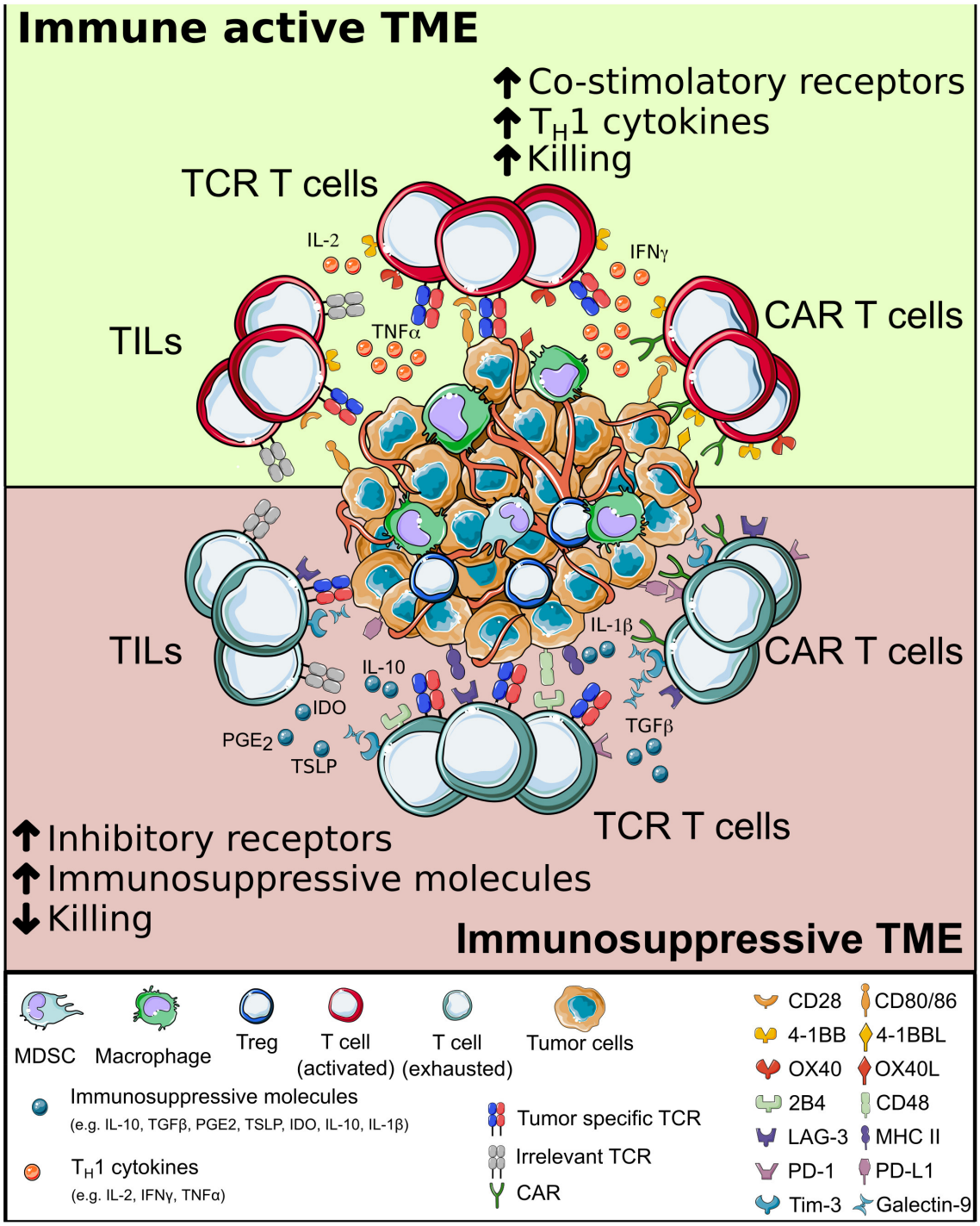

FIGURE 4 | The interplay between T cells and tumor microenvironment. When adoptively transferred T cells (either tumor-infiltrating lymphocytes, Chimeric Antigen Receptor or T cell Receptor-redirected T cells) infiltrate the tumor, they interact with a complex environment, in which a combination of intracellular signals compete. When inflammatory signals dominate, T cells can perform effector functions and potentially eradicate cancer cells; else, they may become exhausted, have limited survival and fail in killing tumor cells. CAR, chimeric antigen receptor; TCR, T cell receptor.

2,3-dioxygenase (IDO) (373), Interleukin-1β and thymic-stromal lymphopoietin (TSLP) (374, 375), and prostaglandin E2 (376)], or (iii) alteration of pH and oxygen levels (377–380). Among the soluble cytokines, the role of TGF-β gained particular attention. TGF-β is released by neoplastic cells of different origins (381) and its secretion is linked to common cancer genetic mutations (382).

At the tumor site, TGF-β acts as a local immunosuppressor, thus reducing the effect of immunotherapy on cancer cell growth. In ACT models, the infusion of CD8 T cells genetically manipulated to resist TGF-β outperformed TGF-β sensitive cells in mediating tumor control (383–387). Interestingly, in a recent clinical study 4 out of 8 patients with Hodgkin lymphoma treated with tumor-specific T cells engineered to express a dominant negative form of TGF-β receptor type II (388) experienced an objective clinical response (389).

To help engineered T cells in counteracting the immunosuppressive microenvironment, different strategies are currently under scrutiny. CAR-T cells have been genetically modified to secrete Interleukin-12 or Interleukin-18 (TRUCK cells) upon CAR engagement (390). A deeper understanding of the expression profile associated to functional CAR-T cells has been now translated in new manipulation processes, involving the overexpression of transcription factors, such as c-Jun (391), or the deletion of inhibitory molecules such as REGNASE-1 (392). These newly proposed strategies could lead to the generation of T cell products endowed with early differentiated phenotypes and enhanced anti-tumor functionality.

FINAL REMARKS

Adoptive T cell therapy represents a unique and innovative therapeutic pillar for cancer treatment. T cells couple the ability to circulate and home at different sites, to sense and respond to the surrounding environment and to persist long-term, thus providing immunosurveillance against residual malignant cells. Each of these characteristics, intrinsic to T cell biology, is however challenged by several immune escape mechanisms active in cancer patients.

Table 1 summarizes the efficacy and toxicity profiles of TCR-redirected T cells reported in clinical trials. Results demonstrate the feasibility of the approach, indicating its therapeutic potential, but also underline the challenges that TCR-based ACT needs to face. Firstly, a suboptimal efficacy of TCR-based studies has currently been observed in patients and no clinical results have been published yet with engineered T cells targeting neoantigens or MiHA. Both evidences underline the difficulty in isolating high affinity tumor-specific TCRs that might be exploited for treating a large number of patients. Secondly, extensive *in vitro* and *in vivo* assays are necessary to lower the incidence of adverse events and increase the safety profile of the infused T cell products.

An interesting point of discussion is whether CAR-T cell therapy should be preferred to TCR-based T cell therapy or vice versa. The answer likely lies in the middle and might envisage the alternated or even the combined use of TCR- and CAR-T cells in different clinical settings, according to the antigenic profile of each tumor type. Combinations could exploit the strength of both strategies. Despite CAR-T cells can count on the extensive level of knowledge acquired on cancer cell phenotypes, testable targets remain few. It's possible that this effect underlies the intrinsic limitation of CAR-T cells, able to target surface proteins but at present unable to recognize all the intracellular mutated or overexpressed proteins in cancer cells. An engineered TCR, instead, has the ability to virtually recognize every tumor antigen, independently of intracellular localization, including mutated molecules, intracytoplasmic proteins, and transcription factors. Hence, finding suitable targets for TCR-engineered T cells is theoretically easier. However, the HLA-restriction of TCR-based immunotherapies needs to be taken into consideration.

In terms of efficacy, CAR-T cells showed optimal results in the context of relapsed/refractory ALL. TCR therapy seems promising in liquid tumors, but both CAR and TCR-engineered T cell therapies showed less than satisfactory results in solid tumors other than melanoma when compared to TILs therapy.

In terms of signaling, TCRs are sensitive to much smaller epitope densities than CARs (393) and T cell activation is finely tuned by the affinity and the avidity for the ligand itself. These differences may prove important when dealing with low antigen density and also in promoting immunological memory while avoiding T cell exhaustion. Even if the precise role of the different signals conveyed in the immunological synapse are still not completely understood, TCR signaling might be more rewarding in the ability to balance T cell activation, possibly solving the limitation in T cell persistence and functionality reported in ACT with CARs.

The possibility of genetically engineering T cells by redirecting their specificity toward cancer by employing CARs or TCRs has already produced relevant clinical results in patients affected by selected tumor types. By combining T cell therapy with alternative therapeutic approaches (i.e., checkpoint blockades) and by implementing multiple genetic manipulation (i.e., by genome editing) in T cells, the efficacy of cancer immunotherapy is further increasing, and we might envisage its successful extension to a larger range of tumor types. As the field progresses, several challenges, including manufacturing complexity, regulatory issues, and sustainability will need to be faced, with the ultimate aim of offering this new therapeutic tool to all patients who could benefit.

AUTHOR CONTRIBUTIONS

AP, BC, ET, ER, FM, and MN did the primary research and wrote the manuscript. AP, BC, and FM edited the figures. FM and ER oversaw the preparation of the manuscript. FC, AB, CB, and ER edited the final draft. All authors contributed to the article and approved the submitted version.

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Externally-Controlled Systems for Immunotherapy: From Bench to Bedside

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Immunotherapy is a very promising therapeutic approach against cancer that is particularly effective when combined with gene therapy. Immuno-gene therapy approaches have led to the approval of four advanced therapy medicinal products (ATMPs) for the treatment of p53-deficient tumors (Gendicine and Imlygic), refractory acute lymphoblastic leukemia (Kymriah) and large B-cell lymphomas (Yescarta). In spite of these remarkable successes, immunotherapy is still associated with severe side effects for CD19+ malignancies and is inefficient for solid tumors. Controlling transgene expression through an externally administered inductor is envisioned as a potent strategy to improve safety and efficacy of immunotherapy. The aim is to develop smart immunogene therapy-based-ATMPs, which can be controlled by the addition of innocuous drugs or agents, allowing the clinicians to manage the intensity and durability of the therapy. In the present manuscript, we will review the different inducible, versatile and externally controlled gene delivery systems that have been developed and their applications to the field of immunotherapy. We will highlight the advantages and disadvantages of each system and their potential applications in clinics.

Keywords: immunotherapy, gene therapy, externally controlled, inducible, ATMPs, transgene expression, cancer, autoimmunity

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INTRODUCTION

Immunotherapy has drastically evolved since the past 30 years, providing diverse approaches for boosting the intrinsic power of the host's immune system to target different diseases, especially cancer. This field includes a broad spectrum of strategies that includes the administration of cytokines, chemokines, monoclonal antibodies, cell lysates, and living cells (1–7) to directly or indirectly boost the immune system to fight cancer or to defuse it for mitigating transplant rejection (8), autoimmune diseases (9), or chronic inflammation (10).

Immunotherapeutic molecules can be delivered systemically or locally into the patients through different systems such as non-viral or viral strategies that can be administered through *in vivo* or *ex vivo* strategies (11). Immuno-gene therapy is a new strategy of immunotherapy that involves genetic modification of cells in order to control immune responses. Some of the most successful immuno-gene therapy applications target tumor cells (12–14) and reduce autoimmune/inflammatory disorders (8, 9, 15).

The re-administration of T cells that are genetically modified to recognize and kill specific cell types (Chimeric Antigen Receptor, CAR-T cells) are particularly successful immunotherapeutic lines to fight refractory tumors (7, 16). Nowadays, Kymriah (Tisagenlecleucel) and Yescarta (Axicabtageneciloleucel, Axi-cel) CAR-T cells became the first two advanced therapy medicinal products (ATMPs) approved in 2017 for the treatment of refractory CD19+ acute lymphoblastic leukemia and aggressive B-cell lymphomas, respectively (17). A third potential ATMP, JCAR017 (Liso-cel) has received the Food and Drug Administration (FDA) breakthrough designation and priority access to medicine program by the European Medicine Agency (EMA) for Relapsed/Refractory Large B-cell Lymphoma (16) and expected to be clinically approved in 2020 (18).

Besides the excellent clinical outcome reported for several immuno-gene therapy approaches, the continuous expression and secretion of potent active molecules [such as IL-12, interferons (IFNs)] can generate adverse clinical events that can lead to life-threatening organ damage and death. This toxicity also limits efficacy, due to the impossibility to reach the appropriate concentrations in target organs. There is therefore a clear necessity to develop fine-tune strategies capable of modulating immune cell activity in order to improve safety and effectiveness of immunotherapies. In this sense, gene therapy field has developed multiple strategies to control the potency and duration of the immune responses by controlling transgene expression.

Several autonomous and externally-control strategies for regulating activity in immuno-gene therapy have been developed [reviewed in (19–21)] (**Figure 1**). First autonomous systems are self-regulated and respond to signals such as stress, inflammation, cytokines, or endogenous hormones. However, those strategies do not allow clinicians to control the intensity and durability of the therapy.

On the contrary, remote-controlled systems allow the modulation of activity and associated side effects. Those approaches rely on the co-administration of an inductor, which should fulfill certain characteristics in terms of pharmacokinetics, tolerability and biodistribution (**Table 1**). There are various systems for controlling gene expression or managing toxicities at different levels (**Figure 2**). For example, inducible suicide herpes simplex virus tyrosine kinase (HSV-TK) or human thymidylate kinase (TMPK) systems trigger cell death upon a small molecule administration [reviewed in (27)] but are irreversible systems. On the other hand, several systems have been developed to control CAR-T activity (28–31). Despite their clinical potential, they are CAR-specific and not able to control other immuno-gene therapy strategies.

In this review, we will focus on externally controlled, reversible (on/off switchable) and versatile inducible systems which can constitute potential tools for improving immunotherapeutic application. We will discuss the benefits and weaknesses of every emerging approach regarding their state of development, safety, on/off dynamics, inductor properties and closeness to clinics.

PRINCIPLE OF EXTERNALLY CONTROLLED SYSTEMS

Gene therapy provides us a robust, safe and heterogeneous platform of gene transfer for clinical applications. This field has generated a wide range of long-term, stable (or transient, if required) tools, with reduced immunogenicity for modifying immune cells by using non-viral and viral delivery systems.

Although multiple inducible systems have been developed, we will focus on those that are externally controlled, are able to regulate any transgene and are potentially applicable to humans. In order to compare the different versatile available systems for clinical applications, several characteristics must be considered including the inducer properties, vector architecture, and origin, single or dual systems, promoters, target cells, leaking (basal expression in absence of the inducer) and potential risks parameters. For clarity, we will classify the different externally controlled systems on (1) those based on the administration of drugs and (2) those based on the application of physical inductors (light, ultrasounds or irradiation).

Drug-Inducible Systems

Inducible systems controlled through the administration of drugs are designed to trigger conformational changes on target proteins so they induce (ON-systems) or block (OFF-systems) transcription of the desired transgenes. OFF-systems have the disadvantage of continuous administration of inductor, necessary for silencing transgene expression. Permanent-high levels of antibiotics, for example, can lead to several complications for the patients and have therefore very limited applications in clinics. In this review, we will focus on the ON-systems (**Figure 2**). These systems require, in general, two key components: (1) a chimeric transcription factor which contains a DNA-binding domain and a drug-binding domain; and (2) a regulated minimal promoter, with very low basal activity, followed by the gene of interest. This promoter includes several copies of a non-natural DNA-binding site in which the chimeric transcription factor binds in the presence of the drug.

Tetracycline-Regulated Expression Systems

Tetracycline (Tet)-based gene expression control systems have been established as the systems par excellence for gene induction due to ease of handling, high efficiency and minimal side effects. This system has been designed to have three different configurations: (1) the system based on the original tetracycline repressor, TetR (Tet-ON) (32–34). In these configurations, TetR-binding sites (Tet operator-tetO) are inserted between a constitutive promoter and the gene of interest blocking its activity. The addition of tetracycline or its derivatives [such as doxycycline (Dox)] promotes a conformational change in the TetR that makes it incapable of tetO binding, allowing transcription to proceed. (2) tTA-based systems (Tet-OFF) (35). These systems are based on a chimeric protein formed by the fusion of TetR and a domain of VP16 (derived from *herpes simplex virus type 1*). Contrary to the TetR-based system, here the inducible transgene is placed downstream

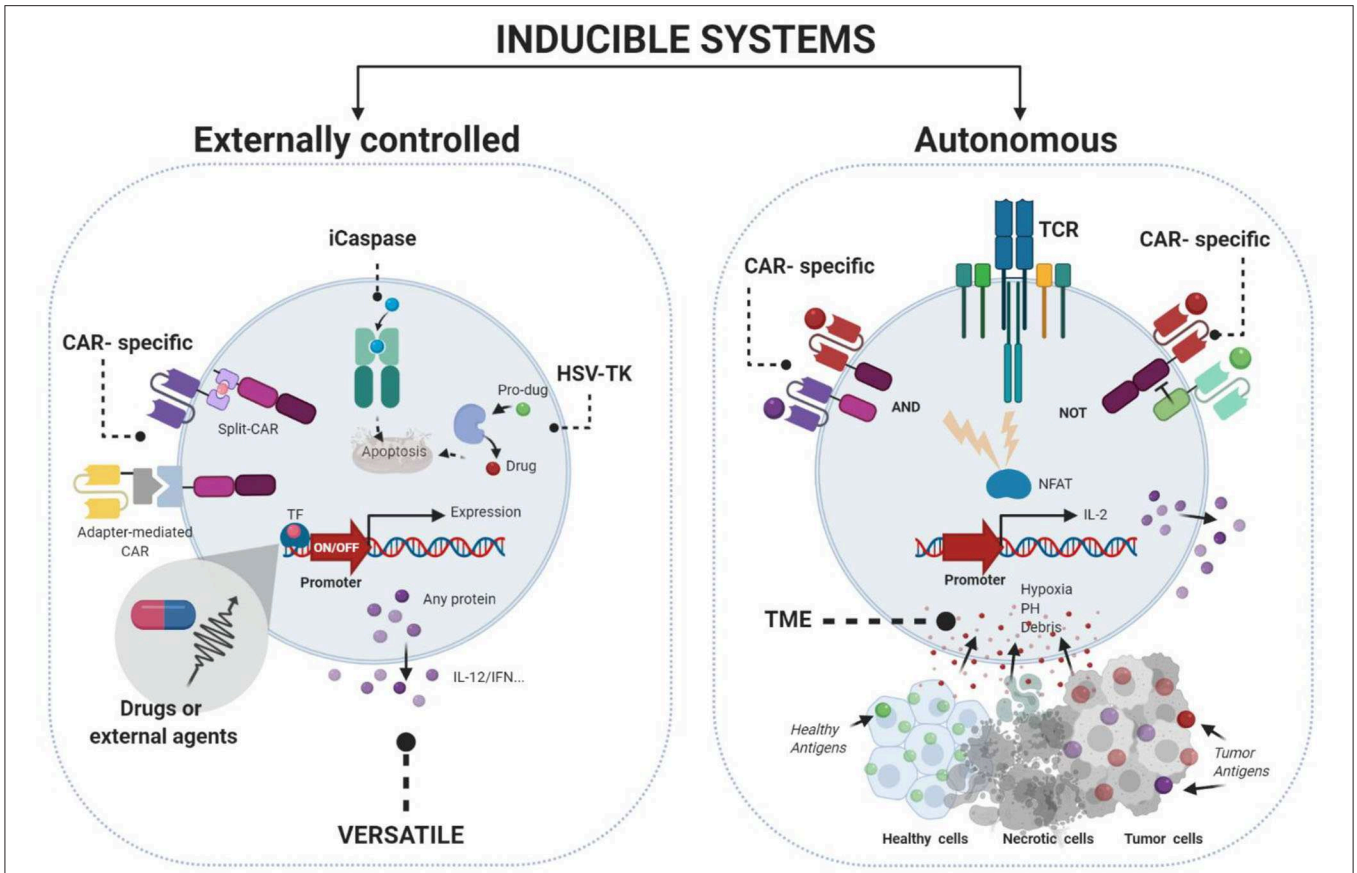

FIGURE 1 | Gene therapy strategies to control immunogene therapy using inducible systems. Externally controlled systems (left) require the addition of an external stimuli (chemical or physical) to modulate the expression of the desire transgene. Autonomous systems (right) are designed to control the expression of the transgene in function of different cellular situations such as inflammation, cytokines, hypoxia, or pH. Figures were created with BioRender.com.

TABLE 1 | Characteristics of Dox-inducible Tet-On CARs.

System	Target	Delivery	Population	Doses[a]	*In vivo* induction?	Leaking	rtTA?	ClinicalStage	Ref
Tet-On 3G (TaKaRa Bio)	CD19	Single	Selected	100 ng/ml	Yes (pre-induced)	Yes	Yes	Pre-clinical	(22)
Tet-On (Sangon Biotech)	CD19	Single	Bulk	4 g/ml	No	Yes	Yes	Pre-clinical	(23)
Tet-On 3G(Clontech)	CD38	Dual	Selected	1,000 ng/μl	No	No	Yes	Pre-clinical	(24)
Tet-On 3G (TaKaRa Bio)	CD147	Single	Bulk	1,000 ng/ml	Yes(pre-induced)	Yes	Yes	Pre-clinical	(25, 26)

[a]*Doses in vitro; Ref, reference.*

of a minimal (inactive) promoter harboring tetO sequences. Only if tTA bind to the tetO sequences, will the promoter be active through the activity of the VP16 domain, and will express the transgene. In this configuration, the addition of tetracycline also makes the tTA unable to bind to tetO and transcription stop. (3) rtTA-based systems (Tet-ON) (**Figure 2**, bottom-right). As mentioned before, Tet-OFF systems have limited applications in clinics. Different groups have therefore designed Tet-On systems based on mutational modifications of tTA in order to allow its binding to the tetO only in the presence of tetracycline. In these new systems, transcription requires the presence of tetracycline, becoming a Tet-ON system. The first Tet-On system (36) based on the rtTA had high leaking, but new developments improved the control of the expression (37–39). However, these systems, as we will discuss in detail, still have important drawbacks for clinical applications due to the presence of transactivators. In this direction, new developments of the original TetR systems have managed to control transgene expression in the absence of transactivators in most cell types analyzed (33, 34), including primary T cells (40). These developments could open new opportunities in the field of immunotherapy.

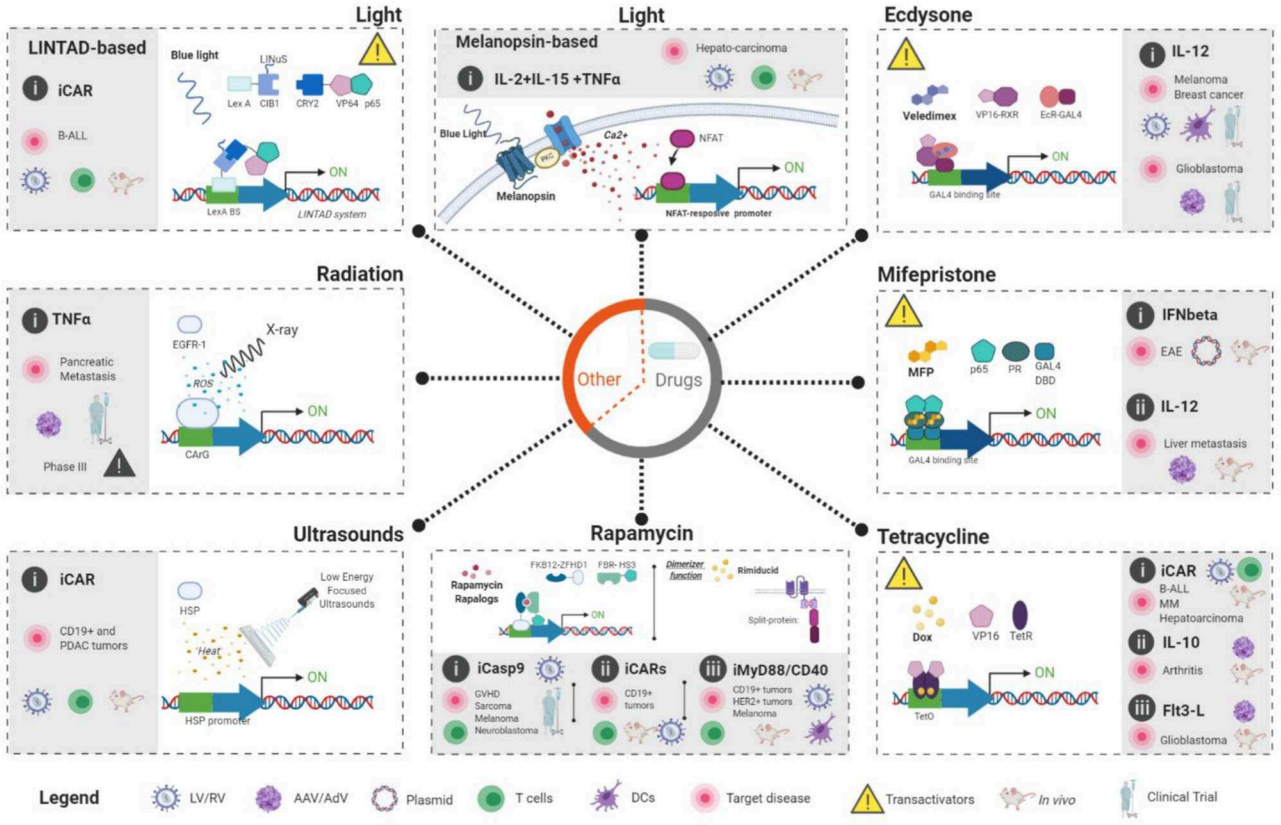

FIGURE 2 | Externally-controlled inducible systems applied to immunogene therapy applications. Two main groups can be established: (1) Drug-based systems (Gray circle in the middle) which include ecdysone, mifepristone, tetracycline, and rapamycin systems. In these systems, clinicians could control the activity of the immunogene therapy through the administration of a drug that will, usually, activate the expression of the desired transgenes. (2) Physical-based systems include two light-based (melanopsin-based and LINTAD), one radiation-based (TNFerade) and one ultrasound-based (FUS-CAR). White area of the each dashed-line square shows the rational of each approach to achieve externally-controlled transgene expression. Key molecular players (inductors and regulatory-proteins) in each system are also indicated. Gray area inside the dashed-line squares indicate published immunogene therapy approaches for each system and the current status; *In vitro* studies (indicated by the absence of a mice or human drawing), *in vivo* studies (indicated by the presence of a mice) and clinical trials (indicated by a drawing of human figure). In addition, the target disease, therapeutic gene, modified cells and vector type are also shown. The legend at the bottom of the figure illustrate the meaning of the different symbols used in the figure. Figures were created with BioRender.com.

An important advantage of these systems is that tetracycline and its derivatives, such as doxycycline, have been widely used as antibiotics in humans for decades and have been very well-characterized clinically (41). With 93% of oral absorption efficient, 14–22 h of half-life and deep tissue penetration, including blood-brain barrier (BBB) (**Table 2**), they are ideal inducing agents for a rapid increase in expression, long-term and rapid decrease of the desired transgene.

Immunotherapeutic application

CAR-T cells. A number of studies utilizing Tet-regulatory systems to regulate CAR expression have been carried out. CAR-T therapy is a promising approach in antitumor therapy, with remarkable results obtained so far in hematological diseases. However, there are important limitations due to uncontrolled responses as a consequence of constitutive expression of the CAR molecules on the surface of T cells. For this reason, a temporary and reversible CAR expression, in which CAR-T cells response can be turned on/off, would be a convenient and eligible solution. Sakemura et al. (22) used the all-in-one pRetroX-TetOne-3G vector in which the CD19CAR-tEGFR sequence was expressed in an inducible manner in primary CD8+ T cells using the rtTA system. CAR+ cells were first selected for obtaining an almost 93% pure population. Maximal CAR expression in SUP-T1 cells was achieved with 100 ng/ml Dox and the expression went down after 20 h of Dox removal, although it did not reach zero in the absence of Dox. Clear differences regarding antitumor efficacy *In vitro* between (Dox+) Tet-CD19 CAR-T cells and (Dox-) Tet-CD19 CAR-T cells were found, but the system exhibited a significant CAR expression in the absence of Dox. For *in vivo* experiments, only Tet-CD19CAR-T cells incubated with Dox prior to inoculation suppressed tumor growth. Following a similar strategy, Gu et al. (23) generated an all-in-one vector expressing the rtTA2S-M2 protein (an improved version of the rtTA) and CD19-CAR (23). In this case, a concentration of 4 μg/ml of Dox was necessary to induce CAR expression 5-fold.

TABLE 2 | Pharmacokinetics of the small molecules used as inductors for inducible immunotherapy.

Inductor	Type	FDA-approved	FDA-Dose[a]	Oral	T_{max}	$T_{1/2}$	BBB	Ref
Doxycycline	Antibiotic Tetracycline	Yes, for bacterial infections	200 mg/day	Yes	1–3 h	18–22 h	Yes	(41)
Veledimex	Diacylhydrazine	Investigational, Fast Track-FDA, as Inductor	10–20 mg/ml	Yes	2.5–5.5 h	18–27.5 h	Yes	(42)
Mifepristone	Progestational and glucocorticoid antagonist	Yes, abortive, contraceptive	4.5 mg/kg	Yes	1–2 h	15–30 h	Yes	(43)
Rapamycin (Sicrolimus)	Antibiotic macrolide	Yes, as immunosuppressant	2–5 mg/day	Yes	1–6 h	57–68 h	Yes	(44)
Rimiducid (AP1903)	Antibiotic macrolide	Investigational, Orphan-Drug designation	0.4 mg/kg	Yes	N.D	5 h	Yes	(45)

T_{max}, peak in blood after administration; $T_{1/2}$, elimination half-life of the drug; BBB, blood-brain barrier; N.D, non-determined; Ref, reference.
[a]FDA approved or used in the current clinical trial in adults.

Those inducible CAR-T cells also presented better killing of tumor cells in the presence of Dox, although they produced killing also in its absence. The efficiency of the TetOn system has been also tested for multiple myeloma (MM), using CD38 antigen as the target of Dox-regulated CAR T cells (24). Here, the authors used two vectors, the pRetroX-TRE3G vector to control the expression of the CD38-CAR and the pRetroX-TET-On 3G for expression of the rtTA transactivator. CAR-expressing cells were selected by puromycin to obtain a pure population. Maximal tumor lysis *In vitro* was assessed with 1,000 ng/ml and the prompt reversion of the CAR activity was better achieved after a short exposition (24 h) with 10 ng/ml Dox. There are also pre-clinical assays using the Tet-On 3G system in solid tumors, specifically for hepatocellular carcinoma (HCC) treatment (26). Zhang and co-workers constructed the Tet-CD147-CAR lentiviral vector to generate Tet-CD147-CART cells. With a Dox concentration of 1,000 ng/ml CAR expression reached the peak at 24 h and returned to baseline level at 48 h after removal of Dox, but expression never reached zero. CART cells exhibited higher lytic activity in the presence of Dox, but residual lysis as a consequence of CAR leaking was observed. In an HCC mouse model, mice treated with pre-induced (Dox+) Tet-CD147-CART significantly reduced tumor volume and weight compared with those of mice that received (Dox-) Tet-CD147-CART (26), but *in vivo* leaking was noticeable.

Others immune-gene therapy approaches. Tet-On systems have also been applied to control cytokine expression in order to boost or control immune responses in a doxycycline-dependent manner. One of the first demonstrations of the potential of this strategy used two adeno-associated vectors (AAVs), one AAV harboring the Tet-responsive promoter driving the expression of interleukin-10 (IL-10) and the other expressing rtTA (46). The authors showed therapeutic efficiency over *In vitro* human rheumatoid synovium from rheumatoid arthritis patients as well as *in vivo* mice model, after intramuscular injection of both AAVs.

In another approach, the group of Dr. Castro developed a combined strategy that used Adenoviral vectors (AdV) to express HSV-TK constitutively and FLT3L in a Dox-dependent manner (47) for the treatment of Glioblastoma multiforme (GBM), a primary malignant brain cancer with very poor prognosis. This strategy aims to induce apoptosis in dividing cells in the presence of ganciclovir, and to stimulate the recruitment of DCs to the site of HSV-TK-mediated tumor killing through Dox-induction of FMS-like tyrosine kinase three ligand (Flt3L). The authors observed significant therapeutic benefits in rat models of GBM after intracranial inoculation of the AdV vectors and after treatment with Dox. Of note, a dose of 300 mg/day Dox was more effective than 200 mg/day equivalent, showing the high Dox concentrations required in this Tet-On system. Interestingly, rats were able to generate adaptive immune responses against the implanted tumors (48). Based on these studies, a clinical trial was approved in 2013 (ClinicalTrials.gov Identifier: NCT01811992) and currently ongoing Phase I (updated on April 2020).

Ecdysone-Regulated Expression System

Another interesting system to control gene expression in a rapid, robust, precise, and reversible way are based on the use of steroids-based regulatory domains from insects. Steroids present very interesting properties as inducers of externally-controlled systems: they can penetrate all tissues and are quickly metabolized. The group of R.M. Evans developed the first regulated system based on the ecdysone receptor of *Drosophila melanogaster* to regulate transgene expression on mammalian cells (49). Different versions of these systems have been published since with different success in different cell types and tissues (50, 51). Of all the systems, the RheoSwitch (52) has been the most successful, with applications even in clinical trials.

The RheoSwitch Therapeutic System® (RTS) consists of a series of inter-dependent functional components for gene induction (**Figure 2**, top-right): (1) two transcription factors (VP16-RXR and Gal4-EcR), (2) an inducible promoter and (3) an activating small molecule ligand. The first factor arises from the fusion between the ligand binding domains of a chimeric RXR and the transcriptional activation domain of VP16 of HSV1, that acts as a co-activation partner. The second consists of a DNA-binding domain of the yeast transcription factor Gal4 fused with the hinge domains of the mutated ecdysone receptor (EcR) of the Spruce budworm (*Choristoneurafumiferana*), where the

ligand is bound. To achieve regulation, both proteins must be constitutively expressed. The addition of the ligand promotes the stabilization of the heterodimeric complex which binds the responsive-promoter through Gal4 and leads to transcriptional activation thanks to the VP16 domain. In the absence of an inducer, the complex is destabilized and transcription is blocked.

The RTS system has been clinically validated for the control of IL-12 expression through clinical trials (53). Previous studies using IL-12 were based on the use of strong constitutive promoters, such as CMV or EF1-α to achieve high expression levels. However, IL-12 plays crucial roles in naive T cells differentiation into cytotoxic T-lymphocytes (CTLs) via IFN-γ production. It does need therefore a clear control in order to achieve the desired therapeutic benefits minimizing side effects.

Different groups have also investigated the most appropriate ligand to be used in clinical settings (54). Ecdysteroids are contained in vegetables thus its safety for humans is well-proven. Veledimex is a synthetic analog of ecdysone used as the ligand of the RheoSwitch system and is currently under investigational in the Fast-track line of FDA due to its pharmacokinetics features (42, 55) (**Table 2**).

Immunotherapeutic applications

The VP16-RXR and Gal4-EcR sequences was adapted into an AdV vector to express IL-12 under the control of the RTS [reviewed in (56)]. Using this rationale, two strategies were followed: (1) to transduce dendritic cells (DCs) *ex vivo* and introduce them into the patients, and (2) to introduce the Ad-vector *in vivo* (56). In the first strategy, a complete tumor regression was reached in a subcutaneous B16F0 melanoma model by delivering mIL-12-DCs intratumorally (57, 58). Using the second strategy, between 73 and 90% of tumor regression was obtained using the melanoma model and tested successfully against other tumoral models (56, 59). In all the cases, IL-12 increased DCs life, generated a high infiltration into the tumors of cytotoxic CD4+ and CD8+ T cells producing high levels ofIFNγ. Based on these data, the first-in-human clinical trial was approved that used externally-regulated gene therapy intervention (NCT00815607). This first study aimed to analyze safety, regulation of the IL-12, tolerance, response rate, and immunological effects. Patients enrolled received 5×10^7 DCs transduced with Ad-RTS-hIL12 and oral administration of inducer ranging from 0.6 to 200 mg. A second phase I clinical trial was also approved using the second strategy. Patients were injected with 1×10^{12} Ad-RTS-IL-12 particles into accessible lesions in combination with oral inducer administration. Patients included had stage III-IVmelanoma (NCT01397708) and metastatic breast cancer (NCT01703754, NCT02423902). Although only a minority of the patients achieved a partial regression, a veledimex dose-dependent increment of mRNA IL-12 intratumorally as well as serum IFNγ levels were manifested (60). Unfortunately, several patients experienced serious toxic effects but were rapidly solved after veledimex discontinuation (60, 61). In another phase-I study targeting Glioblastoma (NCT02026271), the authors showed a significant improvement in patient's survival (55). In this study, several patients experienced severe adverse events (CRS or neurological-related) that were quickly controlled after suspension of veledimex uptake. Today, there are four open clinical trials to evaluate the intratumoral injection of Ad-RTS-hIl-12 and activated with oral veledimex (20 mg/day during 15 days) as a therapy to treat patients with recurrent or progressive glioblastoma (alone or combined with anti-PDL1 monoclonal antibody (mAb), NCT04006119, NCT03679754, NCT03330197, NCT03636477).

Mifepristone-Regulated Expression System

The first development of Mifepristone (MFP)-based systems (62) took advantage of the modular nature of functional domains of steroid receptors. The authors generated a Mifepristone-responsive regulator (pGL-VP) by fusing the ligand-binding domain of the human progesterone receptor, the DNA-binding domain of yeast GAL4 protein and the VP16 transactivation domain of the HSV protein. They showed that this chimeric protein was able to promote transcription of minimal promoters containing GAL4-binding sites after administration of MFP *In vitro* and *in vivo*. Importantly for these systems, the MFP (RU486) concentration required for transgene activation is lower than that required for antagonizing progesterone action.

A later development consisted in a chimeric regulator, GLp65 composed of a mutated ligand-binding domain (LBD) of the human progesterone receptor, the DNA-binding domain of yeast GAL4 protein and the activator domain (AD) from the human p65 protein, part of the nuclear factor kappa B complex (63). This system was commercially named as the GeneSwitch™ (GS) platform (64). GS needs two expression cassettes: the first one expressed constitutively (normally through the CMV promoter) the GLp65 transactivator protein, and the second cassette includes the inducible promoter, which contains at least four sequences for GAL4 binding, and the gene of interest. When MFP is present and binds to the LBD, a conformational change allows the GLp65 transactivator to dimerize and binds to the GAL4-promoter, activating transcription through the p65 domain (**Figure 2**, middle-right). The elimination of the VP16-domain from the system reduced expression levels but also reduced leaking, improved safety and reduced immunogenicity.

MFP is a clinically approved drug, with anti-progestin and anti-glucocorticoid properties (65). The long-term use of this drug in both females and males is under current investigation in phase III for psychotic depression (66) but MFP appeared safe and well-tolerated at the doses required to activate transcription (**Table 2**). However, the potential side effects as a glucocorticoid antagonist should be further characterized. Its progesterone antagonistic activity could be a problem on human T cells, which present progesterone receptors in the membrane, and T cell proliferation was inhibited after 5 mM of MPF (43, 67). Altogether, suggest that dose-escalation for future clinical application should be carefully validated.

Different GS system has been developed for inducible gene therapy approaches to fight liver cancer (68–70), as well as for the treatment of neurological diseases (71, 72).

Another important aspect to consider of inducible systems is the alterations provoked by the constitutive expression of

the chimeric regulators. Reboredo et al. (73) analyzed the effect of GLp65 and rtTA2(S)-M2 in the liver's transcriptomics. They found that while rtTA2 expression induced alterations in 69 genes, GLp65 caused an altered expression of 1,059, although functional analysis showed only mild alterations.

Immunotherapeutic applications

GS system has also been applied to regulate the expression of potent cytokines such as IL-12 with the aim to control its activity while keeping their therapeutic potential. In an elegant study, Wang et al. developed a strategy to achieve hepatic-specific expression of IL-12 that also responded to the control of MFP. The authors developed an AdV harboring the sequences for GAL4 binding into a hepatic-specific promoter driving the expression of IL-12 (6). Direct administration of these AdVs enabled controlled hIL-12 expression in the liver for more than 48 weeks when MFP was administered every 24 h. In addition, this system achieved complete tumor regression in an aggressive model of liver metastases *in vivo*. Whereas, using a specific-liver promoter seems to be useful for preventing immunogenicity, the IL-12 production in the liver was associated with a moderate inflammatory reaction opens the possibility that higher doses of AdV-MFP could induce-IL-12-related severe inflammation.

In a different approach, MFP-GS was used to express IFN-beta for the treatment of a murine model of multiple sclerosis, experimental autoinflammatory disease (EAE) (74). In that model, a single intramuscular administration of the inducible mIFNβ vector delivered as DNA plasmid was sufficient to decrease significantly the onset of disease. The procedure was well-tolerated and the overall severity of the disease scores was reduced in the presence of MFP (74).

Rapamycin-Regulated Expression System

Rapamycin-regulated system is a human platform designed by Rivera et al. (75, 76) that is based on the interaction between two cytosolic proteins that only dimerizes in the presence of rapamycin. FK506 binding protein (FKBP12) is a 12 kDa cytosolic protein and FKBP12-rapamycin-binding protein (FRB) is a 11 kDa domain derived from mammalian target of rapamycin (mTOR). The original system contained three copies of FKB12 fused to a DNA-binding domain (zinc finger homeodomain transcriptional factor 1, ZFHD1) composing the DBD and FRB was fused to the DNA activation domain (AD) of Nuclear Factor Kappa B p65 subunit, driven expression of the gene of interest in a three-plasmid system. Transgene expression was induced after a 24 h incubation with 10 nM of rapamycin (75) but induction failed when DBD was incorporated in a retroviral vector (77). A new and more potent AD domain, called SH3, containing sequences from human heat shock factor one (HSF1) and p65, overcomes that problem even with only one copy of FKB12 in a single vector and placing the target gene cassette in reverse orientation achieves no leaking. In this case, 1 μM rapamycin or analog AP1903 was necessary for maximal induction (**Figure 2**, bottom-middle).

Rapamycin (Sirolimus) is a macrolide antibiotic with potent immunosuppressant activity used for allograft rejection in renal and cardiac transplantation (45). This immunosuppressive action occurs by targeting calcineurin and IL-2 production in T cells by Rapamacyin-FKB12 and inhibiting mTOR, thus affecting cell proliferation and metabolism via Rapamycin-FBR. In order to improve safety, a mutation in the FKBP domain was generated (FKBP12-F36V) (78) to allow the design of novel rapalogs (AP1903/AP20187) that bind the mutated but not the wild-type FKBP protein. Therefore, AP1903 (Rimiducid) is a safe and well-tolerated drug that can be administered up to 1 mg/kg (44, 79) (**Table 2**).

Immunotherapeutic applications

Since these systems are based in human-derived components, they present minimal immunogenicity and have been efficiently adapted to immunotherapy (**Table 3**). In addition, the inductor is able to cross the BBB (84) and required low concentrations (78, 84) (**Table 2**).

One of the most important uses of this system has been adapted to induce the activation of the proapoptotic enzyme caspase 9 (85), initially adapted to kill tumor cells, it did translate soon for suicide and irreversible T-cell depletion (86) to treat GVHD (BPX-015, Phase I/II). iCasp9 or CaspaCIDE is based on the homodimerization of mutated FKB12 fused to the signaling domain of caspase 9 after the treatment of AP1903/Rimiducid. This system can eliminate 85 to 95% of circulating CD3+ T cells within 30 min [NCT01494103 (84)]. A phase I trial had demonstrated long-term-persistence of transduced T cells (up to 3.6 years) without compromising proliferation. However, a single clone of iCasp9-transduced T cells caused a delayed CRS in one patient that developed *de novo* Epstein–Barr virus-associated post-transplant lymphoproliferative disease (EBV-PTLD), being unresponsiveness to Rimiducid (87).

iCasp9 have been also included in TCR-restricted (88) and CAR-T cell therapies as a safety measure (89). iCasp9 showed efficient clearance in anti-CD19 CAR-T cells co-expressing IL-15 (90) and in third generation anti-CD20 CAR-T cells, where the 90% of engineered T cells were depleted *in vivo* in only 12 h (91). GD2-specific and iCasp9-expressing CAR (GD2-iCAR) T cells have reached clinical trials against advanced melanoma (CARPETS, ACTRN12613000198729), neuroblastoma (GRAIN, NCT01822652), sarcoma (VEGAS, NCT01953900), and other GD2+ solid tumors (NCT02107963). Of note, while iCasp9 can rapidly reverse toxicity, sacrifices the long-term antitumor efficacy. Stavruo et al. (78) have exploited the same Caspase 9 strategy in CAR19-T cells but using original FRB/FKBP system to generate homodimers of Casp9 after rapamycin addition (RapaCasp9), which is indeed a clinically-drug approved, exhibiting a similar response of rapaCasp9 to iCasp9 at 1 nM (78).

Another elegant FKBP/FRBmut system specific for CARs, are the "ON-switch" CARs (28), where the CAR structure is split into two chimeric polypeptides: CAR-I encloses the antigen recognition domain, transmembrane and 4-1BB costimulatory domain and CAR-II harbors the main CD3zeta-ITAMS signaling domain. Both chimeric proteins are fused to intracellular FKBP/FRBmut. Only when AP21967 was administered, an anti-tumoral effect was observed in mice, but due to its shorter life, another heterodimer system would be desirable for a more-suitable future clinical application.

TABLE 3 | Systems for controlling transgene expression applied to immunotherapy.

System	Inductor	Gene	Model/Disease	Product	Administration	Clinical stage	Ref
Tet-On 3G (TaKaRa Bio)	Dox	CAR-CD19	CD19+ Raji cells (Burkitt's lymphoma)	All-in-one RV-T cells	Cells[a]: intravenously Inductor: pre-induced *ex vivo*+ oral	Pre-clinical	(22)
Tet-On (Sangon Biotech)	Dox	CAR-CD19	CD19+ Raji cells (Burkitt's lymphoma)	All-in-one LV-T cells	*In vivo* experiments were not conducted	*In vitro*	(23)
Tet-On 3G (Clontech)	Dox	CAR-CD38	CD38+ cell lines (Multiple myeloma)	Dual system RV-T cells	*in vivo* experiments were not conducted	*In vitro*	(24)
Tet-On 3G (TaKaRa Bio)	Dox	CAR-CD147	CD147+ cells (Hepatocellular carcinoma)	All-in-one LV-T cells	Cells[a]: intratumoral Inductor: pre-induced *ex vivo*	Pre-clinical	(26)
Tet-On	Dox	IL-10	DBA1 mice (Rheumatoidarthritis)	All-in-one AAV vp	Vector[b]: intramuscularly Inductor: oral	Pre-clinical	(46)
Tet-On	Dox	FLT3L	Glioblastoma multiforme	All-in-one Ad	Vector[b]: intracranial Inductor: oral	Phase I	(47)
RheoSwitch (RTS)	Veledimex	IL-12	Stage III or IV melanoma	All-in-one Ad-DCs	Cells[a]: intratumoral Inductor: oral	Phase I	(57)
RheoSwitch (RTS)	Veledimex	IL-12	Stage III-IV melanoma Metastatic breast cancer	All-in-one AdV	Vector[b]: accessible lesions Inductor: oral	Phase I/II	(56)
Gene Switch	MFP	IL-12	MC-38 mice (Livermetastases)	All-in-one AdV	Vector[b]: intravenous Inductor: intraperitoneal	Pre-clinical	(68)
Gene Switch	MFP	IFN-β	EAE mice (multiple sclerosis)	DNA plasmid	Plasmid[c]: intramuscular Inductor: subcutaneous	Pre-clinical	(74)
Light- pNFAT	Blue light	IL-2, IL-15, TNF-α	SK-HEP-1 mouse (Hepatocellular carcinoma)	All-in-one LV-T cells	Cells[a]: subcutaneous Inductor: externally applied	Pre-clinical	(80)
LINTAD	Blue light	CAR-CD19	CD19+ Nalm6+ mice (B-lymphoblastic leukemia)	Dual system LV-T cells	Cells[a]: subcutaneous Inductor: externally applied	Pre-clinical	(81)
TNFerade	Radiation	TFN-a	Metastatic pancreatic cancer	All-in-one Deficient AdV	Vector[b]: intratumor Inductor: externally applied	Phase III	(82)
FUS-CAR	Ultra-sounds	CAR-CD19	CD19+ Nalm6+ cells (B-lymphoblastic leukemia) PC3 cells (Prostatecancer)	Dual system LV-T cells	Cells[a]: subcutaneous Inductor: externally applied	Pre-clinical	(83)

[a]Ex vivo transduction. [b]in vivo transduction. [c]Direct plasmid injection Dox, doxycycline; MFP, mifepristone; CAR, chimeric antigen receptor; IL-10, interleukin 10; FLT3L, FMS-like tyrosine kinase 3 ligand; IL-12, interleukin 12; IFN-β, interferonβ; IL-2, interleukin 2; IL-15, interleukin 15; TNF-α, tumor necrosis factor α; EAE, experimental autoimmune encephalomyelitis; RV, retroviral vector; LV, lentiviral vector; AAV, Adeno-associated vector; vp, viral particles; Ad, adenoviral vector; Ref, reference.

In a different configuration but with a similar idea, the dimerizing agent–regulated immunoreceptor complex (DARIC)-T cells is also composed of two CARs (92): the CAR-I is composed by the ScFv-FRB-TM domains and the CAR-II by the FKPB-TM-41BB-CD3ζ domains. In both CARs, the FKBP/FRBmut domains are located extracellularly. DARIC-T cells also allow the application of a plugin for targeting another antigen (a subunit of the ScFv and FRB). In addition, Tacrolimus, which has a high affinity for FKBP12, can be used as a rapamycin competitor, which could be interesting as a safe method for reducing severe CRS or persistent neurotoxicity (92).

Controlling the ScFv presentation at the cell surface after AP21967 addition is another FKB16/FRBmut design, where the FRB and FKBP12 domains were placed between the CD8a hinge and the scFv domains, modulating the cytotoxic properties of this "transient" CAR-T cell (93).

Rapamycin-based systems have also been developed to activate immune cells such as dendritic cells (DC) or T cells, regulating the synergy of TLR/IL1R (through MyD88) and CD40 signaling within the context of an immunological synapse. In this case, rimiducid-inducible MyD88 and CD40 (iMC) system is composed by aTIR domain-deleted version of MyD88 fused to tandem copies of the modified FKBP12V36 and a myristylation-targeting sequence for membrane anchoring, whereas the same fusion structure was used for the cytosolic domain of CD40. Autologous iMCs-DCs showed a strong antitumoral effect *in vivo* (94, 95).

More recently, this iMC strategy has been applied for the CAR-T field against HER2+ solid tumors. In the presence of Rimiducid, T cells expressing HER2–CARζ and this FKBP12 iMyD88/CD40/FKBP12 system exhibited potent antitumor activity in pre-clinical models, allowing their remote-control post-infusion (96), becoming a very promising platform. Duong et al. (97) engineered CAR-T cells with this Rimiducid iMC-signaling system for CAR T cell activation in combination with the rapamycin-induced caspase-9-based safety switch (iRC9) for controlling potential risks. This dual-switch (DS) system generated higher CAR-T cell expansion in a drug-dependent

manner while triggering apoptosis to avoid severe toxicities if required (97). However, escalation doses of Rim to *in vivo*/clinical application should be carefully evaluated since 100x more of the Rim dose for activate iMC counterpart (1 nM), could also trigger the iRC9 system *in vitro*.

Future Drug-Inducible Systems for Immunogene Therapy Applications

Here, we will briefly describe systems that fulfill the above criteria of versatile, reversible and inducible system but have not been used yet for immunotherapeutic applications.

Antibiotics

Other antibiotic-based systems found in different bacterial strains have been modified and adapted to control gene expression in mammalian cells such as streptogramin (PipOFF/PipON) and macrolide (EON/EOFF) based gene regulation systems (98). Those four systems have been tested *In vitro* using different cell lines and different transgenes, obtaining a fast (<24 h) and great induction (until 100-fold) of transgene and low leaking (98, 99). Both the macrolide and the streptogramin antibiotic families present interesting clinical properties, such as excellent bioavailability, optimal pharmacokinetics, and human compatibility (100, 101).

Another system that has not been applied yet to immunotherapy approaches is the system based on the original tetracycline repressor, TetR (32–34). These systems have several advantages over the traditional Dox-based system that use transactivators such as the absence of toxicity, the low leaking, and the low Dox requirements.

Quorum Sensing

A chimeric transcription factor controlled by an acylated homoserine lactone (AHL), getting up to 1000-fold induction and low basal transcription in different human cell lines have been developed (102). However, AHL signaling molecules can influence the behavior of eukaryotic cells and tissues and it is unknown its pharmacodynamics *in vivo* (103, 104). Looking ahead, it is possible to engineer other transcription factors from different bacterial species and develop inducer compounds with improved characteristics.

Physically-Induced Systems

Light-Based Systems (Optogenetics-Based)

Optogenetics rely on light-sensitive proteins that have a physiological role of regulating the behavior of living cells. Most optogenetic tools are based on light-sensitive ion channels, but there are also other types of molecules able to respond to light, such as enzymes and protein interaction modules (105–110). This variety of tools has opened the opportunity of modulating gene transcription and to use it for gene therapy applications (111–113). Transgene inducible-systems based in Optogenetic are a very promising approach due to their high spatial-temporal control capacity (114) compared to other systems and because light can be applied locally without affecting other organs. However, the low penetrance of blue light may be a limiting factor for future clinical application. Two main optogenetic strategies have been used in immunotherapy: Melanopsin-based (calcineurin-NFAT-based) and biLINuS-based (nuclear translocation induced by light).

The Melanopsin-based system (**Figure 2**, top-middle) relies on the ability of this protein to induce calcium influx under blue light illumination. Intracellular Ca^{++} increment activates calcineurin that triggers the nuclear translocation of NFAT (111), a transcription factor involved in the expression of multiple genes related to effective immune responses (115). For the system to achieve light-response into target cells we need to express the melanopsin and introduce an expression cassette harboring an NFAT-responsive promoter (**Figure 2**) (111). Once all the components are into the target cell, light will activate Ca^{++} influx through the melanopsin that is expressed in target cells. This Ca^{++} influx initiates a signaling cascade that leads to NFAT-nuclear translocation, activation of NFAT-promoter and expression of the desired genes.

In the biLINuS system (**Figure 2**, top-left), the light will expose the NLS motif to cause nuclear translocation of the complex (generally harboring transcriptional activators) required for transcriptional activation. These systems are based in the light-inducible nuclear localization signal (LINuS) from the LOV2 domain of Avena sativa phototropin 1 (ASP-1), a small tag that can be added to different proteins and cell types (116). In particular, the LINuS system has been used in combination with the blue light–based CRY2-CIB1 (used for blue light-dependent transgene expression) (117) but that had a high background. Huang et al. (81) developed the LINTAD gene activation system that rely in two chimeric proteins and a light responsive promoter (**Figure 2**): (1) The LexA-CIB1-biLINuS (LCB) protein combines the CRY2-CIB1 pair with the LOV2 domain reducing non-specific CRY2/CIB1 dimerization, (2) the CV protein contains the NLS from CRY2PHR (*Arabidopsis CRY2* photolase homology region) and a strong VPR transcription activator (a tripartite VP64-p65-Rta), and (3) the light-inducible promoter harbors several LexA-binding sequence (LexA BS) and a minimal promoter that require the presence of transactivators to be active. The LCB remains in the cytoplasm, while the CV remains in the nucleus. When stimulated by blue light, biLINuS in the LCB is activated, exposing the NLS motif to cause nuclear translocation of the SCB. At the same time, the CRY2PHR domain in CV is activated by blue light and can bind to the CIB1 domain of LCB with high affinity. Therefore, the LCB-CV complex is directed to the LexA BS in the reporter cassette so that the VPR is very close to the minimal promoter, which triggers transcription of the target reporter gene. This would generate a strong activation of the gene by stimulating blue light with a high signal-to-noise ratio.

Immunotherapeutic applications

Following the two main strategies described above, optogenetics have pursued two main strategies for immunotherapy: to increase the expression of NFAT-targeted genes (mainly cytokines), key regulators of T cell function (80, 112, 115, 118) and to induce CAR expression through the biLINuS system (80, 116).

Expression of NFAT-targeted genes. Based on the melanopsin-based system, Zhao et al. (80) designed a light control system for the inducible expression of three molecules: IL-2, IL-15, and TNFα. The system relies on the expression of melanopsin on T cells in order to change T-cell functions via the NFAT-calcium pathway. The T cells must also be modified to contain a NFAT-responsive promoter expressing the desired factors. The author included all these components into T cells using Lentiviral vectors and generated T cells that express increased amounts of IL-2, IL-15, and TNFα after blue light stimulation. In this system, light activates melanopsin to induce Ca2+ input triggering NFAT nuclear translocation and cytokine NFAT-dependent gene expression of IL-2, IL-15, and TNFα. This enhances the tumor killing activity of T cells, a crucial factor for immunotherapy to be effective for solid tumors. The system was tested on pan-T cells, and checked for up-regulation of IL-2, IL-15, and TNFα by messenger RNA and protein after light stimulation during 12 h of continuous exposition. Maximum reporter expression (determined in 293T) was achieved 1-6 h after point-light stimulation and reached basal levels at 48 h, but not light-kinetics were performed over primary T cells. This group demonstrated *in vivo* light-controlled antitumor efficacy in a subcutaneous model of hepatocellular carcinoma, SK-HEP1 in NSG mice. Engineered T cells were introduced intratumorally, and blue LED light applied for 7 days, showed significant tumor regression (80).

Light-inducible CAR-T cells. As described above, Huang et al. (81) generated a blue-light transgene inducible system (LINTAD) with low background that allowed the generation of a light-inducible CAR. Indeed, Huang et al. demonstrated that they could regulate the expression of several genes (including CAR) by blue light administration *In vitro* and *in vivo*. The authors engineered T cells to express constitutively the LCB and CV light-responsive proteins and to integrate a light-inducible promoter to express a CAR targeting CD19. These light-inducible CAR-T cells were able to lyse Nalm-6 cells (CD19+) 7.3-fold more efficiently after providing light. Importantly, the system also worked *in vivo* since significant differences in tumor regression was observed under blue-light conditions (1 s-pulse every 30 s during 12 h) with respect to the dark state (81).

Radiation-Controlled System

Radio-genetic therapy takes advantage of radiation and gene therapy for cancer applications, controlling by radiation the expression of a therapeutic gene. Ionizing radiation (IR) induces DNA damage such as double strand breaks (DSBs) and reactive oxygen species (ROS) that activate certain signaling pathways of mammalian cells (SAPK/JNK or DNA-PK) in order to offer an early (expression of transcription factors) and late response against that dangerous stress. IR induces the expression of TNF-alpha, IL-1 and other growth factors and metabolic enzymes for DNA repair, mutagenesis, apoptosis, and proliferation. Those inducible promoters constitute interesting tools such as transcription factors AP-1, NFkB, or Early growth response-1 (EGFR) that respond to ROS thanks to the upstream presence of the CArG box, a sequence composed by CC-(A+T rich)-6GG. Hallahan and his team developed the first AdV that controls TNF-alpha under six radio-inducible CArG boxes of *EGFR-1* gene designed as TNFerade (119) and showed a tumor regression in several xenograft models (see below) (**Figure 2**, left-middle).

Other groups have developed other artificial X-ray inducible promoters in order to overcome the cell type-dependent limitations of natural physiological promoters (120, 121) for prostate cancer. A combination of different cis-elements based on NF-kB, AP-1, NF-Y and CArG, among others, produced a candidate promoter able to regulate luciferase with a peak at 6–10 h post 10 Gy radiation, exhibiting certain antitumor efficacy but with high leaking.

Radiation systems should be further improved in order to achieve minimal basal activity if considered for other clinical applications or methods of administration and obtain more-sensitive promoters, thus 10 Gy dose is relatively high for radiotherapy (fractions of 1.8−2Gy per day are normally used in adults) (122). A treatment of several low-fractionated doses of radiation will be desirably employed for minimizing severe damage, that would depend on the tumor-type and kinetics of the therapeutic gene.

Immunotherapeutic applications

TNFerade, developed by GenVec, is a deficient AdV designed for intratumoral administration and that regulated human TNF-α under a radiation-inducible promoter, approved for phase I clinical trials by FDA in 2000 (82). TNFerade has been used over a dosage range of 4×10^7-1×10^{12} vp with 30–70 Gy synergistic radiation for several types of solid cancer such as pancreas, esophagus, rectum, breast, lung, skin, head-neck carcinoma, and soft tissue sarcoma, demonstrating improved overall and progression-free survival of cancer patients in phase I (82). Unfortunately, phase III clinical trial for locally metastatic pancreatic cancer was discontinued in 2011, when TNFerade did not increase patient survival in comparison to standard treatment (123). Instead of its proven safety, other limitations of TNFerade include its administration, only feasible to accessible cancers; a spillover out of tumor neighborhood can be a serious issue and moreover, a possible immune response against adenovector may accelerate the metabolism of TNFerade and therapy became ineffective (82).

Ultrasounds

Focused ultrasounds (FUs) can be also used as inductor for externally control of gene expression, penetrating with a depth of centimeters into tissues. Therapeutic FUs-controlled by Magnetic Resonance Imaging (MRI) have been applied into clinics for vasodilation, neuromodulation, heat-ablation of tumors and as an adjuvant therapy for drug, gene delivery (124) and tumor vaccination *in situ* (125). Low-energy focused ultrasounds (LO-FUs, with normal intensities of 0.1–2 W/cm^2 and frequencies of 0.5–3 MHz) generate rapid oscillating pressures that lead to non-invasive hyperthermia. In response, mechanical and thermal stresses are manifested transiently without killing the cells and several genes are upregulated such as heat-shock proteins that translocate from cytoplasm to cell surface (125). Based on this response, the use of heat-shock protein's (HSP) promoters have

been used for local control gene expression in several models (126) but not for immunotherapy. However, this rationale has been recently applied for CAR-T regulation induced by low-intensity FUs (83) (**Figure 2**, bottom-left). In this case, a HSP promoter drives the transgene expression upon FUs stimulation controlled by Magnetic Resonance Imaging (MRI) thermometry. This is a reversible system that generates oscillatory patterns of expression after repeated stimulation of 10 min-FUs every 48 h in T cells, thus preventing short- and long-term side effects.

However, several authors have developed acoustogenetics systems for maintaining a sustained expression, making it irreversible but avoiding FUs pulses to minimize cell death and facilitate treatment application through the adaptation of the Cre-LoxP system in a dual approach.

Immunotherapeutic applications

Based in the FU-based Cre-LoxP system, Wu et al. (83) developed a two-vector system in which the HSP promoter drives de expression of Cre recombinase in one inducible LV vector whereas a second vector allow the CAR production after the excision of a LoxP-flanked "STOP" cassette (83). *In vitro* CAR expression in primary T cells was detected 24 h later after a 15 min-pulsed FU, reaching 43°C. Minimal cell death with pulsed-FUS was observed when compared to continuous stimulation during the same period. In addition, this dual system was able to control the cytotoxic potential of inducible CAR-T cells against subcutaneous models of CD19 + Nalm6 cells and human prostate cancer PC3 cells, treated with three pulses of 5 min-FUs, whereas FUs alone did not had tumoricidal effect.

This specific design overcomes the continuous requirement of inductor, whose application is not as easy as a drug-based inducer, but making it irreversible and not allowing a safer control of CAR-T against CRS and neurotoxicity in CD19+ leukemia. Moreover, HSP are translocated to membranes after LO-FUs exposition in cancer cells. This HSP complexes can activate natural killer cells, being interpreted as danger signals for DC activation and cross-presented for generated immunity (125). Whereas, this response is very desirable for tumor treatment for the generation of immune priming and activation of the TME, should be further studied in primary T cells, in order to analyze the worst scenario of a non-desirable immunogenicity against CAR-T cells that can compromise the persistence of the therapy.

DISCUSSION

Immuno-gene therapy has revolutionized the treatment of chemo/radio-refractory cancers, sometimes reaching the frontline. As mentioned above, adoptive cell transfer based on the use of CAR-T cells has enabled the rescue of many patients by boosting their immune system. Novartis' Kymriah has achieved a complete tumor regression of 60% and Kite's Yescarta, between 36 and 54% (16). But this is not risk-free, due to severe side effects and rapid exhaustion of T-cells by uncontrolled expression of CAR (127). On the other hand, these treatments have been focused on aggressive leukemias and lymphomas, since their efficacy in solid tumors is very low, which requires alternative approaches to convert a "cold" to a "hot" battlefront into the tumor microenvironment (TME) (31, 128).

There is a lot of work to do yet, and this is where expression control systems by external stimuli become important. Being able to externally control the expression of different molecules of interest (CAR, immune-checkpoint inhibitors, cytokines...) opens new ways of re-orienting immunotherapies toward safety and efficacy (19–21). Controlling the expression of CAR will allow reducing CRS, on-target/off-tumor effects and T-cell exhaustion. In addition, controlling other molecules of interest will increase the effectiveness in the treatment of resistant lymphomas and solid tumors. In fact, CAR has already been co-expressed with interleukins (IL-12 or IL-18) and/or with antibodies against PD-1/CTLA-4, in a constitutive way (129–132). Controlling the expression of these molecules may not only enhance therapy in solid tumors, but also avoid the devastating side effects of continued expression in patients.

In this review, we have focused on inducible systems that meet four characteristics: (1) the expression of the transgene can be externally-controlled, (2) they are reversible systems, they can be turn on and off multiple times. (3) They are versatile, they can control the expression of any transgene and (4) they have been adapted for immunotherapy. We found four different systems that meet these criteria: drug, light, radiation and ultrasound-based systems.

Below we discuss different aspects that must be considered when applying these systems for immunotherapy strategies: the properties of the inductor (penetrance, viability, stability toxicity, immunogenicity, etc.), the characteristics of the elements required to achieve external regulation (toxicity, secondary effects, immunogenic, etc) and finally, the ability to deliver all components into the desired cells or tissues.

Inductor Properties

Bioavailability

Doxycycline, veledimex and mifepristone have a similar persistence of 20 h ($T_{1/2}$ in **Table 2**), good to achieve continuous stimulation for 1 day and also for a quick elimination upon drug removal. However, rapamycin has a $T_{1/2}$ of ∼60 h, which makes the system "off" slower, while rimiducid, with a $T_{1/2}$ of 5 h, has a very fast switch-off, but require frequent drug administrations. Due to their small size and polar properties, all these inductors are able to cross the BBB.

Side Effects

In general, mid-high and continuous doses of most of the inductors mentioned before are not ideal. Antibiotics can generate a serious antibiotic resistance. Tet systems that have been used to express the CAR require a Dox concentration (*In vitro*) between 10–1,000 ng/ml, that overlaps with the doses required to kill bacteria and will therefore generate resistance if prolonged or intermittent exposure. The same could apply for rapamycin-regulated systems. Therefore, long-term studies should be performed to investigate in both cases how microbiota can also be altered.

Another important aspect to consider is the effect of the inductor in immunomodulation. In fact, most of the drugs

used as inductors have an immunomodulatory effect; Rapamycin can efficiently immunosuppress activation and proliferation of T cells (133). To avoid this problem, rapamycin analogs such as Rimiducid can be used as inductors of the mutated-rapamycin system, thus reducing the affinity for the natural FKBP domain (78). Doxycycline has an anti-inflammatory effect by targeting NF-kB (134) and could therefore interfere with the immunotherapy strategy. It is therefore important to reduce the Dox concentration not only to avoid antibiotic resistance, but also to reduce the possibility of immune-suppression. In this direction, new Dox-regulated systems based on the original TetR could be an important resource due to the low Dox concentrations required for activity (34, 40). Finally, MFP acts as an antagonist of glucocorticoid receptors in non-reproductive cells, also present in T cell membrane, and can therefore exert a role blocking the T- activation (67).

Physical Inducers

Compared to drugs, the use of light or FUs as inductors has advantages and disadvantages. Firstly, light is, in theory, an ideal induction agent, since it allows the most precise spatial-temporal regulation, simply by applying light to a localized body region the induction takes place in that area. However, the system relies on blue light which is ideal for safety reasons but limits the penetration into the tissue. Probably, red or infrared light systems will overcome the penetration concern, but at the moment are less efficient and require additional cofactors plus photo-activatable domains. Radiation has emerged as an alternative induction agent, although its safety must be considered since actual systems use 10 Gy radiation, 3–4 times higher than the doses used for radiotherapy. Finally, the use of short-pulse pattern stimulation of FUS minimizes the side effects of ultrasound exposure, such as hyperthermia or induction of severe immune responses. In fact, the reversible FUS-inducible system may prevent the on-target/off-tumor toxicity of canonical CAR-T therapy. This occurs because T-cells that leave the tumor environment do not receive FUS again (since the induction is localized), so they will gradually lose membrane CAR molecules. Ultrasound pulses also allow precise temporal control of gene expression. Of note, different companies are manufacturing wearable ultrasonic emitter patches that could be useful for induction through this system (83).

Characteristics of the Elements Required to Achieve External Regulation

For an externally controlled system to be successful, the most important part is probably the characteristics of the different components that are required. Simplicity as well as low toxicity and immunogenicity are the three most important characteristics to compile after, of course, a high inducibility and a low leaking.

Toxicity of the Components

Although, in general, the expression of new proteins into a cell can alter its intrinsic properties, most of the proteins are well-tolerated and allow the cells to fulfill the functions that the scientist expect from them. However, chimeric proteins harboring transactivators, present in several of the described systems, exhibit a great capacity to recruit and sequester different transcription factors [e.g., TATA-binding protein (135)]. The majority of the Tet-based systems, the mifepristone-based, the light-inducible LINTAD system and the ecdysone-based ATMP require either viral or human chimeric transactivators (VP16, VP64, p65). In-deep studies have been performed in this regard with the Tet-platforms showing that the TetR-VP16 protein may be toxic by altering cell physiology and binding to pseudo-TetO sites which can activate undesired genes (136, 137). Furthermore, several studies (138–140) have shown a misinterpretation of the data due to the high toxicity of transactivators. In this direction, Benabdellah et al. (33, 34) have developed the first and only all-in-one, transactivator-free Dox-inducible system based on the original bacterial system. Studies are undergoing to investigate the advantage of these systems for immunotherapy applications.

Immunogenicity of the Components

The immunogenicity of all the elements required to achieve the transgene induction is another key point in the success of the system for clinical applications. In general, human proteins are going to be less immunogenic than viral and bacterial components, although in some cases this could not always be the case. The only system that is 100% human is the platform based on rapamycin. All other systems include yeast, bacterial, or viral components that are generally highly immunogenic and to which healthy individuals will mount an immune response. In particular, the immunogenicity of Tet-transactivator-dependent systems has been studied in detail finding that both cellular and humoral responses are mounted when using viral (141, 142) and non-viral (143–145) systems for *in vivo* delivery. Therefore, final strategies using these systems should include strategies that achieve immune tolerization of these components if a durable effect is desired. In fact, different approaches are being investigated to avoid these responses (146), including different routes of administration (147) that successfully achieved long term regulation in animal models.

AUTHOR CONTRIBUTIONS

MT-M and PJ-L designed this study, wrote the first draft of the manuscript, first screening of papers, and revised the manuscript. NM-P screened papers and contributed to the writing and revision of the manuscript. MC-G and KB wrote and revised the manuscript. FM designed this study, contributed with critical revision, writing of the manuscript, and paper screening. All authors approved the final version of the manuscript.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Using Gene Editing Approaches to Fine-Tune the Immune System

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Genome editing technologies not only provide unprecedented opportunities to study basic cellular system functionality but also improve the outcomes of several clinical applications. In this review, we analyze various gene editing techniques used to fine-tune immune systems from a basic research and clinical perspective. We discuss recent advances in the development of programmable nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas-associated nucleases. We also discuss the use of programmable nucleases and their derivative reagents such as base editing tools to engineer immune cells via gene disruption, insertion, and rewriting of T cells and other immune components, such natural killers (NKs) and hematopoietic stem and progenitor cells (HSPCs). In addition, with regard to chimeric antigen receptors (CARs), we describe how different gene editing tools enable healthy donor cells to be used in CAR T therapy instead of autologous cells without risking graft-versus-host disease or rejection, leading to reduced adoptive cell therapy costs and instant treatment availability for patients. We pay particular attention to the delivery of therapeutic transgenes, such as CARs, to endogenous loci which prevents collateral damage and increases therapeutic effectiveness. Finally, we review creative innovations, including immune system repurposing, that facilitate safe and efficient genome surgery within the framework of clinical cancer immunotherapies.

Keywords: immunotherapy, CARs, gene editing, graft-vs-host disease, base editors

GENE EDITING TOOLS: AN UPDATE

The well-established field of genome editing (GE) facilitates precise genomic modifications to enable genetic diseases to be studied and treated. The precise modifications induced by GE tools generate small (1–10 bp) and large (up to 20 kb) changes using different strategies. These technologies can be classified into two main groups: (1) traditional approaches that generate double-stranded breaks (DSBs) in DNA at the desired genomic loci followed or not by the introduction of exogenous DNA; and (2) approaches enabling the genome to be modified without

requiring double-stranded DNA (dsDNA) cleavage either by introducing small molecules forming a triplex structure or by combining deaminase enzymatic activity with specific impaired catalytic endonucleases. In this review, we evaluate the different techniques used to engineer immune cells in the treatment of primary immunodeficiencies and acquired diseases such as cancer and infectious diseases.

DSB-Based Gene Editing Approach

Over the last three decades, the following major specific endonucleases (SENs) have been successfully developed for both basic research and clinical purposes: meganucleases (MGNs), transcription activator-like effector nucleases (TALENs), megaTAL nucleases, ZFNs and, more recently, clustered regularly interspaced short palindromic repeat (CRISPR)-Cas-associated nucleases. The success of SENs is evidenced by the 43 on-going clinical trials using ZFNs (14), CRISPR/Cas9 nucleases (23), and TALENs (6) to treat infectious diseases (HIV-1, HPV), cancer, as well as blood and metabolic disorders (Clinicaltrials.gov June 2020). This field began to develop in 1994 when Dr. Maria Jasin and her team discovered that the generation of double-stranded breaks (DSBs) in mammalian DNA favors homologous recombination (HR) repair and that DSBs can be directly repaired by non-homologous end joining (NHEJ) (1–3). These discoveries laid the foundations of SEN-based gene editing research (4). The first SENs used to create specific DSBs for genome editing were meganucleases (MGNs), a group of endonucleases that recognize 12–45 bp DNA sequences (5–9). More versatile GE tools were developed some time later, with the use of FokI catalytic and zinc-finger domains to generate the first ZFNs (10). Soon afterward, TALENs were designed based on the bacterial system of TAL effectors (TALEs). TALENs have two different domains: a DNA-binding domain, the TALE proteins, that can be designed to bind the desired sequence (10), and a Fok1 endonuclease domain (11). With its simple design, the CRISPR/Cas system, the most versatile gene editing tool, is derived from bacteria, particularly from an adaptive immune system found in prokaryotes, which provides defense against viral infections and plasmids. CRISPR/Cas proteins form a complex with RNA molecules which guides Cas endonucleases to the target DNA to be cleaved (12). RNA nucleotides are the only part of the system that needs to be changed to specifically cut a new target site in the genome (13, 14).

Programmable Editing Without Double-Stranded DNA Cleavage

As mentioned earlier, the introduction of DSBs by SENs, followed by the activation of cellular repair machinery, can generate unwanted side effects such as off-target site indels, large deletions, and translocations. In addition, multiplex editing can lead to relatively frequent translocations and/or chromosomal rearrangements (15, 16). The following alternative systems can be devised to edit the genome without generating DSBs (**Figure 1**): (1) viral episomal vectors, such as adeno-associated viruses (AAVs), containing donor DNA (17–19); (2) triplex-forming oligonucleotides (TFO), which are able to deform DNA strands, triggering repair mechanisms without inducing DNA breaks (20–22); (3) mutated Cas9, where the RuvC nuclease domain that targets the non-complementary DNA strands and the HNH nuclease domain that targets the complementary strand are mutated converting Cas9 into a DNA nickase (23, 24); and (4) base editing (BE), a genome editing method that generates exact point mutation in both genomic and RNA sequences without DSBs (25). Two different base editors, the cytosine base editor (CBE) and the adenine base editor (ABE), have been developed over the last 3 years. The CBE, based on cytidine deamination, a common natural DNA and RNA modification, is involved in several normal biological processes (26). The principal deamination enzymes are the apolipoprotein B mRNA editing enzyme, a catalytic polypeptide-like 3 G (APOBEC), and the activation-induced cytidine deaminase (AID) enzyme (27). These enzymes can be combined with the versatile CRISPR/Cas system for genome base editing. A substantial number of studies have been published on improvements made in areas such as specificity and efficacy (28). *Petromyzon marinus* cytidine deaminase 1 (PmCDA1), identified in the lamprey genome, was used to increase BE versatility (29). The substitution of SpCas9 by *Staphylococcus aureus* (SaCas9) or *Lachnospiraceae bacterium* Cpf1 (Cas12) facilitates base editing in AT-rich organisms and interrogation of more genomic loci (30). Unlike the CBE, ABEs have no natural eukaryotic adenosine deaminase enzymes capable of acting on single-stranded DNA (ssDNA). The first ABEs were developed using adenine deaminase from *Escherichia coli* TedA (31). Over the last 2 years, several modifications to evolving ABE variants have been made: modified nuclear localization signals (NLSs) and codon usage, inclusion of ancestral deaminases resulting in BE4max, AncBE4max, and ABE max, as well as ABE8s with no significant levels of off-target adenine deamination in genomic DNA (32, 33).

Regardless of the system used, given that GE technologies need to be sufficiently specific for use in clinical treatments, the specificity of any proposed technique needs to be accurately assessed (34). Two articles in a recent issue of *Science* highlight the high rates of off-target mutation associated with base editing in two disparate rice and mouse organisms (35, 36). Therefore, to be used in clinical settings, improvements have been made to increase BE specificity. Both CBEs and ABEs have been optimized, resulting in BE variants (**Figure 1**) with improved efficacy and specificity (32, 37, 38). Finally, the more powerful and precise genome editing technique, prime editing (PE), has an impressive array of applications. PE involves the fusion of two proteins from a Cas9 nickase domain and an engineered reverse transcriptase domain (39, 40). The possibility of introducing a point mutation at a specific location offers greater targeting potential than other SENs, especially with regard to non-dividing cells such as those in the nervous system (41).

ADVANCES IN GENETIC ENGINEERING OF IMMUNE CELLS

The remarkable progress made in GE tools in recent years has made it possible to engineer different immune cell types for

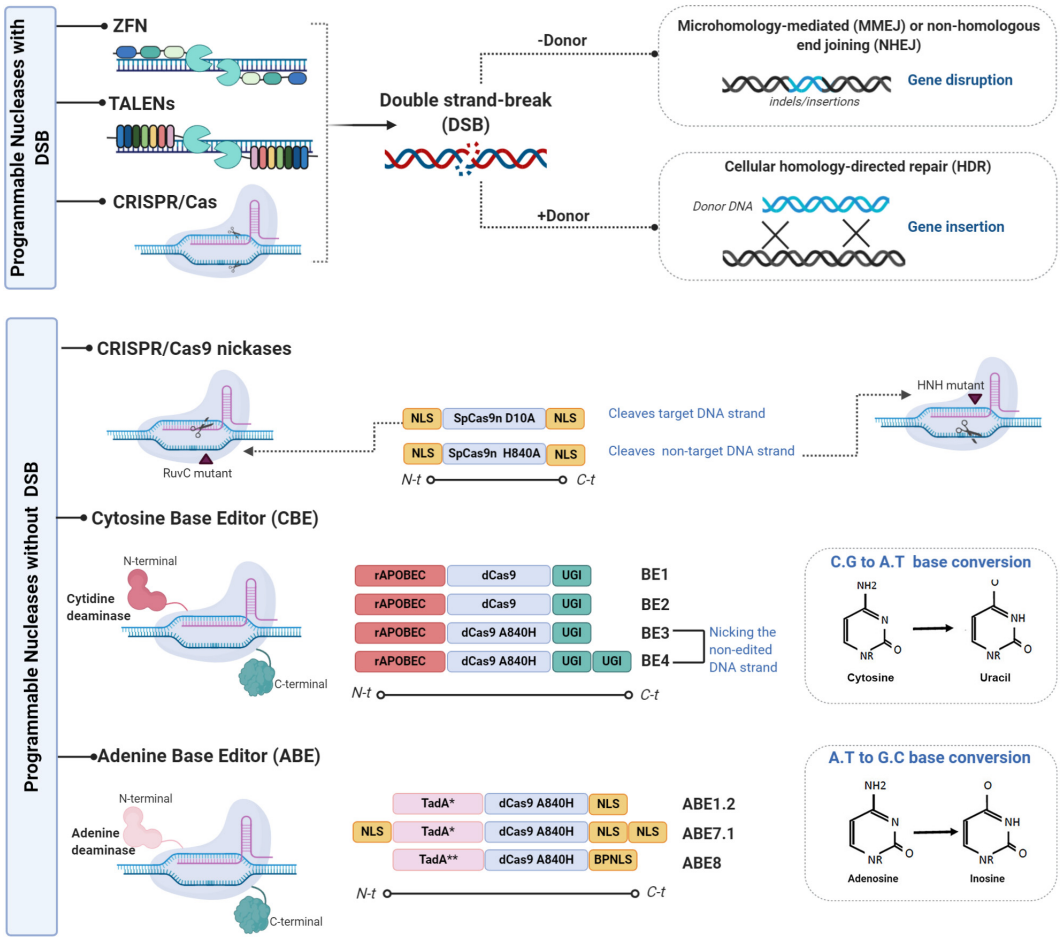

FIGURE 1 | Representative scheme of the different genome editing tools used to improve the immune system. There are two variants of genome editing technologies: those that introduce double-stranded breaks (DSBs) into DNA and those that enable genome editing without DSBs. The first variant is mainly composed of the components ZFNs, TALEN, and CRISPR/CAS which are used to enhance immune system capacity. ZFNs are chimeric proteins containing a DNA binding domain (3–5 zinc-finger domains) and a Fok1 endonuclease domain (114). Each zinc-finger domain is specifically designed to bind to virtually any DNA sequence. ZFN cleavage activity needs to be dimerized given that Fok1 acts as a dimer. Two ZFNs therefore need to be designed, each targeting a DNA sequence separated by a short sequence from the recognition site of the other ZFNs in a head-to-head fashion. As ZFNs, TALENs contain two different domains: the DNA binding domain of the TALE protein designed to bind the desired sequence (10) and the Fok1 endonuclease domain (11). As TALENs only act as dimers, two TALENs must be designed to bind to the target locus in a face-to-face fashion to cleave the target sequence (110). CRISPR/Cas, the last described SENs, is the easiest to design and the most versatile gene editing tool. It is derived from the adaptive immune system of prokaryotes, which provides a defense mechanism against certain viral infections and plasmids. The CRISPR/Cas protein forms a complex with the RNA molecules crRNA (CRISPR RNA) and crRNA (tracrRNA) which guides the Cas protein to the target DNA and produces the cleavage (12). The only part that needs to be changed to specifically cut a new target site in the genome is 20 crRNA nucleotides (13, 14). Various modifications to the gRNA design and to the Cas9 protein have been made to reduce off-target activity. A mutated Cas9 nickase has been generated to expand the CRISPR genome editing system (111). The second group is mainly composed of CRISPR-CAS nickases variants and of two base editor (BE) variants, the cytosine base editor (CBE) and the adenine base editor (ABE). The first variant is based on simple APOBEC deaminase system named BE1, which fuses APOBEC1 and dead Cas9 from *Streptococcus pyogenes* with D10A and H840A mutations (28). Its lower efficiency is attributable to uracil DNA glycosylase (UDG) which catalyzes the removal of U from DNA in cells and initiates base-excision repair (BER), thus converting the U:G pair to the C:G pair (115). The uracil-DNA glycosylase inhibitor (UGI) was fused to the C terminus of BE1 to create the second-generation BE2 system, with an improved base editing yield of 50%. A further improvement was implemented for the third-generation BE3 system. The improved BE4 base editor contains a rAPOBEC cas9 linker expanded to 32 amino acids, a Cas9n-UGI linker expanded to 9 amino acids, as well as the addition of a second copy of UGI to the C terminus of the constructs (116); BE3 and BE4 have been validated for use as base editors of human primary T cells (55). The replacement of the APOBEC1 component in BE3 with natural adenine deaminase *Escherichia coli* TedA led to the creation of the first adenine base editor ABE which was followed by ABE1.2 After several target mutations and optimizations, the ABE7.1 base editor was released. This was followed by the latest version ABE8 with its base editing facility particularly for HSPCs and human primary T cells (33). Figures were created by BioRender.com.

use in immunotherapy (**Figure 2**). Cells previously considered highly resistant to genetic modification can now be gene-edited very efficiently using these new technologies. This enables the behavior of different immune cells to be fine-tuned by deleting or enhancing endogenous gene expression and by inserting new genes in safe harbor loci. HSPCs, T cells, B cells, macrophages,

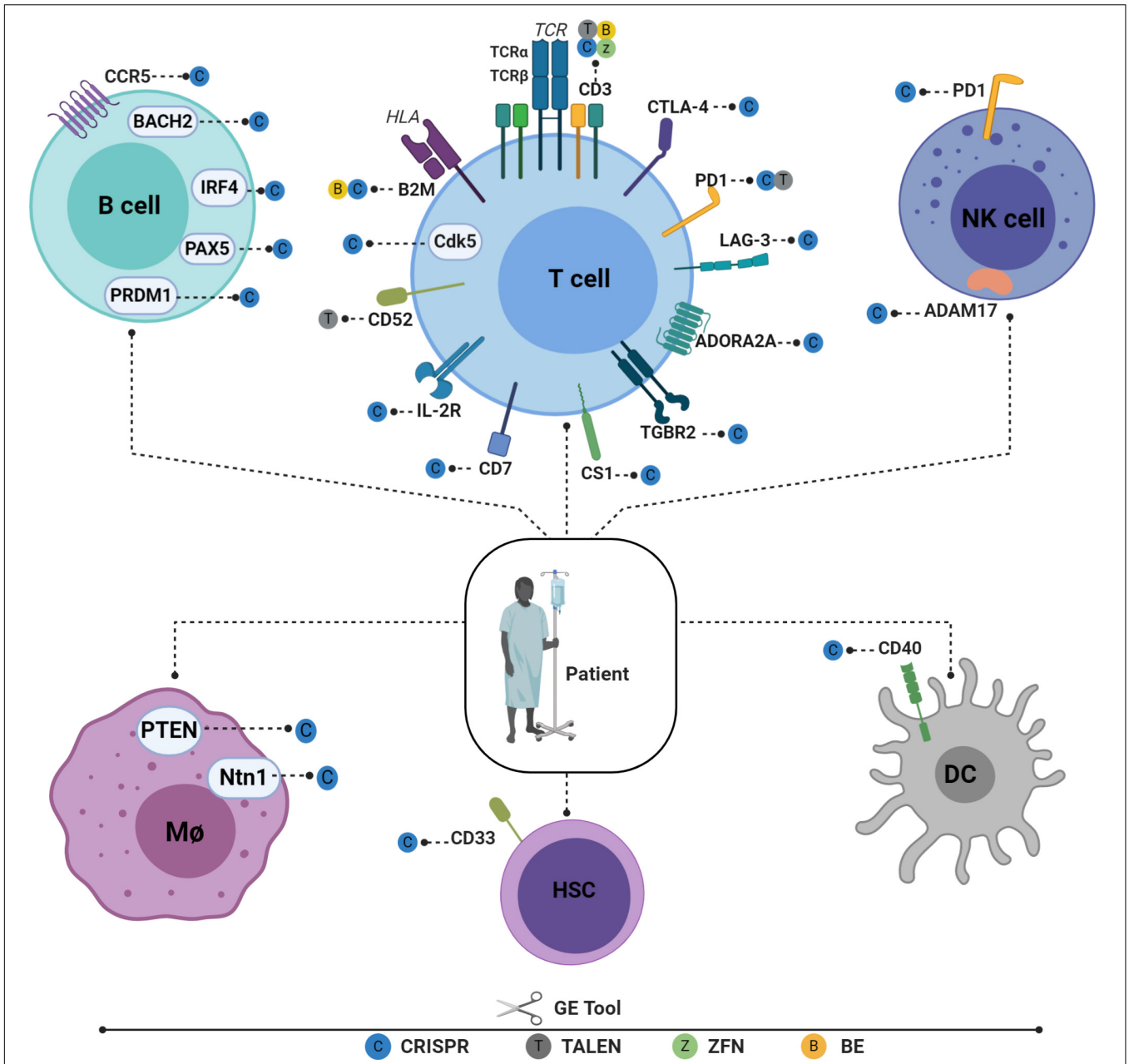

FIGURE 2 | Recent advances in engineering different immune cell types for immunotherapy applications. Engineered T cells in the B2M gene have lowered HLA class I antigen expression on the cell surface and have reduced the possibility of graft rejection. TCR/CD3 cells have been knocked out to reduce GVHD and to enable physiological CAR expression, thus enhancing CAR T potency. Tumor-suppressive microenvironments have been overcome by downregulating CTLA-4, PD1, and LAG-3. T cells have also been engineered to ignore suppressive signals by expressing dominant negative TGF beta receptors (TGBR2). On the other hand, to engraft T cells under lymphodepleting preparative conditions, the elimination of CD52 is required to enable T cells to resist alemtuzumab-mediated lymphodepletion. Targeting CD7 prevents fratricide and enables the expansion of CD7 CAR T cells without compromising their cytotoxic function. IL-2R has also been engineered to facilitate IL12P70 expression in a controlled manner. Cyclin-dependent kinase 5 (*Cdk5*), a serine/threonine kinase, whose inhibition confers antitumor immunity, has been identified to regulate the PD-1/PD-L1 pathway. HSPCs are also gene edited to enhance adoptive immunotherapy. CD33-deficient human HSPCs resistant to CD33-targeted approaches have been produced to mitigate CART33 toxicity, to maintain myelopoiesis, and to prevent on-target off-tumor toxicity. Immune NK cells play an important role in host immunity against cancer and viral infections. Despite the low efficiency of viral and non-viral delivery methods, several NK cells can be edited to enhance their persistence, cytotoxicity, and tumor targeting (117). Dendritic cells (DCs) play a critical role in T-cell response instructions, with triple knockout established as proof of concept (118). A similar approach is used to target the costimulatory molecule CD40, whose disruption significantly inhibits T-cell activation, thus reducing graft damage and prolonging graft survival (88). Macrophages can also be edited using CRISPR-CAS9 by targeting USP7 and USP47, two genes that regulate inflammasome activation (119). The Ntn1 gene, thought to be involved in cell migration disruption, can also be targeted *in vivo* using nanoparticles encapsulating CRISPR/Cas9 RNPs under the control of the CD68 promoter (89). To elucidate its role in inflammation, the NEU1 gene can be targeted in macrophages using CRISPR-CAS9, thus demonstrating the role of NEU1 macrophages as inflammation enhancers (120). It is also possible to engineer B cells to express mature broadly neutralizing bNAb antibodies targeting IgH loci or safe harbor CCR 5 in the case of FIX. Figures were created by BioRender.com.

natural killers (NKs), and dendritic cells (DCs) can now be efficiently manipulated to boost their potency (**Figure 2**). By editing different immune cell types, a specific cellular circuit can be shut down or specific endogenous immune pathways repurposed for new functions. Below, we review recent advances in GE methods that have been successfully applied to different immune cell types.

Hematopoietic Progenitor and Stem Cells

Hematopoietic progenitor and stem cells (HPSCs), some of the most desirable target cells in adoptive immunotherapy, either by the introduction of genes encoding T-cell receptors (TCRs) and chimeric antigen receptors (CARs) that target tumor-associated antigens. Unlike other hematopoietic cells, HPSCs, with their long-term engraftment capability, could provide a sustained source of effector cells. TCRs and CAR-HPSCs, which constantly produce T-lymphocyte progenitors, potentially increase the development of immunological memory. However, the engineering of HPSCs with CARs has two major drawbacks: (1) the increased likelihood, as compared with T cells, of insertional oncogenesis observed in previous clinical trials (42, 43). This can be countered by targeting CAR and TCR constructs at specific loci to raise physiological gene expression levels and to reduce the risk of insertional mutation; (2) as with other CAR strategies, the absence of cancer cell surface markers which increases on-target off-tumor toxicity. For example, the targeting of CD123 and CD33 in myelodysplastic syndrome (MDS) and in acute myeloid leukemia (AML) patients, respectively, results in toxicity due to the elimination of normal myeloid cells. MDS belongs to a group of heterogeneous diseases which is induced by defective hematopoiesis and characterized by bone marrow dysplasia and cytopenia. CAR T-cell therapy can be used to treat high-risk MDS patients, using CD123 to delineate malignant stem cell markers. Nevertheless, despite its upregulation in MDS stem cells, many studies have shown that CD123 is expressed in subpopulations of healthy non-malignant HSPCs (44). Thus, therapies, including CAR T therapy, envisaged for targeting CD123, need to take into account the on-target off-tumor effect (45, 46). Clinical trials have begun on the treatment of blastic plasmacytoid dendritic cell neoplasm (BPDCN) (NCT03203369) and relapsed AML (NCT03190278) using universal T-cell targeting of CD123. Thus, to reduce these undesirable effects, effective complementary gene editing techniques include targeted removal and reductions to below the CAR T activation threshold, while maintaining normal CD123 expression in donor HSPCs. The myeloid differentiation cell surface marker CD33 can be targeted in CAR T AML therapies. This immune-targeting approach has been designed to target CD33 which is a myeloid differentiation antigen predominantly expressed on leukemic blasts in most AML patients (85–90%). However, this marker is not only present on leukemic cell surfaces but also in healthy cells, as clearly evidenced by the withdrawal from the market in 2010 of gemtuzumab, a conjugated anti-CD33-antibody, due to bone marrow toxicity. CD33-deficient human HSPCs resistant to CD33-targeted therapy mitigate CART33 toxicity to sustain myelopoiesis and to prevent on-target off-tumor toxicity (47). AML and MDS strategies can also be used for other cell-surface antigens in CAR settings and in all antigen-specific immunotherapies.

B Cells

B cells are key regulators of humoral responses in adaptive immune system. Mature B cells, also called plasma cells, which reside in bone marrow, are in charge of producing and secreting antibodies. Most gene editing of B cells relies on the development of cellular humoral vaccines, thus avoiding the need for repetitive administration of antibodies (48). The replacement of heavy and light chain B-cell receptors with sequences encoding a suitable monoclonal antibody in an allergenic manner could improve immunization (49, 50). Based on this hypothesis, GE could be used to express recombinant antibodies under the control of endogenous regulatory elements, enabling strict regulation of gene expression in response to specific antigens. This would facilitate the production of appropriate concentrations of the corresponding antibodies (49). Genome editing of human B cells mediated by homology-driven repair (HDR)/CAS9 has also been used to produce anti-HIV-1 broadly neutralizing antibodies (bNAbs) (51) and to boost the capacity of long-lived plasma cells to physiologically produce proteins with therapeutic applications (30). Hung et al. have generated active FIX-secreting human plasma cells at the CCR5 safe harbor locus using the HDR-mediated method. The engineered B cells expressed the active recombinant FIX gene, demonstrating the possibility of editing primary B cells with high specificity and efficacy. This approach paved the way for future clinical applications involving primary B cells (30). Cas9-mediated disruption has also been tested on naïve B cells for IRF4, PRDM1, PAX5, and BACH2 genes, with over 80% efficacy, thus proving the feasibility of gene knockdown in this cell type and its potential use in immunotherapeutic treatments (30).

T Cells

Given their central role in immune responses, primary human T cells have great potential for use in immune cell gene therapies. Genetically modified T cells have been investigated for many years in adoptive cellular immunotherapies for the treatment of cancer, infectious diseases, and immunodeficiencies. However, although GE technologies were initially successful in other cell types, their use in primary human T cells was hampered by low-efficiency delivery methods and the vulnerability of these cells to physical and chemical challenges. Nevertheless, as mentioned earlier, new developments have led to increased efficiency and reduced damage overall, specifically in T cells. T cells from different sources, such as peripheral blood, tumors, and bone marrow, with different phenotypes, including αβ, γδ, and NKT, have been successfully edited and used in various immunotherapeutic approaches (see next section). Almost all the aforementioned GE tools have been applied to T cells for different purposes. However, target genes such as programmed cell death-1 (PD1), TCRs, human leukocyte antigens (HLAs), and β2-microglobulin (β2M) have been used in multiple studies

given their potential to increase or to control T-cell activity. It is important to note that both GE strategies, SENs and BEs, are now reaching efficacies over 80% of the desired modifications without a significant effect on T-cell physiology other than the desire. As with other cell types, two principal approaches, gene silencing and gene insertion, are used to fine-tune T-cell activity.

Gene Expression Silencing, Aimed at Blocking Gene Expression, Which Blocks T-Cell Activity or Interferes With Its Survival in the Host

(i) Programmed cell death-1

T cells, including tumor-infiltrating lymphocytes, were successfully gene edited using the three specific endonucleases (SENs), as well as the BEs described earlier, to target programmed cell death-1 (PD-1). By the electroporation with a clinical-grade mRNA encoding for ZFNs specific for PD-1, Bean et al. achieve an average modification frequency of 74% bulk population reducing to a minimal level the PD1 surface expression. Importantly, the authors do not report any adverse effects affecting T-cell functionality or phenotype (52). Other approaches based on TALEN and CRISPR/Cas9 methodologies were also considered. TALEN-mediated PD-1 gene inactivation in melanoma $CD8^{++}$ T cells was triggered using an optimized mRNA-electroporation protocol (53, 54). More recent ABE- and CEB-based approaches were also considered to significantly reduce the risks associated with unintended genomic alterations and genotoxicity (33, 55).

(ii) Human leukocyte antigen

Human leukocyte antigen (HLA) expression on CAR T cells can lead to immediate rejection by the host recipient through recognition of non-self HLAs. GE-based methods are used to eliminate HLA molecules from T-cell surfaces. As with PD-1, ZFNs (56), and the CRISPR system, base editing and mRNA electroporation have been used to eliminate HLA expression (9, 33, 55, 57, 58). Another possible strategy to eradicate HLA expression is the elimination of b2-microglobulin (B2M). HLA proteins are covalently associated with B2M in the endoplasmic reticulum, an association which is crucial for the formation and trafficking of functional HLA molecules on cell surfaces. However, some concerns have been raised with regard to the possible targeting of these HLA class I negative cells by NK cells (59, 60).

(iii) T-cell receptor

As discussed in the clinical applications section, TCR knockout has profoundly revolutionized immunotherapy. Genome editing of this locus has, on the one hand, consolidated the off-the-shelf nature of CAR T therapy by reducing graft-versus-host disease (GVHD) toxicity and has, on the other hand, facilitated the physiological regulation of CAR function and enhanced anti-tumor activity. The study by Berdien et al. was the first to completely shut down endogenous TCRs through the transfection of TALEN pairs specific to human TCR α and β chains (61). As noted later, TCR KO has become a well-established feature of clinical treatments (62–64).

(iv) Transformation of growth factor beta receptor 2 (TGFBR2) proteins

Another major issue is the complexity of the tumor microenvironment (TME) which inhibits CAR T therapies. One of the most important regulators in the TME is TGF-β. There are three TFG-β ligands, TGF-β1, TGF-β2, and TGF-β3. The receptor most commonly upregulated in tumor cells is TGF-β1, which negatively regulates CAR T-cell cytotoxic function via TGF-β receptors. With an attempt to enhance CAR T cells using CRISPR/Cas9, Tang et al. managed to knock down TGFBR2 and to improve the *in vivo* CAR T cells in an animal model (65).

(v) Cyclin-dependent kinase 5 (Cdk5)

The serine/threonine kinase Cdk5 has been identified to regulate the PD-1/PD-L1 pathway. Its inhibition confers antitumor immunity due to interference with interferon regulatory factor 2 (IRF2) and interferon regulatory factor-binding protein 2 (IRF2BP2) (66, 67). Consistent with this model, the depletion of CDK5 by shRNA leads to the hyperphosphorylation of IRFBP2, increased IRF2 expression, and lower PD-L1 levels (68, 69). In addition, CRISPR-mediated disruption of CDK5 in cancer cells results in suppression of tumor growth (69, 70).

(vi) Multiplex gene knockout

Targeting two or more loci simultaneously is a pre-requisite for certain therapeutic approaches (71). For example, the CD52 locus targeted by alemtuzumab, along with the TCR and PD1 loci, can be targeted simultaneously to facilitate engraftment of CAR T cells resistant to alemtuzumab and PD-L1. CD52/TCR KO T cells were successfully obtained by TALEN mRNA electroporation (72), and this approach has recently been used in clinical therapies (73). Similarly, TCR/PD1 KO T cells were obtained using highly specific non-conventional TALEN technology for multiplex genome editing (74). The Ren research group carried out a quadruple gene ablation using CRISPR system to generate dual inhibitory resistant universal CAR T cells deficient in TCR, HLA-I, PD-1, and CTLA-4. The multiplex genome editing of CAR T cells with the one-shot CRISPR system was found to be highly efficient (57, 58). Despite these impressive results, undesirable effects, such as chromosomal re-arrangement and translocation, are highly possible. However, both genome base editing and the nickase variant of the CRISPR system enable several genes to be targeted simultaneously without any adverse effects. This multiplex approach using BE tools has been used successfully to target TCR, B2M, and PD-1 genes in a single round of electroporation. All these genes were silenced without DSBs while preserving their capacity to mediate target cell killing (33, 55).

Insertion of New Genes Into Desired Loci: Dual Knockin Effect

Recent advances in gene editing technologies have enabled the knockout of endogenous genes and at the same time the introduction of large DNA sequences into specific loci to integrate new genetic instructions into specific endogenous loci to modulate T-cell function and specificity. These strategies were successfully adapted to CAR T therapy, simultaneously knocking out one gene and expressing another. This approach takes

advantage of the tightly regulated nature of immune pathways, enabling the endogenous genes to be precisely repurposed. In this regard, Roth et al. reported a virtual total loss of endogenous TCRs which were replaced by specific tumor-associated antigen TCRs using CRISPR/Cas9 ribonucleoproteins (RNPs) co-transfected with dsDNA HDR templates (75). With the aid of this feature, Eyquem et al. developed a similar strategy to incorporate the CAR construct into the TRAC locus (76). As part of a more sophisticated approach, Sachdeva et al. used the signaling pathways of TCR, CD25, and PD1, three major players involved in T-cell activation, to express CAR and IL12-P70 genes in a controlled manner (77). The highly regulated nature of immune pathways enables therapeutic genes to be expressed in a tumor cell–dependent manner, thus reducing several side effects associated with uncontrolled continuous expression (76, 77).

Natural Killer Cells

Natural killer (NK) cell lymphocytes play an important role in the innate immune system due to their ability to kill a variety of target cells, including cancer cells, and can rapidly respond in a thymus-independent manner without previous recognition of the antigen (78). Their effector capacity depends on the balance between activating and inhibiting signals triggered by specific receptors that recognize ligands on stressed cells such as tumoral and infected cells (79). Recent studies show that NK cells can be genetically engineered to express chimeric antigen receptors (CARs) (80–82). However, few studies have reported gene editing modifications in NK cells due to their low rates of transduction with viral systems and the difficulty of NK expansion *in vitro* (83, 84). These issues are well illustrated by the first study to demonstrate efficient HDR genome editing in NK cells which appeared in 2020 (84). Another study, using the CRISPR/Cas9 RNP system, showed that NHEJ-GE KO of ADAM17 and PDCD1 (85) induces enhanced killer phenotypes via ADCC and non-ADCC pathways, respectively.

Dendritic Cells

Dendritic cells (DCs), considered to be at the center of the immune system, provide a crucial link between innate and adaptive immune responses. These cells, first described by Steinman and Cohn, have a unique immunomodulatory capacity. The few studies in the literature on dendritic cell editing mainly focus on graft rejection mediated by activated T cells. CD40, a key molecule involved in DC activation and maturation, is deeply implicated in communication between DCs and T cells. This interaction relies on antigen presentation and subsequent helper and cytotoxic T-cell priming (86, 87). Thus, any CD40 reprogramming of DCs will allow transplant tolerance, and with this in mind, Zang et al. used a novel delivery method consisting of poly(ethylene glycol)-*block*-poly(lactide-*co*-glycolide) (PEG-*b*-PLGA)-based cationic lipid-assisted nanoparticles (CLANs). This method is capable of delivering of delivering Cas9 mRNA (mCas9) and a guide RNA targeting the costimulatory molecule CD40 (gCD40) both *in vivo* and *in vitro*. CD40 knockdown significantly reduced T-cell activation, thus alleviating graft damage and prolonging graft survival (88).

Macrophages

Macrophages and their monocyte precursors are members of the innate immune system and form part of the mononuclear phagocyte system. Macrophage gene editing is currently being tested for use in the therapeutic treatment of several diseases. Luo et al. have developed CRISPR/Cas9 vectors using a plasmid delivery system under the control of the CD68 promoter which is capable of boosting gene expression specifically in monocytes and macrophages. In addition, these plasmids were encapsulated in PEG-*b*-PLGA-based CLANs, which are redirected to B cells, neutrophils, monocytes, and macrophages (89). Luo et al. performed *in vivo* targeting of the *NTN1* gene, whose overexpression is associated with type 2 diabetes (T2D) and monocyte/macrophage-specific expression, with no off-target effects and an editing efficiency of 10.1% at a dose of 1 mg/kg and of 19.6% at 2 mg/kg as compared with 1.2 and 2.3% for indels detected in neutrophils, respectively (89). Macrophages were also genetically edited for cell-based cancer immunotherapy through the elimination of CD47:SIRP-α interactions by knocking out SIRP-α using the CRISPR-Cas9E20 system and by introducing an E20 tag at the protein N terminus, thus facilitating self-assembly with arginine-coated gold nanoparticles (ArgNPs). This novel complementary strategy, which enables over 30% of genes to be edited, provides a novel and complementary approach to cancer immunotherapy (90).

GENOME EDITING STRATEGIES TO IMPROVE CAR T CANCER IMMUNOTHERAPY

Cancer, which is a complex disease caused by a variety of genetic and epigenetic changes, is a major threat to human life and public health. Conventional strategies, such as surgery, radiotherapy, and chemotherapy, have made significant advances in the treatment of cancer. However, manipulation of the immune system has become one of the most promising therapeutic approaches. Among the vast repertoire of effective antitumoral molecules and drugs, genetically modified immune cells have emerged as a truly effective therapeutic approach. One of the most promising anticancer treatments is CAR T-cell therapy, which mainly involves a redirection of immune cells against tumors. Although CAR-based therapy has already proved its efficacy in certain hematological malignancies, several challenges remain to be addressed: (1) the biological characteristics of autologous T cells; (2) the expansion of CAR T cells and their potency; (3) the persistence of CAR T cells; (4) the determination of optimal CAR expression; (5) the determination of optimal T-cell subpopulations. In the following section, we describe different GE approaches for generating next-generation CAR T cells with improved characteristics, as well as the potential clinical applications of CAR T gene editing products.

Off-the-Shelf Engineered T Cells

Autologous CAR T cells are ideal for adoptive T-cell therapies due to the absence of allogeneic reactions. However, several limitations are associated with autologous T cells, mainly related

to the cost and failure of personalized production processes. Some patients may not be able to meet numbers and quality of T cells required for the production process. Thus, autologous T cells may not be effective in the case of intrinsic dysfunctions associated mainly with previous treatments. To avoid these drawbacks, various groups have been investigating the production of suitable CAR T cells generated from healthy donors. To generate the CAR T cells to be administered to allogeneic transplant patients, the issue of the T-cell alloreactivity levels that trigger allograft rejection and GVHD needs to be resolved. As discussed earlier, GE can be used to resolve these potential issues by eliminating TCRs to prevent GVHD and by eliminating MHC class I and/or MHC class II molecules to prevent allograft rejection. Since the first study in 2012 in which TCRs were deleted using ZFNs (56), different approaches have been used to eliminate endogenous TCRs alone or combined with other target molecules to improve T-cell properties. Poirot et al. applied multiplexed gene editing to primary T cells for the first time using TALENs which simultaneously knocked out TCR and CD52 molecules (72). CD52, a surface protein expressed on immune cells, is targeted for lymphodepletion to delay allograft rejection. These edited T cells did not cause GVHD *in vivo* and were resistant to the anti-CD52 monoclonal antibody alemtuzumab. When equipped with a CD19 CAR, T cells are capable of destroying $CD19^+$ tumor cells in *in vitro* and *in vivo* experiments. A similar strategy is used for expressing BCMA CAR T cells in multiple myeloma treatments, leading to the generation of *in vivo* activity similar to that of their wild-type counterparts (91) in clinical trials (NCT04093596). Liu et al. generated *TRAC*, *B2M*, and *PD-1* triple knockout T cells using the CRISPR/Cas9 system. They compared how double (TRAC, B2M) and triple (TRAC, B2M, and PD-1) knockout affected CD19-CAR T-cell activity, demonstrating *in vitro* that the triple CART showed higher efficiency than double knockout cells (92). As clinical proof of concept, TALEN-mediated TRAC/CD52 KO CD19 CAR T cells (UCART19) were administered to two infant patients with relapsed acute lymphoblastic leukemia (ALL), both of whom presented tumor remission, which was followed by successful allogeneic stem cell transplantation (allo-SCT), with no significant GVHD observed (73). Three multicenter clinical trials are ongoing on the safety and efficacy of UCAR19 cells in children with ALL (NCT02808442, pediatric ALL), adults with ALL (NCT02746952, CALM), and both age groups with lymphoid malignancies (NCT02735083). Other clinical trials have been initiated using universal CD123 CAR T cells generated by TALEN gene editing for BPDCN (NCT03203369) and relapsed AML (NCT03190278). The following clinical trials using CRISPR-edited T cells are also ongoing: TRAC/B2M KO CD19 CAR T cells for B-cell leukemia and lymphoma (NCT03166878; NCT03229876); TRAC/PD1 KO mesothelin CAR T cells for solid tumors (NCT03545815); TRAC KO dual CD19/CD22 and CD19/CD20 CAR T cells for B-cell leukemia (NCT03398967); CD19 TCR-CAR T cells for non-Hodgkin lymphoma and B-cell ALL using meganucleases (NCT03666000). However, as mentioned earlier, multiplex gene editing could involve chromosomal aberration and translocations. To prevent these major constraints, Webber et al. used CBEs to generate triple-negative CD19 CAR T cells (TRAC, B2M, PDCD1). These cells showed improved antitumoral capacity against target cells *in vitro* (55). It is important to note that the initial concerns regarding the off-target effects of BEs appear to dissipate with the development of new-generation BEs. The eighth generation of adenine base editors, ABE8, provides for multiplexed editing of B2M, CIITA, and TRAC genes, with an editing efficiency rate of over 98% (33). TCR gene editing also benefits immunotherapy approach based on cancer-specific TCR surface expression, in which the presence of both endogenous TCRs and cancer-specific CARs induces the formation of heterodimers which, in turn, reduces therapeutic effectiveness. Legut et al. therefore co-transduced primary T cells expressing cancer-specific αβ and γδ TCRs, with a lentiviral vector encoding a CRISPR/Cas9 system designed to target TRBC*1* and *TRBC2* genes. The response of the double-transduced T cells to target B-LCL and melanoma cell lines was more sensitive, strong, and efficient than that of standard TCR-transgenic T cells (63). These preliminary findings were clinically corroborated in a phase I clinical trial using autologous NY-ESO-1 TCR T cells after CRISPR-Cas9 editing of *TRAC*, *TRBC*, and *PDCD1* loci began (NCT03399448). Although the edited T cells in patients resisted for up to 9 months, only one patient experienced tumor regression (71). TCR genes were also knocked out to produce fratricide-resistant CD3-specific CAR T cells to treat T cell ALL, whereas TCR $CD3^-$CAR T cells created by TALEN editing generated strong antileukemic effects on a xenograft mouse model (93). To treat T-cell malignancies, other markers such as CD7 were knocked out. Using the CRISPR/Cas9 toolkit, CD7 CAR T cells (UCART7) mediated the efficient destruction of malignant T cells with no significant T-cell fratricide reported (94–96).

Expansion, Persistence, and Potency of CAR T Cells

Although CAR-modified T cells have been successfully used in the treatment of hematologic malignancies, several limitations still need to be resolved, especially with regard to solid tumors, which are mainly associated with an unfavorable TME, which includes the upregulation of inhibitor receptors (IRs), leading to the activation of intrinsic inhibitory pathways. Modulation of TME responses by antagonizing tumor-associated negative immune regulators such as PD1, TGF-β, and adenosine is considered a desirable treatment strategy. One of the most extensively characterized T-cell IRs is programmed cell death 1 (PD-1), also known as CD279, which is a cell surface immunoinhibitory receptor expressed by a wide range of immune cells such as T cells, B cells, dendritic cells (DCs), natural killers (NKs), and myeloid cells (97). Its activation depends on the PD-L1 and PD-L2 ligands present in tumor cells, whose interaction with PD-1 inhibits T-cell activation and proliferation by inducing energy and an immunosuppressive process in consonance with the TME (98). PD1 blockage is being tested as a novel immunotherapeutic target in different cancers. FDA-approved PD-1 and PD-L1 antibodies, such as nivolumab, pembrolizumab, and MPDL3280A, are indicated in the treatment of melanoma, metastatic bladder cancer, and

glioblastoma (99–101), although PD-1 gene editing may increase CAR T potency. The GE tools described, including TALEN, CRISPR-Cas9, and ABE, have all been used to target *PD1* loci (54, 57, 58, 71, 102, 103). In all cases, the disruption of this protein enhances cellular immune responses, thus increasing cancer cytotoxicity and enhancing cancer immunotherapy. Clinical trials with PD-1 knockout autologous T cells are currently under way for the treatment of cancers such as prostate cancer (NCT02867345), bladder cancer (NCT02863913), and renal cell carcinoma (NCT02867332). Other clinical trials on where PD-1 genes are downregulated to fight lung cancer (NCT02793856), esophageal cancer (NCT03081715, phase II), and multiple tumors (NCT03545815, NCT03747965, NCT03399448) are ongoing. TGF-β (types 1, 2, and 3) is secreted by stromal cells such as cancer-associated fibroblasts, blood endothelial cells, mesenchymal stem cells, lymphatic epithelial cells, and pericytes. TGF-β, one of the most important immunosuppressive molecules in the TME, contributes to cancer initiation and progression. TGF-β favors the conversion of $CD4^+$ effector T cells into $CD4^+$ Tregs and blocks the secretion of cytotoxic molecules and cytokines in $CD8^+$ T cells. In CAR T cells, the presence of TGF-β1 accelerates the exhaustion of CAR T cells by upregulating the PD1- and FOXP3-dependent Treg-like phenotype of CAR T cells (65). *TGFBR2* knockout enhances the antitumor efficacy of CAR T cells *in vivo* in cell line–derived xenograft and patient tumor–derived xenograft (PDX) mesothelin pancreatic carcinoma. After the PDX tumor was eliminated by *TGFBR2*-KO CAR T cells, the treated mice were re-challenged with novel contralaterally reinoculated patient-derived tumor cells, showing a persistence and antitumor properties (65). The adenosine A2A receptor, also known as ADORA2A, plays a regulatory role in the adaptive immune system. Adenosine, which is generated by tumor cells, strongly inhibits endogenous antitumor T-cell responses by activating ADORA2A. ADORA2A ablation could block adenosine signaling inhibition in T cells, thus providing a feasible way to study hostile TMEs, one of the major barriers to inhibiting immune reactions (104). This could be used to enhance CAR T-cell efficiency. Beavis et al. have highlighted the potential of targeting A2AR-mediated suppression to enhance CAR T-cell activity, particularly against solid tumors, which has, so far, been less impressive and in which adenosine-mediated immunosuppression is more prevalent due to the hypoxic environment (105). A2AR antagonists have undergone phase III clinical trials for Parkinson's disease and are currently in phase I trials for oncology, indicating the highly translational nature of this approach (ClinicalTrials.gov, NCT02655822). A2AR was knocked down using retroviral shRNA to downregulate the target receptor and a second retroviral vector with an anti-HER2 CAR.

Persistence of CAR T Cells

Robust *in vivo* expansion and persistence of genetically modified T cells are considered prerequisites for positive responses in hematologic malignancy patients. On the one hand, cytotoxic T lymphocyte-associated protein 4 (CTLA-4 or CD152) is a protein receptor expressed in activated T cells and regulatory T cells that bind to B7 ligands in antigen-presenting cells to transmit inhibitory signals to T cells following activation, downregulating IL-2 and reducing cell division. CTLA4 disruption with CRISPR/Cas9 increases TNF-α and IFN-γ secretion, leading to a significant increase in the apoptosis of human adenocarcinoma and bladder carcinoma tumor cells (106, 107). On the other hand, LAG-3, a major inhibitory receptor belonging to the IgG family, has 20% similarity with CD4 and binds to MHC class II receptors with higher affinities than CD4. LAG-3 expression occurs on activated CD4 and CD8 T cells, regulatory T cells (Tregs), NK cells, B cells, and plasmacytoid dendritic cells. LAG-3 is upregulated on exhausted T cells as compared with effector and memory T cells (108) in cancer, chronic infections, and autoimmunity (109). Blocking both PD1 and LAG-3 pathways with monoclonal antibodies synergistically reverses T-cell exhaustion (110) and increases memory T-cell formation (111). In addition, clinical trials blocking of LAG-3 alone or combined with anti-PD1 for the treatment of solid tumors are currently under way (NCT01968109). Recent clinical reports highlight the relationship between individual responses and the frequencies of infused $CD8^+$ CTL19 cell expressing PD-1 TIM3 and LAG-3. Analysis of PD-1 co-expression with LAG3-3 and TIM-3 revealed that $CD8^+LAG3^+$ cells expressing PD1 are associated with poor responses, whereas patients in full remission were infused with products containing significantly lower frequencies of these cells (112). Although several preclinical studies have been carried out to evaluate the effect of lymphocyte activation gene 3 (LAG3) ablation, no definitive data are available on, for example, the bulk population of 70% of LAG-3 KO CAR T cells, which show no significant improvement, as compared with CAR T cells, with regard to efficiency or T-cell exhaustion in a xenograft $CD19^+$Raji-NSG mouse model (113).

CONCLUSION

The possibility of modifying the genome of human cells in general and immune system cells in particular opens up a whole range of opportunities hitherto unimaginable. The generation of large deletions/insertions, transcription factor targeting, and epigenetic markers have enabled scientists and clinicians to modulate gene expression with unprecedented precision. Despite some remaining limitations with respect to specificity and efficiency, various several studies carried out in this field have produced encouraging results and feasible clinical applications such as those utilizing available gene editing tools to generate off-the-shelf CAR T cells. A new system, in which endogenous TCR and HLA molecules on allogeneic T cells are eliminated, leading to a reduction in GVHD and rejection by the transplant recipient's immune system, has also been developed.

These off-the-shelf–based therapies are of special interest to patients who, due to previous treatments or disease progression processes, have T cells deficient in either number or quality. Universal T cells can be generated and stored for later immediate use. GE approaches have also used immune checkpoint inhibitor therapies. Several immune checkpoint molecules expressed on immune cells have been successfully targeted using SENs to enhance the effect of CAR T cells, particularly CTLA-4, PD1, and LAG-3, with the elimination of PD1 most extensively studied

in clinical settings. Blockage of the PDL1-PD1 axis enhances the cytotoxicity of T cells, an effect which is intensified when inhibitory checkpoints, such as CTLA-4, PD-1, and LAG-3, are simultaneously targeted. Although these strategies are being tested in several clinical trials, one of the major shortcomings of the multiplex strategy is associated with the simultaneous presence of multiple DSBs, which dramatically increases the likelihood of non-lethal but potentially toxic translocation. This problem is likely to be solved by the use of emerging efficient base-editing technologies to drastically reduce genotoxicity and to eliminate the possibility of genomic translocation. All these emerging technologies open up new possibilities and applications in the field of biomedicine. Although the results of early phases of clinical trials evolving these systems are encouraging, the limited number of patients involved hinders for the moment a definitive conclusion concerning the safety of those approaches.

AUTHOR CONTRIBUTIONS

KP, MT-M, NM-P, and MC-G: manuscript writing and figure processing. SS-H and PJ-L: manuscript writing. MDC and CH: manuscript review. FM and KB: manuscript writing and final approval of manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Identification of Targets to Redirect CAR T Cells in Glioblastoma and Colorectal Cancer: An Arduous Venture

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The chimeric antigen receptor (CAR) is an artificial molecule engineered to induce cytolytic T cell reactions in tumors. Generally, this molecule combines an extracellular single-chain variable fragment (scFv) able to recognize tumor-associated epitopes together with the intracellular signaling domains that are required for T cell activation. When expressed by T cells, the CAR enables the recognition and subsequent destruction of cancer cells expressing the complementary antigen on their surface. Although the clinical application for CAR T cells is currently limited to some hematological malignancies, researchers are trying to develop CAR T cell-based therapies for the treatment of solid tumors. However, while in the case of CD19, or other targets restricted to the hematopoietic compartment, the toxicity is limited and manageable, the scarcity of specific antigens expressed by solid tumors and not by healthy cells from vital organs makes the clinical development of CAR T cells in this context particularly challenging. Here we summarize relevant research and clinical trials conducted to redirect CAR T cells to surface antigens in solid tumors and cancer stem cells with a focus on colorectal cancer and glioblastoma. Finally, we will discuss current knowledge of altered glycosylation of CSCs and cancer cells and how these novel epitopes may help to target CAR T cell-based immunotherapy in the future.

Keywords: solid tumor, CRC (colorectal cancer), CAR T cells therapy, CSCs, GBM, MAbs

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INTRODUCTION

The three traditional pillars of cancer treatment, surgery, radiotherapy and chemotherapy are still the therapy of choice for most patients (1). The immunotherapy treatments approved in recent years has widened the arsenal to the fight against cancer (2, 3), particularly for the use of monoclonal antibodies (mAbs) and genetically modified cells recognizing tumor-associated antigens (TAAs) (4). In some cases, immunotherapy results in significant improvement of the patient survival, even when the disease was particularly resistant to the traditional therapies (5, 6). Among the different cellular immunotherapy strategies, the adoptive transfer of T cells directed against tumor antigens is a new and particularly promising approach for the rapid generation of many tumor-specific lymphocytes (7). The transduction of T cells with a chimeric antigen receptor (CAR) recognizing TAAs is an effective method to target tumor cells in an MHC-independent manner. The clinical outcome of the CAR T cell approach in solid tumors depends on several parameters (8) such as

CAR architecture (9); lymphodepletion before the administration of CAR T cells (10); efficient tumor homing and persistence in the tumor environment (11, 12); toxicity (13); specificity for the target (14). Most of these parameters are extensively reviewed in the cited articles. In this review we will give first a brief overview about the molecular composition of the CARs and then concentrate on the tumor targeting and the lack of specific antigens as one of the biggest difficulties in the generation of CAR T cell therapy in general and particularly in solid tumors such as colorectal cancer (CRC) and glioblastoma (GB) (7).

THE MOLECULAR COMPOSITION OF CHIMERIC ANTIGEN RECEPTOR-AN OVERVIEW

Under physiological conditions, the specificity of T cells is strictly dictated by the recognition of major histocompatibility complex (MHC)-presented antigen by the T cell receptor (TCR) and subsequent clonal expansion of antigen-specific (e.g., tumor-specific) cells. Using recombinant DNA technologies and retro- or lentiviral transduction, T lymphocytes can be engineered to express CARs. These consist of an extracellular domain that serves for antigen recognition and an intracellular domain for signal transduction. In the majority of CARs, the central component used for the signal transduction is derived from the CD3 zeta chain (CD3z) of the TCR complex, while the antigen recognition is directed by a single-chain variable fragment (scFv) engineered from antibody heavy and light chains (9). These structures combine the specificity of MHC-independent antibody recognition with the anti-tumor potential of T lymphocytes and open the possibility to generate T lymphocytes of any antigenic specificity. CARs using only the CD3z chain for signal transduction are defined as first-generation (15, 16). T lymphocytes expressing these constructs show strong anti-tumor activity *in vitro*, but they have limited efficacy *in vivo* (17). These observations led to the design of second-generation CARs, which are engineered with an additional intracellular costimulatory domain often derived from either CD28, 4.1BB, ICOS, or OX40 molecules. The transduction with second-generation CARs produces T cells that have a greater capacity for cytokine production and expansion (18, 19). The combination of three signal domains (e.g., CD3z-CD28-4.1BB or CD3z-CD28-OX40) further increased the activity. These constructs are subsequently called third-generation CARs (20–22). The so-called fourth-generation CARs or TRUCKs (CAR T cells redirected for universal cytokine killing) have shown to increase T cell activation, proliferation, and persistence, through the combination of two costimulatory domains and the engineered capability of enhanced cytokine secretion (23, 24). However, although third and fourth generation CARs were shown to have advantages in preclinical model systems, their superiority compared to second-generation CARs in the clinical setting still has to be proven. We also like to mention that the only two FDA approved CAR therapies, tisagenlecleucel (KYMERIAH) and axicabtagene ciloleucel (YESCART) are both based on second-generation constructs. In addition to the classification by how the activating signal is transduced, the CAR can be differentiated based on its capacity to recognize a single or several TAAs. To increase the versatility, universal CARs (UniCARs) and tandem CARs (tanCARs) were developed. UniCARs have an extracellular moiety that binds to a soluble adaptor, which in turn defines the specificity against a certain TAA. Several different versions of UniCARs with adaptable specificity are available. These include antibody-dependent cytotoxicity receptors such as NKp30 (targeting B7H6) (25), CD16 (26), and NKG2D (27). The anti-Tag CARs also belong to the UniCARs. These receptors utilize scFvs targeting molecular tags or chemically conjugated peptides, which in turn bind to tumor antigens (28) and are supplied either systemically or intratumoral in the experimental animal. A similar strategy is followed by the biotin-binding immune receptor CAR (BBIR CAR) that employs the biotin-avidin system to bind CAR T cells to an antigen (29) In these constructs, the extracellular scFv part is replaced by a biotin-binding protein (e.g., avidin). This allows for the simultaneous targeting of multiple antigens by exogenous addition of different biotinylated ligands recognizing TAAs (e.g., antibodies). BBIR CAR T cells have been shown to result in tumor suppression, both *in vitro* and *in vivo* (29, 30). The split, universal, and programmable (SUPRA) CARs follow a similar strategy by linking the antigen-binding molecule (scFv) with the help of a leucine-zipper oligomerization system to the transmembrane and intracellular activation domain of the CAR. This system was shown to be very versatile as several ligands can be employed (31). However, although the versatility of the UniCARs is intriguing, their transfer into the clinical setting may be impaired by several caveats. For the generation of SUPRA CARs, the transduction of several expression cassettes is needed. This may lead to substantial technical problems in the generation and standardization of the cells. Furthermore, the potential immunogenicity of the leucine zippers is likely to be higher as of standard scFv-CARs. This problem of the increased immunogenicity and thus neutralization may also affect the BBIR CARs that consist of a non-human, potentially highly immunogenic biotin-binding domain and the tags needed by the ligands for the anti-Tag CARs (32). TanCARs can be used to overcome these problems. TanCARs induce distinct T cell reactivity against two different tumor-restricted antigens and result in a synergistic enhancement of effector functions when both antigens are simultaneously encountered (33–35). A major advantage of this system is that the tandem CAR preserves the cytolytic ability of T cells even upon loss of one of the target molecules and thus, reduces the risk of antigen escape that is a substantial problem for CAR T cell therapy.

By the time of this review, clinical benefits of CAR T cell treatments have mainly been observed in B cell malignancies such as relapsed B cell acute lymphoblastic leukemia (B-ALL) and diffuse large B cell lymphoma (DLBCL) (36, 37). Apart from the comparable easy accessibility of the tumor cells, the nature of the antigens that serve as targets for the CARs has strongly contributed to the therapy success. Most CARs generated for these tumors target the CD19, CD20, and CD22 (35), that are highly expressed on the tumor cells and thus enable a potent on-target/on-tumor effect of the CAR T cells. However, these

molecules are also present during B cell development and the most evident on-target/off-tumor effect of the treatment results in B cell depletion. Fortunately, this effect can be managed by immunoglobulin replacement, and the clinical benefit of the massive anti-tumor function justifies the risks of side effects (38).

CAR T CELLS TARGETING TUMOR-ASSOCIATED ANTIGENS IN COLORECTAL CANCER AND GLIOBLASTOMA

The identification of suitable surface antigens in solid tumors is more complicated and currently under heavy investigation (39). Of over 671 ongoing clinical trials in the CAR T field, the U.S. National Library of Medicine (ClinicalTrials.gov) database listed 189 CAR T cell trials targeting solid tumors at the time of this review. To reduce the complexity, we will here concentrate on CAR T cell targets explored in clinical trials of two important solid tumor entities: GB that represents the most aggressive form of brain tumors and CRC, which is the third most deadly tumor type worldwide (40, 41). **Tables 1**, **2** give an overview of ongoing CAR T cell trials for CRC and GB that are currently recruiting patients in the United States, Europe and China. Here we will introduce the CAR T cells and their targets that are currently being investigated in these clinical trials.

CAR T Cells Targeting CRC

The surface protein ERBB2, epidermal growth factor receptor 2 (HER2) is a member of the tyrosine kinase receptors family and is highly expressed by many cancer cells (42). NC03740256 is a phase 1 trial in combination with an oncolytic adenovirus (CAdVEC). CAdVEC supports the immune system including HER2-specific CAR T cells to react against the tumor by promoting a pro-inflammatory microenvironment. Another member of the family of tyrosine kinase receptors, epidermal growth factor receptor (EGFR) also appears to be a good target for CRC (43) and also GB (see below). Recently two clinical trials were launched to evaluate the targeting of this protein in phase I and phase II (NCT03152435 and NCT01869166 CRC). However, by the time of this review, no results of these studies were available.

Several clinical trials are investigating, the use of carcinoembryonic antigen (CEA) CAR T cells in different tumors including CRC. Zhang et al. demonstrated the safety and efficacy of a CAR T cell therapy targeting CEA-positive CRC patients with lung and liver metastases in a phase I trial. They demonstrated that CEA CAR T cell therapy was well tolerated in CEA+ CRC patients even in high doses, and some efficacy was observed in most of the treated patients (44).

In this dose-escalation trial seven out of 10 patients initially showed stable disease by PET or CT analyses. In two of them, the tumor growth was inhibited for more than 30 weeks (44).

In another clinical trial, the feasibility of delivering first-generation CAR T cell therapy to patients with advanced CEACAM5+ malignancy was determined (NCT01212887). Unfortunately, no objective clinical responses were observed. Instead, the on-target/off-tumor toxicity against pneumocytes and lung-associated macrophages was so high that the trial had to be closed (17).

The epithelial cell adhesion molecule (EpCAM) is aberrantly expressed in several epithelial-derived tumors including CRC (45, 46) and also suggested as a target for CAR T or NK cells. In preclinical studies, an EpCAM second-generation CAR was constructed and transduced into NK-92 cells by lentiviral vectors. Synergistic effects of regorafenib and EpCAM CAR NK-92 cells were analyzed in a mouse model with human colorectal cancer xenografts. The CAR NK-92 cells specifically recognized EpCAM-positive colorectal cancer cells, released cytokines including IFN-γ, perforin, and granzyme B, and showed cytotoxic activity *in vitro* (47). These results encouraged the launch of a clinical trial with CAR T cells recognizing EpCAM positive cells in CRC as well as hematological malignancies (NCT03013712). This trial was designed as phase I/II and is still ongoing.

Hedge et al. reported a clinical trial with patients with metastatic CRC who have been treated in two phase I trials with first-generation retroviral transduced CAR T cells targeting tumor-associated glycoprotein (TAG)-72. Both trials (C-9701 and C9702) were not successful, and the limited persistence of the cells was supported by the finding that the tumor-associated TAG-72 expression is non-uniform. Unfortunately, the data from these CART72 trials did not give any insight some insight into whether coadministration of IFN-α can result in sufficient TAG-72 upregulation to avoid the loss of antigen (48).

Finally, a Mucin-1 (MUC1) CAR T cell therapy was proposed for metastatic colorectal adenocarcinoma. It was shown to be safe in humans (49) and is now investigated in a phase I/II trial (NCT02617134) with over 73 participants. This trial consists of multi-target-gene-modified CAR/TCR T cells.

CAR T Cells Targeting GB

At the time of this review, several CAR T cell trials targeting different proteins in GB are ongoing (**Table 2**). By now, published results of these trials are only available for some of the targets.

A robust anti-tumor efficacy following regional intraventricular delivery of HER2-CAR T cells for the treatment of multifocal brain metastases and leptomeningeal disease was described (50). The HER2-CAR T cells persisted for 6 weeks without evident toxicities. Although this therapy was designed to target breast cancer metastases, the data demonstrated the safety and feasibility of intraventricular HER2 CAR T cell administration and showed encouraging signals of clinical activity (51), thus setting the stage for studies that combine HER2-CAR T cells with other immune-modulatory approaches to enhance their expansion and persistence (51, 52). The re-stimulation of antiviral immunity via defined peptides from common pathogens provides a unique therapeutic avenue for cancer immunotherapy. Reactivating the virus-specific memory T cells (VSTs) arrested the growth of checkpoint blockade-resistant and poorly immunogenic tumors in mice after injecting adjuvant-free non-replicating viral peptides into tumors (53). These results extend recent observations of virus-specific T cells

TABLE 1 | Selected CAR T cell clinical trials for CRC.

Target	Identifier	Tumor	Country	N	Results
NKR2	NCT03018405	CRC	USA/Europe	146	Recruiting, not disclosed
	NCT03310008	mCRC	Europe/Belgium	36	Active, non-recruiting
	NCT03370198	mCRC	Europe/Belgium	1	Active, non-recruiting
NKG2D	NCT03692429	mCRC	Europe/Belgium	36	Recruiting, not-disclosed
CD133	NCT02541370	CRC	China	20	Completed, not-disclosed
CEA	NCT02349724	CRC	China	75	Unknown, not-disclosed
	NCT03682744	CRC	United States	18	Active, not recruiting
EGFR	NCT01869166, NCT03152435	CRC	China	60	Unknown, not disclosed
EGFRvIII	NCT03267173	CRC	China	10	Unknown, not disclosed
EpCAM	NCT03013712	CRC	China	60	Recruiting, not disclosed
MUC1	NCT02617134	CRC	China	20	Unknown, not disclosed

Natural-killer 2 Receptor (NKR2); Natural-killer group 2, member D receptor protein (NKG2D); CD133; Carcinoembryonic antigen (CEA); Epidermal growth factor receptor (EGFR); Epidermal growth factor receptor variant III (EGFRvIII); Epithelial cellular adhesion molecule (EpCAM); Mucin 1 (MUC1).

TABLE 2 | Selected CAR T cell clinical trials for GB.

Target	Identifier	Tumor	Country	N	Results
NKG2D	NCT04270461	GB	USA	10	Not yet recruiting
CD147	NCT04045847	GB	China	31	Not recruiting
B7H3	NCT04077866	GB	China	40	Not yet recruiting
EGFRVIII	NCT02844062	GB	China	20	Unknown, not disclosed
	NCT02664363	GB	United States	3	Terminated, not disclosed
	NCT03726515	GB	United States	7	Active, not recruiting
	NCT01454596	GB	United States	18	Completed, results (closed)
EpHA2	NCT02575261	GB	China	60	Completed, not disclosed
GD2	NCT03252171	GB	China	60	Completed, not disclosed
HER2	NCT01109095	GB	United States	16	Completed, not disclosed
	NCT03389230	GB	United States	42	Recruiting, not disclosed
IL13Rα2	NCT04003649	GB	United States	60	Recruiting, not disclosed
	NCT02208362	GB	United States	92	Recruiting, not disclosed

B7H3; CD147; Epidermal growth factor receptor variant III (EGFRvIII); EPH receptor A2 (EpHA2); Disialoganglioside 2 (GD2); HER2; Interleukin 13 receptor α2 (IL13Rα2), Natural-killer group 2, member D receptor protein (NKG2D).

in GB. In a clinical study of 17 patients with progressive HER2-positive GB, autologous HER2-specific CAR-modified VSTs were infused without prior lymphodepletion (NCT01109095). The treatment with VSTs was safe and well-tolerated, with no dose-limiting toxic effects. Seven patients showed stable disease after CAR T cell treatment and three showed long term responses of more than 2 years without progression (54). While these studies are very encouraging for intracranial applications, the systemic treatment with high-affinity HER2-CARs can also be dangerous. A patient with metastatic colon cancer received an infusion of CAR T cells targeted to the antigen HER2 (ERBB2) and died 5 days later (55) due to the massive on-target/off-tumor toxicity of the CAR T cells for lung cells that express low levels of HER2. Moreover, in an animal model, similar problems were also observed for CAR T cells with high affinity for Disialoganglioside 2 (GD2, glycolipid antigen) (56) which has been identified as an immunotherapy target in melanoma and neuroblastoma about 10 years ago (57, 58). Although this antigen serves as a bona fide model that the affinity of the targeting may be tightly associated with unwanted toxicity, the treatment with lower affinity CAR T cells showed much promise in recent studies in diffuse midline gliomas (DMGs) with mutated histone H3 K27M (H3-K27M). If the results can be translated into humans, it could be a valuable immunotherapeutic strategy for children with H3-K27M-mutant DMGs (59).

Another intensively studied GB associated tumor antigen is interleukin 13 receptor α2 (IL13Rα2) (60) which was described as a potential CAR target more than 10 years ago (61). Subsequent studies showed efficacy in animal models (62, 63). Interestingly, one of these studies showed a higher expression of IL13Rα2 on stem-like vs. differentiated glioma populations, indicating that IL13Rα2-directed immunotherapeutic approaches could be useful for eradicating therapeutically resistant glioblastoma stem cell (GSC) populations (62). IL13Rα2 was the primary target in two important clinical studies showing safety and efficacy in humans (60, 64). In contrast to most CARs, of which some

also target IL13Rα2 (65), the binding domain of the construct used in these studies was not an scFv. but based on IL13 fused to the intracellular signaling domains. Thus, these CARs also recognize interleukin receptor 13 alpha 1 (IL13Rα1), and this dual specificity most probably resulted in a strong therapeutic effect. In the study of Brown et al. the described patient was a participant in an ongoing dose-escalation safety study to evaluate the role of intracranial CAR T cell therapy targeting IL13Rα2 in patients with malignant gliomas. However, after stunning initial responses, the tumor relapsed most likely due to the antigen loss (60, 66).

Epidermal growth factor receptor deletion mutant variant III (EGFRvIII) is a tumor-specific antigen expressed in GB and its expression is often associated with survival, invasion, angiogenesis and resistance to radio- and chemotherapy (67). Sampson et al. developed a third-generation, EGFRvIII-specific murine CAR, and performed tests to determine its efficacy in a fully immunocompetent mouse model of malignant glioma. They showed that CAR-treated, cured mice were resistant to rechallenge with EGFRvIII negative tumors, suggesting the generation of host immunity against additional tumor antigens (68). These results in a refined syngeneic mouse model suggested that EGFRvIII-targeted CAR T cells may provide a highly specific, promising therapeutic candidate for patients with tumors in the CNS and a phase I clinical trial (NCT01454596) was launched. Unfortunately, this study with 10 patients failed. Two of the patients treated with the highest doses of CAR T cells experienced severe hypoxia and one of these patients died. No objective responses were detected nor persistent CAR+ cells were identified (69). While the molecular reason for the toxicity remains unclear, an explanation for the lack of clinical efficacy may be the heterogeneity of antigen expression (70, 71) and a different activation of bystander immune cells in mouse and human. Thus, antigen loss would be one of the main reasons for the lack of therapeutic efficacy observed in the clinical trials. Furthermore, studies by Maus and colleagues showed that a single dose of peripherally infused CAR T cells targeting EGFRvIII resulted in marked antigen loss and reduced clinical efficacy of this treatment (72).

In summary, the clinical results obtained for CAR T cell therapy in CRC and GB are much less promising when compared to B cell leukemia. The studies indicate that besides other T cell-intrinsic or environmental factors (73–77), the risks of on-target/off-tumor toxicity and antigen loss are two of the main problems hindering a long-lasting therapeutic success. Thus, strategies to optimize CAR T cell function in solid tumors including the discovery of new targets remain an important goal and will be discussed in the sections below.

STRATEGIES TO IMPROVE THE TARGETING OF CARs TO CRC AND GB

A precise tumor targeting and the lack of specific antigens is one of the biggest difficulties in the generation of CAR T cell therapy in general and particularly in solid tumors (7). The absence of cancer-specific targets increases the potential risk of significant on-target/off-tumor toxicity in case that antigens are also expressed in healthy tissues (78). These problems and some of the potential solutions are summarized in **Figure 1**. A potential solution for this problem can be the use of CARs modified to bind antigens highly expressed by tumor cells and present, although at lower levels, in healthy tissues with lower affinity. By introducing mutations in the antigen-binding regions of the scFv, Liu et al. generated CARs binding to HER2 and EGFR with lower affinity. The resulting CAR T cells still killed the TAA overexpressing tumor cells efficiently but are likely to be much less toxic for healthy cells. A similar result was shown by two EGFR-targeting CARs generated with the scFvs from two monoclonal antibodies of different affinities for the antigen (79, 80). These results together with the route of application (e.g., intracranial vs. systemic) may also explain why some HER2-targeting therapies are well-tolerated, while others showed fatal side effects (54, 55). A strong impact of the affinity on potential toxicity was also observed with GD2-CAR T cells. Richman et al. showed that inserting a single amino acid exchange in the scFv (E101K) was generating GD2-CARs with 10-fold more affinity to the target. Compared to the CARs with the lower affinity scFv, these cells were much more efficient in killing GD2-expressing cells *in vitro*. Unfortunately, the treatment with these high-affinity CAR T cells resulted in fatal encephalitis in the mouse model, while the CARs generated with the wt scFv were not toxic (56). Other ways to enhance the safety of CARs recognizing TAAs also expressed by healthy tissues is a stringent control of their expression levels either by transient expression methods using mRNA transfer instead of stable transduction (81) or the integration of the transgene in a defined genetic locus enabling controlled expression levels using the CRISPR/Cas9 technology (82).

Another problem is that the few known highly tumor-specific TAAs are often lost during the treatment, which reduces their therapeutic value. To overcome some of these problems, the concept of utilizing bi-specific tanCARs is very attractive for fighting solid tumors. The ideal antigen should be selected based on high cell surface expression in cancerous tissue and low cell surface expression on healthy tissue. As an example, HER2, MUC1, and EpCAM are not highly expressed by normal colon tissues and their co-expression should be limited to cancerous tissue. Using this approach, T cells were transduced with both a CAR that provided suboptimal activation upon binding of one antigen and a chimeric costimulatory receptor (CCR) that recognized a second antigen (83). Although this is a very interesting concept, CCRs are so far still in the preclinical stage. To increase the specificity of CARs targeting GB, two or even three antigens were used. In one study, the antigen escape observed upon treatment with IL13-CARs was approached by the construction of second-generation tanCARs targeting IL13Rα2 and human epidermal growth factor receptor 2 (HER2) (66). These tanCAR T cells recognized tumors distinctly and effectively and improved persistence in the presence of both antigens (84). The single universal (U) tricistronic transgene (UCAR) T cells are generated by the expression of three independent CAR constructs in one T cell. Constructs for contemporary targeting of IL13Rα2, HER2, and EphA2 have shown some preclinical functions in

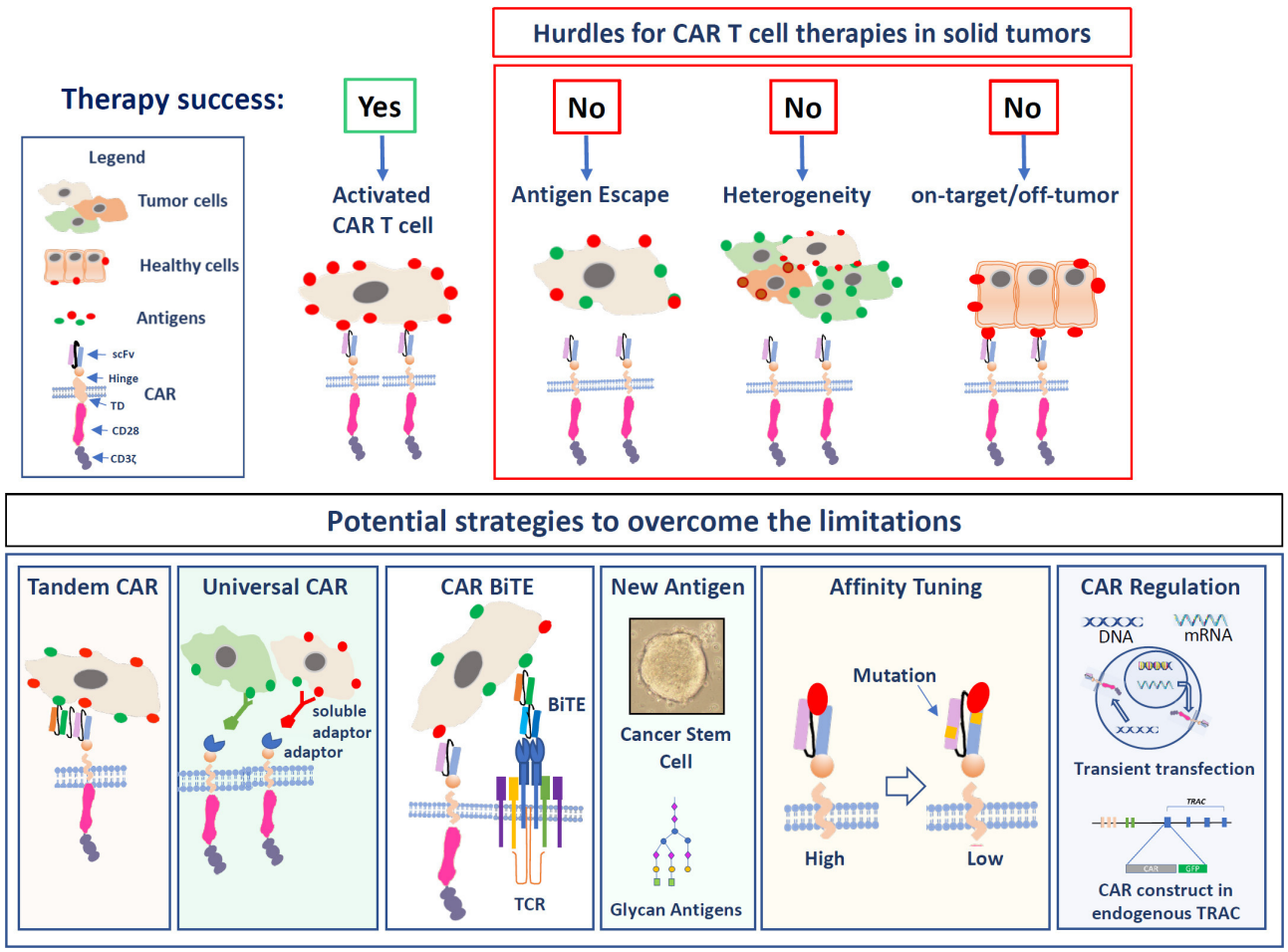

FIGURE 1 | The figure illustrates the hurdles of solid tumor targeting as well as potential strategies to overcome these limitations. Upper panel: The major hurdles are: Antigen escape, tumor cells that lose the expression of the antigen; Heterogeneity due to the expression of different TAAs on solid cancers; On-target/off-tumor toxicity in the case that antigens are also highly expressed in healthy cells. In the lower panel, the strategies to optimize CAR T cell function in solid tumors are illustrated: Targeting the T cells with Tandem CARs, universal CARs, or BiTEs. Targeting alternative antigens, Affinity tuning, and the regulation of the CAR expression levels.

mouse models (85). However, although this approach may be useful to overcome the antigen heterogeneity in GB and other tumors, it is not very likely that these treatments can be translated into the clinical setting. The transgenes are very large and complicate the generation of high titer virus and the integration of the viral genome needed for the genetic modification of the primary T cells under current clinical settings.

The problem of antigen escape can also be addressed by other CAR approaches. These include the induced expression of bi-specific T-cell engagers (BiTEs) or the use of UniCARs. BiTEs typically consist of two scFvs, one specific to CD3 (T cell co-receptor) and the other one specific to a tumor antigen, connected by a flexible linker. Thus, these molecules can physically link a T cell to a tumor cell (86). Choi and colleagues recently showed that BiTEs can enhance CAR T cell efficacy *in vivo*. They found a clearance of heterogeneous EGFRvIII/EGFR expressing GB cells in mouse models, by using a bicistronic construct to drive expression of a CAR specific for EGFRvIII, and a BiTE against wild type EGFR (74). The secreted EGFR-specific BiTEs were able to re-direct CAR T cells and recruited non-transduced bystander T cells against wild-type EGFR (74). Thus, BiTE-secreting CAR T cells hold much promise for the treatment of solid tumors and can provide an advantage over CAR T cells (28). As another strategy to improve the versatility and the safety of CAR T cell therapies, several groups used a CAR platform termed UniCAR system consisting of two components: UniCAR-modified T cells and tumor-specific target modules (TM). The bivalent α-EGFR-EGFR TM has shown to redirect UniCAR T cells to tumor cells expressing low levels of EGFR. According to PET experiments *in vivo*, the increased avidity of the bivalent α-EGFR-EGFR TM improves the enrichment at the tumor site (87).

While these approaches can help to increase the efficacy of CAR T cell therapy against known antigens, the identification of more robust targets with high potential to help the eradication of the tumor is still a major task in the fight against solid tumors.

SEARCH FOR NOVEL ANTIGENS: TARGETING OF CAR T CELLS TO CANCER STEM CELLS (CSCs) IN SOLID TUMORS

The heterogeneity and thus high risk of antigen escape in solid tumors belong to the main cavets in the design of efficient CAR T cell therapies. A potential solution may be the selective targeting of tumor cell subpopulations that drive tumor growth. For GB and CRC, it is generally accepted that tumor growth is fueled by a subpopulation of CSCs that promote tumor progression and are highly resistant to conventional therapy (88). Thus, the extinction of these cells by CAR T cells represents a promising anti-tumor therapy. Interestingly, the primary cultures enriched in CSCs may be responsible that these cells keep many features of the primary tumor, including some tumor antigens (89). In the last 15 years, it was shown that CSCs from different solid tumors express various surface proteins at levels substantially higher when compared to the healthy or bulk tumor cell population (90). While all these markers may represent potential targets, by today only a limited number of CARs recognizing GB- and CRC-CSC surface markers are under investigation and will be discussed in this section.

Our group discovered that CRC metastases arise from disseminated colorectal cancer stem cells (CR-CSCs). Todaro et al. showed that CR-CSCs express CD44 variant 6 (CD44v6), which is required for their migration and generation of metastatic tumors (91).

CD44v6-CAR T cells have been generated to target leukemia and myeloma cells. These CAR T cells display potent *in vitro* and *in vivo* anti-tumor reactivity (92–94). However, because CD44v6 is also highly expressed in some normal tissues, especially in the skin, the safety of this treatment has to be proven before applying this therapy to humans.

EPH Receptor A2 (EphA2) is a tyrosine kinase (95) capable of activating multiple diverse signaling pathways involved in tissue homeostasis and cancer (96) and described as being a functional CSC marker in GB (97). CAR T cells targeting EphA2 showed a dose-dependent cell killing of esophageal squamous cell carcinoma (ESCC) cells and have been optimized for the adoptive T cell therapy of EphA2+ glioblastoma for further clinical development (98). Based on these results a clinical trial with EphA2-CAR T cells in GB was launched (NCT02575261).

In a preclinical study, the effect of NKG2D-CAR T cells on GB and GB stem cells was investigated and confirmed the high expression of NKG2DLs in all the samples. The NKG2D-BBz CAR T cells efficiently lysed GB cells and CSCs *in vitro* and produced high levels of cytokines, perforin, and granzyme B. The CAR T cells markedly eliminated xenograft tumors *in vivo* and did not exhibit significant treatment-related toxicity in the treated mice. In conclusion, NKG2D-CAR T cells targeted GB cells and CSCs, support the use of CAR T therapy in GB (99) and let to the design of a clinical trial (NCT04270461).

CD133 is a marker expressed by CSCs of various origins, including GB and CRC, and another attractive therapeutic target for cancers. The potential danger of CD133-CARs was unveiled in a study of Bueno et al. The authors treated mice with B-ALL and detected strong myeloablative toxicity upon CD133-CAR T cell transfer. Most probably this was due to the high expression of CD133 on the mouse hematopoietic stem cells (100). This toxicity was not detected in a phase I clinical trial (NCT02541370). Wang et al. showed the feasibility, controllable toxicities, and effective activity of CD133-CAR T transfer for treating patients with CD133-positive and late-stage metastatic malignancies. In this trial, 14 of 23 patients showed stable disease upon treatment and 3 even partial remissions. As described above, different affinities of the CARs targeting the murine and human protein may be responsible for the different toxicities (101).

While the concept of killing selectively the cells responsible for tumor growth and dissemination is very appealing, the targeting of CSCs by CAR T cells is being complicated by several factors. The vast majority of CSC-markers are also expressed on the surface of tissue-specific stem cells (102). This raises the likelihood of strong and potentially non-controllable on-target/off-tumor effects. A second problem may be that the low percentage of CSCs is surrounded by the tumor bulk and thus not accessible for the CAR T cells. Furthermore, there is accumulating evidence that CSCs can shift between stem and differentiated states depending on cell-intrinsic or microenvironmental factors. This "CSC plasticity" is also reflected by the expression of the stemness markers (103) that may be lost although the cells contain the capacity to self-renew and drive tumor growth. Thus, additional alternative antigens selectively expressed by the majority of the tumor cells need to be identified.

SEARCH FOR NOVEL ANTIGENS: TARGETING OF ALTERED GLYCAN STRUCTURES IN CANCER CELLS

Many tumor- or CSC-selective monoclonal antibodies (mAbs) directly bind to the sugar chains of glycolipids (e.g., SSEA-3/4, GD2) or glycosylation-residues of proteins (e.g., some CD133 mAbs, CA 19-9). Compared to healthy tissue cancer cells have an altered metabolism leading to different repertoires of metabolites and activities of enzymes catalyzing glycosylation. This ultimately results in aberrant glycosylation patterns on their cell surface and secreted glycoproteins (104). Thus, these structures may represent a class of potential CAR antigens that, by now, received little attention. Glycans have fundamental mechanisms in controlling cancer development and progression (105). Changes in the cellular glycosylation are associated with malignant transformation of cancer cells, tumor progression and metastasis formation (106). Furthermore, glycans have a major impact on the interplay between cancer cells and the tumor microenvironment (104, 106, 107).

Compared to healthy tissue, CSCs and cancer cells have increased levels of truncated O-glycans (T and Tn antigens) and fucosylation, increased Lewis antigen expression and increased sialylation. All these altered structures could be new targets for CARs based on specific mAbs (48, 108–117). Some interesting mAbs targeting truncated O-glycan structures Tn and sialyl-Tn are characterized by relatively high affinity (∼10–9 M

TABLE 3 | Selection of mAbs targeting glycosylation-related tumor-associated epitopes discovered in the last 20 years.

MAB	CLASS	ANTIGEN	REFERENCE
LpMab-21	O-Glycopeptide	Sialyl-PDPN	(119)
PankoMAb	O-Glycopeptide	MUC1	(120)
2D9	O-Glycopeptide	Tn-MUC1	(111)
6E3	O-Glycopeptide	Tn-MUC4	(121)
5E5	O-Glycopeptide	Tn-MUC1	(122)
mAb237	O-Glycopeptide	Tn-OTS8	(110)
5G2	N-Glycan	Le^a Le^c	(115)

range) and little or no reactivity against the peptide with elongated O-glycans or the non-glycosylated peptide (118) (**Table 3**).

Schietinger et al. found that a wild-type transmembrane protein can be transformed into a TAA by a change of the glycosylation pattern. A somatic mutation in the chaperone gene Cosmc abolished the function of a glycosyltransferase, disrupted O-glycan Core 1 synthesis, created a tumor-specific neo-epitope consisting of a monosaccharide and the wild-type protein sequence. This epitope induced a high-affinity, highly specific, syngeneic mAb with anti-tumor activity (110). Sato et al. generated antibodies by directly immunizing mice with spheroids from human CRC. They obtained a functional mAb recognizing glycan structures that were lost in conventional cell lines. These results show that cancer tissue-originated spheroids can be a useful antigen for generating novel anti-cancer antibodies (123).

MUC1 is a large O-glycan-carrying protein over-expressed by most adenocarcinomas (124). MUC1-CAR T cells have been engineered based on the mAb 5E5 and have shown efficacy in eliminating pancreatic cancer cells (111). In a paper by Posey et al., the authors demonstrated the therapeutic efficacy of CAR T cells directed against Tn-MUC1 and presented aberrantly glycosylated antigens as a novel class of targets for tumor therapy with engineered T cells (114).

CD171 is an abundant cell surface molecule on neuroblastomas and a glycosylation-dependent tumor-specific epitope is recognized by the CE7 mAb. CE7-CAR T cell therapy was successful in 4 out of 5 neuroblastoma patients in a phase I study. All four CE7-CAR T cell products demonstrated *in vitro* and *in vivo* anti-tumor activity (117).

CAR T cells targeting stage-specific embryonic antigen 4 (SSEA-4) were also generated (125). The overexpression of SSEA-4 in several cancers including GB, the relatively restricted expression in normal tissues and anti-tumor effects of the antibody in preclinical mouse models in the absence of toxic side effects made it an interesting target. Unfortunately, the CAR T cell treatment in mice resulted in strong on-target/off-tumor effects especially in the hematopoietic stem cell pool (126).

Liau et al. produced an IgM antibody that is capable to distinguish malignant ovarian carcinoma cells from benign ovarian epithelia by binding specifically to cancer cell-associated glycans (127). Kaneho et al. developed and characterized anti-glycopeptide mAbs against human podoplanin hPDPN that is expressed in cancer cells or cancer-associated fibroblasts indicating poor prognosis (128).

Finally, disialoganglioside 2 (GD2, glycolipid antigen) (129, 130) has been identified as an immunotherapy target in melanoma and neuroblastoma about 10 years ago (57, 58). As reported above, this antigen serves as a bona fide model that the affinity of the targeting is tightly associated with unwanted toxicity (56). However, the treatment with lower affinity CAR T cells showed much promise in recent studies in diffuse midline gliomas (DMGs). Currently, a clinical trial targeting GD2 in GB was is recruiting patients (NCT03252171). In preclinical approach, a CAR targeting GD2 was also used to direct tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expressing mesenchymal stem cells into experimental GB. Although the results have still to be confirmed in more relevant systems, this approach shows potential new venues on how to fight GB with CARs (131).

CONCLUSION AND FUTURE DIRECTIONS

In recent years, CAR T cell immunotherapy has achieved encouraging results in the treatment of onco-hematological pathologies. Despite significant progress, some important challenges have not yet been resolved in treating solid tumors, especially in terms of specificity, persistence, safety and immunosuppressive microenvironment. In particular, the lack of tumor-selective antigens hinders the development of an efficient CAR T therapy for solid tumors. Although the expression of tumor-specific antigens is likely to be patient-specific and thus reliable biomarkers are needed to guide the therapy decisions, we assume that the identification of novel targets is one of the main keys to improve CAR T cell therapy for solid tumors such as GB and CRC. Besides modern gene expression-based approaches, we suggest applying primary tumor cultures enriched in CSCs to generate and screen for highly specific mAbs as for the engineering of novel CARs. We are convinced that CARs with mAbs targeting altered structures of cancer cells and CSCs offer a valid opportunity to develop new therapeutic options. Although significant barriers remain and hider the broad clinical application of CAR T in solid tumors, numerous studies are underway and more specific and safer CAR T cells can be expected in the future.

AUTHOR CONTRIBUTIONS

EP designed the review. EP and TLH wrote the main manuscript. RDM and TLH critically revised the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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Manufacturing and Management of CAR T-Cell Therapy in "COVID-19's Time": Central Versus Point of Care Proposals

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The COVID-19 pandemic, caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), has generated a significant repercussion on the administration of adoptive cell therapies, including chimeric antigen receptor (CAR) T-cells. The closing of borders, the reduction of people transit and the confinement of the population has affected the supply chains of these life-saving medical products. The aim of this mini-review is to focus on how the COVID-19 pandemic has affected CAR T-cell therapy and taking into consideration the differences between the large-scale centralized productions for the pharmaceutical industry versus product manufacturing in the academic/hospital environment. We also review different aspects of CAR T-cell therapy and our managerial experience of patient selection, resource prioritization and some practical aspects to consider for safe administration. Although hospitals have been forced to change their usual workflows to cope with the saturation of health services by hospitalized patients, we recommend centers to continue offering this potentially curative treatment for patients with relapsed/refractory hematologic malignancies. Consequently, we propose appropriate selection criteria, early intervention to attenuate neurotoxicity or cytokine release syndrome with tocilizumab and prophylactic/preventive strategies to prevent infection. These considerations may apply to other emerging adoptive cell treatments and the corresponding manufacturing processes.

Keywords: chimeric antigen receptor, adoptive cell immunotherapy, SARS-CoV-2 coronavirus, manufacturing process, good manufacturing practice

Abbreviations: SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus-2; CAR, chimeric antigen receptor; ALL, acute lymphoblastic leukemia; NHL, non-hodking lymphoma; CART19, anti-CD19 CAR T-cell; FDA, Food and Drug Administration; DLCL, diffuse large B-cell lymphoma; GMP, good manufacturing practice; CRS, cytokine release syndrome; ICU, Intensive Care Unit; TCZ, tocilizumab; PBMCs, peripheral blood mononuclear cells.

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INTRODUCTION

The SARS-CoV-2 coronavirus has generated an unprecedented global impact on multiple aspects of society, economy and health. SARS-CoV-2 was reported as a new emerging zoonotic pathogen in Wuhan (China) in December 2019, and declared as a pandemic by the World Health Organization in March 2020 (1). Its rapid human-to-human transmission has affected millions of people (2). Indeed, the routine operation of medical systems has been significantly disrupted generating a health crisis in many countries due to limited resources such as hospital beds and personal protective equipment. In early June, the number of infected people had already risen to more than 6 million globally and the number of new cases detected is increasing daily. There is not pre-existing specific immunity placing at risk humanity as a whole to infection by SARS-CoV-2. Taken together with the severe pulmonary and systemic inflammatory complications associated with the disease, it has caused governments to enforce isolation measures to prevent its rapid spread. Hospitals have been forced to change their usual workflows to cope with the saturation of hospitalized services. The closing of borders, the reduction of the transit of people and the confinement of the population has affected the supply chains of products. Access to life-saving drugs has also been affected, negatively impacting on patients with other life-threatening diseases (3).

Chimeric antigen receptor (CAR) T-cell therapy is a life-saving bioengineered cell replacement therapy against leukemia. This adoptive antitumor immunotherapy, based on autologous T-cells transduced with a genetically engineered receptor for CD19 to redirect their cytotoxicity against native CD19 surface antigens expressed in tumor cells, has completely changed the management of patients with hematologic malignancies such as acute lymphoblastic leukemia (ALL) or non-Hodking lymphoma (NHL) (4–6). Tisagenlecleucel (Kymriah, Novartis) and axicabtagen ciloleucel (Yescarta, Kite/Gilead) are both anti-CD19 CAR T-cell (CART19) commercial products that obtained Food and Drug Administration (FDA) approval in 2017 for the treatment of pediatric and young adult patients with CD19+ relapsed/refractory B-cell ALL, relapsed/refractory B-cell NHL [diffuse large B-cell lymphoma (DLBCL), primary mediastinal B-cell lymphoma and transformed follicular lymphoma]. Other regulatory agencies like the European Medicines Agency (EMA) have also recently approved these commercial CART19 use. Therefore, CART19 is considered a potential curative therapeutic option for CD19+ hematologic malignancies with no response to conventional treatments. In addition, approval of other CAR T-cell products developed commercially against other molecular targets are expected as further options for treatment in the coming months. More than 500 clinical trials[1] are being carried out testing CAR-based products. Some of them are financed by the industry and others are being developed in the academic ambit. The cohabitation of both is inevitable and necessary. Industry production could supply CAR T-cell therapies to reach the whole population using good manufacturing practice (GMP) accredited large facilities to centralize the manufacturing process. On the other hand, academic centers should be dedicated to develop CAR T-cell therapies against less frequent diseases. Independently, both have been seriously affected by the COVID-19 pandemic despite the differences they present in product manufacturing and management processes (7). The management of CAR T-cell therapy is a complex time-consuming process that requires highly specialized personnel and coordinated work systems including medical management of the patient before and after the infusion due to feasible toxicities (8–10).

Owing to the doubtless relevance of CAR T-cell therapy for hematologic patients, it is important to have in mind some considerations within the context of the COVID-19 pandemic. Therefore, the aim of this mini-review is to focus on how the effects caused by the pandemic have affected this therapy taking into consideration the differences between the large-scale centralized production of CAR T-cells by the pharmaceutical industry versus the product manufacturing processes employed by the academic/hospital environment. We also review different aspects of CAR T-cell therapy, including patient selection and resource prioritization performed in our center during the COVID-19 pandemic.

CAUTIOUS CONTINUATION VS DEFERMENT OF CAR T-CELL THERAPY DURING COVID-19 PANDEMIC

Chimeric antigen receptor T-cell therapy has elicited an unprecedented response against B-cell malignancies, but it is associated with significant toxicity, including prolonged cytopenia, cytokine release syndrome (CRS), and neurotoxicity (10–13). Toxicity is normally associated with the T-cell's inherent mechanism of action that has been well-described in academic and industry developed CAR T-cells (14). The use of CART19 cell therapy can result in prolonged B-cell aplasia and therefore inability to develop an antibody response necessary to respond against pathogens such as SARS-CoV-2 (15, 16). Furthermore, these patients are usually managed as in-patients to facilitate intensive monitoring due to the severity and rapidity of CRS, neurotoxicity and frequent need the intensive care unit (ICU) (17).

The COVID-19 pandemic is a threat to interrupt any cell therapy. It is priority that each center carefully review the internal policies and procedures to adopt the recommendations needed according to their healthcare needs. Hospitals have instituted measures to defer multiple medical interventions, including adoptive cell treatments or hematopoietic stem cell transplantation. Another relevant consideration is the inconsistent uniformity of clinical cell therapy protocols. This hampers the establishment of standardized strategies during the COVID-19 pandemic. A large proportion of these patients receive therapy in academic or pharmaceutical clinical trials and many stopped to preserve patient's safety. Indeed, lymphodepletion before final cell-product infusion facilitates the expansion of CAR T-cells as it generates a suitable environment for *in vivo* modified T-cell expansion and survival (18). However, this generates a severe immunosuppression

[1] www.clinicaltrials.gov

that can be seriously complicated by SARS-CoV-2 infection. Nevertheless, delaying cell therapy as a consequence of the COVID-19 pandemic could be fatal for the majority of patients with relapsed/refractory malignancies. Alternative therapeutic strategies are also generally associated with significant immunosuppression and subsequent potential hospitalizations. In fact, CAR T-cells are potentially curative for patients with poor prognosis (19).

Required Resources for Safe Cell Therapy During the COVID-19 Pandemic

Not all centers will be affected in the same way during the COVID-19 pandemic. Therefore, a careful evaluation of resources and infrastructures should be performed between the department of hematology and the hospital emergency planning group. The availability of ICU, hospital beds and mechanical ventilation equipment are limited, but required resources. Moreover, staff shortages due to potential exposure and resource constraints on personal protective equipment affect the management of the patient before and after the treatment infusion.

Some practical aspects should be considered for the safe administration of CAR T-cell therapy during the pandemic (**Table 1**):

- In the case of ICU collapse: establish a triage algorithm to select only patients who are most likely to benefit with no alternative treatment options and in whom the risk of toxicity is lower.
- Guarantee the availability of personnel to perform the leukapheresis and the reception and sample processing in the adoptive cellular immunotherapy unit.
- Initiate lymphodepletion procedure only after receiving CAR T-cell products at the site to avoid potential obstacles on supply chain operations.
- Hospital bed availability should be ensured for the immediate 4 weeks surrounding the treatment. There are few children admitted with COVID-19 so young adult patients who may benefit from this therapy may be transferred to a Pediatric Center with greater availability of beds.
- Guarantee the availability of a member of the medical team with the capacity to respond to complications associated with COVID-19.
- Establish a specific workflow for patients infected with COVID-19.

TABLE 1 | Practical aspects before and after the manufacturing process for the safe administration of an academic CAR T-cell therapy during the COVID-19 pandemic.

Before the manufacturing process of CAR T-cells	
Patient selection	Selection of appropriate patients
	Clear assessment of risk/benefit balance
Screening measures	Assess for signs/symptoms of COVID-19
	Evaluation of risky contacts at relevant time points, including before leukapheresis and before CAR T-cell infusion
	Laboratory qPCR testing for SARS-CoV-2 for every patient before leukapheresis
ICU capacity	Guarantee the availability of ICU beds
	Young adult patients may be transferred to a Pediatric Center to ensure availability of ICU beds
Working protocols	Establish a specific workflow for the management of patients infected with SARS-CoV-2
Availability of personnel	Guarantee the availability of a member of the medical team with the capacity to respond to complications related to COVID-19
After the manufacturing process of CAR T-cells	
Specific measures for infected patients	Final cell product should be tested for SARS-CoV-2 by qPCR before infusion in patients with a positive qPCR prior to leukapheresis
	The manufacturing process would continue if a patient becomes infected after leukapheresis. The infusion should be postponed until patient's clinical improvement

Patient Selection in the COVID-19 Pandemic Setting

The COVID-19 pandemic has altered inclusion algorithms for the proper selection of patients, that is now associated to ethical dilemma. It is imperative to outline criteria to identify optimal candidates who have the potential to achieve significant remission. The benefit-risk balance should be clear, taking into account the risk of delaying CAR T-cell therapy against the risk of progression of the underlying disease. Therefore, a group of experts should evaluate the inclusion of patients considering, among multiple factors, the lack of avalability in the hospital and alternative treatments if complications appear. In our experience at Hospital Clínic de Barcelona, a medium-sized academic institution, we have continued to administer our academic and other commercial CAR-T cell therapy against aggressive relapsed/refractory B-cell NHL and ALL (20, 21).

CELLULAR THERAPY SUPPORT DURING THE COVID-19 PANDEMIC

During the first month of the COVID-19 state of alarm declared in Spain, every CAR T-cell operation was postponed and only one infusion was performed in our center. Ultimately, no treatment was canceled, although the final product infusions were delayed due to the lack of availability of space in the ICU. Different supportive measures to mitigate the risk of COVID-19 in our patients have been taken (**Table 1**). Two young patients (aged 26 and 30) were treated in another major hospital of the city, Sant Joan de Déu Children's Hospital Center. After the first month of the pandemic, one bed at ICU was reserved for CAR T-cell therapy. This allowed the treatment of nine patients at Hospital Clínic de Barcelona. They were patients under the age of 70 with few co-morbidities and 0–2 ECOG functional status. Altogether, six patients were treated with academic CART19 and one patient with CAR T-cell therapy targeting BCMA (20). On the other hand, two patients were treated with CAR-T cells developed by the industry. The feasible impact of the delay on

CAR T-cell treatment outcomes will be analyzed in the medium-long term.

CAR T-Cell Guidance: Screening and Preventive Measures

SARS-CoV-2 viral detection tests have been performed in every patient treated with CAR T-cells at various time-points. Before leukapheresis, patients were screened for SARS-CoV-2 by qPCR given that qPCR is the gold standard test to measure viral loads. The determining factor in the start of the cell therapy manufacturing process is the infection status of the patient and the potential ability to transmit the virus. Serological tests report directly on humoral immunization but serology is not a specific marker of infection. A positive IgM result in the serological test is not always equivalent to an acute infection since it could persist for months without viral load being detected in the patient. As with other infections, serological tests are very useful for screening but should only be used for diagnosis if a gold standard test is not available.

Our patients were assessed for specific symptoms during the whole process. In addition, in-person visits have been avoided as a preventive measure and patients were asked about their potential risky contacts. The major importance of extreme confinement measures has been therefore repeatedly emphasized. The use of EPI facemasks in patient's environment was essential to maximally reduce infection risk. The final cell product should be tested for SARS-CoV-2 by qPCR before infusion in those patients with a positive qPCR prior to leukapheresis.

The special committee formed in our center to evaluate SARS-CoV-2-positive cases initially decided to avoid the CAR T-cell manufacturing process if COVID-19 was diagnosed in order to reduce possible contagions among medical staff and contamination of manufacturing facilities. This decision was controversial considering the low risk of contamination in a closed-circuit manufacturing process and the low viral load in blood samples (22). Currently, we have established to perform the leukapheresis 12 days after a positive qPCR result to ensure that the viral load in patient's samples are virtually undetectable (23).

Diverse complications that could emerge from SARS-CoV-2 infection could actually worsen CAR T-cell therapy outcomes. Thorough monitoring of these patients is required in order to detect eventual neutropenia or other infection. Post-infusion standard antiviral, antifungal and antimicrobial prophylaxis protocols were recommended.

MANUFACTURING TIME FOR ACADEMIC VS PHARMACEUTICAL CAR T-CELL THERAPIES

Autologous CAR T-cell therapy requires a personalized manufacturing process based on several critical steps that demands good coordination between different medical disciplines. For those heavily treated patients, time is crucial and well-established workflows are essential for academic or commercial cell-based therapies. After the theoretical design of the synthetic chimeric receptor and the corresponding pre-clinical studies, the complete process for CAR T-cell therapy includes (i) obtaining the starting patient's cell population by leukapheresis followed by the (ii) *ex vivo* genetic introduction of the synthetic CAR into these cells using mainly lentiviruses or retroviruses, previously generated. These modified T-cells are then (iii) activated and expanded in bioreactors and the resulting product might be (iv) adequately prepared and cryopreserve in infusible media for the final (v) product re-infusion into the patient (24, 25). All these steps can be affected by the pandemic setting. Along with inherent complexity in any cell therapy, limitations and obstacles in academic hospitals and pharmaceutical industry must be added. In this way, the standard manufacturing period of 9–11 days until the final product could be fatally extended (**Figure 1**).

COVID-19 Impact to Delivery and Critical Supply Chains

The main difference between academic and pharmaceutical CAR T-cells, regarding the timeline of the manufacturing process, is obtaining the "living drug." As for academic products, commercial CART19 are based on autologous T-cells and each patient requires his own T-cells. In the academic CAR T-cell manufacturing, all steps are carried out in the hospital facilities enabling a more flexible, personalized and coordinated "patient-friendly process." It allows short transfer times from the leukapheresis site to the production facilities and from the production center to the patient's bed, both being less than 1 hour (26). In commercial manufacturing, the same process takes place in distant geographical locations and depends on delivery protocols established by pharmaceutical companies. Logically, the transfer times from the leukapheresis site to large-scale production GMP facilities and from this production center to the patient's bed could be, at least, 1 day (27).

However, COVID-19 has rapidly constrained travel and mobility, extending the delivery times of commercial CAR T-cell products. Another side effect of reduced mobility are the potential disruptions in the resources supply line essential to the manufacturing process. This may affect both academic CAR T-cell products and those produced by industry. For example, the parallel process to obtain vectors with the CAR transgene like lentiviruses is a compendium of GMP skilled steps where different reagents are needed. Similarly, other specialized products are essential for the activation and expansion of T-cells in a bioreactor like CliniMACS Prodigy (28, 29). These critical steps can be seriously aggravated in countries where the production of these reagents is scarce and are imported from other regions. However, the large-scale production that takes place by industrial facilities ensures a greater storage capacity and a robust supply chain.

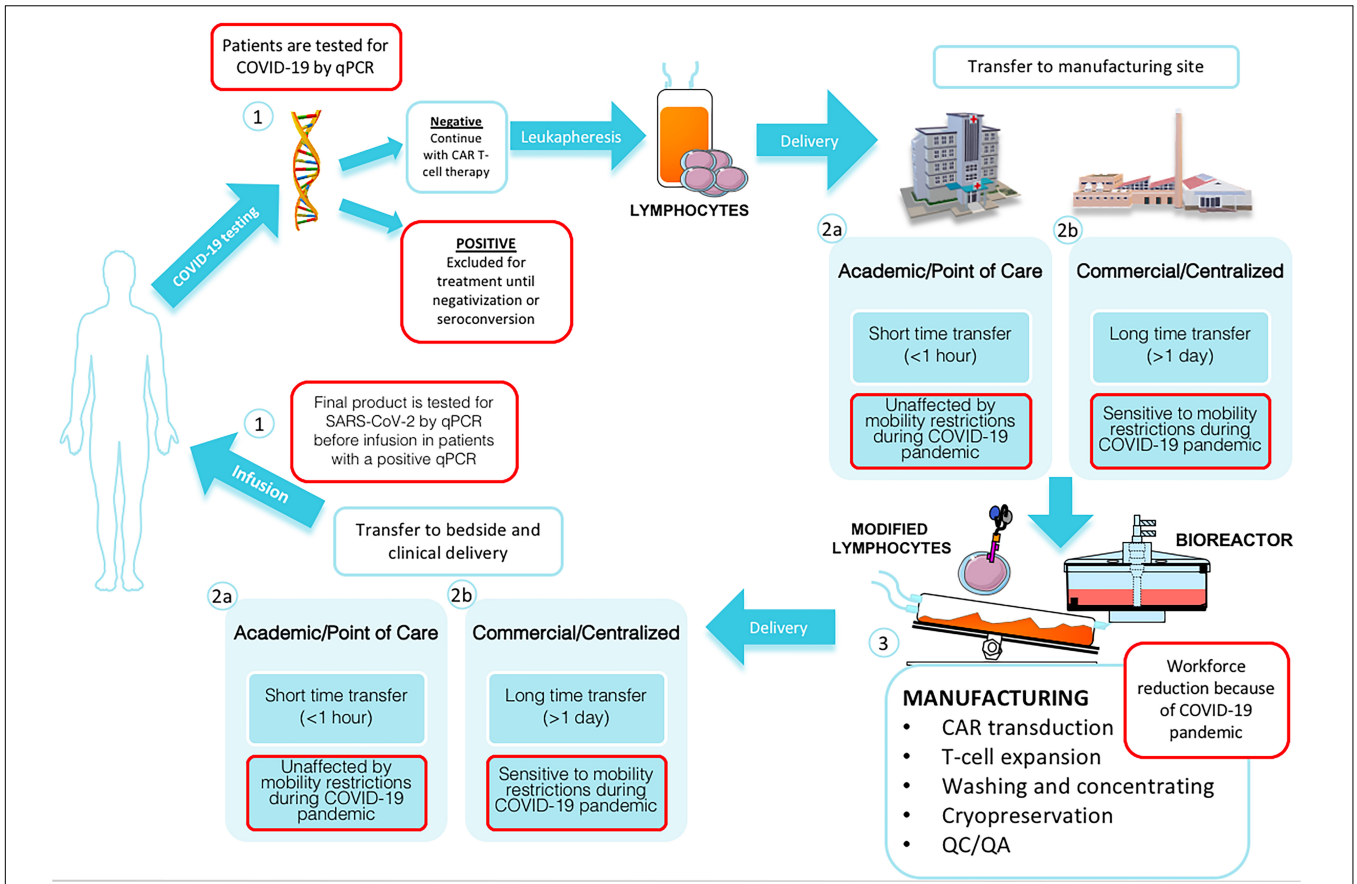

FIGURE 1 | Standardized CAR T-cell manufacturing process during COVID-19 pandemic. Patients have to be tested by qPCR for SARS-Cov2 before leukapheresis (1); in case of a negative result, peripheral blood mononuclear cells (PBMCs) are collected from patient via leukapheresis. Timing of delivery to manufacturing site and the susceptibility to mobility restriction varies between Academic/Point of Care (2a) and Commercial/centralized (2b) production. Manufacturing process is affected by workforce reduction (3). T-cells are selected, activated, transduced with a viral or non-viral vector to express the desired CAR and expanded in a bioreactor. After that, resulting CAR T-cells are isolated and cryopreservated and assessed by quality control (QC) and quality assurance (QA) programs. Finally, the patient's modified own cells are transferred to bedside. Timing of delivery to bedside and the susceptibility to mobility restriction varies between Academic/Point of Care (2a) and Commercial/centralized (2b) production. Before CAR T-cell product infusion, final product is tested for SARS-CoV-2 by qPCR before infusion in patients with a positive qPCR result prior to leukapaheresis (1).

COVID-19 Impact to Availability of Personnel in Every Step of the Manufacturing Process

The reduction of personnel due to possible contagions among the staff, the restructuring of the workforce to treat infected patients and the limited resources and protective equipment has been a cause for concern (**Table 1**). All of these factors can impact equally on the academic or pharmaceutical production modalities.

The staff and the reagent shortage can affect the leukapheresis process and cell processing in the laboratory. There exist strict regulations associateds with cell therapy where personnel who develop "living drugs" must work under sterile and GMP conditions. For this reason, a contagion that could spread between qualified personnel and the consequent imposing quarantine that would be imposed on the rest of the personnel can become a bottleneck in the global production of the adoptive cell therapy. The stringent and immobile regulation has not been amended in the context of the COVID-19 pandemic to ensure the safety of patients, medical staff and laboratory personnel.

Similarly, within the associated legislation, a series of quality controls are imposed and must be complied with before CAR T-cell product infusion. Among them, the confirmation of the product sterility is essential. In addition, it must be ensured that the product is free of endotoxins, adventitious viruses and mycoplasma. There are other non-microbiological controls that must be carried out as the number of copies of the CAR per genome, flow cytometry experiments to determine the percentage of cells with CAR, among others. Having these controls in place for each cell product requires precious time in COVID-19 pandemic setting and is highly dependent on human work. Therefore, any delay in verifying all of these control points may delay the infusion.

CYTOKINE RELEASE SYNDROME AND TOCILIZUMAB PRIORITIZATION

Once the manufactured CAR T-cell product is infused, a resulting CRS is the widest described associated side effect. A compilation of different symptoms from hypotension to fever due to a massive pro-inflammatory cytokine release (IL-6, IL-1β, TNF-α) have been reported (30). Serum IL-6 level has been correlated with CRS severity (15). Hence, tocilizumab (TCZ), an anti-IL-6 receptor monoclonal antibody, has become the drug of choice for the management of moderate or severe CRS (10). This IL-6 cytokine release profile resembles the virally driven hyper-inflammation (CRS-like) suggested as predictor of fatality in COVID-19 (31). This fact has led to the setting-up of several clinical trials with the aim of studying the impact of TCZ administration in the evolution of the acute respiratory distress syndrome and the off-label use of this drug in symptomatic COVID-19 patients.

The management of CRS is extremely relevant given that viral infections (not only COVID-19) may promote CRS (32). CRS requires treatment with TCZ or corticosteroids depending on severity of symptoms. The notable increase in TCZ use has raised a concern about supply during COVID-19 pandemic. However, availability of 2 TCZ doses for each patient is a mandatory condition before CAR-T infusion. Two patients treated with CAR T-cell therapy have received TCZ to treat CRS in our center in the pandemic setting. Tocilizumab administration protocol has not been reconsidered despite supply problems. This protocol consists on early TCZ use at the onset of grade 2 CRS. We have dealt with TCZ scarcity through the use of other drugs such as sarilumab (human anti-IL-6 receptor) or siltuximab (chimeric anti-IL-6) to face the CRS-like complications. If ICU is collapsed, CRS management protocols should initiate earlier to reduce the probability of using ICU. As a practical option, we propose TCZ administration from grade 1 CRS (and not grade 2 as is usually done), or even prophylactic TCZ administration in those patients considered to be at high risk of severe CRS, with high tumor burden. Corticosteroids used as CRS treatment when TCZ has failed could promote potentially disadvantageous effects on COVID-19 outcomes (33). Nevertheless, corticosteroids should be cautiously used in COVID-19 patients that suffer from CRS after CAR T-cell infusion (34).

In fact, the current pandemic has exposed us to an ethical dilemma regarding the prioritization of TCZ. Each case must be thoroughly evaluated to ensure the availability of this drug. Tocilizumab could decrease the duration and severity of COVID-19 symptoms allowing the weaning of the ventilatory support (34). On the other hand, TCZ supply prior to infusion must be ensured for the hematologic patients during CAR T-cell therapy to mitigate the toxicity of an eventual CRS complication.

CONCLUSION

CAR T-cell therapy has been consolidated as a potential curative therapy for patients with refractory/relapsed hematologic malignancies. The COVID-19 pandemic represents an unprecedented challenge to continue safely treating patients with this adoptive cell therapy. Nevertheless, centers should continue offering this potentially curative treatment with CAR T-cell therapy for critical patients using appropriate selection criteria, early intervention to attenuate side effects like CRS with standardized TCZ protocols, and prophylactic/preventive strategies to prevent infection. For those heavily treated patients, time is crucial. Mobility and personnel restrictions add obstacles to a truly personalized therapy in which multidisciplinary teams intervenes. This has affected both academic CAR T-cell therapies and those commercially available. There are several limitations related to delivery and the time-consuming manufacturing processes that create new concepts concerning the benefit of academic point of care proposals. Also, some regulatory rules should be re-evaluated to become more flexible during this pandemic.

AUTHOR CONTRIBUTIONS

MJ and MP proposed, directed, discussed, and revised the development of the manuscript. GM-S, NE, and IOL had equally contributed to the data gathering and writing of the review. VO-M reviewed and collaborated particularly on aspects related to the hematological issue. VB and CG had been in charge of analyzing the technical aspects related to the product manufacturing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cancer Immunotherapy Using Chimeric Antigen Receptor Expressing T-Cells: Present and Future Needs of Clinical Cancer Centers

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Chimeric Antigen Receptor-T cells (CAR-T) are considered novel biological agents, designed to selectively attack cancer cells expressing specific antigens, with demonstrated clinical activity in patients affected with relapsed/refractory B-cell malignancies. In consideration of their complexity, the use of CAR-T requires dedicated clinical setting and health care practitioners with expertise in the selection, treatment, and management of toxicities and side effects. Such issue appears particularly important when contextualized in the rapid progress of CAR-T cell treatment, translating into a constant need of updating and evolution. Moreover, the clinical grade manufacturing of CAR-T cells is complex and implies articulated regulatory and organizational aspects. The main goal of this review is to summarize and provide an accurate analysis of the clinical, logistic, and regulatory requirements of CAR-T cell centers. Finally, we describe a new occupational figure called "CAR-T specialist" devoted to the establishment and coordination of the required facilities and regulatory landscape in the context of cancer centers.

Keywords: CAR-T cells, CAR-T process, CAR-T Unit, CAR-T Specialist, JACIE, GMP, ATMP

INTRODUCTION

The demonstration that the immune system can control tumor growth has been provided for the first time by Thomas and Burnet (1, 2). This evidence has been confirmed after several decades, demonstrating the prognostic role of immune cells infiltrating the tumor lesions (3–10). The crescent knowledge of cancer immunology and immunotherapy has allowed the development of novel biological agents that showed unprecedented clinical results (11). These results contributed to turn into reality the paradigm that patient's immune system represents effective "living drugs" against cancer cells. Among these, adoptive cell therapy (ACT) that implies the isolation and

expansion *ex vivo* of tumor antigen-specific T lymphocytes and their re-infusion in patients has shown promising clinical activity improving the overall survival of cancer patients (12). T-lymphocytes engineered with Chimeric Antigen Receptors (CARs), that consist of an antibody-derived domain for antigen recognition linked to T-cell signaling molecules, can recognize in a MHC-independent manner tumor antigens expressed on tumor cell surface (13–17). These biological drugs are based on the engineering of T-lymphocytes, isolated from patient's or, less frequently, from donor's peripheral blood, with either gamma-retroviral (RV) or lentiviral (LV) vectors encoding CARs linked to co-stimulatory molecules (either CD28 or 4-1BB linked to zeta-chain) (18–20). The development of anti-CD19 CAR-T cells and their application in several clinical trials showed durable clinical responses in both adult and pediatric cancer patients with disease relapse or refractory to other therapeutic interventions. Complete responses (up to 70–80%) and significant improvement of overall survival (OS) were documented in patients with either acute lymphoblastic leukemia (ALL) (21, 22) or high grade non-Hodgkin lymphomas (NHLs), including diffuse large B-cell lymphoma (DLBCL) (23, 24) and mantle cell lymphoma (ML) (25). These clinical trials led to the accelerated approval by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) of two CD19-CAR-T cell medicinal drug products, tisagenlecleucel (Kymriah) and axicabtagene ciloleucel (Yescarta). For the first time, ACT products entered commercial production by few pharmaceutical companies. Nevertheless, the clinical grade manufacturing is complex and with relatively long timeline for cell production, requiring the availability of patients' or donors' peripheral blood mononuclear cells (PBMCs) to be shipped to good manufacturing practice (GMP) facilities, if not locally available, and the return to clinical sites for infusion into patients. One of the challenging factors of CAR-T cells is represented by the short-term associated toxicities arising in patients immediately after infusion. Cytokine release syndrome (CRS) represents the most common side effects of this type of therapy with a range of incidence among the different clinical studies of 50–90% (26). The mechanistic hypothesis behind CRS still needs to be fully dissected; however, it is a life-threatening situation that requires timely and effective interventions (27). Other common side effects observed after CAR-T cell therapy for B-cell malignancies involve neurotoxicity, B-cell aplasia and hypogammaglobulinemia (26). CAR-T cells have been also investigated for the treatment of solid tumors with limited clinical responses, thus suggesting that this therapeutic strategy might be promising, but still needs optimization. One of the principal limitations is represented by the choice of target antigens; the majority of solid tumors have epithelial origins with many of tumor-associated antigens (TAAs) being shared with normal tissues (28, 29). This translates into high risk of inducing off-tumor toxicities following the infusion of CAR-T cells specific for these TAAs. In addition, the homing of CAR-T cells to tumor site for solid tumors showed low efficiency due to the complexity of the tumor microenvironment (TME) (30). Multiple studies are aimed at addressing the choice of target molecules, such as IL-13alpha2, B7-H3, CSPG4, CD44v6, MUC1, Mesothelin, EGFRvIII, Her-2, GD2 *etc.*, allowing to specifically redirect the CAR-T cells to tumors with selected histological origins (31–37). A remarkable number of clinical trials are ongoing worldwide to assess the clinical activity of these immunobiological drugs against solid tumors and the optimal clinical setting for cancer patients (28, 29, 35).

The organizational and regulatory aspects of CAR-T cell clinical center represent novel and challenging topic within a rapidly evolving field. These involve different clinical expertise and departments, regulatory and quality management, laboratory dedicated to the tissue collection, processing and storage, and the accurate and timely coordination of logistics. The complexity of this organization requires personnel with specific background and continuous interactions and coordination among them. This manuscript aims at addressing the clinical, regulatory, and organizational requirements of clinical centers, including hematological and oncological departments, dedicated to CAR-T cell therapies called CAR-T Unit. In this review a model of CAR-T Unit is proposed, as well as the need of a new figure called "CAR-T specialist". The connection between the CAR-T Unit and the involved facilities, either within the clinical centers (*e.g.*, clinical unit, apheresis unit, processing unit, pharmacy, intensive care unit) or external (*e.g.*, pharma companies, clinical research organizations) is also described.

THE MULTIFACED EVOLUTION OF CAR-T CELLS

In order to achieve clinical efficacy, the CAR-T cell therapy should have four features: i) high effect or activity, even in the immunosuppressive tumor environment; ii) high proliferation capacity, even after multiple encountering with cancer cells; iii) long-term persistence; iiii) absence or low degree of toxicity after cell infusion.

Despite the documented clinical success of anti-CD19 CAR-T cells, the broad clinical usage is still limited due to several factors, such as the non-optimal persistence, the low efficacy in solid tumors, the safety profile and the high costs associated with the complex manufacturing. Although anti-CD19 CAR-T cells can lead to complete response rates for B-cell malignancies, a consistent proportion of patients relapse through various resistance mechanisms, including loss of expression of the target antigen (CD19-negative recurrences) (38). To overcome this drawback and reduce the risk of disease recurrence, T cells have been engineered with multiple CARs to target simultaneously two tumor-associated antigens (bispecific CAR-T cells) (39–41). This strategy may allow decreasing the toxicity while augmenting the tumor specificity of CAR-T cells. To improve their anti-tumor efficacy, they have been implemented with molecular features that can target the tumor microenvironment (TME). These are represented by the fourth generation "armored" CAR-T cells that include T cells redirected for Universal Cytokine-mediated Killing

cells (TRUCK) through the incorporation of genes encoding for cytotoxic cytokines (42). Moreover, allogeneic or "universal" CAR-T cells are produced from T cells isolated from healthy donors, representing "off the shelf" engineered T cells. This strategy allows the viability of CAR-T cells for a high number of patients and the decrease of the costs regarding clinical grade production (43). Great emphasis is also placed in the development of CAR-T cells for the treatment of solid malignancies; to date results showed limited clinical efficacy (44–48). The challenges associated with the effectiveness of CAR-T cells in this setting are: i) the selection of target antigens, specifically expressed by tumor and not by normal cells; ii) the homing of CAR-T cells and their interaction with TME and the vascularization; iii) the production of immunosuppressive molecules; iiii) the high metabolic rate of both tumor cells and TME (49). To date in fact, a candidate target antigen expressed by solid tumors that is comparable to CD19, a lineage antigen over-expressed on B-cell malignancies which counterpart expression on normal cells limited to B cells is not available. Due to the high heterogeneity of cells within solid tumors, the "armored" CAR-T cells allow promotion of a universal cytokine-mediated activation, targeting even those cancer cells that would be invisible to CAR-T cells.

Notably, the ability to penetrate the TME, the biodistribution, survival, and proliferative features of CAR-T cells need to be considered for the efficient redirection of these cells to tumor cells. The antigen expression and localization on tumor cells is one of the limiting factors for the anti-tumor activity of CAR-T cells; nevertheless, the immunomodulatory activity of the TME can influence the fate of immune responses and the outcome. The combination of CAR-T cells with immune checkpoint blockade or other immunomodulatory agents represents a promising therapeutic intervention that is currently under evaluation (37, 50). The *in situ* administration of CAR-T cells could overcome their limited migration to solid tumors (51). In neuro-oncology this strategy can be applicable through intralesional and intraventricular administrations of the cells since the peculiarity of the cerebral circulatory system allows the spreading of the CAR-T cells (51).

CAR-T CELL PROGRAM

CAR-T cell therapies have been classified in the regulatory category of Advanced Therapy Medicinal Products (ATMPs) under the definition of a gene therapy medicinal product, although they represent cellular therapies. To assure the reliability and safety of this type of therapy, it is important to define the optimal conditions of administration to patients. Therefore, an organizational structure with accreditation path needs to be identified. In 2009, a specialized committee was created by the EMA, the Committee for Advanced Therapies (CAT) (Regulation (EC) No. 1394/2007of the European Parliament and of the Council of November 13, 2007), aimed at providing opinion on the quality, safety, and efficacy of studies utilizing CAR-T cells. Details of the structure, experience, and regulatory requirements of clinical centers where CAR-T cell therapy can be administered are discussed in the text below. The initial indication has been established for the hematology field, for which approved medicinal products are available. To guarantee the safety of patients, the requirements for CAR-T cells centers have been identified as similar to those for early-phase clinical trials for novel immune therapies, with the designation of clinical centers with documented experience in allogeneic hematopoietic stem cell transplantation (HSCT) program, from alternative donors and sources, as reference for this type of therapy. This implies the availability of on-site clinical hematology unit, with intensive care unit, neurological department, emergency department, pharmacy, and transfusion center.

Long-term trained and experienced multi-disciplinary teams working together to care for intensively treated cancer patients are mandatory. Nevertheless, regulatory and logistic landscape of this cell-based therapy displays high complexities. Therefore, an accurate planning of infrastructures and identification of specific guidelines are needed to develop cell-therapy programs. The details of organizational and regulatory requirements are explained in the following paragraphs and sub-paragraphs. Of note, the administration of CAR-T cells to patients with either hematological or solid tumors requires joint transplant programs that include different figures, organization charts, and quality systems. Given the complexity of this scenario, the so called "CAR-T specialist" has great expertise in regulatory aspects and therefore has a relevant role in identifying, controlling, and implementing the specific requirements that a "CAR-T Unit" should obtain.

REGULATORY GUIDELINES

In order to analyze the regulatory aspects of CAR-T cell therapy, the following premises are of relevant value: i) CAR-T cells have to be considered "Drug Products"; ii) prior to their production, a deep knowledge of the legislation concerning the whole process (from lymphocytes collection to cells infusion). Lymphocytes are both blood components and cells; therefore their manipulation is controlled by legislation on blood components, tissue, and cells.

Taken into consideration the above points, the process of CAR-T cell generation requires the compliance with the regulation summarized in **Table 1**.

TABLE 1 | Regulatory entities for the production of CAR-T cells.

	Tissueregulation	Bloodregulation	ATMP	JACIEStandards	GMPregulation
Collection of tissue and cells	X	X		X	
Production			X		X
CAR-T Infusion			X	X	

Tissue Regulation

Lymphocytes are cells and are regulated by the European guidelines for tissue and cells. The legal framework defining the safety and quality standards for tissues and cells is based on the *Directive 2004/23/EC*, also referred to as the "European Tissues and Cells Directive", adopted in 2004 by the European Parliament and Council. It comprises the process for transplant, including donation, procurement, testing, processing, preservation, storage, and delivery. The *Directive 2004/23/EC* has been implemented by the following:

- *Commission Directive2006/17/EC* providing technical requirements for the donation, procurement and testing of human tissues and cells.
- *Commission Directive2006/86/EC* addressing the traceability, notification of severe adverse reactions and events, coding, processing, preservation, storage, and delivery of human tissues and cells to facilities and clinical centers.
- *Commission Directive 2015/565* amending the Directive 2006/86/EC regarding the coding of human tissues and cells.
- *Commission Directive 2015/566* implementing the Directive 2004/23/EC with the procedures for verifying the equivalent standards of quality and safety of imported tissues and cells.
- *Commission Decisions 2010/453/EC* and Commission Directive 2012/39/EU, as well as Commission Decision C (2015)4460

The *Directive 2004/23/EC* specifies that (introduction, point 7):

> "Tissues and cells intended to be used for industrially manufactured products, including medical devices, should be covered by this Directive only as far as donation, procurement and testing are concerned, where the processing, preservation, storage and distribution are regulated by other Community legislation. The further manufacturing steps are covered by Directive 2001/83/EC of the European Parliament and of the Council of 6th November 2001 on the Community code relating to medicinal products for human use(4)".

Thus, only donation and donor selection/testing are regulated by the Directive 2004/23/EC, in particular: i) criteria for donor selection and evaluation, as the document defines how to manage the inform consent of donors and the procedures for data protection and confidentiality; ii) the procurement requirements are illustrated in chapter 15 (*Directive 2004/23/CE*). *"The activities related to tissue procurement shall be carried out in such a way as to ensure that donor evaluation and selection is carried out in accordance with the requirements referred to in Article 28(d) and (e) and that the tissues and cells are collected, packaged and transported in accordance with the requirements referred to in Article 28(f).*Authorization

An important point of the Directive 2004/23/CE is paragraph 6:

1. "Member States shall ensure that all tissue establishments where activities of testing, processing, preservation, storage or distribution of human tissues and cells intended for human applications are undertaken have been accredited, designated, authorized or licensed by a competent authority for the purpose of those activities.
2. The competent authority or authorities, verified that the tissue establishment complies with the requirements referred to in Article 28(a), shall accredit, designate, authorize or license the tissue establishment and indicate which activities it may undertake, and which conditions apply. It or they shall authorize the tissue and cell preparation processes which the tissue establishment may carry out in accordance with the requirements referred to in Article 28(g). Agreements between tissue establishments and third parties, as referred to in Article 24, shall be examined within the framework of this procedure."

These guidelines identify that only accredited, designed, authorized or licensed institution can manipulate tissues for cell therapy. Each State Member identifies the criteria for the accreditation, and variability takes place in terms of the authorization criteria applied among different state members in EU. Once tissue isolation has been licensed, accredited, or authorized, it will be registered on the dedicated EU website.

Regulation for Blood Collection

This topic has multiple guidelines according different regions or countries. In some cases (*i.e.* Italy), this activity is under the responsibility of Transfusion Center in terms of collection of peripheral blood-derived cells. National regulatory agencies provide local procedures and guidelines. This introduces further complexity in the regulatory field of CAR-T cell therapy.

Joint Accreditation Committee ISCT-Europe (JACIE) Standard Requirements and Accreditation

CAR-T cell production and clinical management have peculiar characteristics that can be associated with HSCT; therefore in most of the Countries in EU the Regulatory Agencies have identified that their administration should occur in a Bone Marrow transplant Unit. This activity is regulated by the Foundation for the Accreditation of Cellular Therapy-Joint Accreditation Committee of the International Society for Cellular Therapy (ISCT) and the European Group for Blood and BMT (FACT-JACIE). The European Society of Blood and Marrow Transplantation (EBMT) implemented the FACT-JACIE international standards for HSCT programs including specific requirements for CAR-T cell preparation and treatment, intended as guidelines for the usage of this novel therapeutic approach ("*The Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) is Europe's only official accreditation body in the field of hematopoietic stem cell transplantation (HSCT) and cellular therapy. It promotes high-quality patient care and medical and laboratory practice through a professional-driven and voluntary accreditation scheme*". JACIE has developed quality standards to evaluate the quality level of the transplant programs in all the phases, including the collection of stem cells,

the activities of cell processing, and the infusion in patients. The latest version of the JACIE Standard included the accreditation for the administration to patients of Immune Effector Cells (IEC), including CAR-T cells. It is important to note that the JACIE standard is limited to the qualification of centers dedicated to the *stem cell transplant programs* and for this reason only CAR-T products connected to the stem cell unit and utilized for the treatment of hematological malignancies are included. CAR-T cell products for the treatment of solid tumors are not included, and this can generate controversies in the organization of clinical centers for the CAR-T cell therapy of this type of tumors. According to the JACIE standards, the requirements to apply for the CAR-T products are the following:

- Clinical requirements;
- Collection requirements;
- Processing requirements.

The FACT-JACIE standard is a voluntary standard of excellence and aimed at accrediting and qualifying stem cell transplant programs; therefore institutionally it does not cover the activities for solid tumors. However, an HPC-A transplant program includes several units: the clinical unit that performs the transplant, the aphaeretic collection center, and the cell manipulation laboratory that processes the products. The clinical unit takes care of the therapeutic aspects while the aphaeretic collection unit and the cell manipulation laboratory are not strictly dependent on the clinical hematological activity. The activities of the aphaeretic collection unit and the cell manipulation laboratory are focused on the donor and the cellular product; therefore, these activities can be performed also for oncological clinical units for the CAR-T cell therapy of patients with solid tumors. The requirements of the FACT-JACIE standards related to apheresis and cell manipulation are relevant for the cell product collection and manipulation regardless the type and origin of tumor.

Clinical Requirements

The JACIE standard defines the clinical requirements for the compliance of BMT centers. The clinical requirements for CAR-T cells therapies are: i) donor selection and evaluation; ii) infusion of the IEC product; iii) recipient care; iiii) evaluation and training of the personnel dedicated to the above activities; iiiii) product management.

Collection Requirements

JACIE standard provides guidelines to perform the apheresis for the collection of lymphocytes. In particular, the following points are discussed:

- Donor selection and evaluation;
- Aphaeretic product collection;
- Aphaeretic product labeling;
- Aphaeretic product release to the laboratory dedicated to its manipulation;
- Apheretic product transport;
- Evaluation and training of the personnel.

Processing Requirements

According to the EC Directives, only a processing laboratory recognized as tissue establishment (TE) can release the product and coordinate the transfer to the GMP facility for CAR-T cell manufacturing. JACIE standard identifies the criteria and guidelines for: 1) the receipt of apheretic product; 2) final product (CAR-T product) labeling; 3) final product (CAR-T product) release; 4) final product (CAR-T product) transport; 5) evaluation and training of the personnel.

Advanced Therapeutic Medicinal Products Regulation

CAR-T cell therapies are included in the category of ATMPs within the definition of gene therapy medicinal product. According to the Committee for Advanced Therapy, the CAR-T Product is an ATMP product: "*products consisting of cells or tissues may scientifically be at the border between Tissues and Cells directive (Directive 2004/23/EC) and the ATMP regulation"*. The classification of an ATMP as a biological product determines a wider regulatory framework and the subsequent requirements for the development and the marketing authorization. These need to be considered in association with the specific framework for ATMPs, *Regulation 1394/2007/EC*, which was approved on December 30, 2008: "*ATMP are authorized centrally through the European Medicine Agency (EMA). They benefit from a single evaluation and authorization procedure. EMA continues to monitor the safety and efficacy of ATMPs following their approval and commercialization. This Agency also provides scientific support to the developers of these products in the design of pharmacovigilance and risk management systems to monitor the safety of these medicines*". This regulation provides the overall framework on ATMPs for those products, which are intended to be placed in the market of EU Member States. In addition, *Directive 2009/120/EC* updated the definitions and detailed scientific and technical requirements for advanced therapies. ATMPs are regulated by both guidelines of medicinal products and that of medical devices. On the 25th of May 2017, two additional regulations on medical devices were identified (*European Commission, 2017a*). For the development of advanced therapies in EU, the development and approval of clinical trials are in charge of the individual national competent authorities. However, for marketing authorization, all ATMPs are evaluated through centralized procedures ensuring that they benefit from a single evaluation and authorization across EU. Two committees are responsible for the scientific evaluation and approval of ATMPs: the CAT and the Committee for Medicinal Products for Human Use (CHMP) (European Medicines Agency, 2018a). The CAT is the EMA committee responsible for classifying, assessing the quality, safety, efficacy of ATMP, in order to follow the scientific progress in the field and to provide an opinion on each ATMP application submitted to the EMA, supporting the final decision by the CHMP.

THE CAR-T CELL PROCESS

The process for the manufacturing and infusion of CAR-T cells is relatively complex not only for the clinical aspects, but also from an organizational and regulatory point of view. The production

and infusion of CAR-T cells require a specific organizational structure; the clinical unit task force includes physicians and nurses, and numbers of other clinical and healthcare personnel with long-term expertise in the care of patients with either hematological malignancies or solid tumors and in HSCT procedures. Therefore, it is important to have a detailed list of the personnel and multi-disciplinary expertise involved in this process and their role and relationship.

Patient Evaluation and Selection

The clinical CAR-T unit is in charge of the evaluation and selection of patients eligible for CAR-T cell therapy. This represents a critical task since the demand of patients in need of such treatment will increase overtime while the availability of CAR-T cells are still limited—at least so far—to a relatively low number of clinical centers. Stringent eligibility criteria are required in order to select those patients that may benefit from CAR-T cell administration. The decision to treat a patient with CAR-T cell therapy should be made in the context of a multi-disciplinary team. The main factors that influence the suitability of a patient to CAR-T cell treatment are represented by the medical history, the clinical conditions, the disease burden, and life's expectancy. In particular, disease burden at the time of evaluation represents a critical consideration, as patients with low tumor burden experience less treatment-related toxicities and have superior clinical responses (22). However, in real life, most patients have aggressive malignancies that need to be carefully managed along with the timeline and processes, such as insurance authorization, apheresis procedure, and manufacturing, to obtain the medicinal product for infusion. Moreover, clinicians should take into account the risk of disease progression and the related complications that could occur until the expected date of CAR-T cell infusion. Finally, also manufacturing failures should be foreseen, since in this case alternative therapies have to be considered.

Lymphocyte Collection

The apheresis unit is in charge of the patient/donor suitability assessment for the procedure. The collection procedure follows the clinical protocol guidelines. The following steps involve the labeling of the apheresis product and the delivery to the cell processing laboratory.

Lymphocyte Processing (Minimum Manipulation)

The TE may perform the manipulation to isolate and cryopreserve lymphocytes according to standardized and validated procedures. The cryopreserved lymphocytes are then delivered to the good manufacturing practice (GMP) facility that can be either part of a pharmaceutical company or an independent site. Alternatively, fresh collected lymphocytes may be sent directly from the TE to the GMP facility that will proceed to lymphocyte isolation and subsequent processing.

GMP Production and Transport

The GMP facility is responsible for the manufacturing, control, and release of the drug products, according to ATMP regulation. The product is then released and shipped to the CAR-T unit. CAR-T cells are shipped in the form of frozen cells to the clinical center. The pharmacy unit will receive, register, and handover the cells to the TE for temporary storing. The final process involves cell thawing for the administration to the patient. This step may be performed either by the TE that will provide the cells to the clinical unit or directly by the clinical unit.

Treatment of Patient Before the Infusion of CAR-T Cells

CAR-T cell infusion is administered after a brief course of chemotherapy. The aim of such chemotherapy is to control, at least in part, the disease during the period between lymphocyte collection and the final infusion of the CAR-T cell product (52). However, it is advisable that the treatment with CAR-T cells might be performed before the achievement of disease complete remission, since the target molecules of CAR-T cells might be no longer detectable. "Bridge" chemotherapy plays an important role in acute lymphoblastic leukemia (ALL) as leukemic blast burden at the time of CAR-T cell infusion correlates with an increased risk of cytokine release syndrome (CRS). The overall tumor burden and the involved sites are additional factors to be considered also in lymphoma patients: many regimens are indicated in this phase, and the choice must take into account the type of histology, the burden of disease, previous treatments, and the fragility of the patient. However, the "bridge" chemotherapy should be given only after leukapheresis in order not to affect the quality of CAR-T cell product (53). Two to seven days before CAR-T cell infusion, patients receive a lymphodepleting conditioning regimen to get rid of immune cells and factors endowed with immune suppressive properties and to enhance CAR-T cell functions and engraftment (54). The optimal conditioning regimen has not been defined yet, but the majority of studies employed a combination of fludarabine and cyclophosphamide. Experiences at the NCI (55) and Fred Hutchinson Cancer Center (56) demonstrated that aggressive lymphodepleting regimens are associated with the increase of both CAR-T cell expansion and toxicity. Of note, certain drugs such as check point inhibitors or other biological drugs (*i.e.*, alemtuzumab, daratumumab, and brentuximab vedotin) should be avoided since they may interfere with expansion and persistence of CAR-T cells after infusion (53). Infectious complications and organ toxicities should be minimized during bridging chemotherapy (57).

CAR-T Cell Infusion

CAR-T cell infusion requires an appropriate clinical setting with dedicated gene therapy rooms, medical and nursing standard of practice protocols, documentation and verification procedures for administration. CAR-T cells' targeted therapies frequently induce toxicities that can be mitigated by a planned hospital organization. Comprehensive training should be provided to all categories of personnel including scientists, nurses, and physicians, and close collaborations with a range of other specialists, especially intensive care unit (ICU) staff and the neurology/neuroimaging services, are required (58). Indeed, the management of patients undergoing CAR-T cells requires two necessary conditions: a structured clinical unit with well-

established procedures to take in charge of patients developing acute immunological complications and intensive care unit (ICU) close to the clinical unit. Protocols for the interactions between the two units have to be established at each center.

Post-Infusion Follow-Up

During the pre- and early post-infusion period, patients are generally admitted to inpatient unit, as the rapid *in vivo* proliferation of CAR-T cells may be associated with adverse events, such as CRS and neurotoxicity (59–61). Currently, rapid advances have been made in the identification of clinical and biological predictors and in the design of appropriate standards of care to mitigate the severity and long-term consequences of these complications (62). CRS is the most common side effect following CAR-T cell therapy, occurring in 30 to 100% of cases, with CRS of grades 3–4 in 10–30% of patients (63). CRS may present with a spectrum of manifestations from low-grade fever to fulminant multiorgan failure (64–66). In some studies, approximately 10–15% of CAR-T cell patients required vasopressor support for hypotension and/or mechanical ventilation (61, 67). The mainstays of treatment for severe CRS include the anti-IL6-R antibody (tocilizumab), anti-IL6 antibody (siltuximab), high-dose steroids, and supportive care delivered in ICU (59, 61). Patients treated with CAR-T cells may develop concomitantly with CRS or as an isolated feature, a potentially fatal neurological toxicity, including delirium, encephalopathy, nerve palsies, and seizures, whose mechanism seems to be related to systemic inflammation due to rapid CAR-T cell expansion (61, 64). Different products have variable rates and patterns of toxicity, and clinical judgement remains the best method to assess CRS. Tisagenlecleucel induced severe CRS (grade 3 or 4 by the UPenn toxicity criteria) in 48% of pediatric and young adult ALL patients in the ELIANA trial (21) and 28% of adult patients with diffuse large B cell lymphoma (DLBCL) in the JULIET trial (52), while the ZUMA-1 trial reported a 13% rate (by NCI Common Terminology Criteria for Adverse Events) for axicabtagene ciloleucel in adult DLBCL patients (23). Nurses and ancillary medical personnel should be adequately trained about the recognition of CAR-T cells' specific care and toxicities in order to expedite the evaluation for the transfer to ICU and treatment with tocilizumab. The second most common adverse event is the immune effector cell-associated neurotoxicity syndrome (ICANS) (59). The symptoms and signs are non-specific, and the severity is correlated with the increase of specific biomarkers such as C-reactive protein, ferritin and IL-6 (59, 68). ICANS incidence has been reported in 12–55% of cases. Patients are monitored by nursing tools to identify early manifestations of CRS or neurotoxicity. In the latter case, serial cognitive testing and neurological expertise are needed. In addition to infectious events, CAR-T cell recipients are at increased risk of potential medium-term complications, depending on the type of CAR-T cell product, including delayed tumor lysis syndrome, delayed hemophagocytic lymphohistiocytosis/macrophage activation syndrome and CRS, B-cell aplasia, hypogammaglobulinemia. Neutropenia, thrombocytopenia, and anemia are commonly observed in CAR-T cell treated patients, but generally resolve over several months through the administration of growth factors following the observation at early stages of adverse events.

THE CAR-T UNIT

In order to guarantee the processes illustrated above and the regulation compliance, it is necessary to establish a dedicated organization that comprises different units within the clinical center. The clinical unit has the responsibility to select the patient, to evaluate the donor, to perform the infusion of CAR-T cell product, and to manage the follow-up activities. The apheresis unit collects mononuclear cells and transfers them to TE. The TE performs (if required according to the specific protocol) the processing, labeling, and storage of the aphaeretic product and organizes the shipping to the GMP facilities. Under the responsibility of the pharmacy department, the TE receives the CAR-T product, performs the temporary storage, and transfers the product to the clinical unit for infusion. The pharmacy has the responsibility to receive the drug product and to delegate the TE to store the product. ICU supports the clinical unit in the management of the patients in case of adverse events, together with several medical support consultants.The Organizational Model of the CAR-T Unit

A CAR-T unit can be organized according to either a centralized or decentralized structure. A CAR-T center requires the work of a multi-disciplinary team.

The centralized CAR-T unit is a model based on the centralization of the activities with a single unit. This structure is functional for the organizational entities where knowledge and different expertise of the staff are in a single unit. Usually in this organizational modality, the CAR-T unit corresponds to the HSCT Program (**Figure 1**). This model is useful where there is a single Unit of Hematology or Onco-hematology.

The second model, the decentralized CAR-T Unit, is a model suitable for entities where the infusion of cell therapies occurs under the responsibility of different subjects. This model is implemented in complex organizational structures in which the types of IECs utilized for cell therapy are different and are administered to patients with either blood malignancies or solid tumors (**Figure 2**). This model is useful in Hospitals and Institutes with separate business units for Oncology and Hematology even within a single CAR-T center. This is useful for the current different values of Oncology and Hematology and the continuous evolution of their relationship. Furthermore, this model allows having a CAR-T unit even in the context of subdomain of Oncology, such as Neuroncology.

THE CAR-T SPECIALIST

The CAR-T specialist represents a critical profile in the multi-disciplinary and multi-professional team of the CAR-T unit. This profile is dedicated to the compliance with regulatory and organizational requirements for ATMP therapies. Therefore, the CAR-T specialist is a key person within the CAR-T Unit with knowledge of the scientific principles of CAR-T cells, regulatory

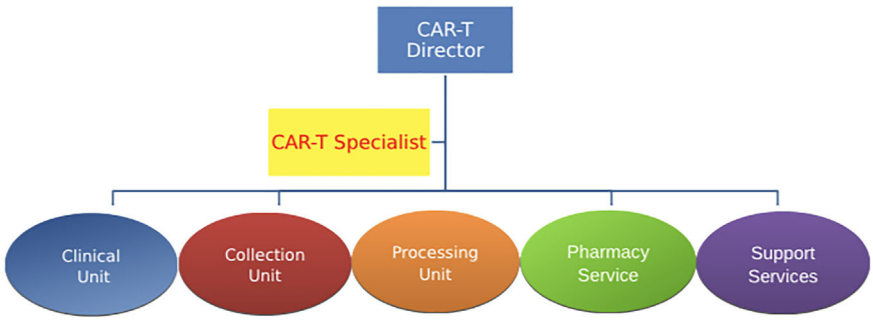

FIGURE 1 | Centralized CAR-T unit This model is based on the centralization of the activities with a single unit, and it is useful for a single unit involved in CAR-T cell process. CAR-T specialist is a unique figure coordinating the whole process.

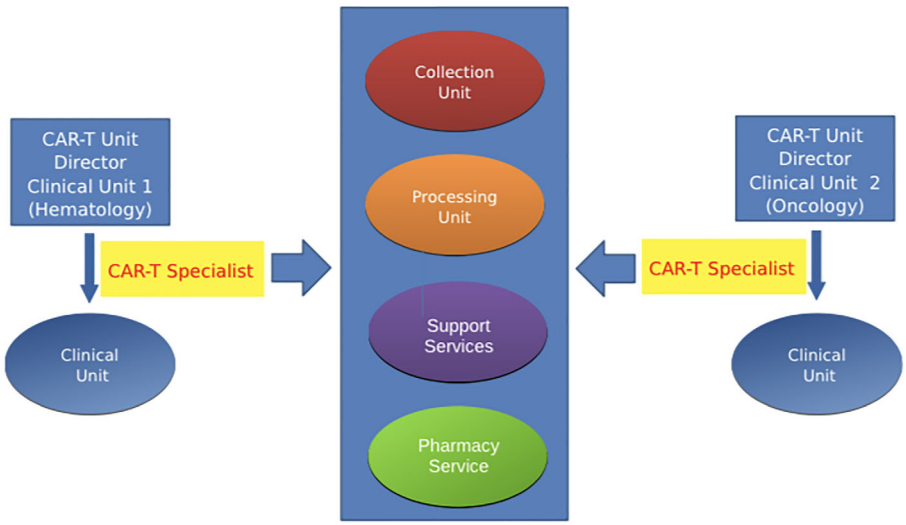

FIGURE 2 | Decentralized CAR-T unit This model is suitable when cell therapies occur under the responsibility of different subjects (complex organizational structures with separate business unit for Oncology and Hematology). CAR-T specialist is present in each involved unit.

and quality requirements, management and administrative skills, organizational knowledge and experience in clinical trial. The CAR-T Unit Director or Transplant Program Director can identify one person on the team (Centralized) or more than one (Decentralized) with responsibility for the organization and quality system of the CAR-T unit. The CAR-T specialist can be represented by the staff responsible for managing the quality system of the BMT program but with documented experience in the aforementioned topics.

Education and Knowledge of CAR-T Specialist

The requirements of the quality manager for BMT centers are detailed in the JACIE Standard, while for the CAR-T specialist those requirements need to be extrapolated and implemented. In particular, the CAR-T specialist has scientific knowledge of CAR-T cells and cell-based therapies, and in addition to the skills listed above, a minimum of 10 h of training and educational activities related to the IEC and the management of quality is mandatory. This role can be covered by a person with the following requirements:

a. Scientific degree (physician, biologist or pharmacist);
b. Experience of 5 years in the field of stem transplant center or in a processing laboratory or in apheresis or oncology unit;
c. Good knowledge of the quality requirements in a tissue establishment, transplant centers (*i.e.* JACIE-FACT) or blood centers;
d. Good knowledge of the leukapheresis process;
e. Good knowledge of the national requirements of the tissue and blood regulations.

The Job Description of CAR-T Specialist

CAR-T specialist bears the responsibility for the organizational management of the CAR-T unit. The following are the major tasks:

- Coordinating the organizational activities of the CAR-T center;
- Developing the organization system;
- Managing quality system;
- Managing personnel training and education;
- Creating a project plan for the management of the CAR-T cell therapies;
- Planning and managing audits for the CAR-T center;
- Collecting data and indicators;
- Performing training related to the procedures for managing the CAR-T cell therapies;
- Coordinating the facilities' activities in the CAR-T program.

The important role of the CAR-T specialist is to coordinate the activities with the different units involved in the CAR-T process. Particularly, the following will be involved:

- *Transplant Program (clinical unit, collection unit and processing unit):* the CAR-T specialist will manage the relationship between the facilities involved in the transplant program according to the CAR-T requirements and JACIE requirements;
- *Pharmaceutical companies:* the CAR-T specialist represents the bridge between the pharmaceutical company and the GMP facility. The CAR-T specialist maintains the relationship with the pharmaceutical company and GMP facility for sponsored studies or for commercial usage of cell-based therapies. Moreover, this person will supervise the education and training activities of members of the teams;
- *Other internal facilities:* the CAR-T specialist guarantees that the team members involved in the activities of the CAR-T unit have the necessary knowledge of their specific tasks. In addition, the CAR-T specialist coordinates with the pharmacy, the clinical trial office, and the ICU.

The CAR-T specialists are responsible for developing and managing the quality system of the CAR-T Unit according to: i) national regulations; ii) requirements of the GMP Facility; iii) requirements of the FACT-JACIE Standards.

Moreover, the CAR-T specialist is in charge of coordinating the preparation of documents for the registration of the activities of the facilities, to maintain the quality system, and to perform internal audits in the CAR-T Unit.

Because the CAR-T unit is incorporated in several facilities, the CAR-T specialist should be in charge of a multi-disciplinary and/or multi-professional group composed of:

a. Quality manager of the Transplant Program
b. Quality Manager of the Apheresis unit
c. Quality Manager of the processing unit
d. Quality Manager or Qualify Person of the GMP Facility (if present)

According to the specific organization, the role of the CAR-T specialist could be managed by the Transplant Program Quality Manager. The role and responsibilities of the CAR-T specialist will vary for the two models: in the first model, the CAR-T specialist will manage all the activities of the team(s), while in the second model each facility will have a CAR-T specialist (one for the oncology clinical unit, one for the hematology unit, and one for the processing and collection unity). A plan of coordination and collaboration between these CAR-T specialists will be required.

CONCLUSIONS

The manufacturing and management of CAR-T cells comprise complex processes, various facilities, including clinical, processing and apheresis units, intensive care unit, pharmacy, medical consultants, GMP facilities, and pharmaceutical companies. Moreover, the CAR-T process has to be compliant, at European level, with different regulations, including tissue and cells, blood and ATMP legislation. According to the complexity of the process and the multi-disciplinary implications, the role of the CAR-T specialist becomes critical and indispensable. This expert figure guarantees that all the requirements are applied along the flow and processes within the CAR-T Unit, including the transfer of knowledge, the interconnections among multiple facilities, and the coordination and management of all the organizational aspects. The scientific and clinical developments of CAR-T cells are under rapid evolution and require specialized and long-term expertise personnel, accurate connections, and coordination of multiple facilities to make such living drugs available to a broad number of cancer patients.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Genetic Engineering of Natural Killer Cells for Enhanced Antitumor Function

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Natural Killer (NK) cells are unique immune cells capable of efficient killing of infected and transformed cells. Indeed, NK cell-based therapies induced response against hematological malignancies in the absence of adverse toxicity in clinical trials. Nevertheless, adoptive NK cell therapies are reported to have exhibited poor outcome against many solid tumors. This can be mainly attributed to limited infiltration of NK cells into solid tumors, downregulation of target antigens on the tumor cells, or suppression by the chemokines and secreted factors present within the tumor microenvironment. Several methods for genetic engineering of NK cells were established and consistently improved over the last decade, leading to the generation of novel NK cell products with enhanced anti-tumor activity and improved tumor homing. New generations of engineered NK cells are developed to better target refractory tumors and/or to overcome inhibitory tumor microenvironment. This review summarizes recent improvements in approaches to NK cell genetic engineering and strategies implemented to enhance NK cell effector functions.

Keywords: natural killer cells, tumor, genetic engineering, transduction, transfection, chimeric antigen receptor-natural killer cells, activating receptors, inhibitory receptors

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INTRODUCTION

Natural killer (NK) cells are part of the innate immune system. Discovered more than 40 years ago, they kill virus-infected cells, counteract tumor formation and initiate innate immune responses (1). Lower NK cell counts and reduced cytotoxicity are associated with higher cancer risks (2, 3), as NK cells kill aberrant somatic cells with downregulated major histocompatibility complex class I (MHC-I) molecules that escape T-cell scrutiny (1). Immunotherapy is a powerful biological therapy for boosting the patient's immune system, helping it to fight cancer off. Currently, immunotherapy options include compounds like monoclonal antibodies, cancer vaccines, and checkpoint inhibitors, and more recently cellular products like T cells, dendritic cells or NK cells. Early results showed that NK cells can be a safer alternative over T cells due to reduced side effects. Currently however, NK cell *ex vivo* expansion technologies are laborious, and their persistence *in vivo* is limited. Genetic engineering is a valuable tool to overcome these limitations and improve NK cells target specificity and cytotoxicity. NK cells were difficult to genetically modify but recently, NK cell engineering has become efficient and reproducible. This review will summarize recent improvements of NK cell engineering and discuss their use in increasing antitumor efficacy and *in vivo* persistence through improved tumor homing, and higher target specificity and cytotoxicity (**Figure 1**).

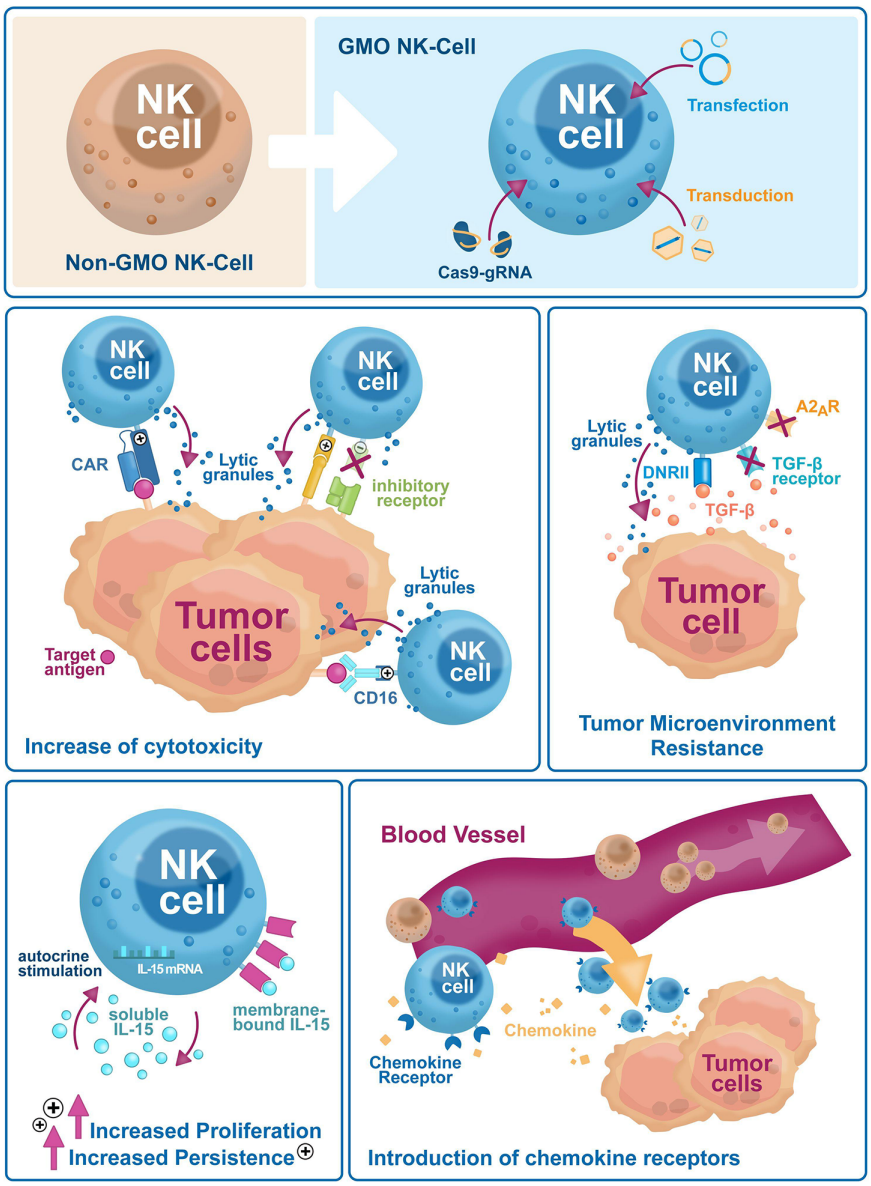

FIGURE 1 | Genetic engineering has broad applications to enhance NK cell-based immunotherapies efficacy against tumor escape mechanisms. Genetic engineering is used on NK cells to improve their cytotoxicity (i.e. exogenous expression of CARs and activating receptors, or selective downregulation of inhibitory receptors), reduce sensitivity to the tumor microenvironment (i.e. downregulation of inhibitory cytokine and small molecule receptors, the introduction of dominant-negative receptors), increase *in vivo* proliferation and persistence via autocrine cytokines stimulation and tumor homing (i.e. expression of chemokine receptors).

FUNCTIONALITY AND MECHANISM OF ACTION OF NATURAL KILLER CELLS

NK cells represent the main innate lymphocyte cell type. They mediate both anti-tumor and anti-viral responses. Only anti-tumor effects will be the subject of this review. NK cells are generally classified as $CD56^{+}CD3^{-}$ lymphoid cells and further subdivided into two major subpopulations based on CD56 and CD16 receptor surface expression: $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$ cells (4, 5). Circulating $CD56^{dim}CD16^{bright}$ NK cells are quiescent but become highly cytotoxic upon recognition of target cells, $CD56^{bright}CD16^{dim}$ cells, that reside in secondary lymphoid tissues, constitutively produce cytokines (6, 7). NK cells killing ability is tightly regulated by a wide range of inhibitory and activating receptors (1). The most prominent inhibitory receptors are the inhibitory killer-cell immunoglobulin-like receptors (KIRs), that bind polymorphic classical MHC-I molecules (HLA-ABC), universally expressed on healthy cells (8). A major role is also played by the inhibitory heterodimer receptor CD94-NKG2A that binds the non-classical MHC-I molecule HLA-E (9, 10). NK cell activating receptors comprise DNAM-1, NKG2D, CD94/NKG2C, CD94/NKG2E,

natural cytotoxicity receptors (NCRs) like NKp30, NKp44, NKp46, and CD16 (11–14) and activating KIRs. All these recognize specific ligands on the surface of target cells: the CD94/NKG2C and CD94/NKG2E heterodimers recognize HLA-E molecules, the NKG2D receptor recognizes the MHC class-I-chain related proteins A and B (MICA and MICB) and UL16 binding proteins (ULBPs), and DNAM-1 recognizes nectin-2 (CD112) and nectin-like proteins (15, 16). Upon activation, NK cells release lytic granules containing perforin and granzyme B within the immunological synapse to kill target cells (17). NK cells can also exert antibody-dependent cell cytotoxicity (ADCC) by recognizing antibody-coated cells through the low-affinity receptor for the Fc portion of IgG_1 antibodies (FcγRIIIa or CD16). Activated NK cells also secrete soluble factors like tumor necrosis factor α (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) to trigger apoptosis in target cells (18). And finally, they secrete interferon γ (IFN-γ) (19), growth factors (GM-CSF), immunoregulatory cytokines (IL-15, IL-10, and IL-13) and chemokines (20, 21). These cytokines modulate both innate and adaptive immune responses, such as dendritic cell (DC) maturation and $CD4^+$ to Th1 T cell differentiation, respectively (22–24).

TUMOR CELLS ESCAPE NK CELLS SURVEILLANCE

NK cells prevent tumor formation and metastases (25). Blood NK cell counts positively correlate with lower risk for cancer development (2), whereas higher tumor tissue NK cell infiltration correlates with improved treatment outcomes (26, 27). This antitumor effect has been comprehensively summarized elsewhere recently (21). However, solid tumors develop escape mechanisms to avoid NK cell recognition (28). They can upregulate MHC-I expression and thereby engage inhibitory NK cell receptors. For example, HLA-E upregulation increases engagement of the NKG2A/CD94 heterodimer in IFN-γ-stimulated ovarian cancer cell lines, dampening NK cell activity (29). Another resistance mechanism involves the downregulation or shedding of NK cell activating receptor ligands (30). Recently, it was found that NK cells express the immune checkpoint inhibitor PD-1 and that their cytotoxic activity is reduced upon PD-1 engagement in PD-L1 expressing tumors (31). Another recognized key player for tumor cell survival and immune system escape is the tumor microenvironment (TME). The TME comprises tumor-associated non-malignant cells and extracellular matrix components. It secretes suppressive cytokines, like transforming growth factor (TGF)-β (32) and IL-10 (33), or suppressive factors like prostaglandin E2 (PGE2) and adenosine (34, 35) that prevent NK cell mobilization and target tissue infiltration. Most of these immunosuppressive factors are secreted by tumor-associated cells, mainly regulatory T cells (T-regs) (36), M2 macrophages and myeloid-derived suppressor cells (MDSCs) (37). Additionally, the TME can be hypoxic by the tumor's high metabolism and poor vascularization, impairing NK cells cytotoxic activity (38). In conjunction, hypoxia favors the selection of the phenotypically most aggressive clones, and tumors become capable of sustained proliferation and metastatic potential that can no longer be controlled by NK cell action (39). Therefore, novel approaches for sustaining NK cells antitumor action are crucial for the development of effective tumor therapy.

NK CELL GENETIC MODIFICATION FOR SUSTAINED FUNCTIONALITY IN CANCER IMMUNOTHERAPY

Several clinical trials have confirmed the safety profile and efficacy of adoptive NK cells as treatment for hematological malignancies (21). NK cells do not mediate severe toxicities like graft-versus host disease (GvHD) or cytokine release syndrome (CRS) and, therefore, do not require stringent HLA matching (40, 41), the bane of T cell therapies. Consequently, NK cells derived from a single donor can be used to treat several patients. This "off-the-shelf application" dramatically improves therapy access and reduces production times and costs. The trials however also highlighted a relatively limited effectivity against solid tumors (21). This is caused by low tumor homing and infiltration, short *in vivo* persistence and impaired NK cell activity in the cancer patients, this by tumor antigen downregulation and the immunosuppressive TME (28). To overcome these drawbacks, genetic modifications of NK cells has been suggested, and will be discussed in detail in this study. Resting primary NK cells from peripheral blood (PB) or umbilical cord blood (UCB) are difficult to engineer by commonly used approaches, like lentivirus, as low transduction efficiencies were always reported (42). Reduced transduction efficiency rates may be explained by the strong antiviral mechanisms NK cells possess (43). Lentiviral transduction can activate innate immune receptor signaling and trigger NK cell apoptosis. Retrovirus showed higher transduction rates in NK cell lines (especially NK-92) or *ex vivo* activated and expanded NK cells, mostly PB-NK cells (44) (**Table 1**). Standard retroviral transduction methods (γ-retroviruses), though not known to alter NK cell phenotype and function (45), carry the risk of insertional mutagenesis due to their preference of inserting into active gene promoters, and thereby represent a yet unresolved safety concern, especially if high multiplicity of infection (MOI) rates are used. Besides, NK cell viability after retroviral transduction has been seldom reported and data are scarce. On the other hand, lentiviral transduction methods do not require actively dividing cells, conversely to standard retroviral systems (57). All viral systems are limited in insert size (< 10 kb). All in all, transduction efficiencies remain variable, depending on the NK cell source and MOI (which is seldom reported), and sufficient transduction may require either multiple transductions rounds or post-transduction cell enrichment (**Table 1**). Alternatively, high transgene expression levels in both primary and *ex vivo* expanded NK cells (58, 59), can be

TABLE 1 | Genetic modification of NK cells with viral methods.

NK source	Pre-stimulation	Method	Envelope	Construct	Target(s)	MOI	Efficiency	Reference
PB	Co-culture with K562-mbIL15-41BBL and IL-2 10 IU/ml	Retrovirus	RD114	αCD19-BB- CD3ζ CAR	CD19	n.r.	43%–93%	(44)
PB	Co-culture with K562-mbIL15-41BBL	Retrovirus	RD114	αCD19-2B4- CD3ζ CAR	CD19	n.r	24% ± 7.4%	(45)
PB	Co-culture with K562-mbIL15-41BBL and IL-2 10 IU/ml	Retrovirus	RD114	Membrane-bound IL-15	none	n.r.	40%–63%	(46)
PB	Co-culture with K562-mbIL15-41BBL and IL-2 100 IU/ml	Retrovirus	RD114	hTERT	none	n.r	51%–65%	(47)
PB	Co-culture with EBV-LVL	Retrovirus	gp100-specific T cell receptor	NGFR CXCR2	none CXCL1-2-6-8	n.r.	26%–93%	(48)
PB	Co-culture with K562-mbIL15-41BBL and IL-2 10 IU/ml	Retrovirus	Murine stem cell virus	NKG2D-DAP10- CD3ζ	MICA, MICB, ULPB	n.r.	28%	(49)
PB	Co-culture with K562-mbIL15-41BBL and IL-2 400 IU/ml	Retrovirus	Murine stem cell virus	Anti-NG2A PEBL	NKG2A	n.r.	49%–82%	(50)
UCB	IL-15 10 ng/ml	Retrovirus	RD114	DNRII	TGF-β	n.r.	61%–88%	(51)
UCB	Co-culture with K562-mbIL15-41BBL and IL-2 200 IU/ml, IL-15 15 ng/ml	Retrovirus	n.r.	Dominant negative TGF-β receptor (NKA)	TGF-β	n.r.	43% ± 27%	(52)
PB	IL-12 50 ng/ml	Lentivirus	VSV-G	αNKG2A shRNA and eGFP	NKG2A	10	50% ± 20%	(53)
PB	IL-15 10 ng/ml	Lentivirus	VSV-G	αCD19-CD28-CD3ζ CAR	CD19	5–10	0%–34 %	(42)
		α-retrovirus lentivirus	RD114-TR	αCD19-CD28-CD3ζ CAR	CD19	10	48% 41%	
PB	IL-2 500 U/ml IL-15 10 ng/ml	Lentivirus	BaEV	αCD19-4-1BB-CD3ζ CAR	CD19	10	70%	(54)
			VSV-G	eGFP	none	0.1–10	<3%	
CD34+ HSC from UCB	Quiescent	Lentivirus	VSV-G RD114/TR BaEV	eGFP	None	10	0% < 5% 15%–30%	(55)
	SCF/TPO/Flt-3l		VSV-G RD114/TR BaEV	eGFP	None	10	< 5% 30%–50% 80%–90%	
NK-92	IL-2 50 IU/ml	Lentivirus	VSV-G	IL-15 and eGFP	None	n.r.	4% (eGFP)	(56)

Studies based on NK cells lines other than NK-92 have not been reported in this table, as they have not been employed in clinical trials yet. Studies where the transduction efficiency was not reported, or post-transduction enrichment/selection was performed without mentioning the original transduction efficiency were excluded. RD144, serotype of endogenous feline type C virus; n.r., not reported; DNRII, dominant negative TGF-β RII; NGFR, Nerve Growth Factor Receptor; PEBL, protein expression blocker.

achieved with transfection methods like electroporation and lipofection, that are not limited in insert size (**Table 2**). Transgene expression after transfection is however not stable over time, strongly reducing its appeal for clinical applications. Other non-viral genetic integration systems like transposons exist with the advantage to not be limited in insert size. Sleeping Beauty and PiggyBac are the most common and well-characterized transposon systems but have so far mainly been tested in T cells, and NK cell studies are still rare and lack safety data. Alternatively, the CRISPR/Cas9 system has been extensively investigated for targeted engineering, with already some studies in NK cells (65). Although CRISPR/Cas9 can be delivered by both viral and non-viral systems, non-viral delivery of a ribonuclear protein (RNP) complex made up by the Cas9 nuclease and the single guide RNA (sgRNA) is preferred, since it limits off-target effects due to viral DNA integration. However, *ex vivo* non-viral delivery requires optimization, as efficiency is often very limited and viability a concern (61). Of note, CRISPR/Cas9 can be used to efficiently screen effective synthetic constructs electroporated into T cells (66), significantly speeding up the discovery of constructs for reprogramming adoptive NK cell functionality and specificity.

Until recently, these limitations delayed NK cell genetic reprogramming for large scale applications compared to T-cells reprogramming. Novel transduction enhancers (e.g. RetroNectin and vectofusin-1) and studies on alternative viral envelopes, mostly genetically modified versions of baboon endogenous retrovirus (BaEV) and RD114 feline retrovirus glycoprotein (42, 54, 55), contributed to improved NK cell transduction efficiency, rekindling the interest in genetic manipulation of NK cells (**Table 1**). The BaEV envelope consistently displayed efficient NK cell transduction and might replace the standard VSV-G envelope in NK cell applications. NK cells can efficiently be differentiated from CD34+ hematopoietic stem and progenitor cells (HSPCs) (67) derived from UCB, human embryonic stem cells (hESCs), or induced pluripotent stem cells (iPSCs) (68, 69). Both CD34+ HSPCs and iPSCs can be effectively modified with VSV-G, RD114, and BaEV-

TABLE 2 | Genetic modification of NK cells with non-viral methods.

NK type/ source	Pre-stimulation	Method	Construct	Targets	Efficiency	Reference
PB	Non-expanded Co-culture with K562-mbIL15-41BBL and IL-2 100 IU/ml (1000 IU/ml 24h before electroporation)	Electroporation	αCD19-4-1BB-CD3ζ CAR	CD19	18%–59% 28%–92%	(58)
PB	IL-2 600 U/ml	Electroporation	αCD19-4-1BB-CD3ζ CAR CCR7 CD34 CCR7 and CD16-158V	CD19 CCL19-CCL21 none CCL19-CCL21 and Fc receptors	40% 20% > 80% n.r.	(59)
PB	Co-culture with EBV–SMI–LCL and IL-2 500 IU/ml	Electroporation				(60)
NK-92	IL-2 100 IU/ml	Cas9 RNP nucleofection	SFFV promoter	CD16 DNAM-1	1.2 % 3.5%	(61)
iPSCs	Maintained in Matrigel-coated plate for 5 days before nucleofection	Nucleofection	αMesothelin- 2B4-CD3ζ CAR	Mesothelin (ovarian cancer)	n.r.	(62)
NK-92	IL-2 1000 U/ml	Nucleofection	DNTβRII	TGF-β	20% ± 2.5%	(63)
NK-92	IL-2 400 IU/ml	Not specified	NKG2D-DAP10-CD3ζ CAR	MICA, MICB, ULPB	n.r.	(64)

N.r., not reported; DNTβRII, dominant negative TGF-β RII.

pseudotyped lentiviruses and then differentiated into mature NK cells, and offer another attractive source to generate modified NK cells (55).

In summary, since integrative genetic modification systems are preferred in clinical settings, retrovirus-based genetic engineering has been the established platform to modify NK cells so far. The γ-retroviral systems are being gradually overtaken by lentiviruses after the discovery of new NK cell-specific envelopes and are set to be the mainstay in NK cell engineering for several years. Studies implementing non-viral strategies have emerged in the last years as well, although they are as of yet mostly limited to pre-clinical settings (**Figure 1**).

STRATEGIES TO INCREASE TUMOR-SPECIFIC NK CELL CYTOTOXICITY

NK Cells Expressing Chimeric Antigen Receptor

Chimeric antigen receptors (CARs) are antibody-based receptors designed to recognize specific ligands on the surface of target cells. All CAR constructs contain an extracellular antibody single-chain variable fragment (scFvs) fused to a transmembrane region and intracellular immune cell activation domains. The first-generation CAR contained only the intracellular CD3ζ stimulatory domain of the T cell receptor (TCR) as activation domain. The second and third generations include one or two additional co-stimulatory domains, respectively (e.g. 4-1BB and/or CD28). CAR technology was first applied on T cells and although CAR-T cells exhibited strong antitumoral clinical responses, their clinical application is severely curtailed by severe toxicities (GvHD and CRS). This allows only autologous applications for CAR-T cells, which decreases the speed of intervention (negatively affecting therapeutic outcomes), increases production costs and thus decreases treatment accessibility. In addition, T cells for autologous use are necessarily derived from heavily pre-treated patients, which impinges on their functionality. CAR-NK cells, however, can be derived from allogeneic sources, without apparently causing neither GvHD nor CRS in the recipient, potentially related to their short *in vivo* persistence and lack of clonal expansion. Off-the-shelf CAR-NK cells can, therefore, have a huge advantage in terms of manufacturing time, costs and accessibility. Besides, allogeneic NK cells are derived from healthy patients and thus retain their normal activity. This allows CAR-NK cells to still exert their anti-tumoral effect in case the CAR expression is decreased or lost, in contrast to CAR-T cells. Only a few CAR-NK phase I/II trials have been started in the last years. Most registered clinical trials employ the NK-92 NK cell line, and the CAR constructs used were based on targets developed for CAR-T cells. This knowledge gap is largely caused by the huge success of anti-CD19 CAR-T cells and the vast amount of clinical data available from CAR-T cell therapies, and as of yet precludes critical assessment of clinal CAR-NK applications. Currently, two CAR-NK cell trials targeting CD19 for leukemia treatment are ongoing. The first one is at the MD Anderson Cancer Centre of the University of Texas (NCT03056339) and is based on UCB-NK cells transduced with a CD19-targeting scFv, interleukin 15 (to enhance NK cells persistence) and an inducible caspase-9 (iC9) suicide gene as a failsafe mechanism. Preliminary results demonstrated that the approach is safe, despite only partial HLA matching between donor and recipient, and potency is high (seven out of 11 patients achieved complete remission) (70). Of note, CAR.19/IL-15/iC9-NK cells were detected at low levels up to 12 months after the beginning of the treatment, whereas they normally disappear within 2 weeks. Unfortunately, no data about exhaustion is reported, and the influence of pre-conditioning treatment could not be established. The second trial (NCT02892695) is led by PersonGen BioTherapeutics (Suzhou, China), and is based on the NK-92 cell line transduced with a

third-generation CAR (4-1BBL-CD28-CD3ζ co-stimulatory domains). A third phase I study targeting CD19 with haplo-identical PBNK cells for B-ALL treatment has been completed (NCT00995137), but no results are available so far. The NK-92 cell line is employed in most of the other phase I/II studies, targeting CD7 (NCT02742727) for lymphoma and leukemia, CD33 for the treatment of Acute Myeloid Leukemia (NCT02944162), HER2 against glioblastoma (NCT03383978) and Mucin-1 (MUC1) in MUC1-positive relapsed or refractory solid tumor-like colorectal carcinoma (CRC) and gastric carcinoma. These and other trials are listed and summarized in **Table 3**. Efforts directed against multiple myeloma, with CD138 (71) and SLAMF7 (72) as main targets, are still in the preclinical phase. CAR-NK cells targeting solid tumors are now also being explored in preclinical settings. For this, NK cell-specific co-stimulatory domains are being explored to replace T-cell specific domains in an attempt to increment NK cell-specific activation. Two promising approaches target the prostate stem cell antigen (PSCA), highly expressed on primary prostate tumors and metastases (73), and the epidermal growth factor type III (EGFRvIII), expressed on glioblastoma cells, using the DNAX-activation protein (DAP)12 stimulatory domain instead of one of the commonplace T-cell domains like CD3ζ (74, 75). DAP12 expression in NK cells induces NKG2C and NKp44 expression upon stimulation. Another group generated an NKG2D-DAP10-CD3ζ construct (49). The DAP10 domain induces NK cell activation upon phosphorylation through NKG2D-mediated ligand binding (76, 77). Although NKG2D is technically not an scFv, the construct is referred to as a CAR, because of its receptor structure. Recently, the NKG2D transmembrane domain has been combined with an anti-mesothelin scFv and the NK cell-specific signaling domain 2B4 and CD3ζ to target ovarian cancers (62). This construct, cloned in a PiggyBac transposon system, was expressed on iPSC-NK cells, and showed improved specificity and cytotoxicity against the mesothelin-expressing ovarian cancer cell line A1847 and in an ovarian cancer xenograft mouse model. In this mouse model, CAR-iPSC-NK activity was compared with an anti-mesothelin CAR-T expressed in primary T cells and showed similar anti-tumor activity but significantly lower toxicity and prolonged survival. Of note is also a platform that combines NK cell and T cell advantages, by expressing TCR-CAR chimeric constructs on the NK cell surface (78). Recently, the biotech companies Glycostem Therapeutics and Zelluna Immunotherapies announced a partnership to further develop this field.

NK Cells With Downregulated Inhibitory Receptors

Cancer cells can throttle immune responses by stimulating key regulators on the surface of immune effector cells known as inhibitory checkpoint molecules. Identification and targeting of inhibitory checkpoints significantly boost immune responses and is therefore of major interest in cancer immunotherapy. During *ex vivo* expansion of NK cells, some inhibitory receptors, like NKG2A, are still highly expressed (79–81), suggesting a critical role in NK cell maturation. On the other end, inhibitory receptors curb NK cell cytotoxic activity and reduce therapeutic efficacy in clinical settings. Indeed, hyporesponsive NKG2A-expressing NK cells are prominent within the TME, thus stressing the importance of this receptor in reducing NK cells activity (82). NKG2A dimerizes with CD94 to bind HLA-E molecules loaded with tumor peptides. While HLA-E surface expression in tumor cells is very weak, IFN-γ produced by NK cells can cause its overexpression (29, 50, 83). Once the peptide/HLA-E complex is stabilized and binds NKG2A, NK cell activity is dampened. In contrast, RNAi-mediated inhibition of NKG2A expression by shRNA improved NK cell *in vitro* activity against an HLA-E expressing B-lymphoblastoid cell line (53). Notably, NK cell cytotoxicity was also enhanced against the AML-derived, HLA-E-negative cell line K562. This increased HLA-E

TABLE 3 | Ongoing clinical trials with CAR-NK cells.

CAR Target	Condition	Study Phase	NCT
CD19	B-Lymphoid Malignancies/ALL/CLL	I/II	NCT03056339
CD19/CD22	B-Cell Lymphoma	I	NCT03824964
CD19	B-Cell Lymphoma	I	NCT03690310
CD19	ALL/CLL	I/II	NCT02892695
CD19	ALL	I	NCT00995137
CD33	AML	I/II	NCT02944162
CD7	AML/T-cell leukemia	I/II	NCT02742727
CD22	B-Cell Lymphoma	I	NCT03692767
BCMA	MM	I/II	NCT03940833
HER-2	Glioblastoma	II	NCT03383978
Mesothelin	Ovarian cancer	I	NCT03692637
PSMA	Prostate cancer	I	NCT03692663
ROBO1	Pancreatic cancer	I/II	NCT03941457
ROBO1	Solid Tumors	I/II	NCT03931720
ROBO1	Solid Tumors	I/II	NCT03940820
NKG2DL	Solid Tumors	I	NCT03415100
MUC1	Solid Tumors	I	NCT02839954
N.A.	NSCLC	I	NCT03656705
ACE2/NKG2DL	COVID-19	I/II	NCT04324996

ALL, Acute Lymphocytic Leukemia; AML, Acute Myeloid Leukemia; CLL, Chronic Lymphocytic Leukemia; MM, Multiple Myeloma; NSCLC, Non-small Cell Lung Cancer.

independent cytotoxicity was probably caused by increased activating NKp30 receptor levels in the NKG2A-negative cell. Unfortunately, these cells have only been tested on a small set of HLA-E positive/negative cell lines. Another group downregulated NKG2A function in PBNKs and NK-92 cells by linking an anti-NKG2A antibody to an endoplasmic reticulum-retention domain, and achieved increased cytotoxicity against both HLA-E-positive and -negative cells derived from Ewing's sarcoma, osteosarcoma and AML, as well as prolonged survival in immunodeficient mice expressing HLA-E tumors (50). Blocking of inhibitory receptors represents a feasible approach within the field of biotech industry, although the studies focused on NKG2A only, and their number so far is very limited. Additionally, data comparing NKG2A-negative NK cells and CAR-NK cells activity against the same tumor type are lacking, precluding a comparison between both strategies.

NK Cells With Modified ADCC

FcγRIII (CD16)-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) plays an important role in tumor clearance. The CD16 isoform expressed on NK cells (CD16a) has two allelic variants with a phenylalanine (F) or valine (V) at amino acid 158, resulting in low (CD16a-158-F/F) and high affinity (CD16a-158-V/V) Fc receptor isoforms. Several studies showed that patients that express the CD16a-F/F variant have better therapy response than patients that are either heterozygous (CD16a-158-V/F) or homozygous for V upon mAb treatment (84, 85). CD16a expression is downregulated upon NK cell activation, mainly due to matrix metalloproteases shedding (86). NK-92 cells, an attractive and cheap source for clinical applications of NK cells, cannot mediate ADCC as they lack CD16 expression. In an attempt to constitutively enhance ADCC activity of NK-92 cells, these cells have been modified to express a CD16a chimera fused to the CD28 and 4-1BB co-stimulatory domains. Expression of the construct enhanced their cytotoxic activity and restored ADCC activity against CD20-positive tumor cells (87). In another attempt, ADCC potency of NK-92 cells was improved by transduction of the high-affinity CD16a-V158 mutant that is resistant to ADAM17-mediated cleavage and shedding (88, 89). This cell line, (haNK, developed by NantKwest), showed improved killing capacity compared to PBNK cells from healthy donors and is now in clinical trials against breast cancer (NCT03387085), Merkel cell carcinoma (NCT03853317) and squamous cell carcinoma (NCT03387111). CD16a-158V expression in NK-92 and iPSCs-derived NK cells led to enhanced NK cell activation in the presence of rituximab (90), as well as in PBNKs, although overexpression was transient and lasted no more than 3 days (60). CRISPR/Cas9 has been applied in NK-92 cells by nucleofection to restore endogenous CD16 and DNAM-1 expression by introducing a new promoter upstream the endogenous genes (61). Although this approach was successful, the low nucleofection efficiency and its very high toll on NK cell viability currently preclude its application in *ex vivo* expanded NK cells, limiting it to NK cell lines capable of autonomous and indefinite growth. As CD16 overexpression markedly improved NK cell activity and cytokine secretion, combinatorial approaches are currently under investigation. For example, NantKwest is employing CAR technology on haNK cells, and has also initiated a phase I clinical study against PD-L1 expressing non-Small Cell Lung Cancer.

NK Cells With Increased Persistence and Proliferation Potential

In vivo persistence of NK cells strictly depends on exogenous cytokines. Allogeneic NK cell survival is typically restricted to a couple of weeks, necessitating multiple infusions to achieve therapeutic effects (40). Administration of recombinant IL-2 (rIL-2) in clinical settings resulted in severe toxicities at high doses and activation of inhibitory T-regs at low doses (91). To circumvent these drawbacks, NK-92 cells have been transduced with the IL-2 gene, abolishing NK-92 growth dependence on exogenous IL-2 (92). Transduced NK-92 cells have been tested in nude mice with 3-day-established liver metastases, without exerting side effects after 6 months of treatment. With the discovery that IL-15 has higher potency and lower toxicity than IL-2 (93), the focus has shifted to this cytokine. IL-15 exists in both a soluble and membrane-bound isoforms complexed with IL-15 Rα (94, 95). NK cells growth dependence on IL-15 has been circumvented by retrovirally transducing PBNK cells with the mbIL-15 membrane-bound isoform, increasing *in vivo* NK cell persistence without the need for exogenous IL-2 or IL-15 (46). Stimulatory cytokine signaling has also been recently combined with CAR-NK technology. Briefly, CB-derived NK cells were transduced with a retrovirus encoding a CD19 CAR, soluble IL-15 and the iC9 suicide gene (96). These NK cells had prolonged *in vivo* survival and were able to control tumor progression significantly better than non-modified or CD19 CAR-only NK cells. The MD Anderson Cancer Centre phase I clinical study followed, as mentioned above. A CAR construct, targeting the EpCAM carcinoma antigen epithelial cell adhesion molecule, that also encoded IL-15 has been transduced into NK-92 cells (56). The IL-15 transgene induced strong proliferation signals, allowing transduced NK-92 cells to grow in the absence of stimulatory cytokines and, additionally, acting as a selection marker for the transduced cells. The CAR gene selectively improved NK cell cytotoxicity against EpCAM-expressing cell lines.

NK cells have low proliferative potential, possibly because of progressive telomere shortening during division cycles. The process can be partially overcome by overexpressing the telomerase reverse transcriptase protein (TERT) responsible for telomere end restoration, normally very lowly expressed in primary cells. Indeed, NK-cell lifespan has already been extended to several months by hTERT overexpression in PBNKs (47). However, transduced PBNKs were still not capable of autonomous growth and proliferation remained dependent on cell-to-cell contacts with feeder cells. Such "immortalized" NK cells can be useful for clinical applications, where large amounts of NK cells need to be injected, and donor availability and/or variability is limiting. Nevertheless, the use of feeder cells in clinical manufacture is not accepted by all regulatory authorities, as it might pose safety concerns for patients. Consequently, feeder cell-free culture systems need to be developed for worldwide

implementation. Also, hTERT itself can pose safety concerns, as it might drive uncontrolled proliferation, and might require failsafe mechanisms like a suicide gene.

NK Cells With Increased Tumor Homing

Immune cell homing to and infiltration of tumors is a fundamental prerequisite for effective tumor killing. NK cell ability to infiltrate into the tumor stroma is limited, negatively affecting NK cell therapy efficacy (97). The chemokine receptor CCR7 redirects NK cells preferentially to lymph node-associated chemokine CCL19. CCR7 mRNA transfection into PBNKs by mRNA electroporation improved their *in vitro* migration towards CCL19 (60). Another group used primary NK cells transduced with retrovirus encoding CXCR2 to improve trafficking towards renal cell carcinoma (48). The NK-like YT cell line was transduced with lentivirus encoding an anti-EGFRvIII CAR and the chemokine receptor CXCR4 (75). The CXCR4 receptor promoted specific chemotaxis to glioblastoma cells secreting the CXCL12/SDF-1α chemokine, while the αEGFRvIII-CAR improved the killing specificity and cytotoxicity. The approach also increased tumor regression and survival in xenograft mouse models.

These studies, although limited and mainly *in vitro*, demonstrate that NK cell homing to specific tumor sites can improve NK cell-mediated tumor clearance, especially if combined with strategies to enhance NK cell functions after migration to the tumor site.

NK Cells With Increased Resistance to the Tumor Microenvironment

One of the major immunosuppressive factors within the TME is TGF-β, produced by various stromal cells, T-regs, MDSCs, and the tumor cells. TGF-β interferes with NK cell activation by counteracting several important activating receptors. It downregulates NK cell NKG2D and NKp30 surface expression (98, 99) and inhibits CD16-mediated IFN-γ production and ADCC *in vitro* (100). RNAi-mediated knockdown of SMAD3, a TGF-β receptor signal transducer, by transduction of NK-92 cells with a lentivirus encoding SMAD3 shRNA increased IFN-γ, perforin and granzyme B expression, and enhanced cytotoxicity, increasing tolerance to TGF-β signaling both *in vitro* and *in vivo* (101). Expression of a chimera consisting of the TGF-β receptor type II (TGFBR2) extracellular and transmembrane domain fused to the NKG2D intracellular domain on NK-92 cells caused tolerance to the TGF-β signaling and improved chemoattraction to TGF-β-secreting tumor cell lines (102). Additionally, these NK-92 cells inhibited naïve $CD4^+$ T cell to T-reg differentiation by IFN-γ signaling. A dominant-negative mutant form of TGFBR2 (DNRII) has been expressed in CB-derived NK cells to block or decrease TGF-β signaling. These NK cells did not show downregulation of activating receptors NKG2D and DNAM-1 or of granzyme B and perforin upon TGF-β stimulation (51). The same group further created an improved version of the DNRII receptor (renamed "NKA" receptor) by fusing its extracellular domain to DAP12, providing NK cell activating signals upon TGF-β stimulation. CB-derived NK cells expressing NKA had enhanced cell cytotoxic activity and persistence against neuroblastoma both *in vitro* and *in vivo* (52). A similar approach with the DNRTβII receptor in NK-92 cells increased their resistance to TGF-β signaling, potentiating antitumor activity in an *in vivo* lung cancer murine model (63).

Adenosine is emerging as another key negative regulator of NK cells within the TME. Adenosine signals via the $A_{2A}R$ receptor and limits NK cell maturation, negatively affecting their proliferation and tumor control (35). Blockage of $A_{2A}R$ with an inhibitor caused anti-metastatic effects in breast cancer and melanoma mouse models (103). mAb-mediated inhibition of CD73 ectonucleotidase, one of the key enzymes responsible for extracellular adenosine synthesis, in combination with NKG2D CAR NK-92 cells generated using a PiggyBac transposon system, improved control of CD73-positive tumors (64). The combinatorial approach has been tested against cell lines of prostate cancer (PC3), lung carcinoma (A549) and glioblastoma (GBM43 and GBM10) expressing high levels of CD73 and a xenograft mouse model of lung carcinoma with A549 cells. Although the mAb-CAR combined approach did not dramatically improve anti-tumor activity of the NK-92 cells, the study is important as a first demonstration that stable CAR integration with a non-viral system in NK cells is feasible, and can be extended to other constructs.

FUTURE PERSPECTIVES AND CONCLUDING REMARKS

NK cell trials have been ongoing for several years by now, demonstrating the safety and efficacy of NK cell-based immunotherapies, especially against hematological malignancies. So far, efficacy against solid tumors is limited, and requires additional technology. NK cells were quite refractory to standard genetic manipulation techniques, resulting in major delays of the first clinical trials with genetically engineered NK cell. Substantial improvements in the last years has led to the first trials. Nonetheless, challenges remain. Viral-based genetic manipulation of NK cells is currently the gold standard to stably express exogenous genes, but transduction efficiency and transgene expression levels are still variable, requiring multiple transductions rounds or post-transduction enrichment. Besides, insertional mutagenesis needs continued safety monitoring. Non-viral delivery methods are still far from routine implementation, as the efficiency and viability can be very low, and the transgene expression can be transient. Low efficiencies are not a great disadvantage with immortalized cells like NK-92, as these can be enriched and then grown indefinitely. But *ex vivo* expanded NK cells have a limited proliferation potential and enter senescence relatively soon (47). For these cells, highly effective engineering strategies are much more important.

As mentioned above, NK cell efficacy against solid tumors is limited compared to hematological malignancies. Introduction of CAR constructs into NK cells, restoring ADCC functions and/or downregulation of inhibitory receptors can dramatically

potentiate their effector functions, helping the patient's immune system in eradicating the disease. The need for NK-specific CAR constructs is now widely recognized, as T-cell-based CARs have reduced activation potentials compared to the former ones, and high-throughput screening techniques will be essential for their identification (66). The limited proliferation potential of NK cells usually does not allow them to persist longer than 2–3 weeks after injection *in vivo*. Furthermore, homing to tumor sites is often hampered by the TME. Consequently, a highly cytotoxic potential could be relatively limited if NK cells do not persist long enough to eradicate malignant cells or home in on tumor sites. Early efforts in providing NK cells with stimulatory cytokines prolonging half-life like IL-15 are encouraging and worth being further developed, especially in combination with CARs or other activating receptors. Similarly, increasing expression of chemokine receptors on the NK cell surface improves NK cells targeted trafficking and tumor eradication, paving the way for combinatorial strategies. Many solid tumors are difficult to target also because they are encapsulated and protected by a thick layer of extracellular matrix (ECM), a mesh made up mainly by insoluble proteins like type IV collagen and heparan sulphate proteoglycans (HSPGs), that reduce the infiltration abilities of NK cells (104). Heparanase is upregulated in activated NK cells, improving migration within tumor stroma and playing an important role in reducing tumor growth and metastases (104). To conclude, understanding NK cell biology is another key factor that will help to improve genetic engineering strategies and overcome tumor resistance mechanisms, and allow to fully unleash anti-cancer NK cell potential.

AUTHOR CONTRIBUTIONS

SM wrote the paper. LK, DG, and JS reviewed the paper. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: SM, DG, JP and LK are employed by Glycostem Therapeutics.

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Development of CAR-T Cell Persistence in Adoptive Immunotherapy of Solid Tumors

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Chimeric antigen receptor (CAR) T (CAR-T) cell transfer has made great success in hematological malignancies, but only shown a limited effect on solid tumors. One of the major hurdles is the poor persistence of infused cells derived from *ex vivo* activation/expansion and repeated antigen encounter after re-infusion. Bcl-xL has been demonstrated to play an important role on normal T cell survival and function as well as genetically engineered cells. In the current study, we developed a retroviral CAR construct containing a second-generation carcinoembryonic antigen (CEA)-targeting CAR with the Bcl-xL gene and tested the anti-CEA CAR-T cell immunotherapy for colorectal cancer. *In vitro*, the anti-CEA CAR-T cells destroyed CEA-expressing tumor cells and sustained survival. *In vivo*, adoptive cell transfer of anti-CEA CAR-T cells significantly enhanced the ability of the CAR-T cells to accumulate in tumor tissues, suppress tumor growth and increase the overall survival rate of tumor-bearing mice in a murine model of colorectal cancer. These results demonstrate a novel CAR-T platform that has the ability to increase the persistence of CAR-T cells in solid tumors through exogenous expression of persistent genes. The data provide a potentially novel approach to augment CAR-T immunotherapy for solid tumors.

Keywords: chimeric antigen receptor, T cells, persistence, solid tumor, immunotherapy

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INTRODUCTION

Immunotherapy especially immune checkpoint inhibitors (ICI) and CAR-T therapy is emerging as one of the most promising approaches for relapse and refractory malignancies in the past decades (1). Only a fraction of patients with solid tumors benefit from ICI with high relapse rates and adverse event rate (2). For CAR-T therapy, although a great progress has been made in

Abbreviations: ACT, adoptive cell transfer; AICD, activation-induced cell death; Bcl-xL, B-cell lymphoma-extra large; BSA, bovine serum albumin; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; DMEM, Dulbecco modified Eagle medium; ICI, immune checkpoint inhibitor; IRES, internal ribosome entry site; PBS, phosphate-buffered saline; PD-1, programmed cell death-1; RT, room temperature; TNFR, tumor necrosis factor receptor.

hematological malignancies (3), but numerous challenges remain unsolved in solid tumors (4). For instance, selecting favorable target antigens to avoid on-target off-tumor adverse effect; enhancing the ability of infused cells to accumulate in the high-density tumor lesion; and improvement of proliferation and/or persistence of the infused cells within the tumor mass (5). Amongst these obstacles, the lack of proliferation and/or persistence may be responsible to a great extent for the poor effect of CAR-T therapy in solid tumors. Moreover, even for hematological malignancies with complete remission rate as high as to 90%, the patients remain at the risk of relapse because of the poor persistence of CAR-T cells *in vivo*. Effective strategies to circumventing these barriers should significantly improve current tumor immunotherapy, thus are urgently needed (6).

Various factors could influence the persistence of CAR-T cells, including patient preconditioning procedure (7), *ex vivo* culture conditions (8), and the molecular design of CARs (9, 10). In addition to improving the patient precondition approaches and *ex vivo* cell culture protocols, optimization of the CAR molecule structural design is the most common strategies to increase the CAR-T function and persistence. It is well established that the costimulation receptor is critical for T cell activation and proliferation, and that modification of CAR molecules with variable costimulation domains is one of the preferred methods to enhance the persistence of CAR-T cells (11, 12). In fact, almost all CARs possess one or more costimulation domains designated the second or third generation of CARs respectively. More recently, the fourth generation of CARs containing inducible transgenes (termed TRUCKs) and capable of constantly secreting cytokines (e.g., IL-12, IL-15, or IL-18) (13–15) has been subsequently tested. The extracellular non-signal structure such as spacers and transmembrane domains could also be modified to improve the persistence of CAR-T cells *in vivo* (16, 17). Even though some progress has been made, antitumor T cell persistence remains the major hurdle of CAR-T therapy for solid tumors in preclinical and clinical research.

The comprehensive mechanism of CAR-T cell persistence remains to be identified. Activation induced cell death (AICD) may play an important role since the therapeutic T cells encounter stimulation all the way from activation, proliferation/expansion, genetically edition to infusion. The immune suppressive microenvironment may be another important mechanism to inhibit the persistence of CAR-T cells in tumor lesion. A number of suppressive regulatory cells and cytokines could either keep CAR-T cells from tumor tissues or induce CAR-T cell apoptosis in site (5). In addition, the tumor microenviroment lacking various cytokines necessary for T cell persistence can accelerate the apoptotic process. However, almost all the methodologies mentioned above could resolve only part of these problems. Nevertheless, enhancing the ability of T cell persistence directly among the entire process of CAR-T production and treatment may be a more favorable approach.

B-cell lymphoma-extra large (Bcl-xL) plays an important role in T cell survival and function (18, 19), especially after activation. We and other group have previously reported that Bcl-xL overexpression could substantially improve the persistence and antitumor ability of antigen-specific T cells (20, 21), another group demonstrated that Bcl-xL expression resulted from costimulation signal contributes to CAR-T persistence and tumor eradication (22). We hypothesized that increasing Bcl-xL expression is a feasible strategy to enhance the ability of CAR-T cell resistant to AICD and the suppressive tumor microenvironment, therefore improving their persistence and antitumor reactivity. In the present study, we included an exogenous Bcl-xL gene into a second-generation anti-CEA CAR retroviral construct. After gene transduction of T cells, we observed a high expression of exogenous Bcl-xL in the transduced cells, and the CAR-T cells containing exogenous Bcl-xL gene promoted its persistence both *in vitro* and *in vivo*. Importantly, adoptive cell transfer of the anti-CEA CAR-T cells in a mouse model of colorectal cancer significantly suppressed tumor growth and sustained mouse survival, suggesting the anti-CEA CAR-T cells had potent antitumor ability. Our strategy of the CAR design used in this experiment may be explored as a new platform to overcome the main obstacle for CAR-T therapy in solid tumors as well as hematological malignancies.

MATERIALS AND METHODS

Cell Lines and Mouse

MC-38 cell line is CEA negative murine colon cancer cell derived from C57BL/6 mice, and MC-38-CEA-2 (MC-32) cells are CEA positive MC-38 cells retrvirally transduced with human CEA gene (23). Both cell lines were obtained from Kerafast Inc. (Boston, MA). C57BL/6 (B6) and Thy1.1 congenic mice (B6.PL-*Thy1a*/CyJ) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

Antibodies

β-actin (#4967), Bcl-xL (#2762), Myc (#14819 and #12855) and peroxidase-conjugated anti-rabbit (#7054) or anti-mouse Ig (#7056) for Western blot, were obtained from Cell Signaling Technology (Beverly, MA). Anti-mouse CD3 (145-2C11) and CD28 (37.51) antibodies, mouse IL-2 and IFN-γ ELISA Kits, all FITC-, PE-, PE/Cy5-, PE/Cy7, and APC-conjugated antibodies, PE Annexin V Apoptosis Detection Kit (640934) and Cyto-Fast™ Fix/Perm Buffer Set (#426803) were purchased from Biolegend (San Diego, CA)

Cell Cultures

Tumor cells were thawed in 37°C and washed with fresh RPMI-1640 media twice before incubation, and cultured with 5% CO_2 at 37°C in complete RPMI-1640 media supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin and 1% L-glutamine. Retrovirus-packaging Plat-E cells expressing retroviral particles pol, gag and env to package the retroviral vector DNA coding sequences to produce retroviruses without the probability of generating replication competent viruses (24) were used for retroviral transduction. The expansion of Plat-E cells began on day 4 before the retrovirus packaging operation and were passaged at the density of 3×10^6 cells in 10 ml of

DMEM per 10cm plate at least 16 h before transfection. Naive $CD8^+$ T cells were purified from the spleen and lymph nodes of C57BL/6 mice by using the murine naive $CD8a^+$ T cell isolation kit (#130-096-543, Miltenyi Biotech, CA) and cultured in complete media additionally supplemented with 0.06% β-mercaptoethanol based on the complete RPMI-1640 media.

Retroviral Transduction

Anti-human CEA (hMN14) (CD28-CD3ζ) CAR and cDNA for Bcl-xL was linked with 2A sequence and subcloned into the retroviral vector MFG (20, 25), which was derived from murine leukemia virus (MLV)-based retroviral vector and had been demonstrated ready to be modified to improve safety and gene expression (26). Three constructs used in the present study are Vector 1: MFG-control vector (empty plasmid without interest gene); Vector 2: MFG-anti-CEA CAR (MFG-based vector subcloned an anti-CEA CAR sequence); and Vector 3: MFG-anti-CEA CAR+Bcl-xL (MFG-based vector with the same CAR with Bcl-xL gene). The CAR structure in the MFG constructs consists of a synthetic single chain variable fragment (scFv, derived from mouse monoclonal antibody, clone MN-14) specific to hCEA epitope expressed on MC38-CEA-2 cells in which a Myc epitope-tagged framework was engineered to enable the tracking of scFV expression, a spacer and a transmembrane domain (both derived from CD28), a single costimulation domain (derived from human CD28), and a T cell receptor (TCR) signaling element (derived from human CD3ζ chain). CAR in Vector 3 contains a Bcl-xL gene with CD3 ζ through 2A element. The parental MFG-based retroviral plasmids were expanded in MAX Efficiency™ DH5α Competent Cells (ThermoFisher, #18258012) and purified with Invitrogen™ PureLink™ Expi Endotoxin-Free Maxi Plasmid Purification Kit (ThermoFisher, #A31231) according to the manufacturer's instruction. All constructs were verified by sequencing.

Retroviral transduction was performed as described before (27). Briefly, Plat-E packaging cells using the ecotropic envelope were transfected with the CAR constructs and supernatants from the transfected packaging cells were collected 48 h after transfection, and used for transduction of T cells. Murine mature T cells from lymph nodes and spleen were used in all experiments. 5×10^5 $CD8^+$ T cells were stimulated with anti-CD3 plus anti-CD28 antibodies. After 2 days, the supernatant was replaced with 1 ml viral supernatant containing 5 μg/ml Polybrene (Sigma), and the cells were spun for 1 h at 32°C and incubated at 32°C for 8 h. This was repeated the following day. Viral supernatant was removed and replaced with fresh medium, and T cells were re-cultured. Expression of GFP was determined by flow cytometry gating on $CD8^+$ T cells. GFP-expressing T cells were purified from cell sorting using a FACS Vantage SE I high-speed cell sorter (BD Immunocytometry Systems, San Jose, CA).

Cytokine Secretion, Cell Recovery, and Proliferation

5×10^5 $CD8^+$ T cells were stimulated with anti-CD3 plus anti-CD28 antibodies in 48-well plates. Cytokines were measured by enzyme-linked immunosorbent assay (ELISA; Biolegend); T cell survival *in vitro* was determined by trypan blue (Sigma) exclusion assay; and proliferation was measured in triplicate cultures by incorporation of ^{3}H-thymidine (1 μCi/well; ICN Pharmaceuticals, Laval, QC, Canada) during the last 12 h of culture (20).

Immunoblotting

Live $CD8^+$ cells were recovered by Ficoll treatment and positive selection with anti-CD8 microbeads (Miltenyi Biotec Inc). Cells lysates were extracted and used for Western blotting as described (28).

In Vitro Cytotoxicity Assay

Target MC-38 ($hCEA^-$) or MC-32 ($hCEA^+$) tumor cells were co-cultured with effector CEA CAR-T cells at the target:effector (E:T) ratio in 1:2, 1:5, or 1:10 in triplicate. For test of background, wells contained target cells only. The plates were incubated at 37°C for 12 h, and the CAR-T cell-mediated cytotoxicity was measured using the Cayman's 7-AAD/CFSE Cell-Mediated Cytotoxicity Assay Kit according to the manufacture's instruction by flow cytometry. In brief, both prepared MC-38 and MC-32 cells were stained with CFSE dye prior to co-culture with CAR-T cells according to the protocols previously described (29). The percentage of specific lysis was calculated as follows: cytotoxicity (%): [100% × dead targets/(dead targets + live targets)] (experiment) − [100% × dead targets/(dead targets + live targets)] (background). In addition, an Amnis ISXII (MilliporeSigma) equipped with 405, 488, and 642 nm lasers with a single camera (six channels) was used to acquire experimental samples using the INSPIRE software.

Adoptive Transfer and Tumor Challenge

T cells were cultured with anti-CD3 plus anti-CD28 antibodies and transduced on day 2 and 3 with retroviral vectors (27). Cells were re-cultured for 2 more days. GFP^+ $CD8^+$ T cells were sorted and 3×10^6 sorted cells were injected *i.v.* into C57BL/6 mice, which were challenged *s.c.* with 5×10^6 MC-38 or MC-32 tumor cells in PBS, or PBS without tumor cells as a control one week prior to the adoptive T cell transfer. The volume of the tumor (mm^3) was measured using a caliper by a blinded investigator and calculated as follows: $V = \text{length} \times \text{width}^2 \times 0.52$. Mice were sacrificed when the tumor size reached 20mm in any direction.

Histology and Immunofluorescence

Tumor tissues were collected from mice at the indicated time points. Samples were collected in embedding cassette and blocked with 10% neutral buffered formalin. Samples were infiltrated with the wax and embedded the infiltrated tissues into wax blocks. Both vertical and horizontal sectioning were prepared for immunostaining.

H&E staining: Routine Hematoxylin & Eosin (H&E) staining was performed at an interval of every five serial sections. Immunological staining: Tissue sections were fixed with acetone (Sigma), and incubated with 3% bovine serum

albumin (BSA; Sigma) to block non-specific protein binding (30). Sections were stained with fluorescein isothiocyanate (FITC) anti-Myc (1:1000; Thermo Fisher #13-2511).

Statistical Analysis

The data were presented as the mean ± standard error of mean (SEM). Differences in means were analyzed by Student's t-test. One-way ANOVA with Tukey *post hoc* tests were used for differences between three or more groups in a single condition or time point. Survival curves were constructed by the Kaplan–Meier method and analyzed with the log-rank test. All tests were 2-sided, with $p < 0.05$ considered to indicate statistical significance. All statistics were calculated using GraphPad Prism 8 (San Diego, CA, USA).

RESULTS

Expression of Anti-Carcinoembryonic Antigen Chimeric Antigen Receptor and Bcl-xL in Primary CD8+ T Cells

MFG-based retroviral vector had been demonstrated a reliable gene-edition system with high transduction efficiency and enable engineered cells highly expressing the interest protein (31). The 2A peptide regions from Picornavirus has been widely used to create an individual fragment encoding multiple proteins (32). To generate reliable and versatile constructs to transduce primary $CD8^+$ T cells that permit the expression of multiple genes, we used a T2A sequence to generate multicistronic retroviral vectors with efficient translation of two cistrons (e.g., the CEA scFv-TM28-CD28-CD3ζ CAR with Myc tag and Bcl-xL) (**Figure 1A**). The CEA scFv was derived from mouse monoclonal antibody clone MN14.

Thus, two fragments of the CEA CAR and Bcl-xL were linked with the 2A sequence and were subcloned into the MFG vector. The new construct MFG-CEA CAR-2A-Bcl-xL was confirmed by DNA sequencing (**Supplementary Data 1**) and GFP expression in the packaging Plat-E and primary T cells (**Figure 1B**). Furthermore, naive $CD8^+$ T cells were infected with the retrovirus-mediated transduction, which led to the surface expression of CEA CAR (Myc^+) (**Figure 1C**) and exogenous expression of Bcl-xL (32 kDa; **Figure 1D**).

Bcl-xL Promoted the Survival of Carcinoembryonic Antigen Chimeric Antigen Receptor-T Cells *In Vitro*

To determine whether enforced expression of Bcl-xL could contribute to the survival of CEA CAR-T cells, we compared the proliferation, recovery and apoptosis rate of transduced $CD8^+$ T cells with the MFG retroviral constructs containing Bcl-xL or not. The primary $CD8^+$ T cells were activated with anti-CD3 plus CD28 antibodies, and after retroviral transduction on days 2-3, T cells were passively re-cultured in the absence of further stimulation and their proliferation was assessed by thymidine incorporation after 1 (day 4) and 3 days (day 6). Bcl-xL-forced expression in $CD8^+$ T cells resulted in enhanced passive proliferation at late times at day 6, as measured by thymidine incorporation, compared to only CEA CAR

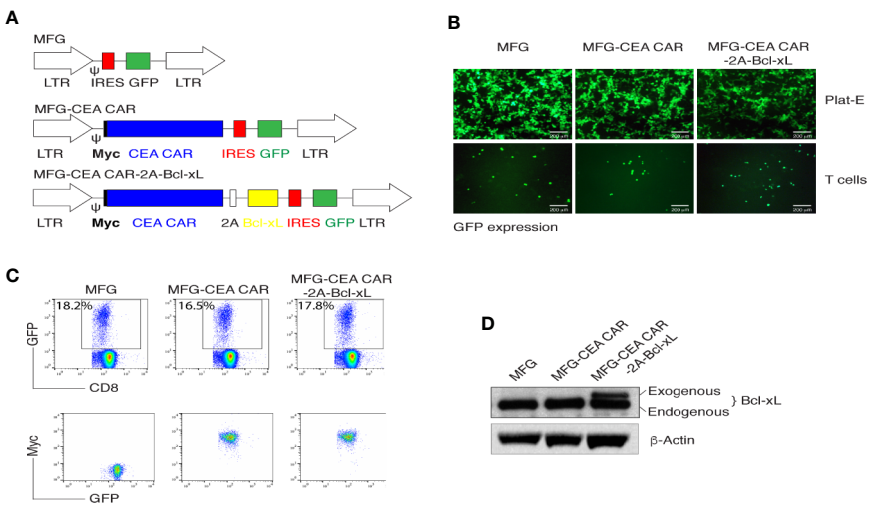

FIGURE 1 | *In vitro* generation of carcinoembryonic antigen (CEA) chimeric antigen receptor T (CAR-T) cells. **(A)** Schematic representation of the retroviral constructs MFG, MFG-CEA CAR, and MFG-CEA CAR-2A-Bcl-xL. CEA CAR is Myc tagged; Ψ, packaging signal; 2A, picornavirus self-cleaving 2A sequence; LTR, Long terminal repeats. **(B)** The packaging Plat-E cells (upper panel) and MFG-transduced $CD8^+$ T cells (lower panel) were visualized by fluorescence microscopy (scale bars: 200 μm). **(C)** $CD8^+$ T cells were transduced with the retroviral constructs and GFP^+CD8^+ T cells (upper panel) or GFP^+Myc^+ T cells gated on GFP^+CD8^+ populations were analyzed by flow cytometry. **(D)** GFP^+CD8^+ T cells were sorted, and the cell lysates were determined for the expression of Bcl-xL and β-actin by western blotting. All data are representative of three independent experiments.

expression (**Figure 2A**). In line with this, enumerating the recovery of live T cells through monitoring GFP expression showed that expression of Bcl-xL allowed $CD8^+$ T cells to expand from day 4 through day 6 over that engendered by transducing only CEA CAR in isolation. Culture over 8 days showed that, Bcl-xL significantly enhanced the ability of $CD8^+$ T cells to survive (**Figure 2B**). In addition, the percentage of apoptosis in $CD8^+GFP^+$ T cells was reduced in anti-CEA CAR+ Bcl-xL transduction group compared to transfection of only anti-CEA CAR (**Figure 2C**).

To investigate whether overexpression of Bcl-xL promote greater recall responses of CEA CAR-T cells, effector $CD8^+$ T cells from C57BL/6 mice expressing CEA CAR or CEA CAR with Bcl-xL from primary naive cultures were sorted based on GFP expression and re-stimulated. $CD8^+$ T cells transduced with CEA CAR with Bcl-xL displayed enhanced recall proliferation (**Figure 3A**), and greater numbers were recovered over time (**Figure 3B**) compared to the introduction of CEA CAR alone in isolation. In addition, effector function was not affected in which production of IL-2 or IFN-γ was unaltered regardless of the forced expression of Bcl-xL (**Figure 3C**). However, the percentage of apoptosis in $CD8^+GFP^+$ T cells transduced with CEA CAR with Bcl-xL was decreased compared to the introduction of CEA CAR alone in isolation. The percentage of early apoptotic cells (Annexin V^+7-AAD^-) reduced less (4.18% with CEA CAR with Bcl-xL *vs.* 5.78% in MFG vector control and 5.16% with CEA CAR alone), but the percentage of late apoptotic cells (Annexin V^+ 7-AAD^+) markedly lessened (9.72% with CEA CAR with Bcl-xL *vs.* 30.2% in vector control and 31.5% with CEA CAR alone) (**Figure 3D**).

Collectively, our results demonstrated that expression of Bcl-xL substantially promotes the survival of CEA CAR-T cells when measuring short-term T cell expansion *in vitro*.

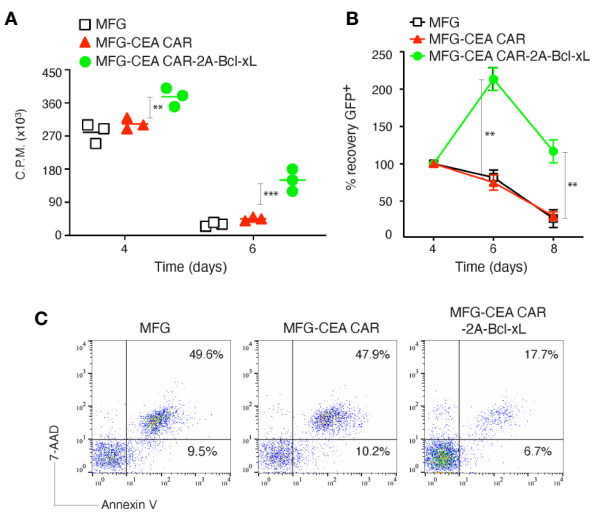

FIGURE 2 | Carcinoembryonic antigen (CEA) chimeric antigen receptor T (CAR-T) cells overexpressing Bcl-xL had enhanced proliferation and survival *in vitro*. Naive $CD8^+$ T cells from C57BL/6 mice were stimulated with anti-CD3 plus anti-CD28 antibodies, and transduced on days 2/3 with retroviral vectors expressing GFP, GFP with CEA CAR, or GFP with CEA CAR and Bcl-xL, and then re-cultured without any further stimulation. **(A)** Primary passive proliferation on day 4 and day 6 were measured in unseparated cultures by pulsing with tritiated thymidine for 20 h. Data are represented with a mean of three independent experiments (**$P < 0.01$, ***$P < 0.001$, Student's unpaired *t*-test). **(B)** GFP^+CD8^+ T cell recovery normalized to take into account differences in initial transduction efficiency between cultures. Numbers of GFP^+ cells present on day 4 were assigned a value of 100%, and numbers surviving on day 6 and day 8 were used to calculate the percentage recovery relative to day 4. The data represent the mean ± S.E.M. percentage change from three separate experiments (*$P < 0.05$, **$P < 0.01$, Student's unpaired *t*-test). **(C)** Apoptosis of GFP^+ $CD8^+$ T cells on day 6 based on staining of Annexin V and 7-AAD and analyzed by flow cytometry. Data are representative of three independent experiments.

The Cytotoxicity of Carcinoembryonic Antigen Chimeric Antigen Receptor-T Cells Was Unaffected by Overexpression of Bcl-xL *In Vitro*

To determine this functional activity of CEA CAR-T cells, we co-cultured the CAR-T cells with murine tumor cells: MC-32 (expressing human CEA) and MC-38 (lacking human CEA) (**Figure 4A**). No apparent cytotoxicity was observed in CEA negative MC-38 cells at the target:effect or ratio of 1:5 for each transduced cell. The control T cells showed no cytotoxicity to MC-32 cells at any target:effector ratio and the CEA CAR-T cells showed significantly increased cytotoxicity to MC-32 cells in the co-culture system at various target:effector ratio of 1:2, 1:5, 1:10 (**Figure 4B**), indicating a CEA-specific antitumor reaction. Furthermore, the CAR-T cells over-expressing Bcl-xL or not displayed a dosage-dependent cytotoxicity to MC-32 cells, but no significant difference was observed between these two groups, indicating that the exogenous Bcl-xL protein did not affect on the overall antitumor function of the CAR-T cells *in vitro*.

Amnis image stream analysis showed colocalization of CAR-T cells and tumor cells, which suggest that the scFv of the CAR expressed on CEA CAR-T could bind with hCEA on tumor cell stably and activated the CAR-T sufficiently, because both CAR-T with or without Bcl-xL were far bigger than T cell transduced with empty MFG vector. The data also indicate that the cytotoxicity of CAR-T is cell-cell direct-contact dependent (**Figure 4C**).

Bcl-xL Promoted the Persistence of Carcinoembryonic Antigen Chimeric Antigen Receptor-T Cells *In Vivo*

To determine whether Bcl-xL was capable of increasing the expansion or persistence of CEA CAR-T cells in response to antigen presented *in vivo*, GFP-sorted CEA CAR-T cells ($Thy1.2^+$), obtained from the *in vitro* cultures in **Figure 3** and adoptively transferred into syngeneic recipients ($Thy1.1^+$). These mice were subsequently challenged with anti-CD3 CD3ϵ F $(ab')_2$ fragment. Activated T cells transduced with either the vector control or the CEA CAR expanded less over 3 days in the lymph nodes and spleen than those CEA CAR-T cells expressing Bcl-xL (**Figures 5A, B**), supporting the *in vitro* results (**Figure 3**).

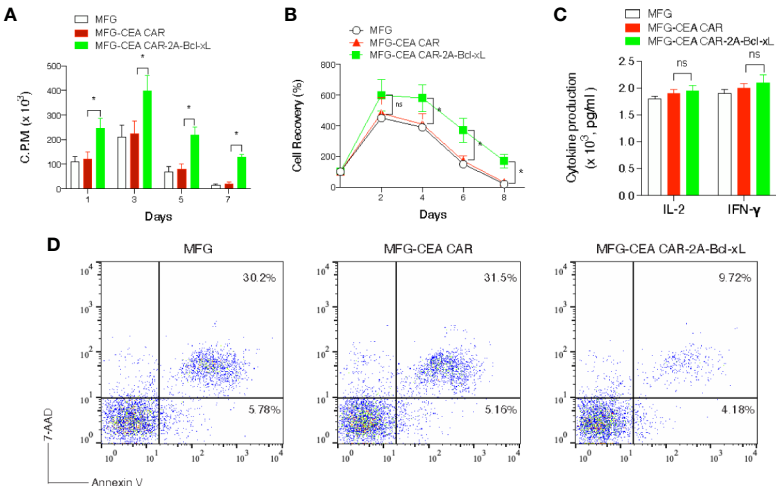

FIGURE 3 | Carcinoembryonic antigen (CEA) chimeric antigen receptor T (CAR-T) cells overexpressing Bcl-xL had a greater recall response *in vitro*. GFP$^+$CD8$^+$ T cells were sorted (**Figure 2**) and re-stimulated with anti-CD3 plus anti-CD28 antibodies. **(A)** Recall proliferation on various days was measured in by pulsing with tritiated thymidine for 20 h. Data are representative with mean ± S.E.M. percentage change from three independent experiments (*P < 0.05, Student's unpaired t-test). **(B)** Recall survival on various days. The data represent the mean ± S.E.M. percentage change from three independent experiments (*P < 0.05; ns, not significant, Student's unpaired t-test). **(C)** Recall IL-2 and IFN-γ production by ELISA at 40 h. ns, not significant. Data are means ± S.E.M. from three experiments. **(D)** Apoptosis of GFP$^+$ CD8$^+$ T cells on day 6 based on staining of Annexin V and 7-AAD and analyzed by flow cytometry. Data are representative of three independent experiments.

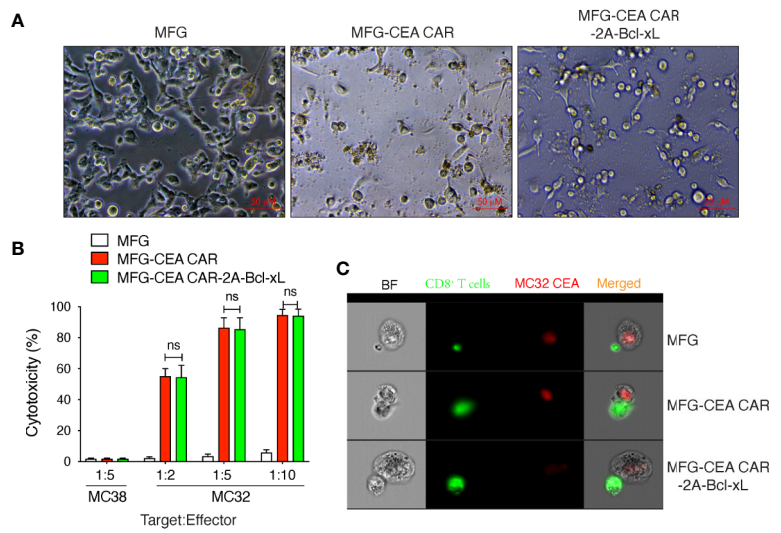

FIGURE 4 | Carcinoembryonic antigen (CEA) chimeric antigen receptor T (CAR-T) cells overexpressing Bcl-xL had modest *in vitro* cytotoxicity. GFP$^+$CD8$^+$ T cells were sorted (**Figure 2**), and co-cultured with MC-38 or MC-32 tumor cells *in vitro*. The *in vitro* cytotoxicity was measured by the Cayman's 7-AAD/CFSE Cell-Mediated Cytotoxicity Assay Kit. **(A)** Representatives of the *in vitro* co-culture of T cells with MC-32 tumor cells at 12 h (scale bars: 50 μm). **(B)** *In vitro* cytotoxicity assay. Data are representative with mean ± S.E.M. from three independent experiments (ns, not significant, P>0.05, Student's unpaired t-test). **(C)** Representatives of the *in vitro* colocalization of CAR-T cells and tumor cells by Amnis image stream analysis. Data are representative of three independent experiments.

The effect of Bcl-xL is long lasting, with enhanced numbers of CEA CAR-T cells not only present a week after the anti-CD3 challenge through the peak of response, but also after two weeks while the secondary *in vivo* response was completed and contraction of T cell populations had followed in all recipients (**Figures 5C, D**). Overall, these data strongly support the conclusion that an action of Bcl-xL sustains the persistence of CEA CAR-T cells.

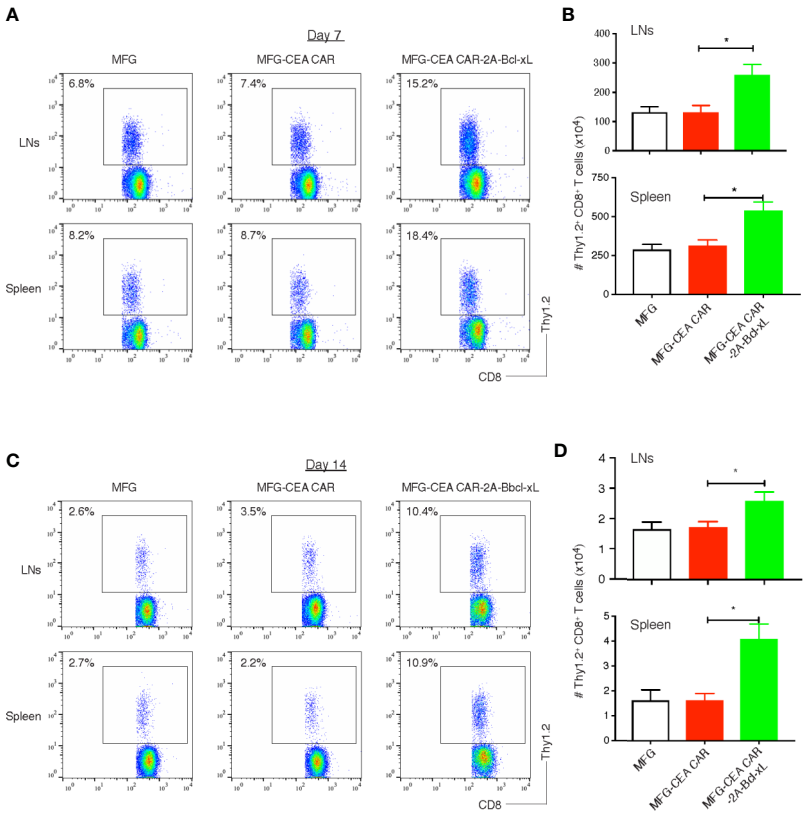

FIGURE 5 | Bcl-xL promoted the persistence of carcinoembryonic antigen (CEA) chimeric antigen receptor T (CAR-T) cells *in vivo*. 3×10^6 GFP^+ $CD8^+$ T cells ($Thy1.2^+$, **Figure 2**) were adoptively transferred into Thy1.1 congenic mice *via* the tail vein, and on day 7 and day 14, $CD8^+$ $Thy1.2^+$ T cells in the lymph nodes (LNs) and spleen were analyzed by flow cytometry. **(A)** Representatives of the $CD8^+$ $Thy1.2^+$ T cells in the LNs and spleen on day 7. Data are representative of three independent experiments. **(B)** Cell numbers of $CD8^+$ $Thy1.2^+$ T cells in the lymph nodes (LNs) and spleen on day 7. Data are representative with mean ± S.E.M. percentage change from three independent experiments (*P<0.05, Student's unpaired t-test). **(C)** Representatives of the $CD8^+$ $Thy1.2^+$ T cells in the LNs and spleen on day 14. Data are representative of three independent experiments. **(D)** Cell numbers of $CD8^+$ $Thy1.2^+$ T cells in the LNs and spleen on day 14. Data are representative with mean ± S.E.M. percentage change from three independent experiments (*P < 0.05, Student's unpaired t-test).

Adoptive Cell Transfer of Carcinoembryonic Antigen Chimeric Antigen Receptor-T Cells Overexpressing Bcl-xL Significantly Increased T Cell Accumulation Within Tumor Tissues and Suppressed Tumor Growth

To demonstrate that the CEA CAR-T cells overexpressing Bcl-xL have the ability in inducing CEA-specific T cell persistence in a physiologically and clinically relevant setting, we used a murine model of colorectal cancer (**Supplementary Data 2**). We *s.c.* injected C57BL/6 mice in the flank region with MC-32 or control MC-38 tumor cells. After a week, we performed *i.v.* adoptive cell transfer of genetically engineered T cells. Three weeks after the adoptive cell transfer, we observed that more CEA-reactive $CD8^+$ T cells accumulated in MC-32 tumor tissues in mice receiving CEA CAR-T cells overexpressing Bcl-xL than those receiving the CEA CAR-T cells without overexpressing Bcl-xL or the non-specific T cell control (**Figure 6**). Mice receiving CEA CAR-T cells overexpressing Bcl-xL had the smaller MC-32 tumor sizes as compared to those receiving the CEA CAR-T cells without overexpressing Bcl-xL or the non-specific T cell control (**Figure 7A**), correlating with their enhanced survival (n=6; **Figure 7B**). Taken together, these findings indicate that the adoptive transfer of CEA CAR-T cells overexpressing Bcl-xL can generate CEA-specific CTL persistence and result in enhanced tumor suppression.

DISCUSSION

CAR-T cell persistence has been associated with better clinical outcome in both patients with blood malignancies and solid tumors (33, 34). The programmed cell death through various mechanisms may be responsible for CAR-T cell apoptosis and short-term persistence (35). Numerous strategies have been tested by different groups and obtained promising results (11–16), however, an optimized condition remains to be identified. We and other group have previously reported that both exogenous

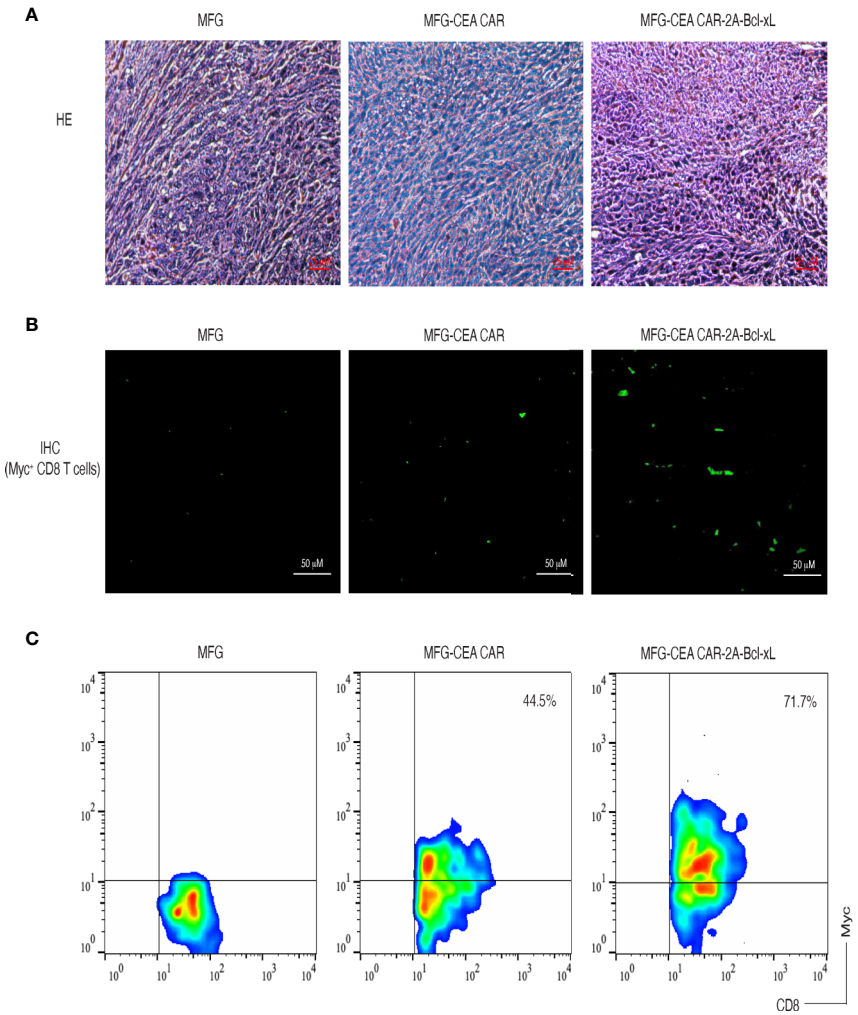

FIGURE 6 | Carcinoembryonic antigen (CEA) chimeric antigen receptor T (CAR-T) cells overexpressing Bcl-xL prominently accumulated in tumor tissues. C57BL/6 mice were adoptively transferred with CEA CAR-T cells expressing with or without Bcl-xL or control cells one week after the mice were *s.c.* injected in the flank region with MC-38 or MC-32 tumor cells. On day 21 to 22 after tumor challenge, tumor tissues were examined for tumor-reactive T cell infiltration. **(A)** H&E staining (scale bars: 25 μm). **(B)** Immunohistological staining (scale bars: 50 μm). Myc^+ CEA CAR-T cells (green) infiltrated in tumor tissues (the dark background). **(C)** Single-cell suspensions from tumor tissues were analyzed for expression of $CD8^+$ and Myc^+ T cells by flow cytometry, after gating on the $CD8^+$ population. Data are representative of three independent experiments.

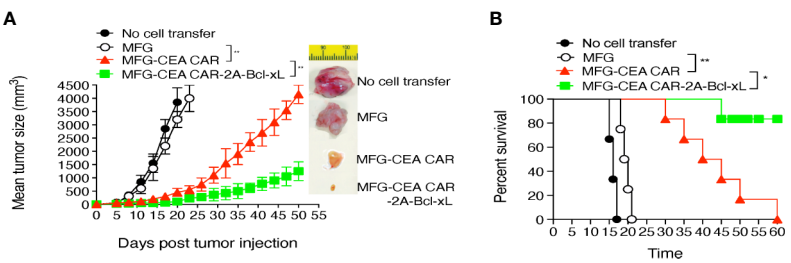

FIGURE 7 | Adoptive cell transfer of carcinoembryonic antigen (CEA) chimeric antigen receptor T (CAR-T) cells expressing Bcl-xL substantially suppressed tumor growth and sustained mouse survival. The tumor challenge and the adoptive cell transfer are described in **Figure 6**. **(A)** Tumor growth was monitored over time with representative tumors on day 18. Data are mean tumor size ± S.D. from six individual mice (n=6) and representative of three experiments. **(B)** Mouse survival was assessed over 60 days (Kaplan–Meier survival analysis). *P < 0.05, **P < 0.01, Student's unpaired t-test.

and endogenous Bcl-xL could improve the persistence and antitumor ability of antigen-specific T cells (20, 21). In the current study, we exploited in a novel approach to enhance the ability of CAR-T cell persistence by overexpressing exogenous Bcl-xL and demonstrated promising results. The constructs used in the study could be an excellent platform for CAR design to increase CAR-T cell persistence in solid tumors.

Endogenous Bcl-xL is encoded by a Bcl-2 gene superfamily, expresses on mitochondrial membrane and binds specifically with the residues of cytochrome C, the process could prevent cytochrome C released from mitochondria to initiate the programmed cell death (36). It was demonstrated that Bcl-xL can rescue T cells from TCR engagement mediated cell death (37). Bcl-xL is the critical driver of T cell expansion, not only through enhancing activated T cell survival, but also increasing T cell proliferation and energy metabolism (38). Bcl-xL could also rescue T cells from PD-1 and/or Fas signal-induced cell death (18, 39), which generally upregulate at the early stage of T cell activation. Several costimulation signals were demonstrated to involve in upregulation of the expression of endogenous Bcl-xL in activated T cells (40–43), further indicating the important role of Bcl-xL in the life cycle of T cells. Therefore, various strategies combining distinct costimulatory domains with the CAR gene have been tried to increase Bcl-xL expression in CAR-T cells and achieved promising results (44). Since Bcl-xL provides an intrinsic mechanism for activating T cell survival, we introduce the Bcl-xL gene with a second-generation CAR through the 2A sequence, then exogenous Bcl-xL can be transcribed along with the CAR. We confirmed high expression of exogenous Bcl-xL protein in MFG-anti-CEA CAR+Bcl-xL transduced T cells and no exogenous Bcl-xL (~32 kDa) is expressed in MFG-anti-CEA CAR transduced T cells as well as empty MFG vector and untransduced T cells. The expression level of endogenous Bcl-xL is comparable amongst variable transduced and untransduced T cells, indicating exogenous Bcl-xL gene incorporating has no effect on the expression of endogenous Bcl-xL. In *ex vivo* experiment, both CEA specific CAR-T cells with or without exogenous Bcl-xL gene displayed significant cytotoxicity than the empty control vector. Bcl-xL did not improve cytotoxicity of the CAR-T cells *in vitro*; however, the *in vivo* study clearly showed that CAR-T cells expressing Bcl-xL considerably enhanced their antitumor ability.

In vivo persistence of CAR-T cells was notably enhanced by exogenous expression of Bcl-xL. IHC images showed increased accumulation of CAR-T cells expressing Bcl-xL, and flow cytometry analysis presented that the frequency of MFG-anti-CEA CAR+Bcl-xL transduced T cells was significantly higher than that of MFG-anti-CEA CAR transduced T cells in tumor-infiltrating lymphocytes (TILs), further indicating improved persistence of CAR-T cells by overexpressed exogenous Bcl-xL. These results may interpret the enhanced antitumor potency of CAR-T cells expressing Bcl-xL. Further analysis comparing the frequency of CAR-T cells in various tissues demonstrated the accumulation of CEA-specific CAR-T cells in the tumor lesion but not in the lymph nodes and spleen, indicating that Bcl-xL-induced CAR-T cell accumulation is antigen-specific.

Targeting antigen selection is another critical issue for CAR-T therapy because almost all of the tumor-associated antigens are shared by normal tissue/organs at a relatively low level. On-target off-tumor effect is one of the major obstacles for CAR-T therapy in solid tumors, and generally results in lethal adverse effect once the antigen expresses on life-important organ even at a very low level (45). The total number of available targeting antigens for CAR-T treatment in clinical trials for solid tumors is very limited, considering the amount of tumor types, it is very few of each type of tumor (46). CEA is considered as a relatively safe and effective target, it is expressed at high levels in embryo phase and decrease rapidly to very low level after birth, and majorly expressed at colorectal mucus apical side. CEA is greatly overexpressed in most epithelial cell-derived cancers (47), and often used as a representative tumor-associated antigen to investigate immunotherapy for solid tumors. In the current study, we demonstrated that CEA is an effective target for CEA-specific CAR-T cells and effectively recruit CAR-T cells into the tumor lesion. Consideration of the CAR designation platform containing human T cell activation signal as well as hCEA targeting scFv, it is ready to test the efficiency of the constructs engineered human T cells against various human solid tumors expressing CEA through *in vitro* system, such as co-culture with organoid as previously described (48). Another advantage of the constructs used in the study is the MFG-based vector, which is the retroviral vector used extensively in clinical trials and ready to be modified by inserting IRES and multicloning sites without adverse effects (26).

The phenotype of persistent CAR-T cells remains to be identified. It had been demonstrated that adoptive transfer of genetically edited memory T cells has long-term persistence (49), and Bcl-xL contribute to sustain effector and memory T cells at later stages of activation through members of the TNFR family (50, 51). We acknowledge the potential oncogenic risk of exogenous Bcl-xL expression. Apoptosis is the general mechanism to eliminate cells with damaged DNA or aberrant cell cycle, anti-apoptotic proteins have therefore oncogenic potential. Although mature T cells seem resistance to gene transfer even for oncogene (52), and we examined that most anti-CEA CAR-T cells did not survive after a month of the adoptive cell transfer, a longer observation of CAR-T cells expressing exogenous Bcl-xL is needed prior to a further clinical trial. As an alternative, the depletion of the transferred CAR-T cells can be achieved by targeting the inserting suicide genes into the CAR construct, or designation of a universal target for accessible defined therapeutic monoclonal antibody in the external domain of the CAR.

In the current study, we confirmed the therapeutic potential of CEA-specific CAR-T cells overexpressing Bcl-xL in the treatment of colorectal cancer. Bcl-xL, a critical regulator for T cell survival, showed the potential ability to increase the persistence of genetically engineered CAR-T cells in the tumor tissues, regressed tumor growth, and increased the overall survival rate of animals. Our strategy of the CAR design may be explored as a new platform to improve CAR-T cell-based immunotherapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Texas A&M University Animal Care Committee (IACUC; #2018-0006).

AUTHOR CONTRIBUTIONS

Conceptualization of the study was by JS. Funding acquisition, methodology, validation, supervision, and manuscript review and editing were done by HC and JS. Data curation, formal analysis, and original draft writing were done by JF, JKD, and XX. Investigation was carried out by JF, JKD, and XX. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.574860/full#supplementary-material

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METHODS
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Genetic Modification of Tumor-Infiltrating Lymphocytes *via* Retroviral Transduction

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Adoptive T cell therapy (ACT) holds great promise for cancer treatment. One approach, which has regained wide interest in recent years, employs antitumor T cells isolated from tumor lesions ("tumor-infiltrating lymphocytes" or TIL). It is now appreciated that a considerable proportion of anti-melanoma TIL recognize new HLA-binding peptides resulting from somatic mutations, which occurred during tumor progression. The clinical efficacy of TIL can potentially be improved *via* their genetic modification, designed to enhance their survival, homing capacity, resistance to suppression, tumor killing ability and additional properties of clinical relevance. Successful implementation of such gene-based strategies critically depends on efficient and reproducible protocols for gene delivery into clinical TIL preparations. Here we describe an optimized protocol for the retroviral transduction of TIL. As the experimental system we employed anti-melanoma TIL cultures prepared from four patients, recombinant retrovirus encoding an anti-CD19 chimeric antigen receptor (CAR) as a model gene of interest and CD19+ and CD19- human cell lines serving as target cells. Transduction on day 7 of the rapid expansion protocol (REP) resulted in 69 ± 8% CAR positive TIL. Transduced, but not untransduced TIL, from the four patients responded robustly to CD19+, but not CD19- cell lines, as judged by substantial secretion of IFN-γ following co-culture. In light of the rekindled interest in antitumor TIL, this protocol can be incorporated into a broad range of gene-based approaches for improving the in-vivo survival and functionality of TIL in the clinical setting.

Keywords: adoptive cell therapy, rapid expansion protocol, T cell subsets, T cell differentiation status, chimeric antigen receptors

INTRODUCTION

ACT with autologous TIL produced from resected melanoma biopsies combined with non-myeloablative lymphodepletion and IL-2 exceeds an overall clinical response rate of 41% [95% confidence interval (CI) 35% to 48%] in patients with highly advanced metastatic melanoma according to meta-analysis (1–5). The TIL protocol consists of three basic steps: (i) patient

preconditioning by lymphodepleting regimen; (ii) Isolation of TIL from tumor biopsies and ex-vivo expansion of generated TIL cultures with the anti-CD3 antibody OKT3, irradiated feeder cells and IL-2; (iii) TIL administration i.v. followed by IL-2 infusion.

We have been applying for over a decade an open label phase II clinical study in stage IV melanoma patients (2, 6–11). Our initial objective was to assess the efficacy and safety of ACT with autologous TIL, which was offered as a second line of treatment following IL-2–based therapy until 2011 and, since, as a salvage treatment following standard of care therapy including other immunotherapy modalities (e.g., anti-CTLA-4 and anti-PD-1 antibodies). Following surgical resection and processing of a metastatic lesion, TIL cultures are typically generated in IL-2–containing medium within 2 weeks to 1 month. Established TIL cultures are then further expanded in a rapid expansion protocol (REP) to treatment levels. REP is initiated with an anti-CD3 agonist, irradiated feeder cells and IL-2, which drives the expansion of TIL to approximately 1000-fold within 14 days. Clinical results of 57 patients treated at our medical center have previously been published (10). Potential predictors for response were shorter time to TIL culture generation, elevated fold large scale expansion, higher total number of cells, higher total number of CD8 T cells and higher frequency of CD8 T cells in the cell product (10).

In spite of the tremendous progress achieved in TIL therapy, tumor specimens may fail to give rise to viable TIL cultures and a high proportion of melanoma patients treated with either TIL-ACT strategy are non-responders (5, 12, 13). Although many responders exhibit long-lasting tumor regression and complete responders only rarely relapse, new strategies are needed for maximizing response rate and duration and, most importantly, for providing a platform for the treatment of other solid tumors. Undoubtedly, a promising route for improving the clinical efficacy of TIL therapy is the enhancement of the functional properties of TIL cultures *via* genetic modification. To achieve this goal we have recently created several classes of "genetic adjuvants," including membrane-attached cytokines, truncated, constitutively active toll-like receptors (caTLRs) and spontaneously homo-oligomerizing, constitutively active tumor-necrosis factor receptors (caTNFRs). Using electroporation of anti-melanoma TIL with in-vitro-transcribed mRNAs encoding membrane IL-2, IL-12, IL-15, caTLR4, or caCD40, alone or in different combinations, we could demonstrate their additive, and often synergistic effects on key parameters concerning TIL function and survival (14–18).

The first clinical assessment of this approach was reported by S. Rosenberg et al. (19) who had successfully introduced the neomycin-resistance gene into anti-melanoma TIL derived from five patients *via* retroviral transduction. Gene-expression in modified TIL could be detected in the circulation of these patients three weeks to two months post-administration. In following studies, the Rosenberg group demonstrated the retroviral transduction of anti-melanoma TIL with the IL-2 gene (20) or with an inducible IL-12 gene placed under the control of a nuclear factor of activated T cells- (NFAT)-responsive promoter (21). While all these studies employed selected anti-melanoma TIL, Forget et al. recently described a protocol for the retroviral transduction of TIL, evaluating the gene encoding the chemokine receptor CXCR2 (22). Here we describe in detail our optimized protocol for retroviral transduction of anti-melanoma TIL cultures and demonstrate its efficacy in endowing transduced TIL with a new antigenic specificity *via* an anti-CD19 CAR.

MATERIALS AND METHODS

Patient Samples, Retroviral Vector, and Cell Lines

TIL samples were obtained from melanoma patients that were enrolled to an open-label and phase II TIL ACT trial for patients with metastatic melanoma at the Sheba Medical Center (NCT00287131). A gamma-retrovirus encoding an anti-CD19 CAR based on an FMC63 derived scFv, a CD28 costimulatory domain and CD3-ζ signaling domain, as well as the following CD19-expressing immortalized cell lines were used: NALM-6 (acute lymphoid leukemia); Toledo (B cell diffuse large cell lymphoma); CD19-K562 cells (CD19-transduced K562 chronic myeloid leukemia cells). NGFR-K562 cells (NGFR- (nerve growth factor receptor)-transduced K562), served as CD19-negative control cells. All cell lines and the retroviral vector were kindly provided by Dr. S. Feldman, NCI. Vector construction and retroviral vector master cell bank production were done at the NCI.

Peripheral blood mononuclear cells (PBMC) of healthy donors (HD) or a lymphoma patient (CAR-T Pt.) were prepared from apheresis products collected at the Sheba bone marrow transplantation unit by single step gradient with Ficoll-Hypaque in lymphocyte separation medium.

Establishment of TIL Cultures

TIL establishment was previously described by us in detail (8–10). In short, fragmentation, enzymatic digestion and cell remnants technique were used to isolate TIL from surgically resected metastatic lesions. During the first week, non-adhered TIL were transferred to a new 24-well plate and cultured separately from the adhered melanoma cells. Cells were cultured in complete medium (CM) containing 10% human AB serum (Valley Biomedical, Winchester, VA or Gemini Bio, West Sacramento, CA), 2 mM L-glutamine (Biological Industries, Israel), Pen/Strep (Biological Industries, Israel) in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA). 3,000 IU/ml IL-2 (Chiron Novartis, NJ, USA) was added to the CM medium during the TIL establishment phase. Cells were split or medium was added every 2 to 3 days to maintain a cell concentration of 0.5–2 × 10e6 cells/ml. TIL cultures were established within 2 to 4 weeks.

Establishment of Melanoma Cultures

The generation of melanoma cell lines was previously described (8–10). Enzymatic digestion and cell remnants technique were

used to isolate melanoma cells from surgically resected metastatic lesions. Melanoma cells were cultures in Target medium contained 10% Fetal Bovine Serum, 25 mmol/L HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate (Biological Industries, Israel).

Standard CAR Protocol (SCP)

This process describes a smaller scale version of our standard expansion protocol of CAR T cells for clinical use (23). On the day of SCP initiation 16×10^6 PBMC or two days prior SCP initiation 8×10^6 TIL were thawed in CM medium with 3,000 IU/ml IL-2. On day 0 of SCP cells were re-suspended at a concentration of 1.0×10^6 cells/ml in Stim medium containing 5% human AB serum (Valley, VA, US), 2 mM L-glutamine (Biological Industries, Israel), 100 U/ml penicillin and 100 mg/ml streptomycin (Biological Industries) in AIM-V medium (Invitrogen, CA, USA, CM). 300 IU/ml IL-2 (Chiron Novartis, NJ, USA) and 50 ng/ml anti-CD3 monoclonal antibody OKT3 (Miltenyi Biotec, Bergisch Gladbach, Germany) were added. After 2 days, 2×10^6 cells were transduced with the CD19 CAR retroviral vector and the rest of the cells discarded. For this purpose, non-tissue culture treated 6-well plates were coated with 10 µg/ml RetroNectin (Takara Bio Inc, Otsu, Japan) in DPBS-Dulbecco Phosphate Buffered Saline (Biological Industries, Israel) for 2 h at room temperature or overnight at 4°C, followed by 30 min blocking with 2.5% human albumin (Bio Products Laboratory Limited, UK) in PBS and washed. Retroviral supernatant was thawed and diluted 1:1 with Stim medium. Four ml of the diluted vector were added per well of the retronectin-coated plates and these were centrifuged at 2,000*g* for 2 h at 32°C. Supernatant was aspirated and 2×10^6 TIL or PBMC in Stim medium with 300 IU/ml IL-2 were added to each well, centrifuged for 10 min at 1,000*g* and incubated at 37°C overnight. On day 3 the transduced cells were transferred to 6-well culture plates and maintained at a concentration of 0.5–2.0×10^6 cells/ml in Stim medium with 300 IU/ml IL-2 and further expanded until day 10, the standard day of CAR T cell infusion.

Rapid Expansion Protocol (REP) With Viral Transduction

This process describes the standard rapid expansion protocol of TIL cells for clinical use, just in a smaller scale (9, 10). TIL were thawed in CM with 3,000 IU/ml IL-2 and allowed to rest for a period of 2 days at a concentration of 1.0×10^6/ml in a 24-well plate. A mini-scale REP was initiated by stimulating TIL with 30 ng/ml OKT3, 3,000 IU/ml IL-2 and irradiated PBMC from non-related donors as feeder cells (5000 rad, TIL to feeder cells ratio = 1:100) in 50% CM and 50% AIM-V medium in T25 flasks. On the day of viral transduction (REP day 7), TIL were harvested, counted and adjusted to a concentration of 0.5×10^6/ml in CM with 3,000 IU/ml IL-2. Four ml of the cell suspension was distributed per well of a 6-well plate layered with viral vector as follows: Plate coating and viral transduction were performed as described above for the SCP. The viral supernatant was then aspirated and 2×10^6 TIL in Stim medium with 3,000 IU/ml IL-2 were added to each well, centrifuged for 10 min at 1,000*g* and incubated at 37°C overnight. The next day the cells were transferred to T75 culture flasks and maintained at a concentration of 0.5–2.0×10^6 cells/ml in Stim medium with 3,000 IU/ml IL-2 until day 14 (potential day of TIL infusion).

Flow Cytometry

Transduction efficacy was determined 4 days after transduction (day 11 of REP and day 6 of SCP) and 7 days after transduction (day 14 of REP and day 9 of SCP) using flow cytometry by labeling CAR-expressing TIL with biotin-labeled, goat anti-mouse IgG, F (ab′)2-specific antibody (Jackson Immunoresearch, West Grove, PA) and streptavidin (APC conjugated; BD Bioscience, San Jose, CA). The following anti-human antibodies were used in addition: anti-CD3 (Pacific-Blue conjugated, BioLegend, San Diego, CA), anti-CD4 (FITC-conjugated; BioLegend) anti-CD8 (PE-cy7-conjugated; BioLegend or APC conjugated; BD Biosciences), anti CD28 (PerCP vio770 conjugated, Miltenyi Biotec), anti-LAG-3 (Vioblue conjugated; Miltenyi Biotec), anti-PD1 (FITC conjugated, BioLegend), and TIM-3 (PE-cy7 conjugated; BioLegend). CD3+/F(ab′)2+ cells were defined as "transduced" cells. For further analysis of memory cell phenotype, cells were stained with antibodies to CD45RA (APC-Vio770-conjugated; BioLegend), CCR7 (PerCP-Vio770 conjugated; BioLegend). TIL were washed and resuspended in cell staining buffer (BioLegend, San Diego, CA). Cells were incubated for 30 min with the antibodies on ice, washed in buffer, and measured using FACS cytometer MACSQuant (Miltenyi Biotec). Samples were analyzed using FlowJo software (FlowJo LLC, Ashland, OR). Cells were gated according to FCS/SSC and singlets. The gating strategy is shown in **Supplementary Figure 1**.

Interferon-γ Release Assay

TIL were co-cultured overnight with target cells in 96-well plates at an E/T ratio of 1:1 (5×10^5 each in a total of 200 µl Target medium). Cells were centrifuged, supernatant was collected, and secreted IFN-γ levels were determined by a sandwich ELISA according to the manufacturer's instructions (BioLegend). Measurements were performed in triplicates.

Cell-Mediated Cytotoxicity Assay

TIL were co-cultured with autologous melanoma cells overnight at 37°C, at an E/T ratio of 1:1 (1×10^5 each in a total of 200 µl Target medium. Levels of lactate dehydrogenase (LDH) in the medium were determined by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, Cat. no. G1780) according to the manufacturer's instructions, using a microplate reader (Antos 2000) at 490 nm. Experiments were performed in triplicates wells. Percent of specific lysis of target cells was calculated using the equation: (Experimental-EffectorSpontaneous − TargetSpontaneous)/(TargetMaximum − TargetSpontaneous) × 100.

Statistical Analysis

Significance of variation between groups was evaluated using a non-parametric two-tailed Student's t test. Test for differences between proportions was performed using two sided Fisher's exact test with $p \leq 0.05$ considered significant.

RESULTS AND DISCUSSION

TIL Transduction During SCP

As an indicator gene for demonstrating TIL transduction efficacy we have chosen the same anti-CD19 CAR we use for treating patients with B cell malignancies (24) rather than, for example, an anti-melanoma CAR. We reasoned that monitoring the newly acquired activity of the anti-melanoma TIL against non-melanoma cells would allow us to determine the net contribution of the CAR gene on TIL function with no background activation or potential bottlenecks along the signaling pathway.

TIL cultures were established from tumor biopsies of melanoma patients within two to three weeks as described before (8–10).

With the aim to develop an optimized TIL transduction method, we first utilized a standard CAR-T cell production protocol (SCP) (**Figure 1**, left panel). In the clinical setting, CAR T production is initiated with PBMC. Here we applied the exact same protocol on TIL cultures derived from three melanoma patients (TIL052, TIL189, and TIL213) and compared fold expansion, transduction efficacy, cell phenotype to results obtained from three healthy donors (HD) PBMC. In short, TIL or HD PBMC were stimulated with OKT3 and IL-2 (23). On day 2, 2×10^6 TIL or PBMC were transduced with an anti-CD19 CAR construct on retronectin-coated plates and further expanded in IL-2-containing medium until day 10, the standard day of infusion.

Cell counts were performed on days 3, 6, 9, and 10. **Figure 2A** presents the fold expansion over time compared to day 2, the day of CD19 CAR transduction. As shown in **Figure 2B**, HD PBMC expanded better than TIL: fold expansion on day 6 was 6.71 ± 0.68 for HD PBMC and 3.11 ± 2.15 for TIL ($p = .0510$) and on day 9, 16.67 ± 2.7 for HD PBMC and 7.71 ± 4.06 for TIL ($p = .0334$)

Transduction efficacy was determinate by $F(ab')_2$ expression on CD3 T cells on day 6 (4 days after transduction) and day 9 (7 days after transduction). A representative FACS dot plot is

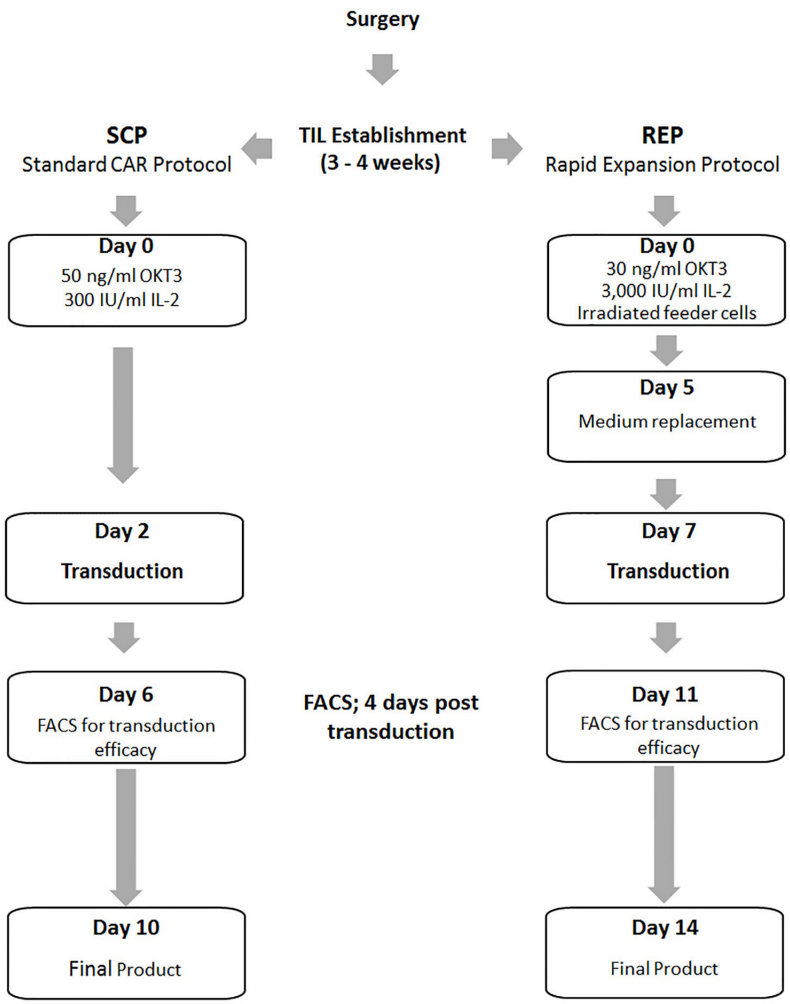

FIGURE 1 | Schematic illustration of the Standard CAR Protocol (SCP) compared to the Rapid Expansion Protocol (REP).

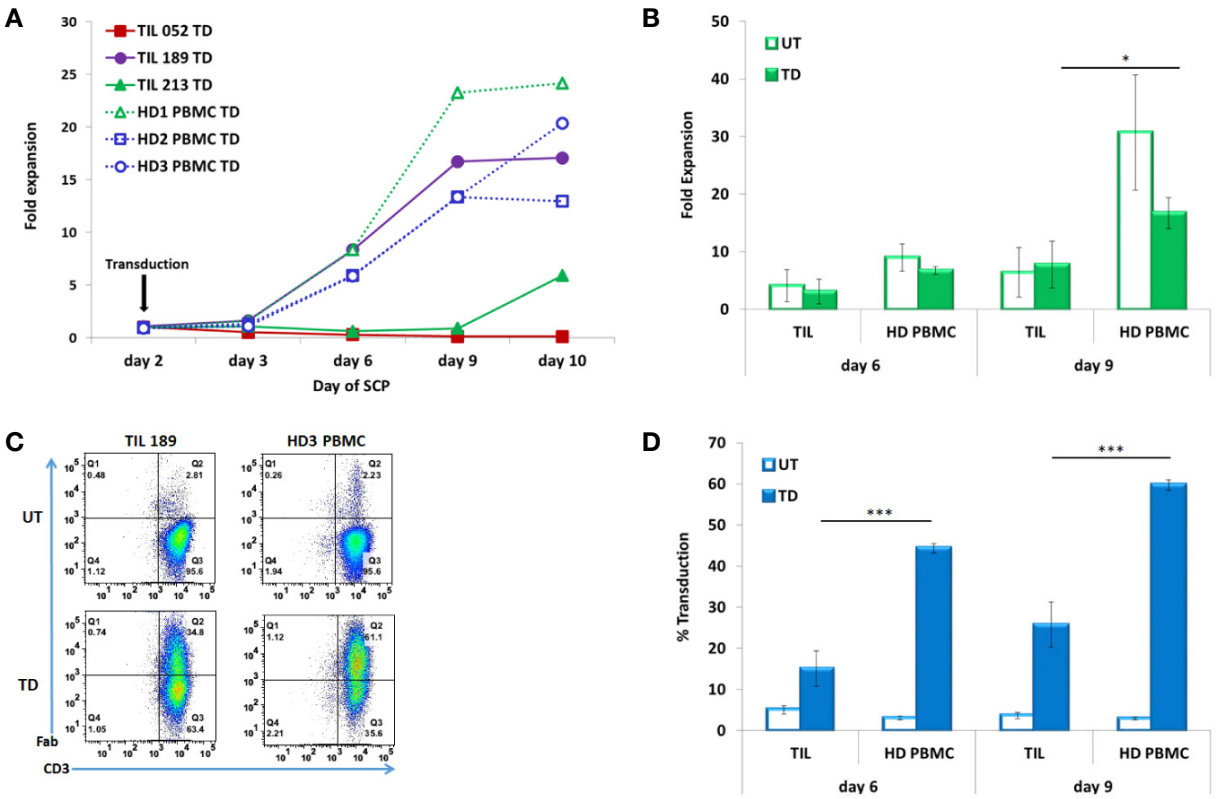

FIGURE 2 | TIL compared to PBMC transduced according to the SCP. **(A)** Fold expansion after transduction (day 2) of melanoma patients derived TIL (TIL052, TIL189, and TIL213) compared to healthy donor (HD) PBMC **(B)** Average fold expansion of transduced (TD) and untransduced (UT) TIL and HD PBMC on days 6 and 9. **(C)** Transduction efficacy determined by CD3/F(ab')2 double positive cells, representative FACS histogram plot **(D)** Average frequency of transduced TIL and PBMC on days 6 and 9. *$p < .05$, ***$p < .001$.

shown in **Figure 2C**. A minor fraction of untransduced TIL and PBMCs were also stained by the same biotin-labeled polyclonal whole goat IgG anti-mouse $F(ab')_2$ antibody, as can also be seen in **Figure 3C**. This staining could be accounted for a small subset of human CD4 T cells naturally expressing FcγRI (25). Transduction efficacy was significantly higher in HD PBMC than in TIL (day 6: 44.4 ± 1.17% for HD PBMC and 15.08 ± 4.24% for TIL, p = .0003; day 9: 59.73 ± 1.26% for HD PBMC and 25.77 ± 5.46% for TIL, p = .0005) (**Figure 2D**). Due to the superior expansion and elevated transduction efficacy in HD PBMC compared to TIL, the total number of transduced cells by day 10 was 10.8 fold higher in HD PBMC than in TIL ($2.42 \times 10^7 \pm 8.67 \times 10^6$ vs. $2.24 \times 10^6 \pm 2.91 \times 10^6$, p = .0141). This phenomenon could reflect inefficient TIL stimulation at the TIL establishment stage in the absence of feeder cells and might correlate with low expression of the co-stimulatory molecule CD28 [(26), see **Table 1**].

T cell subset distribution and differentiation status have been related to clinical response both in CAR-T and TIL therapies (27–29). Additional phenotype analysis performed on transduced and untransduced TIL on day 9 revealed that transduction had no impact on the T cell CD4/CD8 distribution. T cell differentiation status (examining naïve, central memory, effector memory and terminally differentiated effector T cells (T_N, T_{CM}, T_{EM}, and T_{EMRA}, respectively) and the expression of the co-stimulatory molecule CD28 or co-inhibitory molecules LAG-3, TIM-3, and PD-1 (**Table 1** and **Supplementary Figure 2**) were also preserved. With the exception of increased LAG-3 expression on transduced HD PBMC (UT, 1.0 ± 0.2%; TD, 5.0 ± 2.0%; p = .0261), transduction had also no impact on the phenotype of HD PBMC (**Table 1**).

TIL Transduction During REP

Since SCP resulted in a low transduction efficacy and expansion, we tested if transduction during TIL REP could yield superior results (**Figure 1**, right).

TIL expansion during the establishment phase is driven by the addition of IL-2 without direct TCR activation. During REP, we routinely provide the cells with optimal signaling *via* three complementary pathways: Cell proliferation is induced by IL-2, T cell activation is triggered by an anti-CD3 antibody (OKT3) and co-stimulation is delivered by feeder cells. In this study we did not assess the separate contribution of each of these stimuli to TIL expansion or transduction efficacy. We used soluble OKT3 in both SCP and TIL REP as we routinely do in our clinical trials with CAR-T cells and TIL. In both protocols,

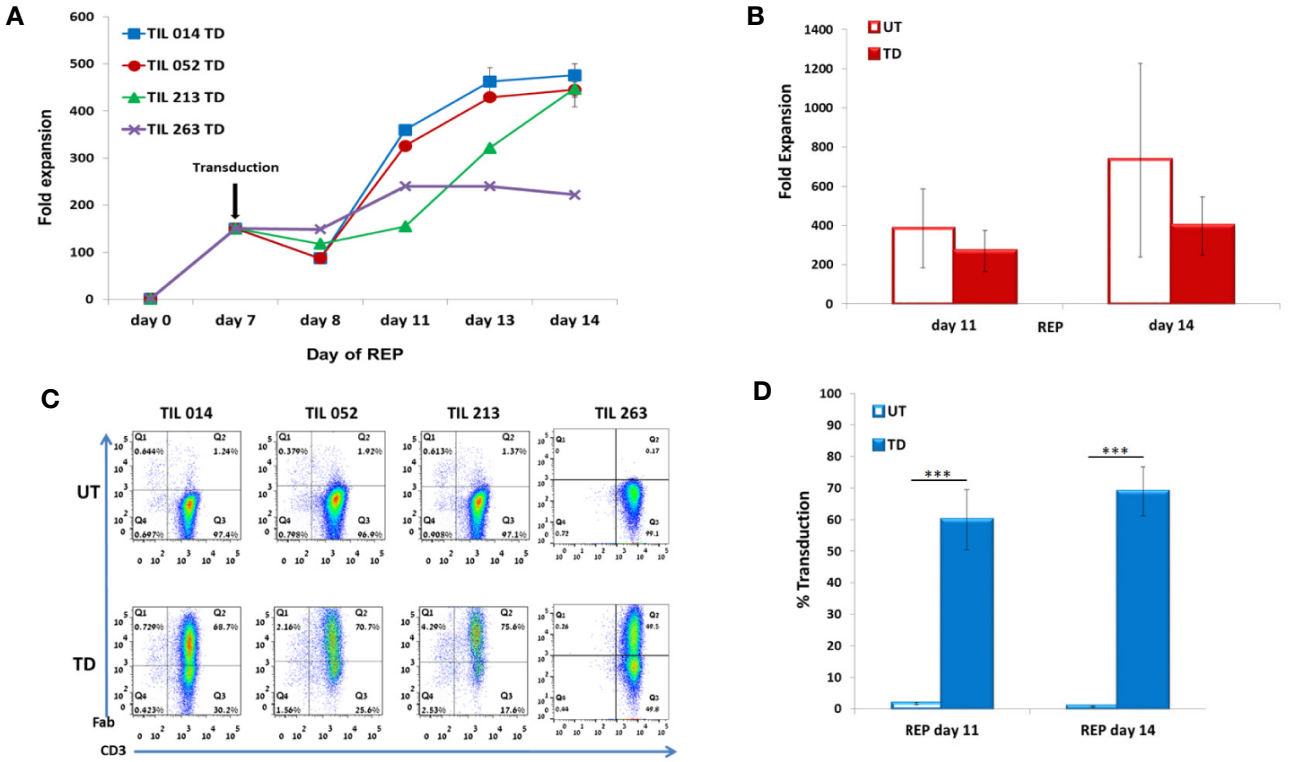

FIGURE 3 | Characterization of REP/d7-transduction protocol. TIL014, TIL052, TIL213, and TIL 263 cultures were transduced with the anti-CD19 CAR on day 7 of REP. **(A)** Fold expansion during REP. **(B)** Average fold expansion of transduced (TD) and untransduced (UD) TIL on days 11 and 14 of REP. **(C)** Transduction efficacy as determined by flow cytometry analysis for the CD3+F(ab')2+ expression; representative FACS histogram plot. **(D)** Average frequency of transduced cells on days 11 and 14 of REP. ***p < .001.

delivering highly proliferative cells is critical for clinical outcome. Indeed, comparison of CD3/CD28 beads to soluble OKT-3 and high concentrations of IL-2 showed that the latter produced more T_{EM} cells with shorter telomeres (30). Yet, employing our protocol we achieved high transduction efficacy during REP, which reflects the ability of soluble OKT3/IL-2 in the presence of feeder cells to induce potent activation of TIL possessing enhanced proliferative capacity.

In contrast to SCP, which results in an average expansion of around 20-fold on day 10 (23), transduction during REP results in about 1,000-fold expansion after 14 days (9). In the clinical setting, day 14 is the day of cell infusion. Based on our previous experience with TIL ACT, at days 6 to 8 of REP, cells possess a high proliferation capacity while the presence of feeder cells, which may decrease transduction yield, is highly reduced. We first demonstrated transduction efficacy of ~70% in REP for day 6 and day 8 cultures (TIL 052: day 6, 71.8 ± 1.4%; day 8, 69.4 ± 0.8%; p = .18). As a compromise between the higher transduction efficacy observed for day 6 and day 8 REP, and the likelihood for remaining feeder cells in the culture we have chosen REP day 7 as the optimal transduction day, as no more irradiated donor feeder cells were present in the culture at that time point. Transduction was performed with TIL samples derived from four melanoma patients (TIL014, TIL052, TIL213, and TIL263).

TIL were counted on days 7, 8, 11, 13 and 14 (7 days post-transduction). Fold expansion over time is presented in **Figure 3A**. Although untransduced TIL expanded significantly better than CD19 CAR-transduced TIL (day 11: UT 383 ± 201, TD 270 ± 104, p = .3567; day 14: UT 734 ± 494, TD 397 ± 149, p = .2408), the average fold expansion of transduced TIL on day 14 reached 397 ± 149 (**Figure 3B**).

Transduction efficacy was determined 4 days and 7 days (day 11 and day 14 of REP) after viral transduction (**Figure 3C**). As shown in **Figure 3D**, the average frequency of transduced TIL was 60 ± 9.6% on day 11 of REP and 69 ± 8% on day 14 of REP. Of note, the transduction efficacy achieved 4 days following transduction during REP was significantly higher than during SCP (REP 69 ± 8%, SCP 15 ± 4.2%; p <.0001). Transduction did not affect the CD8/CD4 subset ratio but had an effect on the differentiation status of TIL (**Table 2**).

The four CD19 CAR-transduced TIL cultures were further analyzed for antitumor reactivity by assessing IFN-γ secretion in response to CD19 stimulation. To this end, TIL were co-cultured with the CD19-expressing cell lines TOLEDO, NALM-6, and CD19-K562 and the CD19-negative cell line NGFR-K562 (**Figure 4A**). Untransduced TIL served as negative control and T cells transduced with the same CAR construct of a lymphoma patient who achieved complete clinical remission following

TABLE 1 | Phenotypic profile of untransduced (UT) vs. transduced (TD) TIL and HD PBMC on day 9 of SCP. Data are presented as average % expression of the indicated markers.

	TIL			HD PBMC		
	UT (n=3)	TD (n=3)	p value	UT (n=3)	TD (n=3)	p value
CD3+	99 ± 0.9	99 ± 0.6	p = 1.00	99 ± 0.2	99 ± 0.4	p = 1.00
CD4+	40 ± 34	45 ± 34	p = .866	42 ± 11	39 ± 13	p = .776
CD8+	60 ± 34	55 ± 34	p = .866	58 ± 11	61 ± 13	p = .776
T_N	6.0 ± 5.3	11 ± 3.6	p = .248	14 ± 4.3	15 ± 2.7	p = .750
T_{CM}	19 ± 6.6	14 ± 8.3	p = .460	21 ± 4.7	21 ± 4.1	p = 1.00
T_{EM}	70 ± 13	63 ± 11	p = .516	54 ± 11	50 ± 2.3	p = .571
T_{EMRA}	5.0 ± 0.7	9.0 ± 2.8	p = .074	12 ± 2.7	14 ± 3.2	p = .455
CD28+CD3+	13 ± 12	5.0 ± 4.0	p = .335	21 ± 19	8.0 ± 6.0	p = .322
LAG3+CD3+	3.0 ± 4.0	3.0 ± 2.0	p = 1.00	1.0 ± 0.2	5.0 ± 2.0	p = .026
PD1+CD3+	31 ± 7.0	31 ± 8.0	p = .925	5.0 ± 2.0	6.0± 2.0	p = .725
TIM-3+CD3+	40 ± 17	25 ± 13	p = .306	57 ± 10	50 ± 10	p = .395

TN (naïve), CD3+CD45RA+CCR7+; TCM (central memory), CD3+CD45RA–CCR7+; TEM (effector memory), CD3+CD45RA–CCR7–; TEMRA (effector), CD3+CD45RA+CCR7–.

CAR-T cell therapy served as positive control (CAR-T Pt.; transduction efficacy: 60.1%). Transduced TIL demonstrated robust anti-tumor reactivity to the three CD19-positive targets as monitored by IFN-γ secretion, (CD19-K562 = 59,708–163,344 pg/ml; Nalm-6 = 11,081–101,483 pg/ml; Toledo, = 5,591 - 73,974 pg/ml), which was comparable, and often superior to transduced cells of the clinical responder. Practically no response was seen against the CD19 negative-cells NGFR-K562 except for TIL263 that showed secretion of 2,839 ± 373 pg/ml compared with the significantly higher secretion against CD19-K562 positive target (163,344 pg/ml) (**Figure 4B**).

A critical component of TIL anti-melanoma response is the ability to kill tumor cells directly. We compared the ability of transduced TIL to respond to their autologous melanoma or unrelated, HLA class-I-mismatched melanoma with that of untransduced cells as judged by target cell killing. As shown in **Figure 4C**, the four TIL maintained their specific killing of autologous melanoma. Transduced TIL014, TIL052, and TIL213 showed an increased killing activity compared to the untransduced cells, and TIL263 maintained its original 100% killing activity achieved in this assay.

We also assessed IFN-γ secretion of four TIL in response to their autologous melanoma. As shown in **Figure 4D**, TIL014 and TIL213 retained their ability to secrete high amount of IFN-γ. TIL052, however, exhibited considerable reduced secretion, although IFN-γ level of 660 pg/ml still exceeds the acceptance criterion of 200 pg/ml that is implemented in selected TIL procedures (2, 7). The reason for this reduction is not clear. Considering the apparent clonal diversity of the initial TIL052 culture and the cell-specific events of viral genome integration, a direct negative effect of transduction on TIL function as seen here is unlikely. TIL263 secreted only little IFN-γ in response to the autologous melanoma even prior to transduction.

All TIL chosen for this study are short-term-cultured or "young" TIL, not selected according to their ability to secrete IFN-γ in response to their autologous tumor (8, 31). In our own experience, only approximately half of the patients have TIL capable of IFN-γ secretion exceeding the acceptance criterion of 200 pg/ml required for selected TIL. Yet, a considerable fraction of non-secreting TIL are still capable of efficient target cell killing, as clearly demonstrated here with TIL263.

To summarize our protocol for retroviral TIL transduction, a scheme of the workflow is presented in **Figure 5**: Following the establishment of TIL cultures and cryopreservation, TIL are thawed and allowed to rest for 2 days before REP initiation. On day 0 of REP, TIL are re-suspended in CM/AIM-V medium with soluble anti-CD3 antibody, IL-2, and irradiated feeder cells. Transduction is performed on day 7, followed by the expansion of the transduced cells until day 14, the planned day of infusion. The transduction protocol did not exert a discernible effect on the CD8/CD4 T cell subset ratio and the differentiation status of TIL cultures. This protocol results in a transduction efficacy of ~ 69% and an average expansion of > 400-fold. Although transduction reduces TIL proliferation capacity by approximately two-fold compared with untransduced cells (**Figure 3B**), the protocol is highly efficient and achieves clinically relevant numbers of genetically modified cells.

Genetically engineered TIL with improved functionality and/or tumor recognition capacity hold great promise in adoptive cell therapy and several laboratories have already provided detailed protocols for retroviral TIL transduction.

This field was pioneered by S. Rosenberg and colleagues, who in 1990 introduced a neomycin-resistance gene to anti-melanoma TIL derived from five patients, to allow the identification of transductants and their offspring in long-term

TABLE 2 | Phenotypic profile UT vs. TD TIL undergoing transduction during REP.

	TIL		
	UT (n=4)	TD (n=4)	p value
CD3+	100 ± 0.05	100 ± 0.1	p = 1.000
CD4+	15 ± 4.7	16 ± 5.4	p = .7893
CD8+	85 ± 4.7	84 ± 5.4	p = .7893
T_N	5.6 ± 3.9	5.5± 2.7	P = .9677
T_{CM}	12 ± 1.3	21 ± 4.1	P = .0058
T_{EM}	76 ± 4.4	64 ± 6.5	p = .0223
T_{EMRA}	5.9 ± 2.9	9.8 ± 3.2	p = .1209

Data is presented as average % expression of the indicated markers. TN (naïve), CD3+CD45RA+CCR7+; TCM (central memory), CD3+CD45RA–CCR7+; TEM (effector memory), CD3+CD45RA–CCR7–; TEMRA (effector), CD3+CD45RA+CCR7–.

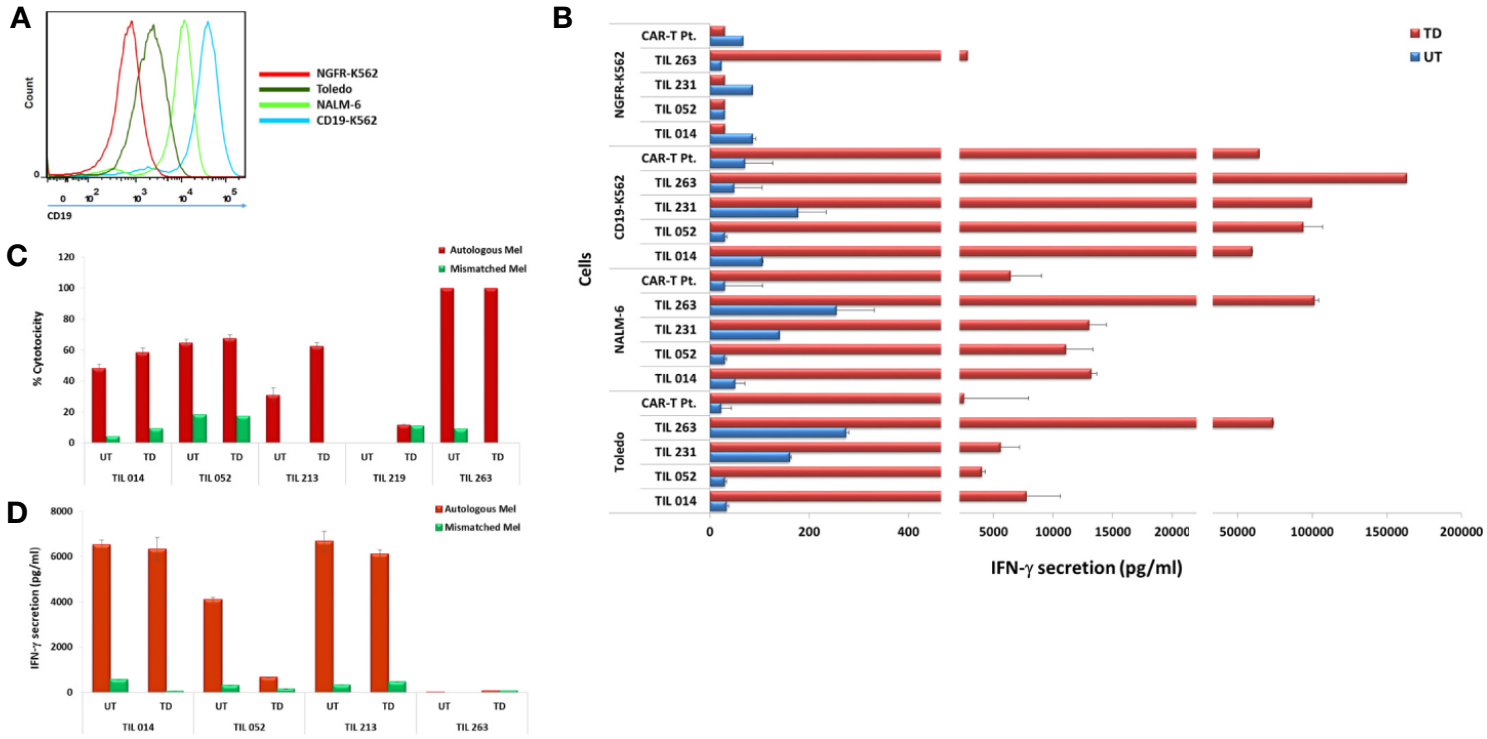

FIGURE 4 | Transduced CAR-TIL are reactive against CD19-expressing cell lines and autologous melanoma cultures. **(A)** Flow cytometry analysis of target cell lines for CD19 expression. **(B)** Transduced (TD) and untransduced (UT) TIL were co-cultured for 24 h with target cells expressing CD19 (K562-CD19, Toledo, NALM-6) or not (K562-NGFR) followed by IFN-γ ELISA. CAR-T Pt. cells served as a positive control. **(C)** Transduced (TD) and untransduced (UT) TIL were co-cultured with autologous melanoma cells overnight at 37°C at an E/T ratio of 1:1. Growth medium was then removed and subjected to an LDH non-radioactive cytotoxicity assay. Experiments were performed in triplicates wells. **(D)** Transduced (TD) and untransduced (UT) TIL were co-cultured for 24 h with autologous melanoma target cells followed by IFN-γ ELISA. IFN-γ secretion by target cell lines alone, T cells alone, and T cells co-cultured with HLA-mismatched melanoma cell lines were below the level of detection and are not shown.

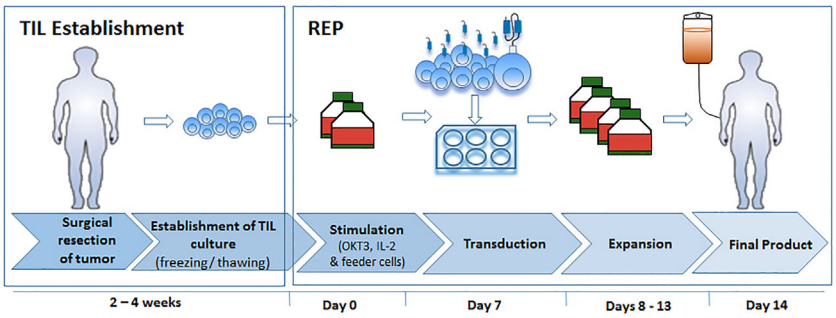

FIGURE 5 | Flowchart of optimized TIL expansion and transduction.

studies of patients (19). In that work, transduction was performed during TIL establishment, i.e., at the pre-REP phase, and entailed lengthy ex-vivo propagation of up to 65 days, which may often result in T cell exhaustion. Transduction efficacy in TIL cultures, which was determined by semi quantitative (sq) PCR, did not exceed 11% and was comparable to the results obtained by a quantitative Southern blot analysis that revealed 4% to 18% efficacy. This work was the first demonstration of the feasibility and safety of human gene therapy through retroviral transduced TIL. In a later study the Rosenberg group investigated ACT of 13 patients who were treated with anti-melanoma TIL transduced with the IL-2 gene employing three different protocols (20). In cohort I (three patients), transduction was performed twice at the pre-REP stage, resulting in ~1000 fold expansion on day 14 and an estimated efficacy of ~21% (as determined by sqPCR). In cohort II and III (five patients each), transduction was performed during first rapid expansion, which was followed by a second expansion phase. TIL of cohort II were transduced twice, on days 7 and 8, and in cohort III TIL were transduced on days 3 and 4. The results for cohort II and III demonstrated ~25% to 35% transduction yield as judged by intracellular staining for IL-2 performed day 14. In comparison, our REP day 8 transduction achieved ~68% efficacy, as monitored by flow cytometry analysis.

Importantly, the lengthy ex-vivo expansion period may have contributed to shorter telomeres, potentially limiting in-vivo survival. In a third report (21), the Rosenberg group reported the transduction of TIL from 33 patients with an inducible IL-12 gene, employing either a dual transduction protocol on REP day 4 and 5 for 21 patients and a single transduction on either day 4 or day 5 for 12 patients. Gene transfer efficiency ranged from 2% to 52%, averaging 14%, with generally diverse, but low expansion rate at day 14.

We observed an average of two-fold reduction in the proliferation capacity of transduced vs. untransduced TIL, which might reflect the increase in T_{CM} and decrease in T_{EM} phenotype in transduced cells (**Table 2**). T_{CM} and T_{EM} cells comprised 12 ± 1.3% and 76 ± 4.4, respectively, in the untransduced TIL compared to 21 ± 4.1% and 64 ± 6.5% in the transduced cells ($p < .0058$ and $p < .0223$, respectively). Our results are in agreement with those of Zhang et al. (21). Nevertheless, T_{CM} represents a population with self-renewal capability, which can be advantageous in ACT as cells derived from the T_{CM} population were shown to persist and expand in-vivo better than those derived from T_{EM} cells (29, 32).

More recently, Forget et al. (22) transduced four anti-melanoma TIL with the CXCR2 gene with transduction efficiency averaging 45%. In their study, transduction was performed on day −1 before REP in the presence of plate-bound, rather than soluble OKT3 at different concentrations and 2 periods of activation of 24/48 h. TIL from one patient contained a considerable proportion of double-negative CD3+ cells that did not expand. In our transduction protocol (see **Figure 5**) we stimulate the cells only during the REP stage (with 30 ng/ml soluble OKT3). Our protocol supports the growth of TIL populations, which comprise exclusively single-positive CD3+ (**Table 2**) and maintains proliferative capacity. Tumor infiltration of CD3+CD8+ T cells is associated with a higher rate of progression-free survival and improved prognosis (5).

Compared to transduction during the TIL establishment phase [as practiced in (22)], transduction during REP requires a greater number of cells, but leads to higher transduction efficacy. It is conceivable that improvement in clinical efficacy achieved by the delivered gene could eventually enable lowering the number of administrated TIL, which ranges between 0.5×10^{10} and 10×10^{10} cells per infusion.

With the growing appreciation of the importance of neo-antigens in the induction of the antitumor response, the field of TIL ACT has been gaining even greater interest in the past several years [e.g., (33–35)]. Here we used TIL transduction with a CD19 CAR as an experimental model. However, this method is not limited to the introduction of an additional antigen receptor and can be used for the delivery of genes designed to prolong TIL survival, confer resistance to suppression, enhance different functional properties, and increase homing to the tumor site, all of which can improve the clinical efficacy of TIL therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Patients signed an informed consent approved by the Israeli Ministry of Health (Helsinki approval no. 3518/2004, NCT00287131). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HW-M, GG, OI, and MB contributed conception and design of the study. HW-M, HB, ML, and OI performed experiments and acquired the data. HW-M and OI wrote the first draft of the manuscript. GG, JS, and MB revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.584148/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Engineering NK Cells for CAR Therapy—Recent Advances in Gene Transfer Methodology

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The development of chimeric antigen receptor (CAR) T cell therapy has introduced a new and effective strategy to guide and promote the immune response against tumors in the clinic. More recently, in an attempt to enhance its utility, this method has been expanded to novel cell types. One of the more successful variants has proven to be the expression of CARs in Natural Killer (NK) cells (CAR-NK). Gene engineering NK cells to express an exogenous CAR receptor allows the innate anti-tumor ability of NK cells to be harnessed and directed against a target tumor antigen. In addition, the biology of NK cells allows the development of an allogeneic cell therapeutic product useable with most or all patient haplotypes. NK cells cause little or no graft versus host disease (GvHD) and are therefore suitable for development of an "off the shelf" therapeutic product. Initial trials have also shown that CAR-NK cells rarely cause cytokine release syndrome. However, despite their potential NK cells have proven to be difficult to engineer, with high sensitivity to apoptosis and low levels of gene expression. The creation of optimized methods to introduce genes into NK cells will promote the widespread application of CAR-NK in research laboratories and the clinics.

Keywords: natural killer cells, chimeric antigen receptor, gene engineering, transduce, electroporation

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INTRODUCTION

NK cells are a subpopulation of lymphocytes central to the innate immune system and the innate response to viruses. In peripheral blood, ~10% of mononuclear cells are NK cells and are thus readily isolated from density gradient preparations of peripheral blood mononuclear cells (PBMC). NK cells are intrinsically unreactive to foreign major histocompatibility (MHC) molecules. This has made them an attractive alternative for generation of therapeutic cell products, as their insensitivity to antigens presented by MHC allows them to be used in an allogenic context with minimal risk of graft versus host disease (GvHD). Although NK cells carry activating and inhibitory receptors for MHC molecules, the MHC mismatch between graft and host is usually not sufficient to contribute to pathology (1). Recent research indicates, however, that host-specific factors may be important to optimize their potential (2). Furthermore, improvements increasing homing to sites of tumor growth such as bone marrow or to the tumor itself by enhanced chemokine receptors have shown promise (3, 4).

Chimeric antigen receptors (CARs) are receptor proteins that have been engineered to allow specific recognition of a target protein and induction of secondary signaling. The recognition domain is usually derived from the antigen-binding regions of an antibody. This is presented on the cell surface by a

so-called hinge or spacer region providing flexibility. This is then bound by a transmembrane domain to intracellular signaling domains, usually CD3-zeta. Costimulatory domains are also often included intracellularly to enhance signaling and longevity post activation. CARs have mostly been used in T cells to retarget them against tumors. The CAR uses the existing T cell cytotoxic machinery to destroy the targeted malignant cells. The fact that other cell types are also known to attack tumor cells prompted recent interest in CAR expression in non–T cells. The most notable success has been the Natural Killer (NK) cell. NK cells were first identified by their unique ability to kill tumor cells without prior antigen priming. NK cells with CAR receptors (CAR-NK) have now been recognized as a potent tool against cancer (5–8). Initially, the creation of CAR-NK cells was undertaken with constructs developed for CAR-T cells; however, recent developments have incorporated adaptions to NK cells. Nevertheless, NK cells pose special and unique problems. Unlike T cells and B cells, NK cells are not typically clonally expanded, making the generation, maintenance and expansion of CAR-NK cells challenging. The most important stage in the generation of NK-CAR cells is the introduction of the genetic element into the NK cell itself (**Figure 1**) and the subsequent expansion of the CAR-NK cells.

SOURCES OF NATURAL KILLER CELLS

Peripheral-blood derived NK cells can be readily isolated from peripheral blood but are difficult to engineer. The reasons behind this are unclear but include low transduction efficiency combined with poor expansion. In order to avoid this bottleneck NK cells have been generated from induced pluripotent stem cells (iPSC), which can yield many more cells and are more permissive to engineering (9). NK cells can also be generated from umbilical cord blood. NK cells from this source are more readily engineered due to their higher proliferative capacity, as was recently demonstrated in the first published clinical trial of CAR-NK cells (10). Despite this success, a possible disadvantage is the relatively immature nature of cord blood derived NK cells. Several trials have used NK cell lines, particularly cell line NK-92 (8). Cell lines are relatively easy to engineer but are undesirable both due to safety considerations and as they must be lethally irradiated before administration, so they cannot persist in the host and cannot therefore give long-term protection. Recently, feeder cell lines have come into common use to expand NK cells *ex vivo*. These MHC-negative cells lines, in particular K562, are often engineered to express

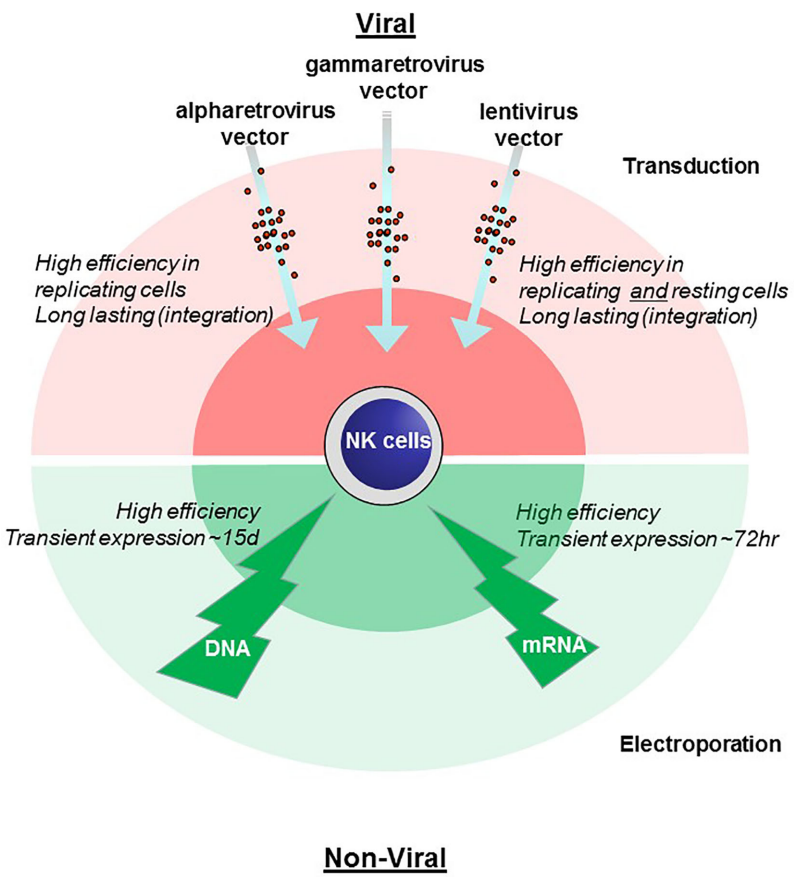

FIGURE 1 | Comparison of methods for genetic engineering of NK cells. Viral transduction (pink, upper) and non-viral electroporation (green, lower) methods of gene engineering NK cells are illustrated with advantages and disadvantages noted.

membrane-bound cytokines (IL-15 and IL-21) and are irradiated before being used (11).

NON-VIRAL CAR-NATURAL KILLER CELL ENGINEERING: mRNA AND DNA TRANSFECTION

In contrast to gene expression *via* viral vectors CAR expression with non-viral–based methods is usually transient, being present for a few days (12). Although long-term expression can be achieved if the sequence can integrate, as is the case with transposon-based systems (13). Typically, nucleic acid introduction is achieved by electroporation, which is a simple and cost-effective method and therefore appropriate for large-scale clinical applications. A major disadvantage is that the permeabilization of the cell membrane by electric pulses can easily result in high cell death rates by unregulated exchange of interior or exterior cell components or the creation of permanent membrane leakage (14).

mRNA TRANSFECTION OF NATURAL KILLER CELLS

Primary CAR-NK generation by mRNA electroporation was initially investigated in 2010 by using an anti-CD19 transgene for transfection in unstimulated and expanded NK cells (15). Expression of the CAR ranged from 33% to 81%. It has been determined that for clinical products expression of a CAR after electroporation is dependent on the dose of mRNA (25–200 µg/ml) and increases the more nucleic acid is applied (16). Interestingly, the viability of expanded cells after one day post-transfection was lower (54%) than for unexpanded cells (64%) (15). However, other groups were not able to transfect peripheral blood or cord blood derived NK cells at transfection rates higher than 10% even with IL-2 stimulation (17). In contrast transfection efficiency for the NK-92 cell line with mRNA encoding an anti-CD19 CAR resulted in higher yields (47.2 ± 8%) (18). More recent protocols have achieved efficient expression of a duration of at least 72 h (3, 19). Recently it has been shown that the co-expression of two transgenes, one being a CAR and the other a chemokine receptor is possible using mRNA electroporation. This yields a transfection rate of 90% modified cells (3). Typically, 5 µg of capped, polyadenylated mRNA is electroporated in a 2-mm cuvette using a voltage of 240–500 V for 4–5 ms (3, 19).

The transfer of mRNA transfection into the clinic has been aided by a recently published protocol for large-scale NK cell expansion in compliance with current Good Manufacturing Practice (cGMP) (20). Large-scale cGMP-compliant electroporation systems such as MaxCyte or CliniMacs are now available for the generation of therapeutic product.

The introduction of CAR mRNA into resting NK cells using a chemical method (charge-altering releasable transporters, or CARTs) has been recently reported (21). This method combined both a higher efficiency compared to electroporation using the 4D nucleofector device and caused less damage and phenotypic change. Finally, CAR-NK cells derived were cytotoxic to CD19+ target cells.

In summary, mRNA transfection is highly efficient but with significant drawbacks. Transfection of complex multigene constructs often necessitates cotransfection of separate mRNA due in part to the difficulty in generating long mRNA sequences. Furthermore, despite its efficiency mRNA is inherently labile, being non-integrative and non-replicative, resulting in a very short period of expression.

DNA TRANSFECTION OF NATURAL KILLER CELLS

Initially, electroporation with DNA was reported successfully for the cell line NK-92 (18) but not for freshly isolated or expanded human NK cells. In a recently published protocol, however, the authors were able to transfect IL-2 expanded primary NK cells by prior optimization of plasmid DNA concentration, target cell number, plasmid size, buffer conditions, voltage, and number and width of pulses (22). Each optimization step contributed to efficiency of transfection with no single element dominating. In contrast, for resting NK cells cell number was of paramount importance with 2–6 × 10^7 cells/ml being the optimal range. After using the new protocol to transfect a first- and second-generation CAR into IL-2 expanded NK cells, 40% transfected cells were observed with a cell viability to up ~60%. This represents a 5-fold increase in efficiency over standard protocols (22). After DNA electroporation the viability of cells is lower compared to mRNA electroporation, probably due to the harsher transfection conditions needed for DNA to reach the NK cell nucleus (18) or due to activation of the innate immune system. Transfected DNA is more durable than mRNA, with expression persisting up to 15 days (23).

To summarize, DNA transfection is less efficient, however complex constructs can be readily introduced and like mRNA non-integrated DNA is self-limiting which thus gives a favorable safety profile compared to viral methods (22).

TRANSDUCTION OF NATURAL KILLER CELLS: VIRAL VECTORS

Transduction refers to the introduction of genetic material *via* viral vectors, including the retroviral and lentiviral-based vectors (24). During the life-cycle of retroviruses viral RNA is reverse transcribed into double-stranded cDNA which is then semi-randomly integrated into the genome of the infected cell (25). For these reasons, this strategy typically takes longer until the gene is expressed. Vectors based on these pathogens have several advantages making it relatively simple to create complex vectors and subsequently reliably introduce them into cells. Typically, these vectors can be up to 10 kb in size without incurring significant

loss of titer during production, allowing inserts of up to 7–8 kbp. Furthermore, as these vectors integrate, this allows the permanent modification of the cell in the absence of antibiotic resistance markers. The modified cells can then be maintained in the host over long periods of time.

The sensitivity of NK cells to foreign genetic material and the stressful process of transduction typically results in low levels of transduction and high apoptosis. Efficiency in transfecting NK cells is therefore relatively low compared to T-cells. This is due to resistance to viral transduction from innate defense mechanisms guided by pattern recognition receptors recognizing foreign genetic material (26, 27). In order to prevent this inhibitory chemicals such as BX795, which inhibits PDK1, can be added during transduction. This blocks activation of signaling pathways mediated by RIG-I like receptors or Toll-like receptor 3 (28). It has been demonstrated that lentiviral transduction efficiency can be enhanced ~4 fold by this strategy (29). Nevertheless, it is sometimes necessary to use multiple rounds of transduction to achieve an adequate transgene expression (30).

In order to increase the safety profile of viral vectors, expression systems have been created where the envelope protein is expressed on a separate plasmid. This allows safe generation of modified viral particles with exogenous envelope proteins, in a process termed pseudotyping. One way of enhancing transduction of NK cells is the selection of the best pseudotyping envelope protein. The commonly used vesicular stomatitis virus (VSV) glycoprotein G is usually highly efficient, as it binds to the LDL receptor which is present in a wide variety of cells (31). However, it is inefficient for infection of lymphoid cells, requiring high viral titers which in turn are often toxic to the cells. The choice of envelope proteins from lymphotropic viruses such as measles or baboon retrovirus, however, has been shown to improve both transduction and integration (32). Successful transduction also depends on diffusion of the virus to the cell surface and adsorption into the target. Enveloped viral particles are typically negatively charged as they derive from the cell membrane. This results in repulsion of virion and cell, interfering with transduction. Cationic polymers such as hexadimethrine bromide (polybrene) or protamine sulfate are used to neutralize the negative charge of the virion, allowing better adsorption efficiency and membrane fusion (33). An alternative strategy is to enhance colocalization of cells and virus by using crosslinking agents such as Retronectin or Vectofusin-1. These have been reported to outperform their cationic polymer counterparts (17, 34). Retronectin, a chimeric peptide derived from fibronectin, promotes the interaction and colocalization between virus particles and counterparts on the cell surface (34). Similarly, Vectofusin-1 facilitates the adhesion and the fusion of the virus with the cellular plasma membrane, although the concrete mechanism remains unclear (35). There is no consensus on which enhancer shows greater optimization of transduction (35, 36).

RETROVIRAL VECTORS

Vectors generated from the related alpharetroviral and gammaretroviral viruses have been used to transduce primary lymphatic cells, including NK cells (30, 37). A significant limitation for retroviral transduction is that retroviral cDNA can only integrate into the NK cell genome during mitosis when the nuclear membrane dissolves. This requirement is particularly problematic in non-replicating primary cells, but less so for activated NK cell lines (38).

LENTIVIRAL VECTORS

Lentiviral vectors are considered genetically more complex and in contrast to their retroviral counterparts can integrate their genetic information into non-dividing cells (39). Recent data shows that the performance of lentiviral vectors in generating CAR-NK cells depends on the envelope protein they express. For the commonly used VSV-G envelope proteins the highest transduction efficiency of primary NK cells using VSV-G pseudotyped particles was found with lentiviral vectors compared to retroviral vectors (34). Which envelope protein has the best performance is unclear as the transduction efficiency for lentiviral vectors pseudotyped with VSV-G or feline endogenous retrovirus envelope protein RD114-TR was similar for primary human NK cells (34). Another group found that a lentiviral/VSV-G vector produced less CD19-CAR expressing cells compared to a RD114-TR pseudotyped lentiviral vector (36). Furthermore, a Baboon envelope pseudotyped lentiviral vector BaEV-LV was significantly better than both the RD-114-TR as well as VSV-G pseudotyped lentiviral vector (32). VSG-V binds to the low density lipid receptor LDL-R which is poorly expressed on activated NK cells (5). In contrast, RD114 binds to the sodium-dependent neutral amino acid transporter ASCT-1 and ASCT-2, as does the BaEV envelope protein (40–42). The ASCT-1 transporter is strongly expressed on NK cells and ASCT-2 is upregulated after activation of NK cells with IL-2 and IL-15 (5). The BaEV envelope protein has also been shown to bind to the glycosylated form of ASCT-1 in contrast to RD-114-TR. This may explain its greater efficiency in transduction (42).

It has been reported that lentiviral transduction can be further optimized by using spinfection, a method where centrifugation at a low RPM is applied. Spinfection transduction rates range from 19% to 73% with CD19 CAR lentiviral transduced cord blood derived NK cells, compared to a range from 12% to 30% for static transduction (17).

In terms of optimization for transduction protocols Müller et al. compared alpharetroviral vectors with lentiviral vectors. A RD114-TR pseudotyped alpharetroviral vector could achieve 82.9% of NKs transduced with CD19 CAR using Vectofusin-1–based transduction (36). A similar comparative study of viral vectors from Suerth et al. confirms the superiority in transfection efficiency when a RD114-TR pseudotyped alpharetroviral vector was used. Although transduction was performed with Rectofusin both transduction enhancers reliably optimized transduction efficiency (34). It is thought that Vectofusin-1 might be beneficial for large-scale expansion due to a simpler usage (36). Because of the stable high rate of transgene expression by using a RD114-TR alpharetroviral vector Kellner et al. established a protocol for

production of genetically modified NK cells compliant with GMP. Following the instructions, transduction procedure resulted in >90% CAR transduced cells (43). In order to achieve this efficiency proliferating NK cells were transduced using retronectin. A possible drawback of this method, however, is the use of the K-562 cell line to expand the NK cells both prior and post transduction. Furthermore, a relatively simple CAR was used to transduce (43). In conclusion, there is no currently available gene transfer method that is universally applicable. All have advantages and drawbacks which we have illustrated in **Figure 1**.

OUTLOOK

Several novel methods to introduce genes into NK cells are under development. These include alternative viral vectors with a higher safety profile such as adenovirus associated virus (AAV) vectors. Advances in mRNA generation and electroporation technology will also bring improved transfection. Combining the two methods, for example with transposons or with CRISPR/Cas9-based integration, would combine long term expression with the efficiency of electroporation. Transposon technology in particular offers integration without complex, expensive, and potentially dangerous viral transduction systems. One disadvantage that remains with this system is its relative inefficiency. Finally, improvement in primary NK expansion protocols will also bring an improvement in gene engineering, as healthy proliferating cells are more readily engineered. The vectors themselves will also be become optimized to the NK microenvironment, for example by using DAP12 or NKG2D signaling domains. Which construct is best suited for NK cells is a focus of current research.

The durability of the CAR-NK cells within the host is also currently a matter of debate. CAR-T cells can persist in the host for years and there is clear data showing that this contributes to tumor clearance. Whether this is the case for CAR-NK cells is unclear. It is currently accepted that allogenic NK cells have a relatively short half-life (44), although persistence of CAR-NK cells *in vivo* for at least 1 year has been recently reported (10). Long-term follow up of clinical trials of primary NK cells will elucidate if persistence of CAR-NK cells contributes to therapeutic efficacy.

CONCLUDING REMARKS

Although NK cells have many desirable characteristics, there remain significant problems in the production of CAR-NK cells for therapeutic purposes. The introduction of foreign genetic material and subsequent expansion of the NK cells is difficult, making the development of feasible and reproducible GMP protocols a challenge. The viability of the CAR-NK cells is central to the success of the therapeutic product, as the long-term persistence of tumor-specific CAR cells in the host is thought to promote the therapeutic efficacy.

Currently, the most successful alternatives for introducing genes into NK cells are either rapid transient expression by electroporation or slow sustained expression by viral vectors. The correct choice of transfection protocol is thus an important element in the design and execution of a successful clinical trial.

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Proceedings From the First International Workshop at Sidra Medicine: "Engineered Immune Cells in Cancer Immunotherapy (EICCI): From Discovery to Off-the-Shelf Development", 15th–16th February 2019, Doha, Qatar

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The progress in the isolation and characterization of tumor antigen (TA)-specific T lymphocytes and in the genetic modification of immune cells allowed the clinical development of adoptive cell therapy (ACT). Several clinical studies highlighted the striking clinical activity of T cells engineered to express either Chimeric Antigen (CAR) or T Cell (TCR) Receptors to target molecularly defined antigens expressed on tumor cells. The breakthrough of immunotherapy is represented by the approval of CAR-T cells specific for advanced or refractory $CD19^+$ B cell malignancies by both the Food and Drug Administration (FDA) and the European Medicinal Agency (EMA). Moreover, advances in the manufacturing and gene editing of engineered immune cells contributed to the selection of drug products with desired phenotype, refined specificity and decreased toxicity. An important step toward the optimization of CAR-T cell therapy is the development of "off-the shelf" T cell products that allow to reduce the complexity and

the costs of the manufacturing and to render these drugs available for a broad number of cancer patients. The Engineered Immune Cells in Cancer Immunotherapy (EICCI) workshop hosted in Doha, Qatar, renowned experts, from both academia and industry, to present and discuss the progress on both pre-clinical and clinical development of genetically modified immune cells, including advances in the "off-the-shelf" manufacturing. These experts have addressed also organizational needs and hurdles for the clinical grade production and application of these biological drugs.

Keywords: cancer, immunotherapy, CAR-T cells, TCR engineered lymphocytes, CAR-NK cells, monoclonal antibody, clinical trial, off-the-shelf development

INTRODUCTION

Cancer immunotherapy is aimed at a driving patient's immune system to attack tumor cells. The great advances achieved in this field during the last two decades, lead to the emerging role of immunotherapy as the "fifth pillar" of cancer treatment, together with surgery, chemotherapy, radiotherapy and targeted therapy (1).

Adoptive cell therapy (ACT) with tumor antigen (TA)-specific T lymphocytes has been clinically developed at an unparalleled pace (2–4). In particular, the approach of the genetic engineering of T or NK cells to target and destroy cancer cells, revealed as powerful and, in some cases, unprecedented in term of clinical success for the treatment of patients with aggressive malignancies, refractory to other therapeutic interventions (5–7).

T cells engineered with chimeric antigen receptors (CARs), that combine the antigen binding region of antibodies and T cell signaling domains responsible for activation (8), represented the breakthrough of cell-based immunotherapy (1, 2, 3). Different CARs have been engineered to target a variety of antigens expressed by either hematologic or solid tumors, that are listed by Sadelain and colleagues (9). The initial clinical application of CAR-T cells was quite disappointing in terms of patients' responses, due to the inefficient expansion and persistence of CAR-T cells *in vivo* (10–12). Additional modifications of the structure of CARs, by including co-stimulatory domains allowed the achievement of clinical benefit through the treatment of patients with B cell malignancies overexpressing CD19 (13–18). Strikingly response rates in the range of 57%–82%, with complete response rate of 52-60%, were detected upon the infusion of CD19-CAR-T cells in patients with B cell malignancies refractory to prior treatments (7, 8, 9). These results led to the accelerated approval by both FDA and EMA of two drug products: 1. tisagenlecleucel/Kymriah for the treatment of children and young adult with acute lymphoblastic leukemia (ALL) (13, 14, 19–21), and for the treatment of adults with relapsed/refractory Diffuse Large B cell lymphoma (DLBCL) (22).

Axicabtagene Ciloleucel/Yescarta for the Treatment of Adult Patients With Relapsed/Refractory Non-Hodgkin Lymphoma (NHL), including **Table 1** (14, 16, 18).

More recently, a third product, brexucabtagene autolucel (Tecartus) has been approved for the treatment of relapsed or refractory mantle cell lymphoma. This approval was granted based on the results of the ZUMA-2 (NCT02601313) clinical trial, showing 87% of ORR, with a complete remission (CR) rate of 62% (23).

CAR-T cells represent promising therapeutic options also for solid tumors, although they are still under clinical development and do not yet have proven their clinical efficacy (**Table 1**) (24). The principal limitations for their clinical activity are: i. paucity of tumor specific antigens and ii. the low efficiency of T cells in penetrating the tumor microenvironment and homing to the tumor site, and iii. their limited functional activity within the tumor (24).

In addition, efforts are ongoing at different leading groups to develop allogeneic CAR-T cell therapies, in order to simplify the manufacturing process, to reduce the costs and rendering these drugs available to larger cohorts of patients (**Table 1**).

Abbreviations: AAV, Adeno-Associated Virus; ACT, Adoptive Cell Therapy; ADCC, Antibody-Dependent Cell-Mediated Cytotoxicity; BMT, Bone Marrow Transplant; CAR, Chimeric Antigen Receptor; CCR7, C-C Chemokine Receptor Type 7; CD1 a, b, c, d, family of glycoproteins expressed on the surface of various human antigen-presenting cells and related to HLA class I molecules; CD19, IgSF surface glycoprotein of 95 kDa expressed on B cells; CD20, B-lymphocyte antigen CD20; CD22, B-lymphocyte cell adhesion molecule; Sialic acid-binding Ig-like lectin 2 (SIGLEC-2); CD28, Cluster of Differentiation 28, T-cell-specific surface glycoprotein; CD40/CD40L, Cluster of differentiation 40/Ligand of CD40; CD52, Campath-1 antigen; CD54, Cluster of Differentiation 54 or Intercellular Adhesion Molecule 1 (ICAM-1); CD58, Lymphocyte function-associated antigen 3 (LFA-3) CD70, Cluster of Differentiation 70; CXCR4, C-X-C Chemokine Receptor Type 4; CD80, Cluster of differentiation 80 (also B7-1); CD86, Cluster of differentiation 86 (also B7-2); Cy, Cyclophosphamide; COSMID, Database of Genomic Structural Variation; CRC, Colorectal Cancer; CRISPR/Cas9, (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas9); CTLA-4, Cytotoxic T lymphocyte Antigen 4; EGFR, Epidermal Growth Factor, Fas, Death Receptor that regulate apoptosis; FcγR, Fc Gamma Receptor; Flu, Fludarabine; GMP, Good Manufacturing Practice; GvHD, Graft versus Host Disease; HLA, Human Leukocyte Antigen; HSCT, Allogenic Hematopoietic Stem Cell Transplant; IL-6, Interleukin 6; IL-7, Interleukin 7; IL-18, Interleukin 18; KO, Knock out; LAG-3, Lymphocyte Activation Gene-3; LDH, Lactate Dehydrogenase; LV, Lentiviral vector; MCP-1, Monocyte Chemoattractant Protein-1; MEGATAL, Meganucleases that have been fused with a Transcription Activator-Like (TA) containing Repeat Variable Residues (RVD); NK, Natural Killer; OS, Overall Survival; PD-/PD-L1, Programmed Cell Death-1/ Ligand; RAS, Rat Sarcoma; RV, Retroviral vectors; scFV, Single-Chain Variable Fragment; TAA, Tumor-Associated Antigen; TCGA, The Cancer Genome Atlas; Tp53, Tumor Protein 53; TSCM, T Cell Memory Stem; TCR, T Cell Receptor; TCR$\alpha\beta$, T Cell Receptor alpha beta; TME, Tumor Microenvironment; TRAC, T-cell receptor α constant locus; TRBC1, T Cell Receptor Beta Constant 1; TRBC2, T Cell Receptor Beta Constant 2; TIM3, T cell immunoglobulin and mucin domain- containing protein 3; UCAR, Universal Chimeric Antigen Receptor; UCB, Umbilical Cord Blood; 4-1BB, activation-induced costimulatory molecule (CD137).

TABLE 1 | Summary of principal clinical studies of CAR-T/NK cells.

Trial registration N.	Sponsor	Country	Agent/ cell source and type	Indication	Note
NCT03601442	Novartis Pharmaceuticals	US	LV CAR-T (CTL019)	B-ALL/B cell lymphomas	Available in the market (Kymriah)
NCT02348216	Kite Pharma	US	RV CAR-T (KTE-C19, Axi-cel)	B-ALL/DLBCL	Available in the market (Yescarta)
NCT02601313	Kite Pharma	US	2nd generation -Autologous anti-CD19 CAR-T cell	Mantle Cell Lymphoma	Available in the market (Tecartus)
NCT01044069	Kite Pharma	US	2nd generation -Autologous anti-CD19 CAR-T cell	CLL,	Phase 1/2
NCT01593696	NCI	US	2nd generation -autologous anti-CD19 CAR-T cell	NHL, ALL, CLL	Phase 1/2
NCT00586391	Baylor College of Medicine, Texas Children's Hospital	US	2nd generation -Autologous anti-CD19 CAR-T cell	Non-Hodgkin's lymphoma, ALL, CLL	Phase 1/2
NCT00924326	NCI	US	Anti-(CD19)-CAR PBL	Primary Mediastinal B-cell Lymphoma, Diffuse Large B-cell Lymphoma, mantle cell	Phase 1/2
NCT01626495	Children's Hospital of Philadelphia, University of Pennsylvania	US	2nd generation -Autologous anti-CD19 CAR-T cell	B-ALL, CD19+ Leukemia and Lymphoma	Phase1/2
NCT03056339	M.D. Anderson Cancer Center	US	Allogeneic CD19-CD28-zeta-2A-iCasp9-IL15-transduced cord blood-NK cells	Refractory B cell Malignancies	Phase 1
NCT02808442	Institut de Recherches Internationales Servier	France, Japan, UK and US	Allogeneic UCAR19	Pediatric patients with relapsed or refractory $CD19^+$ B-ALL	Phase 1
NCT02208362	City of Hope Medical Center	US	Autologous anti-IL13Rα2 CAR-T cell	Glioblastoma	Phase 1
NCT00902044	Baylor College of Medicine	US	Autologous HER2-CAR-T cells	Sarcoma	Phase 1
NCT02107963	NCI	US	3rd generation Autologous anti-GD2-CAR-T cells	Sarcoma, Osteosarcoma, Neuroblastoma, Melanoma	Phase 1
NCT04097301	MolMed	Italy	Autologous CD44v6 CAR T-cells-HSV-TK Mut2 gene	Multiple Myeloma	Phase 1/2
–	National Cancer Institute	US	(EGFRv)III Chimeric antigen receptor (CAR) transduced PBL	Malignant Gliomas Expressing EGFRvIII (Astrocytoma, Glioblastoma, Glioma, Gliosarcoma, Brain Neoplasms)	Phase 1/2
–	Glycostem Therapeutics		Allogeneic cord blood-derived CD19-NK cells (oNKord®)	Elderly AML	Phase 1
NCT03294954	Baylor College of Medicine	US	2nd generation -anti-GD2-$IL15^+$CAR-NKT cells	Neuroblastoma	Phase 1

AML, Acute Myeloid Leukemia; BCMA, B-Cell Maturation Antigen; CD44v6, CD44 Variant 6; GD2, Disialoganglioside; HSV-TK Mut2, Mutated Herpes Simplex Virus Thymidine Kinase; IL13Rα2, Interleukin 13 Receptor Subunit Alpha 2; LV, Lentiviral Vectors; NCI, National Cancer Institute; NKG2D, C-Type Lectin-Like Receptor RV, Retroviral Vectors; UK, United Kingdom; US, United States; ALL, Acute lymphoblastic leukemia; CLL, Chronic Lymphoblastic Leukemia.

Promising results have been obtained also for the alternative approach of engineering T lymphocytes with TA-specific T Cell Receptor (TCR) for the treatment of solid tumors and multiple myeloma, including patients with advanced metastatic malignancies (**Table 2**) (2, 4, 25–31). This methodology has been applied for the generation of high avidity tumor-specific T cells. The choice of the targeted antigens is critical for the efficacy of this type of therapy and to prevent off-target reactivity (32, 33). The targeting of overexpressed antigens and neo-antigens, that are not shared with non-embryonic normal tissues, can lead to the cell-mediated killing of tumor cells without severe toxicities due to the targeting of normal tissue(s) (27–29, 34, 35).

Gene editing through the targeted knock out of genes or the insertion of suicide genes represents an innovative strategy that has been applied to tumor-specific engineered immune cells with the aims of either increasing their specificity and *in vivo* persistence or decreasing the induction of possible toxicities or allogeneic rejection, respectively (36–39). The application of this technique allowed also to generate universal/"off-the-shelf" CAR-T cells using the peripheral blood of healthy volunteers as source of immune cells (40–47). This strategy is currently under clinical development with few clinical trials ongoing in EU and USA (42, 44, 48).

All the topics mentioned above have been addressed in a comprehensive manner in the context of the first international

TABLE 2 | Summary of principal clinical studies of TCR-engineered T cells.

Trial registration	Sponsor	Country	Agent/ cell source and type	Indication	Note
NCT01967823	NCI	US	Anti-NY-ESO-1 mTCR	NY-ESO-1 Expressing Cancers	Phase 2
NCT02111850	NCI	US	Anti-MAGE-A3-DP4 TCR	Various solid metastatic tumors (Targeting MAGE-A3) in HLA-DP04 positive patients	Phase 1/2
NCT02408016	Fred Hutchinson Cancer Research Center/NCI	US	WT1-TCRc4 Tcm/Tn Lymphocytes	Non-small Cell Lung Cancer, Mesothelioma	Phase 1/2
NCT01621724	Cell Medica Ltd/University College, London/Cell Therapy Catapult	UK	WT1 TCR-	Leukaemia (AML CML)	Phase 1/2
NCT03250325	GlaxoSmithKline/ Adaptimmune	US	NY-ESO-1 TCR	Synovial Sarcoma	Phase 1/2
–	GlaxoSmithKline/ Adaptimmune	US	NY-ESO-1 TCR	Non-small Cell Lung Cancer	Phase 1/2
–	GlaxoSmithKline/ Adaptimmune	US	NY-ESO-1	Ovarian cancer	Phase 1/2
NCT03139370	Kite Pharma	US	KITE-718 (MAGE-A3/A6) TCR	MAGE-A3/A6-positive solid tumors	Phase 1/2
NCT03399448	Parker institute for Cancer Immunotherapy/ University of Pennsylvania	US	NY-ESO-1 TCR	Multiple myeloma, Sarcoma, Melanoma	Phase 1/2
NCT02840110	Unum Therapeutics Inc.	US	ACTR TCR	Multiple Myeloma	Phase 1/2
NCT02743611	Bellicum Pharmaceuticals	US	BPX-701/PRAME-TCR	AML, MDS, metastatic Uveal melanoma	Phase 1/2
NCT03247309	Immatics Biotechnologies (Tuebingen, Germany)/ M.D. Anderson Cancer Center	US	IMA-201	Various solid tumors (Head and Neck Squamous Cell Carcinoma, Non-small Cell Lung Cancer)	Phase 1
NCT03912831	NCI/Kite Pharma	US	KITE-439	HPV16+ Tumors (relapsed or refractory)	Phase 1
–	Fred Hutchinson/Juno	US	Anti-mesothelin TCR	Pancreatic cancer	
NCT01567891	Adaptimmune	US	Enhanced TCRs Specific for NY-ESO-1	Refractory Ovarian Cancer	Phase 1/2
NCT03412877	NCI	US	Autologous T-Cells Genetically Engineered to Express T-Cell Receptors Reactive Against Mutated Neoantigens in People With Metastatic Cancer	Glioblastoma Non-Small Cell Lung Cancer Ovarian Cancer Breast Cancer Gastrointestinal/Genitourinary Cancer	Phase 2
NCT03907852	TCR^2 Therapeutics	US	Autologous genetically engineered T cells expressing a single-domain antibody that recognizes human Mesothelin (TC-210 T cells)	Mesothelioma Cholangiocarcinoma Recurrent Ovarian Cancer Non Small Cell Lung Cancer Non Small Cell Lung Cancer Metastatic	Phase 1/2
NCT04323657	TCR^2 Therapeutics	US	Autologous genetically engineered T cells expressing a single-domain antibody that recognizes human CD19 (TC-110 T cels)	Non Hodgkin Lymphoma ALL DLBCL Primary Mediastinal Large B Cell Lymphoma CML FL	Phase 1/2

HPV, Human Papilloma Virus; EGFRVIII, Epidermal Growth Factor Receptor Variant III; NY-ESO-1, New York Esophageal Squamous Cell Carcinoma 1; PRAME, Melanoma Antigen Preferentially Expressed in Tumors; MAGE, Melanoma Antigen; WT1, Wilms Tumor 1; NCI, National Cancer Institute; US, United States; AML, Acute Myeloid Leukemia; CML, Chronic Myelogenous Leukemia; DLBCL, Diffuse Large B Cell Lymphoma; FL, Follicular Lymphoma.

workshop in Doha "Engineered immune cells in cancer immunotherapy: from discovery to off-the-shelf development" (15^{th}–16^{th} February 2019, Doha, Qatar). Renowned speakers from both academia and industry who pioneered the field gathered in Doha bringing high level discussions on scientific and clinical advances and bringing the participants at the forefront of this rapidly evolving topic. A satellite mini-symposium at the initial opening of the workshop, through its educational contents, has provided basic knowledge of cancer immunology, immunotherapy and cell-based therapies to health care practitioners, researchers and students. Poster sessions have also offered the opportunity for active involvement of young researchers and under-graduate students. This report summarizes key data and highlights from each session.

THE CLINICAL EVOLUTION OF CAR-T CELL THERAPY

During the past few years, CAR-T cells, either CD28/CD3ζ (4, 5) or 4-1BB/CD3ζ CARs (6), targeting $CD19^+$ B cell malignancies have demonstrated safety and clinical activity in the context of multiple Phase I/II clinical trials (68). These products have been used for the treatment of either pediatric and adult patients with relapsed or refractory ALL, showing high CR rate (49–54). Similarly, CD19-CAR-T cells showed impressive clinical activity in relapsed/refractory pediatric-adolescent or adult Non-Hodgkin Lymphoma (NHL) with 40-63% of CR (58, 60, 68–70). These unprecedented results lead to the rapid approval by both FDA and EMA and the commercialization of these Advanced Therapeutic Medicinal Products (ATMPs) (61, 62).

Associate Prof. Cameron Turtle (Fred Hutchinson Cancer Research Center, USA) kicked off the first session of the workshop with a keynote lecture summarizing the aforementioned results and focusing on the factors impacting the response to CD19 targeting CAR-T cell immunotherapy in adults with ALL and NHL in a clinical trial at Fred Hutchinson Cancer Research Center, Seattle, WA. Different factors were found to affect the patients' clinical responses, including the dose of infusion of CAR–T cells, their cellular expansion and persistence *in vivo*, the tumor burden, and the conditioning regimen used for lymphodepletion (49, 61). Low levels of circulating CAR-T cells were identified as one of the mechanisms of resistance to CAR-T cell therapies. In some ALL patients, antigen loss was observed in tumor cells following the treatment with CD19-CAR-T cells, as a consequence of either missense mutations or alternative splicing of the CD19 encoding gene (63–65). In ALL patients low levels of lactate dehydrogenase (LDH) and normal platelet counts in the blood prior to lymphodepletion followed by the treatment with cyclophosphamide/fludarabine (Cy/Flu) compared to Cy regimen alone were identified as factors associated with better disease-free survival (DFS) (61). Furthermore, DFS was improved in ALL patients who achieved minimal residual disease (MRD)-negative CR after CAR-T cell therapy and underwent allogeneic hematopoietic stem cell transplantation compared to those who did not undergo transplantation. In NHL patients, high levels of serum LDH before lymphodepletion, and low MCP-1 and IL-7 before or shortly after CAR-T cell infusion were associated with shorter progression-free survival after treatment with CD19-CAR-T cells (66).

CD19-CAR-T cells represented salvage therapy also for patients diagnosed with NHL, including advanced DLBCL, primary mediastinal B-cell lymphoma (PMBCL) and follicular lymphoma (FL). Complete responses (CR), 49%–71%, have been observed in these patients, with a median (overall survival) OS greater than 2 years (16, 18, 22, 59, 67). FDA and EMA approval for axicabtagene ciloleucel in relapsed and refractory DLBCL was granted based on results of the multi-center Phase 2 ZUMA-1 trial, presented in a keynote session by Dr. Frederick L. Locke (Moffitt Cancer Center). Lisocabtagene maraleucel (Liso-cel; JCAR017) represents another CAR-T-cell-based therapy for aggressive NHL patients that is composed by 1:1 ratio of both $CD4^+$ and $CD8^+$ T cells. In the context of the multicenter TRANSCEND-NHL-001 trial, an overall response rate (ORR) of 73% [95% CI, (67–78)] was observed. The CR rate was 53% [95% CI, (47–59)] with a median follow-up of 12 months (67). It appears lower frequencies of toxicities may have occurred in the TRANSCEND trial, as well as the JULIET trial that tested tisagenlecleucel in DLBCL; both of these therapeutic products contain the 4-1BBζ costimulatory molecule, in contrast to axi-cel which contains a CD28ζ costimulatory molecule (58, 67–69). It should be noted that these trials used different toxicity grading systems, so comparisons should be interpreted with caution.

The usage of mathematical and statistical modeling to evaluate the efficacy of CD19-CAR-T cell-based clinical trials, revealed that multiple factors, including the frequency of memory T cells in the circulation, the tumor burden and the inflammatory cytokine profile, can affect the extent and duration of patients' clinical responses (70). The most frequent toxicities attributed to CAR-T cells are the cytokine release syndrome (CRS) and neurologic events. In the majority of cases they are reversible. CRS can usually be controlled by the administration of tocilizumab (anti-IL-6 mAb) with or without corticosteroids (69–71). Therapies for neurotoxicity are less well-defined, but usually involve anti-seizure prophylaxis and corticosteroids, with addition of tocilizumab (anti-IL-6 mAb) only if there is concurrent CRS. Patients presenting with severe pro-inflammatory conditions, quantified by serological increase in C-reactive protein and ferritin, before the injection of CAR-T cells appear to be at higher risk for toxicities and might benefit from treatment with immunomodulatory agents before the toxicities become severe (18, 66, 71–73). Safety cohorts of ZUMA-1 tested at early stage and the prophylactic administration of tocilizumab, showed reduced frequency of severe CRS but not neurotoxicity. In this study, the development of neurological toxicity was associated with higher level of $CD14^+$ myeloid cells and $CD4^+$ CAR-T cells in the cerebrospinal fluid (CSF), again implicating a pro-myeloid inflammatory state associated with CAR T toxicity (66, 71–74). These observations highlight that multiple factors could affect patients' outcome and the development of toxicities; further studies are required to confirm the role of the association of these parameters as biomarkers predicting the risk to develop toxicities upon CAR-T cell treatment.

THE HEMATOLOGY/ONCOLOGY LANDSCAPE IN QATAR

According to registry data from 2015, leukemia represents the 5^{th} most common malignancy in Qatar across genders and nationalities. NHL, including DLBCL, is the 4^{th} most common cancer among males and represents the most frequent lymphoid malignancy in Qatar with about 46% of all lymphoid malignancies. The average OS of DLBCL patients has been reported as 64% between 2013 and 2017. However, 30% to 40% of patients has a high risk of relapse from the first line of chemotherapy treatment, and 10% to develop refractory disease,

with poor prognosis and a median survival lower than 1 year (75). Dr. Ruba Y. Taha (NCCCR-HMC, Qatar) summarized the available therapeutic options for these patients, including the treatment with platinum- and cytarabine-based regimens alone or prior to autologous HSCT (PARMA prospective multicenter study) (76). Patients enrolled in this study with early relapses showed similar clinical outcome as incomplete responders (77). However, these patients have superior clinical benefit from the usage of monoclonal antibodies targeting antigens expressed by blasts (CD19, CD20, CD22, and CD52) as compared to chemotherapy or targeted therapy (53). Therefore, the availability in Qatar of CAR-T cell therapy targeting the aforementioned antigens will provide further options to improve the OS of patients with relapsed/refractory DLBCL. A limited number of patients can benefit from this type of therapy only upon admittance to international clinical centers.

Qatar Cancer Society (QCS) is a charity that was founded in 1997 dedicated to implement the research in this field and orchestrate a community partnership platform to make Qatar among the leading countries in cancer prevention and burden control (Dr. Hadi Mohamad Abu Rasheed, QCS, Qatar). In this context, QCS is facilitating the access of Qatari patients to innovative therapies such as CAR-T cells. A testimonial of a patient with a diagnosis of NHL who underwent a successful CD19-CAR-T cell treatment in US, was presented during the conference, corroborating the need of implementing local efforts and research in this field.

The pediatric cancer genome project showed the differential make-up of driver mutations and related aberrant molecular pathways in these patients as compared to adult cancer patients' profile (78). These evidences indicate the need to deeply understand the disease's mechanisms of children with cancer in an attempt to allow early detection and intervention. The comprehensive biological characterization of pediatric leukemia ongoing at Sidra Medicine (Dr. Chiara Cugno, Sidra Medicine, Qatar) is aimed at identifying dysregulated signaling pathways to contribute to the development of precision medicine treatments. These investigations might also lead to identifying novel target molecules for CAR-T cells or the optimization of targeting multiple antigens in relationship with the evolution of the disease and possible development of resistance to this therapy (79, 80).

TARGETING TUMORS WITH TCR ENGINEERED T LYMPHOCYTES

ACT studies with T cells engineered with TA-specific TCR, provided evidences of their clinical success (**Table 2**) (2, 28–31, 34, 81, 82). Nevertheless, the improvement of the efficacy, the tumor specificity and strategies to overcome the competition in the synthesis of TCR between exogenous and endogenous alpha and beta chains are subjects of multiple novel investigations. In addition, the usage in most of the cases of epithelial-derived TAAs, that are shared with normal tissues can induce "off-target" toxic effects. The accurate choice of TAAs selectively expressed by tumor cells and/or by the associated blood vessels and microenvironment can overcome the unwanted unspecific targeting and the related toxicities.

These topics were addressed by the presentations, that are summarized in paragraphs 4 and 5, discussing advanced tools to implement the engineering of T and NKT lymphocytes and refining the selection of TAs to redirect the cell-mediated responses.

Prof. Chiara Bonini, (San Raffaele Scientific Institute, Milan, Italy) presented the application of genetic knock-out (KO) and gene substitution approach to introduce TA-specific TCRs in T lymphocytes. This aim was achieved through the transient exposure of T cells to alpha and beta chain specific Zinc Finger Nucleases (ZFNs), followed by the introduction of lentiviral vectors (LV) encoding for a "novel" TCR (83). This study was focused on targeting the leukemia-associated TA Wilms' tumor 1 (WT1), that is a zinc finger DNA-binding protein acting as transcription factor and playing an important role in cell growth and differentiation of cells (84). The enrichment of T cell memory stem (TSCM) (85, 86) cells that are "younger" cells with persistence and survival properties and delayed ageing and exhaustion (87) occurred for TA-specific T cells. This population of T cells was found to be enriched in the circulation of patients following HSCT and the infusion with T lymphocytes genetically modified to express the thymidine kinase (TK) suicide gene (88, 89). This phenomenon was observed also following long term treatment with engineered T cells (87), indicating that both antigen exposure and the phenotype of the infused cells can affect their *in vivo* survival and the diversification of their immunological memory (89). ACT can benefit from HSCT platforms followed by the infusion of TCR engineered with gene editing technology in inducing the Graft-versus-tumor (GvT) effect in hematological malignancies (90).

The transfer of lipid specific TCRs into T cells represents another innovative approach for the ACT of leukemia. T cells can recognize lipid antigens presented by MHC class I-related CD1 molecules (CD1a, b, c, d). These T cells are involved in antimicrobial immunity and, in case of reactivity against CD1-presented self-lipids, in autoimmunity and cancer immunosurveillance (91). Dr. Giulia Casorati (San Raffaele Scientific Institute, Milan, Italy) demonstrated that primary acute myelocytic leukemia (AML) leukemia and B-ALL blasts express CD1c and are recognized by a group of CD1c self-reactive T cells specific for methyl-lysophosphatidic acids (mLPAs). mLPAs is a self-lipid antigen highly enriched in malignant cells that might play a role in leukemia growth. Lipid antigens are also less susceptible to mutation and immune mediated selection (92, 93). The anti-tumor activity *in vitro* and the control of the progression *in vivo* of leukemia cells by T cells engineered with mLPA-specific TCRs has been assessed, showing that these T cells could represent novel and efficient tools for ACT for leukemia patients either in the prophylactic setting at the time of HSCT or at post-transplant relapse of the disease (93).

A novel high-throughput technology, XPRESIDENT®, has been applied to identify and qualify peptides derived from TAAs (Dr. Ali Mohamed, Immatics, Tubingen, Germany and Houston,

TX, USA). This platform, through the combination of ultra-sensitive mass spectrometry (LC-MS/MS) with quantitative transcriptomics, was able to identify the differential expression of HLA-bound peptides in tumor vs. normal tissues. Taking advantage of this platform, several T cell-based therapy programs have entered clinical development. These include the targeting of multiple TAAs with T cells expressing either native or exogeneous TCR. A robust clinical grade manufacturing process has been developed for autologous TCR transduced T cells that implied 7–10 days of manufacturing, (IMA201 and IMA202) or 5–6 days (IMA203), shortening the timeline required for the production in autologous setting. An interesting strategy was based on the engineering of allogeneic γδ T cells, that display inherent anti-tumor activity, the ability to infiltrate tumor tissues and MHC-independent reactivity, with exogenous TA-specific αβ TCR (ACTallo® IMA301). Therefore, these cells acquire αβT cell features in the absence of the risk to develop allogeneic reactions or GvHD due to HLA mismatching (94, 95). Additionally, the development pipeline includes T-cell Engaging Receptors (TCER®s). These bispecific TCR molecules are soluble fusion proteins with two binding domains: (i) a TCR domain that recognizes a tumor-specific peptide presented by HLA class I, and (ii) a T-cell recruiting antibody domain, which allows the recruitment and activation of T cells to attack the tumor.

OPTIMIZING THE DELIVERY *IN VIVO* OF CAR-T CELLS AND THEIR REDIRECTION TO TUMORS

The gene transfer platforms for therapeutic application can occur either by isolating *ex vivo* the cells for modification and the subsequent re-infusion in the patients or *in vivo* through viral vectors (LV or adenovirus, AAV). The vector delivery can be controlled *via* particle-receptor interactions. The engineering process for specific targeting involves the mutation of the receptor binding sites and adding the desired target domains in these regions (96). Designed ankyrin repeat proteins (DARPins) can recognize target receptors with high specificities and affinities and when ligated into vectors mediate cellular binding (97). Prof. Christian Buchholz (Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Langen, Germany) discussed the selective delivery of CARs into CD8+ T lymphocytes for the *in vivo* generation of CAR-T cells. LVs that can specifically transduce human $CD8^+$ cells have been used to deliver the CD19-CAR *in vivo* into immunodeficient mice transplanted with human peripheral blood mononuclear cells (PBMCs). The vector administration in fully humanized mice transplanted with human $CD34^+$ cells induced TA-specific CAR-T cells depleting the $CD19^+$ B lymphocytes. Signs of CRS, the presence of tissue-invading CAR-T cells and the complete elimination of the B-lymphocyte-rich zones in spleens were detected (98). More recently, efficient eradication of tumors in transplanted mice was demonstrated upon a single vector injection (99). Although further investigations are desirable, these promising results have demonstrated that the *in vivo* engineering of human $CD8^+$ CAR-T cells could substantially simplify their manufacturing and the development of future cell-based immunotherapies.

Prof. Soldano Ferrone (Massachusetts General Hospital, Harvard Medical School, Boston MA, USA) has dedicated many efforts to elucidate the immunogenic properties of human chondroitin sulfate proteoglycan (CSPG)-4 and its expression on the membrane of different types of cancer cells, including cancer initiating cells (100–104). This molecule is also known as high molecular weight-melanoma associated antigen (HMW-MAA), or neuron-glial antigen 2 (NG2). CSPGs are key bioactive molecules which play a role in cancer cell growth, migration and neo-angiogenesis (105). This antigen can be upregulated on cancer cells incubated under hypoxic conditions and is not or poorly expressed on normal cells with the exception of pericytes in the TME. Prof. Ferrone's team has previously isolated the mAb scFv-Fc C21 specifically reacting with CSPG4 (100, 101) and he pointed out that some of the commercially available antibodies, including those used to generate the information about CSPG4 presented in the Atlas of Proteins, have the wrong specificity. This has generated some of the misleading information presented in the Atlas of Proteins. CSPG4 represents a target TA for T cell-mediated responses (101, 105–107). In addition, the availability of specific mAbs for this antigen has allowed the generation of CAR-T cells targeting either epithelial cancers, such as triple negative breast cancer, squamous cell carcinoma of head and neck and glioblastoma (106, 108–110). Interestingly, cancer initiating cells isolated from these types of cancers could be efficiently recognized and killed *in vitro* by CSPG4-CAR-T cells (108, 110). The anti-tumor activity of CAR-T cells targeting CSPG4 was potentiated through the intratumoral injection of effector cells. These pieces of evidence suggest the relevance of CSPG4 as target TA of CAR-T cells.

The development of novel scFvs, through the generation and screening of monoclonal antibodies targeting TAAs, can be applied to redirect engineered T cells for the targeting of colorectal cancer stem-like cells (CRC-SC; Dr. Eleonora Ponterio, Catholic University and Fondazione Policlinico Universitario "A. Gemelli"-I.R.C.C.S. Rome, Italy; Oral poster presentation). This approach represents a novel framework to implement the efficacy of CAR-T cells for solid tumors. Through the injection into mice of primary CRC-SC, antibody-mediated immune responses selectively binding to CRC tissues and not to normal mucosa have been generated. Upon sequencing of the variable chains of these mAbs, the scFv was tagged with a constant fragment (Fc) and used to isolate, through immunoprecipitation, the recognized TAs. The scFvs have been then shuttled into LVs encoding for either 2^{nd} or 3^{rd} generation CARs and used to transduce the immortalized T cell line Jurkat. These cells, upon the activation of the CAR through the co-culture with CRC-SC, secreted IL-2. The isolated mAbs could represent a powerful new tool to direct T cells against CRC [unpublished data and see (111)].

The deep characterization of the mechanisms for differences in cancer phenotypes could contribute to understand patient's clinical outcome and the responsiveness to immunotherapy. A pan-cancer analysis of the TCGA dataset has revealed that a pre-existing intratumoral T helper (Th-1) immune response can affect the OS of cancer patients with variable outcome depending on the histological origin of tumor. This indicates that cancer-specific pathways modulate the prognostic power of anti-tumor immune response and shape the TME and the type of immune infiltrating cells (Jessica Roelands, PhD Student, Sidra 805 Medicine, Qatar; Oral poster presentation) (112–115). These observations can play a role in the stratification of cancer patients. The clinical relevance of these findings was demonstrated using a dataset of melanoma patients treated with checkpoint inhibitors. A high expression of genes reflecting Th-1 anti-tumor responses in pretreatment samples was associated with improved survival only for samples with high proliferation scores, or with low TGF-β expression (115). The investigation of these parameters in tissues form large cohorts (N=366) of CRC patients represents a valuable tool to either predict the responsiveness of patients to immunotherapy or to identify novel target molecules for immune-based therapies.

MANUFACTURING IMPLEMENTATION OF ENGINEERED T CELLS AND PHARMA/ BIOTECH PERSPECTIVES

The manufacturing of CAR-T cells over the time underwent a rapid evolution, from the 1st until the 3rd generation of CAR-T cells, depending on the structure and number of co-stimulatory molecules included in the signaling portion of the receptor. The choice of 2nd generation CAR-T cell for therapeutic application resulted from the practical need to offset toxicity rather than to achieve greater anti-tumor efficacy. Further advances are the "armored" CAR-T cells. These represent genetically modified T cells bearing CARs that comprise in their structures immunomodulating agents such as: i. proinflammatory cytokines; ii. secreted antibodies or their part bearing antigen specificity; iii. costimulatory ligands (Dr. Renier Brentjens, Memorial Sloan Kettering Cancer Center, USA). Armored CD19-CAR-T cells bearing the murine IL-18 (mIL-18 CAR-T) showed in *in vivo* models enhanced expansion and persistence, anti-tumor activity and prolonged B cell aplasia (up to 150 days after treatment), dependent on autocrine IL-18R signaling (116), even without prior chemotherapy conditioning. Whereas, without preconditioning of mice, the anti-CD19 CAR-T cells failed to prolong the survival of animals in a syngeneic tumor model (117). Through targeted delivery of IL-18 to the tumor, mIL-18 CAR-T cells can modulate the tumor microenvironment, recruit, and activate endogenous anti-tumor immune effector cells, which in turn orchestrate an effective anti-tumor response beyond the CAR-specific targets (116). Programmed cell death-1 (PD-1)/ligand (PD-L1) signaling represents one of the immune checkpoints that can modulate CAR-T cell-mediated responses when they encounter either tumor cells or TME. Armored CAR-T cells endowed with the capacity to secrete anti-PD-L1 scFv showed enhanced in vivo anti-tumor activity, persistence and survival in a PD-L1^{+} tumor model. PD-1-blocking Ab produced by CAR-T cells can also enhance the activity of tumor-specific "bystander" T cells present in TME. Unlike the usage of systemic checkpoint blockade therapy with monoclonal antibodies, the scFv secreted by CAR-T cells penetrate and remain in the TME improving their anti-tumor activity (118). Moreover, the safety of these armored CAR-T cells is superior than checkpoint blockade systemic therapy (118). The third example of armored CAR-T cells comprise the immunostimulatory CD40 ligand (CD40L), that is a type II transmembrane protein belonging to the TNF receptor family. CD40L expressing CAR-T cells displayed enhanced proliferation and secretion of proinflammatory cytokines, e.g., IFN-γ and GM-CSF (119). These armored effector T cells demonstrated enhanced cytotoxic activity both *in vitro* and *in vivo* for both CLL and NHL models (119, 120). All together, these evidences, indicate the promising clinical efficacy of next generation armored CAR-T cells.

The clinical grade manufacturing of CAR-T cells can be challenging due to the complexity of the process. The collaboration between biotech and researchers in academia can result in the development of novel technological platforms to support researchers and physicians from discovery to the clinical development of cell therapy (64, 121). One example is represented by the automated closed system production of engineered T cells that can perform all phases of CAR-T cell generation, from magnetic T-cell enrichment, activation, viral transduction and expansion to downstream harvest for cryopreservation/infusion (CliniMACS Prodigy system). The progress in the generation of this type of system allow standardization of processes facilitating transfer between different manufacturing facilities while the flexibility of the manufacturing platform supports the rapid transfer of innovative cell therapies to the clinic (Dr. Ian Johnston, Miltenyi Biotec B.V. & Co. KG, Germany).

GENETIC ENGINEERING OF CELLS AND GENE EDITING

Genetic engineering can act on the core functioning of cells, including reprogramming, differentiation, migration, fitness, and proliferation. In addition, gene therapy is aimed at correcting genetic and degenerative diseases. Novel tools have been developed for the precise engineering of immune cells.

Retroviral vectors (RVs), one of the most effective approaches to the modification of genes, display the ability to integrate at various positions in the genome. This implies the risk of mutagenesis in the course of the integration of vectors into the genome. Studies on animal models have revealed evidence of leukemia induction in a process of insertional mutagenesis mediated by retroviral vector (122). The utilization of constructs with improved safety, such as the low-risk recombinant alpharetroviral SIN vectors (aRV) can prevent the genotoxicity caused by insertional mutagenesis (Prof. Axel Schambach,

Hannover Medical School, Germany), as SIN-aRV exhibit a neutral integration pattern, lowering the risk of insertional mutagenesis as compared to gammaretroviral and LV (123, 124). Given that aRV vectors do not have the tendency to integrate their cargo into promoter/enhancer regions as well as gene bodies, insertional damages arising from the integration of aRV are lower as compared with other RVs. LVs are considered relatively safe due to their tendency to insert into genes instead to the transcriptional sites that may lead to insertional mutagenesis. The usage of transient retroviral platform, such as the retroviral episome transfer (RET), allows a controllable transgene expression, low or absence of cytotoxicity and the option for cell targeting (123, 124).

Genome editing tools like CRISPR/Cas9, transcription activator-like effector nuclease (TALENs), zinc fingers nuclease (ZFNs), Mega-tal and HEs provide a precise method for the genetic engineering of cells. These platforms introduce double strand breaks (DSBs) in the genome that can be repaired either by non-homologous end-joining (NHEJ) causing disruption of the genes or by homologous recombination which happens at lower rates allowing nucleotide modification of the genome. CRISPR/CAS9 platform requires the co-delivery of a single guide RNA (sgRNA), representing the bottleneck of this strategy. The MS2 bacteriophage RNA packaging mechanism has been applied to the production of non-integrating RVs, GV.MS2-CRISPR/Cas9 all-in-one particles, for efficient gene editing (125, 126). This strategy led to efficient KO of target genes, such as TP53 in human fibroblasts or CXCR4 or CCR7 in Jurkat or T cells, respectively (125–127). These platforms are applicable to the generation of allogeneic CAR-T cells to prevent GvHD and/or to engineer their immune functions.

The usage of ribonucleoproteins (RNPs) as the primary mode of gene editing variants of high-fidelity Cas9 (IDT HiFi SpCas9) enabled better gene-editing by reducing levels off-target INDELs as identified by bioinformatics tools (e.g., COSMID, guide-seq genome-wide profiling of target cells or circle-seq *in vitro* screen for genome-wide CRISPR/Cas9 nuclease off target) (Prof. Matthew Porteus, Stanford University, San Francisco, CA, USA) (128). This represents an efficient tool for CD19-CAR-T cell to lower the associated toxicities and implement the anti-tumor activity. CD19-CAR-T cells employed with the KO of TCRab to avoid GvHD were assessed in the context of infusions before and following TCRab-depleted haploidentical-HSCT (129). This was achieved through the targeting of the locus of TCR (TRAC) with CRISPR/CAS9 non-integrating AAV6 encoding forCD19-CARs. The engineered T cells showed active anti-tumor activity against $CD19^+$ leukemia xenografts (Nalm6 cells in NSG mice). No off-targets indels was detected in these CAR-T cells through the usage of HiFi Cas9 (129). In order to improve further CAR-T cell efficacy and safety, in 2017, Dr Eyquem et al. (130) used complex genome editing tool to demonstrate that TCR-like expression of the CAR improve CAR-T therapeutic activity. However, genome editing tool to demonstrate that TCR-like expression of the CAR improves CAR-T therapeutic activity. In this direction, Prof. Francisco Martin (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research, GENYO, Spain) presented an interesting approach to facilitate clinic translation of TCR-like CAR-T cells. Dr. Martin´s team constructed LVs that closely followed the expression profile of the TCR upon CD3 stimulation and generated CAR-T cells using the LVs (*Patent PCT/EP2019/081346, "Polynucleotide for safer and more effective immunotherapy"* and unpublished data). These physiological CAR-T cells showed strong resistance to exhaustion upon repeated stimulation and showed potent *in vivo* anti-tumor activity. The author proposes the use of these physiological LVs (similar to the one already approved by FDA) to translate TCR-like CAR-T cells into clinic. Another important aspect to improve the potency and safety of CAR-T cells stands on the design of new tools that allow the clinicians to be able to externally control their activity. Dr. Martin presented their latest development on their transactivator-independent doxycycline-regulated LVs (Lent-On-Plus). They showed the potency of this system to induce any transgene in T cells both *in vitro* and *in vivo* with very low doses of doxycycline. As proof of concept, he also presented the ability to generate inducible CAR-T cells that selectively kill $CD19^+$ cells only in the presence of doxycycline. However, the author envision that this approach will be more applicable for future development of inducible TRUCKs (iTRCKs), expressing any desired cytokine (factor) upon the addition of doxycycline.

ALLOGENEIC/"OFF-THE-SHELF" THERAPY WITH CAR-T CELLS

The generation of autologous CAR-T cells for therapeutic treatment implies many challenges related to i. the isolation of circulating lymphocytes from heavy pre-treated patients who might have lymphopenia; ii. the manufacturing of the cells; iii. the logistics of collection of the starting material, preparation and delivery for infusion of the medicinal product into patients; iv the inter-patient variability in the yield and quality of the cell product. The major advantage of generating universal CAR-T cells is their "off-the-shelf availability, without delay in the treatment and suitability for multiple infusions of engineered T cells. Moreover, the manufacturing of universal CAR-T cells allows the implementation of the standardization of batches of cells and to reduce the costs of production. The strategy to generate anti-tumor CAR-T cells starting from peripheral blood lymphocytes isolated from healthy donors, has also the potential to overcome the impairment of effector functions of patient-derived CAR-T cells.

Universal CD19-CAR-T cells (UCART19) represent the first "off-the-shelf" CAR-T cell product targeting CD19 expressing malignancies (Dr. Reuben Benjamin, King's College Hospital, UK). In this product, anti-CD19 scFv-41BB-CD3ζ CAR is expressed in T cells, with KO of TRAC and CD52 genes to prevent GvHD in HLA mismatched patients. The TRAC and CD52 gene KO has been performed through the usage of TALEN (41). In addition, RQR8 epitope, that is a mimotope of CD20 has been introduced into engineered T cells as a safety switch (131).

The safety and tolerability of UCART19 in pediatric and adult patients with diagnosis of ALL have been tested in the context of the PALL/CALM study following lymphodepleting regimens and to evaluate the maximum tolerated dose (MTD). The study revealed that UCART19 has a safety profile, with no acute GvHD and severe toxicities, leading to 67% of CR (40, 43, 132).

Different strategies of gene editing are in place for allogeneic CAR-T cells for the knock-out of genes involved in either GvHD, such as TCRαβ - TRAC, TCRBC1, TCRBC2, rejection molecules (e.g., CD52, β2 microglobulin), or molecules associated with the exhaustion of T cells (e.g., PD-1, CTLA-4) (42, 44). CAR-T cell approaches in infant ALL patients have sometimes encountered difficulties in generating autologous CAR-T cells and, moreover, are associated with the risk of developing CD19-tumor cell escape (Prof. Paul Veys, Great Ormond Street Hospital For Children & UCL GOS Institute of Child Health, UK). UCART19 represented a bridge between lymphodepletion/ CAR-T cell therapy and allogeneic HSCT. This strategy allowed the eradication of $CD19^-$ malignant cells following CAR-T cell therapy during the allogeneic HSCT, followed by reconstitution of donor immune cells and elimination of residual UCART cells. This procedure was followed by a 30-day transient GCSF-dependent recovery of neutrophils and protracted multilineage cytopenia until the second allo-SCT after 12 weeks. The TALEN trial achieved 60% clinical responses in pediatric ALL patients. The effectiveness of TALEN versus CRISPR/Cas9 was compared, indicating that the risk of GvHD might be reduced with CRISPR/Cas9, as CAR insertion and editing of TCR were coupled, leading to reduced potential contamination of the final product with TCR positive cells. The generation of CAR-T cells from umbilical cord blood (UCB) coupled with gene editing has been explored in pre-clinical models as alternative method to generate allogeneic CAR-T cells with increased early differentiation features, including increased proliferative capacity (133). The unmet need is to define the optimal lymphodepletion regimen(s) and whether allo-SCT is required post UCART infusion to extend the clinical benefit of this cell-based therapy.

The usage of UCB as starting material to generate CAR-T cells represented the object of one study ongoing at Sidra Medicine (Dr. Cristina Maccalli, Sidra Medicine, Qatar). UCB offers the unique capacity of broad HLA mismatch between donor and host. The frequency of T lymphocytes in cord blood is lower as compared to peripheral blood representing a limiting factor for the generation of CAR-T cells. The CD19-CD28ζ and CD19- 4-1BBζ CAR-T cells have been isolated and phenotypically characterized utilizing an in-house designed multiparametric IF (including 28 markers) panels. The overall phenotype at different time points (Day 0, + 7, +9, +14) of *in vitro* culture of UCB vs. peripheral blood-CAR-T cells varied in terms of the percentage of $CD4^+$ and $CD8^+$ stem/central memory T cells. In addition, immunomodulating molecules such as PD-1, TIM3, and LAG3 were differentially expressed in CAR-T cells with different origins. The integration of different platforms, such a flow cytometry, EliSpot, and FluoroSpot based cytokine release and transcriptomic profiling (134) allowed the development of a proof of principle study aimed at identifying the UCB-derived CAR-T cell population endowed with superior anti-tumor activity. Further investigations are warranted to validate these results (unpublished data).

CAR-NK cells represent a promising strategy for allogeneic and universal cell-therapy (Dr. Tomas Bos, Glycostem Therapeutics, The Netherlands). NK cells represent the 5%–10% of circulating immune cells and are members of innate immune control with low risk, due to their independence on the recognition of MHC/peptide complexes, of allogeneic rejections or GvHD upon their infusion in cancer patients. The generation of clinical grade NK cells has been optimized in the presence of a liquid cell culture containing differentiation factors and an artificial niche composed of glycosaminoglycans. The NK cell product, denominated oNKord®, showed strong cytotoxicity against CRC, regardless of the mutational status of EGFR and RAS (135), and leukemia both *in vitro* and in mouse models (136). Interestingly, this type of treatment in a phase I clinical trial for AML patients following conditioning with Flu/Cy, showed safety, no toxicities or sign of GvHD and clinical efficacy with improvement of survival (34 months) (137).

The repertoire of available gene editing tools for CAR-T cells has been discussed by Dr. Karim Benabdellah (GENYO, Spain; Oral Abstract Presentation), highlighting that the insertional deficient LV episomes (IDLV) combined with CRISPR/CAS-9 can implement the effciency of CD19-CAR-T cells for the KO of TCR to prevent *in vivo* allogeneic rejection and GvHD. These CAR-T cells can maintain both *in vitro* and *in vivo* the reactivity against tumor cells.

THE USAGE OF maB N CANCER THERAPY AS A COMPLEMENTARY OR INTEGRATIVE APPROACH FOR ENGINEERED IMMUNE CELLS

Several mAbs have been registered as standard of care for different types of tumors. Some of them, through the binding to the Fcγ receptor (FcγR) on effector cells, such as NK and γδ T cells, can activate the antibody-dependent cell-mediated cytotoxicity (ADCC) that can lead to the killing of malignant cells (Prof. Hans van der Vliet, Amsterdam University Medical Center, Cancer Center Amsterdam, The Netherlands). Bispecific Ab, bearing two epitope binding regions, have been designed to bind simultaneously to two target molecules and can be used to target immune effector cells to tumor cells. Trifunctional hybrid antibodies (Triomabs) are formed by combining two halves of distinct mAbs, for example of rat and mouse mAb origin, which, as a result of species preferential heavy-light chain pairing, reduces random pairing of heavy and light chains simplifying manufacturing. In addition, to facilitate the interaction of immune cells with target cells, Triomabs (e.g., catumaxomab, ertumaxomab) can also mediate ADCC through the FcγR (138–141). Notably, other bispecific antibody formats utilize a linking of single chain Fv (scFv) fragments with different antigen specificity,

for which several formats can be used. An example of a scFv based bispecific antibody in a tandem orientation (TaFv) is blinatumomab, an anti-CD3 and anti-CD19 bispecific T cell engager (BiTE) used for the treatment of, e.g., relapsed or refractory B-ALL, that showed an improvement of complete remission rate and of OS as compared with chemotherapy (53, 142). Multiple alternative bispecific antibody platforms (e.g., bispecific diabodies, Fc-containing bispecific antibodies, immunocytokines, prodrug bispecifics, and MHC class I targeting bispecifics) are under development in an effort to further improve the clinical efficacy of such approaches and to extend their application towards other disease indications (143–145). The generation of novel antigen-specific scFv could also complement the function of CARs, either by enhancing the binding to target molecules or expanding the tools to redirect CAR-T cells to tumor sites. In addition, the usage of mAbs, including the bispecific, either in combination with CAR-T cells or encoded simultaneously with CARs by LV vectors, can represent an interesting approach to enhance the homing of CAR-T cells to the tumor site, their penetration into the TME and the anti-tumor activity (e.g., armored CAR-T cells discussed in paragraph 6).

CONCLUSIONS

The development of engineered immune cells, T lymphocytes and NK cells, represents a breakthrough of cancer immunotherapy for both blood and solid tumors (see **Tables 1** and **2**). For the first time, one of these innovative therapeutic strategies (CAR-T cells) has been approved as standard of care for some hematological malignancies. Nevertheless, the optimization of these interventions, in terms of identification of novel TAs, the improvement of the survival and the limitation of differentiation, aging and exhaustion of T cells, as well as of associated toxicities upon *in vivo* infusion and their capacity to penetrate TME are under investigation. Several platforms and approaches have been developed to mitigate the aforementioned limitations of anti-tumor cell-based therapies, contributing to the rapid evolution of the field. Furthermore, novel Ab-based tools designed on tumor cell phenotype and cancer immune setpoints are warranted to design more effective personalized Ab-based weapons for cancer therapy. The development of these approaches has required long path for R&D, however, the gained expertise could facilitate the implementation of the molecular and genomic tools to generate anti-tumor cell therapy with superior clinical efficacy. Nevertheless, the discussion occurring in the context of the conference highlighted the need of specialized manufacturing facilities and clinical centers, including multidisciplinary personnel, for the production, delivery and infusion into patients, as well as their clinical monitoring and follow-up. The development of inter- and intra-regional networks is of high relevance to facilitate the establishment of these cell-therapy centers and to expand the accessibilities to these therapeutic interventions to a broad number of cancer patients. The costs associated with the development of this type of centers and with the clinical grade manufacturing of these cellular medicinal products should also be taken into account. The access of patients to these therapies and their attractiveness for clinical application might increase whether a point-of-care manufacturing model would be designed and established. The role of pharma and biotech has been shown to be critical and to facilitate the clinical grade development of these approaches. The interaction and cooperation between academia and industry were emphasized during the sessions' discussions as in need of being potentiated and initiated even at early phase of R&D. Further investigations are also required to simplify the manufacturing of engineered immune cells and potentiate the development and safety of allogeneic products. The exploration of the combination therapies (such as with immunomodulating agents and/or standard therapies) could represent a strategy to overcome the development of resistance to cell-based therapies and to target a broad variety of tumors. A consensus regarding the need of continuous exchange of information among investigators and clinicians and of the development of international networks and collaborations resulted from the participants to the workshop.

THE EICCI FACULTY GROUP

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AUTHOR CONTRIBUTIONS

BG, ME, AA-M, and DK prepared the draft of the manuscript and the final editing. CM contributed to the preparation, revision, and editing of the manuscript. RBe, CBo, RBr, CBu, GC, SF, IJ, AM, FL, FM, AS, CT, PV, and HV provided critical inputs and revised the manuscript. KBL, TB, EP, JR, HMAR, and RYT contributed to the editing of the text. All authors contributed to the article and approved the submitted version.

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REVIEW
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Empirical and Rational Design of T Cell Receptor-Based Immunotherapies

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The use of T cells reactive with intracellular tumor-associated or tumor-specific antigens has been a promising strategy for cancer immunotherapies in the past three decades, but the approach has been constrained by a limited understanding of the T cell receptor's (TCR) complex functions and specificities. Newer TCR and T cell-based approaches are in development, including engineered adoptive T cells with enhanced TCR affinities, TCR mimic antibodies, and T cell-redirecting bispecific agents. These new therapeutic modalities are exciting opportunities by which TCR recognition can be further exploited for therapeutic benefit. In this review we summarize the development of TCR-based therapeutic strategies and focus on balancing efficacy and potency versus specificity, and hence, possible toxicity, of these powerful therapeutic modalities.

Keywords: T cell receptor, bispecific T cell engager, cross-reactivity, tumor infiltrating lymphocytes, immune mobilizing monoclonal T cell receptors against cancer, peptide- major histocompatibility complexes, T cell receptor mimic monoclonal antibody, T cell receptor-T cell

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INTRODUCTION

Harnessing potent cellular effectors, such as cytotoxic T cells, and soluble molecules of the human immune system has become a successful strategy in the treatment of cancers of a variety of types. While often effective and generally well-tolerated, these effectors are not truly specific for the tumor. Typically, these therapies can either broadly activate cellular effectors, such as with interleukins, interferons, or checkpoint blockade antibodies, or are directed to lineage markers or cell surface differentiation antigens also found on normal cells and tissues. For example, monoclonal antibody (mAb) and chimeric antigen receptor (CAR) T cell therapies have emerged as some of the most successful and important strategies in cancer therapy. However, these modalities are traditionally reactive with a limited repertoire of extracellular antigens. For many cancers, appropriate antigens have not been identified. In contrast, the TCR evolved to detect subtle changes in cellular proteins that can include viral peptides or mutated oncogenic proteins. Thus, TCR-based agents can be directed to the vast majority of truly tumor-specific antigens, or relatively specific tumor-associated proteins, which are derived from intracellular proteins (1–3). Peptides derived from proteins of any subcellular location are presented on the cell surface in the context of major histocompatibility complexes (MHC), known as human leukocyte antigens (HLA) when referring to human MHC, where they are recognized by T cells through their TCRs (3). TCR-based therapies are able to recognize and react to cells expressing these mutated or differentially

expressed, cancer-associated proteins presented on MHC. The exploitation of this powerful modality to treat cancer and possibly other serious diseases is dependent on understanding the unique features of their recognition and effector activities, the types of structures that can be developed to take advantage of these functions, and the possible liabilities that these molecules carry.

Immunotherapeutic modalities that take advantage of the TCR's unique ability to recognize intracellular proteins are both molded by and constrained by key aspects of their structural features and those of their targets, as well as the origins of their antigenic specificity. Critical determinants of success for these agents are (1) the characteristics of the epitope (2); the affinity, avidity, and cellular geometry of the TCR; and (3) the recognition specificity unique to the antigen-TCR interaction. These features of TCRs are markedly divergent from the analogous features of antibodies and must be tackled accordingly to create a successful agent. First, unlike the conformational structure of the molecular targets of antibodies, the potential amino acid sequence epitopes for these TCR agents must be appropriately, expressed, processed, and presented on the cell surface. While peptide presentation on MHC molecules can be predicted in silico, these approaches are inaccurate and ideally, selected epitopes should be validated by using mass spectrometry to verify peptide-MHC presentation and followed by *in vitro* assays to characterize the functionality of target-specific T cells. Second, although unmodified, native TCRs reactive with peptides in context with their MHC proteins are more likely to yield appropriate specificity and functionality that mimic the actions of an endogenous T cell, as compared to a modified TCR, such native TCRs have orders of magnitude lower affinity than antibodies, which can limit their pharmacologic uses. TCRs may need affinity enhancement to increase the peptide-MHC recognition. In addition, native TCRs, unlike antibodies that operate in solution, cooperate as a collection of molecules along with other proteins in a cell membrane synapse on the T cell that vastly alters their effector functions. Third, TCRs, because of their low affinity and the complex structure of their epitope targets, are far more promiscuous than antibodies; strategies to predict toxicities by determining on-target/off-tumor and off-target antigen recognition of TCR-based agents are essential to ensure TCR agent safety, but such methods are currently in their infancy. There are no marketed drugs in the United States that are based upon the TCR. In this review, we will discuss various approaches to identify, address and overcome these constraints to TCR-based agents in order to advance these innovative drugs to clinical trials (**Table 1; Figure 1**).

IDENTIFICATION OF TARGETS OF TCR-BASED AGENTS

Overall, advancements in screening techniques and engineering now provide multiple approaches and formats to achieve the goal of peptide-MHC recognition to target antigens. However, insufficient processing and presentation of the targeted epitope on the cell surface may limit activity. This underlines the importance of validation of target epitopes to ensure high levels of tumor specificity and efficacy. Ideally this can be achieved in advance by immunoprecipitation of MHC complexes and subsequent mass spectrometry identification of the displayed ligands.

The landscape of targets for TCR therapy of non-viral malignancies is comprised of antigens that demarcate aberrant cells, albeit to a highly variable degree (4). This nuance renders TCR target selection non-trivial. For the purposes of this review, we will divide TCR targets into two broad classes: self-antigens, derived from overexpressed proteins, and neoantigens, which exhibit subtle deviations from self due to the malignant state (5). The common feature between these two is that both are derived from the human genome; however, neoantigens arise directly from genomic deviations caused by the genomic instability of cancer.

The earliest class of cancer antigens known to be recognized by TCRs include self-antigens derived from proteins that are over-presented by MHC in tumor tissue. Classic examples that have been extensively studied are MART-1, Wilms' tumor-1 (WT1), PR1, MAGE-A3, NY-ESO-1, carcinoembryonic antigen (CEA) and PRAME (6–8). However, an important distinction is that some of these are cancer-associated by means of their lineage-specificity, such as MART-1 and CEA, whereas others are cancer germline antigens, such as NY-ESO-1 and PRAME, that are only expressed in immune privileged sites such as the placenta or testis, but are re-expressed due to genomic instability in tumor cells (9). Lineage associated antigens require careful consideration of on-target/off-tumor effects associated with TCR therapy (7, 10). In contrast, TCRs targeting cancer germline antigens may confer greater tumor-specific recognition, but may be attenuated by escape mechanisms as these are not typically oncogenes critical for tumor survival (5). As this type of tumor-associated antigen has been studied for decades many of the used targets were also confirmed to be presented on the cell surface by MHC ligand isolation and mass spectrometry which renders them *bona fide* targets (11–13). The growing number of studies utilizing mass spectrometry to verify the presentation of HLA

TABLE 1 | TCR-based agents in development.

TCR-Based Agent	Salient Features
Vaccines (many approaches)	Peptides and immunostimulants activate and expand antigen-specific preexisting T cells.
TILs and native T cells	T cells derived from patients or their tumors; patient-specific; often specific to an individual's cancer.
TCR T cells	A specific TCR (native, foreign, or enhanced) transduced into a T cell.
ImmTac	A defined TCR single chain molecule linked to an scFv to CD3 to redirect T cells to cancers.
TCR mimic antibodies	Immunoglobulins (as IgG, bispecific antibodies, or incorporated into CAR T cells) directed to the peptide-MHC complex.

TIL, tumor infiltrating lymphocyte; ImmTAC, immune mobilizing monoclonal TCRs against cancer; scFV, single chain variable fragment; IgG, immunoglobulin G.

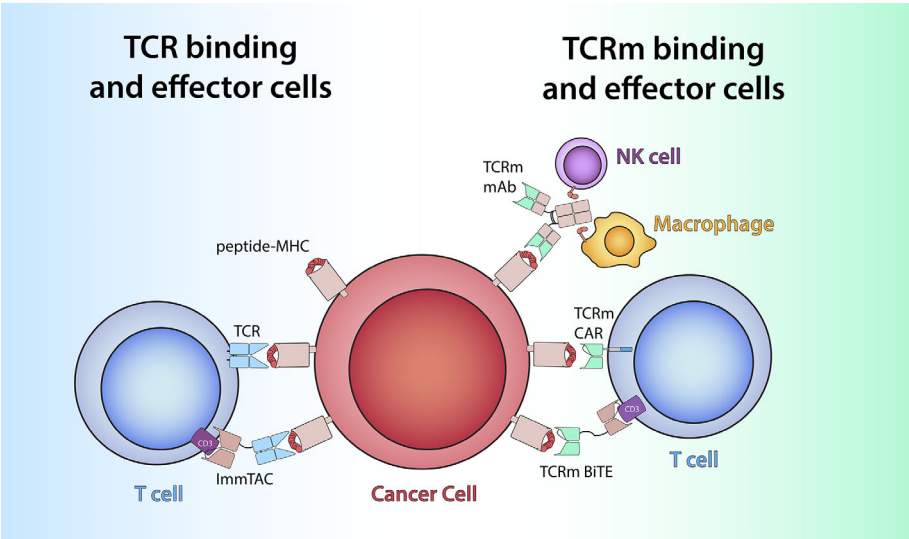

FIGURE 1 | TCR-based therapeutics recognize peptide/MHC antigens (red and pink) on cells by utilizing either TCRs (light blue) or TCRm antigen-binding domains (green). Left: Soluble ImmTAC molecules bind peptide/MHC on cancer cells *via* alpha/beta TCR heterodimer similar to membrane-bound TCR and redirect the T cells by engaging extracellular CD3-epsilon (purple) *via* an anti-CD3 scFv. Right: TCRm mAb recognize peptide/MHC complex *via* its variable region (green) and to engage effector cells such as NK cells and macrophages to elicit Fc-receptor (orange) mediated ADCC or ADCP. TCRm CAR and bispecific mAb leverage TCRm-derived scFv to harness T cell effector function via engagement with intracellular CD3-zeta (blue) or extracellular CD3-epsilon (purple), respectively.

ligands is expected to increase confidence in presented epitopes derived from cancer germline antigens and potentially uncover new epitopes against which new TCR therapeutics can be developed.

More recently neoantigens, peptide antigens that are the result of missense mutations, frameshift mutations, or post-translational modifications, have been exploited as novel targets for TCR-based immunotherapies. However, other types of alterations can produce a neoantigen (14). Neoantigens encoded by missense mutations harbor a nonsynonymous amino acid substitution produced by either a driver or passenger mutation that can be distinguished by T cells by means of an augmented MHC binding affinity or altered TCR recognition. Frameshift mutations produce neoantigens that can be drastically different from wildtype protein-derived peptides, harboring multiple amino acid changes (15). Post-translational amino acid modifications, such as phosphorylation and glycosylation, can exhibit a greatly altered capacity to bind MHC and present a fundamentally different binding moiety to T cells compared to its unmodified variant (16, 17). Careful consideration of a tumor's biologic properties is also necessary to guide effective target selection. For example, for hematologic malignancies self-antigens such as WT1, PR1, or PRAME, remain among the most useful TCR targets for these tumors rather than rare mutant neoantigens (18–22).

Neoantigens derived from frameshift mutations represent a type of shared neoantigen that is particularly prominent in clear cell renal cell carcinoma (ccRCC) in which neoantigens from common tumor suppressors such as TP53, PTEN, MLL2, MLL3 and ARID1A have been observed (23) and found to be recognized by tumor-infiltrating lymphocytes (TIL) (24). Strikingly, the immunogenic frameshift neoantigens were more distinct from the human proteome than were immunogenic missense mutations, a distinction that has also been made for immunogenic frameshifts in microsatellite instability-high tumors. Because of their high degree of sharing between tumors, immunogenicity, and derivation from driver mutations, frameshift-derived neoantigens may constitute a rational TCR target for tumors with adequate frameshift load.

Post-translationally modified peptides are an emerging class of neoantigens, the discovery of which has been accelerated by recent advances in mass spectrometry (25). Early reports discovered shared phosphorylated peptides (phosphopeptides) presented by tumor cell lines (26, 27) and leukemias (28). Phosphopeptides are an attractive target due to their consistently observed immunogenicity in normal donors, enhanced binding properties, and relationship to aberrant cancer metabolism (16, 28, 29). A recent phosphopeptide vaccine trial in melanoma patients elicited T cell reactivity in patients, suggesting that immune responses to these targets can be generated by tumor-bearing hosts (30). Glycosylated and acetylated peptides have similarly been shown to be immunogenic epitopes presented by tumor cells in similar studies (17, 31, 32). Proteomic data are expected to give depth to the cancer-specific modified peptide repertoire and provide a valuable link between post-translationally modified antigen presentation and tumor metabolism with additional classes of tumor-specific intracellular antigens identified and validated.

Although solid tumors present self-antigens, the efficacy of immune checkpoint blockade in highly mutated solid tumors such as melanoma and non-small cell lung carcinoma (NSCLC) has shifted interest to targeting one of the numerous neoantigens.

Correlative studies have repeatedly demonstrated that a critical factor of response to checkpoint blockade is tumor mutational burden, with highly mutated tumors being more likely to respond (33, 34). This paradigm has been further validated by the discovery of T cells that recognize missense-derived neoantigens in responding patients (35). Subsequently, tumor exome sequencing and MHC binding predictions have become an invaluable tool to determine neoantigen load on a patient-by-patient basis (36). However, the accrual of neoantigens as a tumor evolves is unpredictable. Most neoantigens are derived from passenger mutations private to each individual patient or each tumor, rendering scalable TCR targeting infeasible. Furthermore, though some patient specific neoepitopes can be detected by mass spectrometry and their presentation therefore validated, the vast majority of predicted neoepitopes cannot be identified by this technique which further complicates target selection for patient-specific therapeutic approaches (37, 38). Neoantigens derived from shared driver mutations, such as KRAS and PIK3CA missense mutants where presentation has been validated for some HLA alleles (39), might overcome this issue (40–42).

REGULATION OF EPITOPE PRESENTATION

One potential disadvantage of targeting peptides presented by MHC molecules is their paucity of density on the cell surface, which may be 100 to 1000-fold lower than other antigens. This can be further exacerbated by down regulation of MHC by cancer cells as a method of immune escape (43). To deal with this issue, the immunopeptidome can be altered by drug or cytokine treatment to either augment expression or investigate the emergence of novel targets. Physiological alterations by interferon gamma (IFNγ) or tumor necrosis factor alpha (TNFα) favor the presentation of longer peptides as well as ligands which preferably bind to HLA-B alleles (44, 45). In addition to upregulating the HLA expression level, IFNγ also can lead to the specific increase in presentation of a given TCR or TCR-mimic monoclonal antibody (TCRm) peptide target through induction of the cytoplasmic immunoproteasome (46–49). This specific induction of ligand presentation could be utilized to render previously unreactive cells targetable (46).

Interestingly, ALK, RET, MEK, and other mitogen-activated protein kinase (MAPK) pathway inhibitors lead not only to a significant increase of HLA complex surface expression, which can help overcome immune escape *via* downregulation of HLA, but also to a large qualitative change of the displayed peptides (43, 50, 51). Many of which are potentially immunogenic epitopes. Surprisingly, ALK and RET inhibitor treatment can also lead to the presentation of T cell epitopes associated with impaired peptide processing (TEIPPs) which are usually only observed in transporter associated with antigen processing (TAP)-deficient or downregulated cells with low HLA levels. Treatment with the MEK inhibitor trametinib illustrates an example of improved efficacy of a specific TCR-like drug targeting an epitope from the MART-1 protein (50).

The ligandome might be affected to improve presentation of a specific TCR target by use of the proteasome inhibitor carfilzomib, which disfavors presentation of HLA ligands with aromatic C-terminal anchors by altering proteasomal cleavage patterns, as well as the ERAP1 inhibitor, DG013A, which augments presentation of ligands with higher affinity and shorter peptide length by changing endoplasmic peptide trimming (52, 53). Other chemotherapeutic agents, such as gemcitabine can lead to changes through upregulation of HLA-A,B,C complexes and also through immunoproteasome induction (54) or oxaliplatin, which can increase detection by CD4+ T cells through class II peptide presentation (55).

Hypomethylating drugs alone or in combination with histone deacetylase (HDAC) inhibitors are the most effective class of drugs that increase the presentation of specific HLA ligands. This has been most extensively demonstrated in acute myeloid leukemia (AML) for NY-ESO-1, MAGE-A3, and MAGE-A6 (56). Reinduction of cancer germline antigens through reversion of genetic repression marks is also feasible in many solid cancers, e.g. in esophageal squamous cell carcinoma for MAGE-A3 (57), ovarian cancer for protein expressed in prostate, ovary, testis and placenta (POTE) genes (58), mesothelioma for NY-ESO-1, MAGE-A1, MAGE-A3, and XAGE-1b (59) and prostate cancer for NY-ESO-1 (60). Since the presentation of HLA ligands can be altered qualitatively and quantitatively through multiple FDA-approved drugs, therapeutic strategies may benefit from combination therapies of TCR-based agents with HDAC inhibitors and hypomethylating drugs. The synergistic effects of HDAC inhibitors and hypomethylating drugs have been shown to be especially important for immunotherapies as the repressive marks on relevant genes can often only be sufficiently reversed by such a combination treatment (61).

GENERATION OF THERAPEUTIC T CELLS

T cells reactive with tumor associated antigens and neoantigens have been found in TILs and peripheral blood lymphocytes (PBL) (62, 63) of cancer patients, as well as in PBLs of healthy donors (64). Such cells, or their TCRs, would be expected to be an appropriate source for effective therapeutic agents. Despite this, many endogenous T cells, including those found within tumors, are still unable to eradicate tumors presenting their cognate antigen. This failure can be attributed in part to the immunosuppressive tumor microenvironment (TME) (3), low affinity of endogenous T cell receptors (TCR) for tumor associated antigens (65, 66), and possibly other factors. Numerous approaches have been employed to generate a more potent anti-tumor T cell response. Tumor-reactive T cells expressing native TCRs can be stimulated *in vivo* through administration of vaccines, checkpoint blockade inhibitors, or cytokines. Alternatively, reactive T cells can be expanded ex vivo and reinfused for adoptive cell therapy (67). Tumor-reactive T cells can be enriched and used in bulk for treatment, or their individual reactive TCRs can be sequenced and subsequently expressed exogenously in T cells prior to reinfusion.

Cancer Vaccines

Naturally occurring tumor-reactive T cells can be stimulated to boost the anti-tumor T cell response through vaccination with tumor antigens. Vaccines can be peptides (68), DNA or RNA products (69), whole proteins, viruses encoding antigenic peptides (68) or autologous dendritic cells presenting peptide antigens (68, 70). Patients may receive a personalized cancer vaccine, where target peptides are chosen from tumor-specific mutations identified by whole exome sequencing (WES) and filtered through HLA binding prediction (69–71). Some peptides used for vaccination can be modified to further increase their immunogenicity, by substituting peptide residues to result in better binding to HLA molecules. Such "heteroclitic" peptides have been shown to induce stronger T cell responses that cross-react with their native sequences. Characteristic examples are the HLA-A*02:01 restricted peptides, NY-ESO-1 (SLLMWITQC) and WT1 (RMFPNAPYL), where replacing the final amino acid with valine (72), or the first amino acid with tyrosine (73), respectively, increases the HLA binding affinity resulting in increased immunogenicity and enhanced T cell activation. Cancer vaccines have been shown to increase the preexisting anti-tumor T cell response and have proven effective in some patients (68–70, 74–76). Although cancer vaccines are a widely used approach in investigational clinical trials, due to their limited clinical efficacy to date, more potent, passive therapeutic approaches using TCR recognition of tumor antigens have been developed in recent years. This review will focus on these new approaches.

Adoptive T Cell Therapy

Tumor reactive T cells, present either in TILs or PBLs, can be removed from patients for rapid expansion ex vivo, outside of the immunosuppressive TME (77), prior to use for adoptive cell therapy (67). T cells can be stimulated for expansion with resected tumor (67, 78, 79), antibodies targeting CD3 and CD28 (80), or peptide antigens (81, 82). Synthetic peptides of neoepitopes identified by whole exome or RNA sequencing and subsequent HLA binding prediction can be pulsed onto (64, 83–85) or expressed as tandem minigenes (84, 86, 87) on antigen presenting cells, often autologous dendritic cells. For tandem minigenes, mutations are flanked by sequences encoding endogenous amino acids allowing the peptides to be processed and presented on MHC. When using tandem minigenes the reactive peptide and MHC restriction of the respective neoepitope does not have to be identified and it allows multiple antigens to be expressed and presented on the same cell (77, 84, 86, 87). Peptide stimulation has also been employed to generate reactive T cells from the peripheral blood of HLA matched healthy donors (64, 77, 88).

T cells with predefined antigen specificity can be isolated using peptide-MHC multimers (62, 63, 77, 89–94) through either magnetic enrichment or fluorescence activated cell sorting (FACS) when both the antigen and the MHC it binds are known or predicted. Patient specific neoantigens can be predicted based on WES data (94, 95). Multimers have been used to identify antigen specific T cells from patient TILs and PBLs (63, 95) and from healthy donor PBLs (91, 94). In contrast, cell surface biomarkers on T cells can be used to identify reactive T cells without knowledge of the specific antigen or the HLA on which it is presented (96–100). Several biomarkers, including PD-1 (96, 101, 102), LAG-3, TIM-3 (102), OX40 (103), CD137 (84, 97–100, 102–104) and CD107a (105) and cytokine production, such as of IFNγ, indicate the T cell has interacted with its cognate antigen and can be used to isolate T cells (106). Such markers have been used to identify tumor reactive cells from both TILs and PBLs (96). While these approaches have successfully identified tumor reactive T cells, there are limitations. Not all T cells that are multimer positive, and therefore peptide-MHC specific, are able to exert cytotoxic effects against tumor cells expressing these antigens (105). Conversely, multimer staining may not detect all antigen-reactive T cells (107–109). This can be due to decreased TCR surface density or expression of TCRs with low affinity. This can be especially problematic as TCRs reactive with self-antigens and MHC class II antigens tend to have lower affinity for their target. Multimer staining can be enhanced to detect low-affinity TCRs with protein kinase inhibitors, such as dasatinib which decreases TCR downregulation, cross linking antibodies to stabilize multimer binding, anti-coreceptor antibodies, and staining with multimers with more peptide-MHC sites, i.e. dextramers or dodecamers over tetramers (107, 109). Additionally, cytokine production and cytotoxicity are independently regulated, therefor cytokine production does not always correlate with cytotoxic potential (105, 110). These limitations can make it more challenging to accurately identify tumor-specific cytotoxic T cells.

TCR Gene Therapy

Isolated individual reactive TCRs can be transduced and expressed into other T cells, known as TCR-T cells, to broaden therapy to additional patients. Paired TCR alpha and beta chain sequences can be identified from tumor-reactive T cells for subsequent cloning into expression vectors from pooled T cell (111, 112) or single cell (77, 83, 89, 92, 97, 113) sequencing data. Other methods to generate reactive TCRs for subsequent identification circumvent thymic selection to generate high affinity T cells reactive against specific tumor antigens. Immunization of mice expressing human HLA molecules (42, 114) or mice expressing the human TCR repertoire can be used for immunization and isolation of high affinity TCRs (115, 116). High affinity human TCRs with increased activity also can be isolated when human T cells are stimulated ex vivo with tumor antigens on HLA mismatched antigen presenting cells (117–120).

Alternatively, individual TCRs can be affinity enhanced *via* protein engineering to increase their anti-tumor effects (7, 65, 121, 122). As few as one or two amino acid changes in the complementarity determining regions can increase the affinity of TCRs (8, 65, 114, 123), evident by slower TCR off rates (124). High throughput methods such as phage (124, 125), yeast (126, 127), and T cell display libraries (128, 129), along with somatic hypermutation (130), and in-vitro T cell differentiation (131) have been employed to generate high affinity TCRs, sometimes in conjunction with available structure data (132). While increasing TCR affinity has been shown to increase the effectiveness of the T

cell (65, 74, 123), TCRs whose affinities are too high can become less effective (115) and are at higher risk for cross reactivity (74, 115, 123). Drawing any direct correlation between TCR affinity and T cell efficacy can be challenging as affinity itself is determined based on two parameters, the on- and off-rate of TCR binding (133). One model describes T cell responses as requiring long enough dwell time between the TCR and peptide-MHC to stimulate signaling but having a fast enough off-rate to allow for sequential TCR binding and signaling amplification (134, 135). Therefore longer TCR/peptide-MHC half-lives may prevent serial triggering and hamper T cell responses (134). Additionally, mechanisms that decrease the efficacy of T cells have been identified in association with T cells with high affinity TCRs (136). These mechanisms include impaired T cell signaling, upregulation of the inhibitory receptors, such as PD-1, down regulation of costimulatory receptors (135), peripheral deletion, expansion of anergic T cells (136, 137) and TCR down regulation (136).

Finally, T cell therapies can be designed to target patient-specific tumor antigens or public tumor antigens. T cell responses against patient-specific mutated neoantigens have been associated with clinical successes (83, 87, 138) and should be subject to less central tolerance, as such neoantigens are not present in normal tissues (102, 139). Neoantigen-reactive T cells can be highly tumor specific as T cells are able to distinguish between single amino acid changes in peptides, representing either unmutated self or mutant peptide sequences (95). While targeting neoantigens is expected to result in less toxicities (97, 102, 140), finding tumor and patient-specific antigens and reactive TCRs to generate patient specific TCR-T cells is challenging, costly and not currently feasible on a broad scale. Public tumor antigens are not patient or cancer-specific and while they sometimes can be derived from mutant peptides (140, 141), they are often unmutated self-peptides from tumor associated proteins that are minimally or not expressed on normal cells (142, 143). Public antigens have the benefit that the same TCR construct can be used to treat multiple patients (74).

SPECIFICITY OF TCR-BASED THERAPIES

One the most important questions for clinical application of TCR-based agents is specificity, in order to prevent off-target toxicities. T cells and other TCR-based therapies rely on precise recognition of a short linear peptide sequence, typically 8 to 11 amino acids in length in the groove of a largely structurally constrained HLA class I protein (144). Therefore, the TCR must be able to distinguish between the different antigenic peptides derived from thousands of proteins, which may comprise highly similar amino acid sequences, challenging absolute specificity. The estimated 100 million different TCRs expressed by a human is dwarfed by the number of potential sequence targets in the proteome. Therefore, it is speculated that each TCR can recognize hundreds to thousands of different antigens (145). In this way, TCR promiscuity can be a source of both greater scope of protection, but also significant off-target toxicity. Additionally, native TCRs can have low, micromolar affinity for their cognate target, especially if they are targeting non-mutated peptides, due to thymic selection (65, 66). While increasing affinity, as described above, may increase the anti-tumor effects of the TCR, bypassing thymic selection increases the risk for off-target reactivity and toxicity (122) and hence, a balance between TCR activity and toxicity must be struck. The severe off-target toxicities sometimes seen with TCR therapies has emphasized the need for methods to predict reactive off-target peptides and their cells of origin (65, 142).

On-Target/Off-Tumor Toxicity

TCR-based therapies can lead to autoimmune toxicities caused by on-target/off-tumor responses, which occurs when the target antigen is expressed on normal cells. On-target/off-tumor autoimmune toxicity has been seen in some melanoma patients treated with exogenously expanded TILs, which recognized non-malignant melanocytes, as with T cells reactive against melanocyte differentiation antigens, such as MART-1, and with DMF5 TCR-T cells, specifically reactive with the MART-1 antigen (10, 113, 146). Patients variably experienced uveitis, rash, vitiligo, and hearing loss (147). Interestingly these toxicities are seen with DMF5 TCR T cells, but not with T cells expressing the lower affinity DMF4 TCR (143, 147), reactive with the same MART-1 epitope. The higher affinity TCR, DMF5, is hence both more efficacious and more toxic (147). On-target/off-tumor colitis has additionally been seen with T cells using a TCR developed in an HLA-A*02:01 humanized mouse, affinity enhanced for binding to a CEA epitope presented on HLA-A*02:01, likely due to CEA expression on gastrointestinal cells (7).

Molecular Mimicry and Sequence Similarity Toxicity

Molecular mimicry is when a peptide is able to stimulate TCR reactivity due to structural similarities with the target peptide. An example of molecular mimicry was observed with the affinity enhanced a3a TCR, reactive with a MAGE-A3 peptide on HLA-A*01 (65). While pre-clinical screening showed no evidence of cross reactivity, after TCR-T cell therapy patients died from cardiac failure, which was later attributed to cross reactivity with a peptide from the cardiac protein titin (65). The MAGE-A3 peptide target, EVDPIGHLY, and the Titin peptide target, ESDPIVAQY, differ in four amino acid positions, some of which are in the center of the peptide, the area principally responsible for contact with the TCR (66). Existing methods were unable to predict the cross reactivity preclinically (65, 66). Hence, better methods to predict off-target reactivity for TCRs is an unmet need.

An affinity enhanced ImmTAC designed from the same parent TCR as the a3a TCR was also found to have cross reactivity with the titin peptide. Tissue cross reactivity was still observed with a TCR agent that was more specific to the MAGE-A3 epitope and was attributed to high levels of titin expression on myoblasts (66). Therefore, protein expression is another

variable that needs to be considered when testing TCR-based therapies for cross reactivity, as well as affinity and half-life of the TCR/peptide-MHC interaction (66). Additional instances of cross reactivity have been seen due to sequence similarity, as seen with a TCR against a HLA-A*02:01 restricted MAGE-A3 peptide which recognized a peptide unpredicted to be expressed in the brain and led to neurotoxicity including death (114, 142).

Mixed TCR Dimers

If the endogenous TCR alpha or beta chain pairs with an exogenously introduced alpha or beta chain, the resulting TCR, a mixed TCR, could have unknown reactivity with normal peptides (148). These mixed TCRs bypass thymic selection, therefore there is no central tolerance to prevent reactivity with normal tissues (148). To prevent mixed TCR dimer formation, the human constant regions can be interchanged with murine constant regions (122, 147, 149–151), or human TCR alpha and beta constant regions can be interchanged with each other or with the constant regions from gamma-delta T cells, which cannot pair with endogenous alpha-beta chains (152). Additionally, constant regions can be modified to contain cysteines to promote disulfide bond formation and therefor pairing between the alpha and beta chains (85, 148). Other methods to prevent TCR chain mispairing involve transduction of alpha-beta TCRs into gamma-delta T cells (153, 154) and knocking out or down the endogenous TCR chains with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) (155) or small interfering RNA respectively (156–158). TCR mispairing can lead to off-target as well as decreased on-target activity (85, 149). Promoting proper TCR chain pairing increases TCR expression (85, 122, 149), avidity and activity (122).

PREDICTING OFF-TARGETS OF TCR-BASED THERAPEUTIC AGENTS

Prediction of TCR reactivities has proven difficult, as native TCRs are necessarily cross-reactive to enable tissue surveillance. Additionally, TCR reactivity is structurally complex being dependent on the quality and quantity of the expression of the TCR, MHC and presented peptides (1). Steps to detect cross reactive TCRs include screening for reactivity against cell lines known to express the HLA of interest, but not the proteins from which the target peptide is derived (65, 159), as well as HLA mismatched cell lines (159, 160). Reactivity with either of these cells would indicate off-target binding of the TCR and a potential for toxicity. A number of investigations have sought to predict cross-reactivity *via* structural analyses and predictive algorithms. Single amino acid replacement scans, such as alanine scans (**Figure 2A**), are often used to identify peptide residues important for TCR recognition (159, 161–163). Alanine scans involve changing every position in a peptide sequence to an alanine, if reactivity is abolished then that particular amino acid residue and its position are considered necessary for TCR reactivity. However, alanine screens do not identify important interactions if the substituted amino acid is similar to alanine. Changing that amino acid to alanine might still result in binding and therefore alanine screens are biased towards identification of TCR interactions with large and charged residues where the substitution to alanine resembles a major change. A second single amino acid replacement scan can be performed to provide a more complete picture of motif residues important for TCR reactivity (65, 160). This motif can subsequently be compared to human peptides (161), to identify putative cross-reactivities (65).

Other amino acid scans screen additional amino acid combinations to determine the TCR binding motif. X-scans (**Figure 2A**), similar to alanine scans, hold all peptide positions constant except one but change this position to any of the remaining 19 amino acids (162). Combinatorial peptide libraries (CPL) (**Figure 2A**) are another peptide screening method to determine TCR off-targets which allow additional peptide diversity. CPLs are designed so that one position of a peptide is held constant and the remaining positions are changed to any other amino acid (145, 161, 164). Peptides from CPL scans are screened in subpools (145) for TCR reactivity (161, 165). X-scans and CPLs allow for more potentially cross-reactive peptides to be screened compared to alanine scans providing a more complete picture and ranking of potential TCR reactive peptides (161, 162, 165, 166). In the aforementioned amino acid scans, T cell reactivity can be measured in numerous ways including *via* cytokine release (145, 161, 162), typically measuring IFNγ, T cell proliferation (164), target cell lysis (164, 166), detection of T cells activation markers and binding of soluble TCRs to peptide-MHC complexes (166).

Computational modeling methods take previously known TCR and peptide-MHC structural and reactivity data to create models to predict peptide-MHC targets (167–169) and cross reactivities (170). These models are based on the premise that TCRs with common targets will have structural and sequence similarities (167, 171). Characteristics used to compare TCRs include length, charge, hydrophobicity and sequence (171). Peptide-MHC complexes have also been compared to assess cross reactivity based on charge and available surface area (170). These methods are primarily limited by the amount of data available for model development and TCR comparison (167, 168).

However, these structural or predictive methods discussed above are neither comprehensive nor fully accurate, as the rules for binding of TCRs to their peptide/MHC sites are still poorly understood. Therefore, more empiric methods have been applied to the problem. Large libraries, where the peptide target is genetically encoded into expression systems have been used to identify TCR targets and off-targets. Display libraries of peptides have been developed for screening in yeast and baculovirus systems (**Figure 2B**) (172, 173). In these systems the MHC is expressed with the peptide attached by a linker (144, 172–174). For these systems the MHC must fold properly, and the peptide must bind the MHC properly (172). In another library system, known as PresentER (**Figure 2E**), peptides are directed to be loaded onto the endogenous MHC of mammalian cells through an endoplasmic reticulum signaling sequence. Target peptides

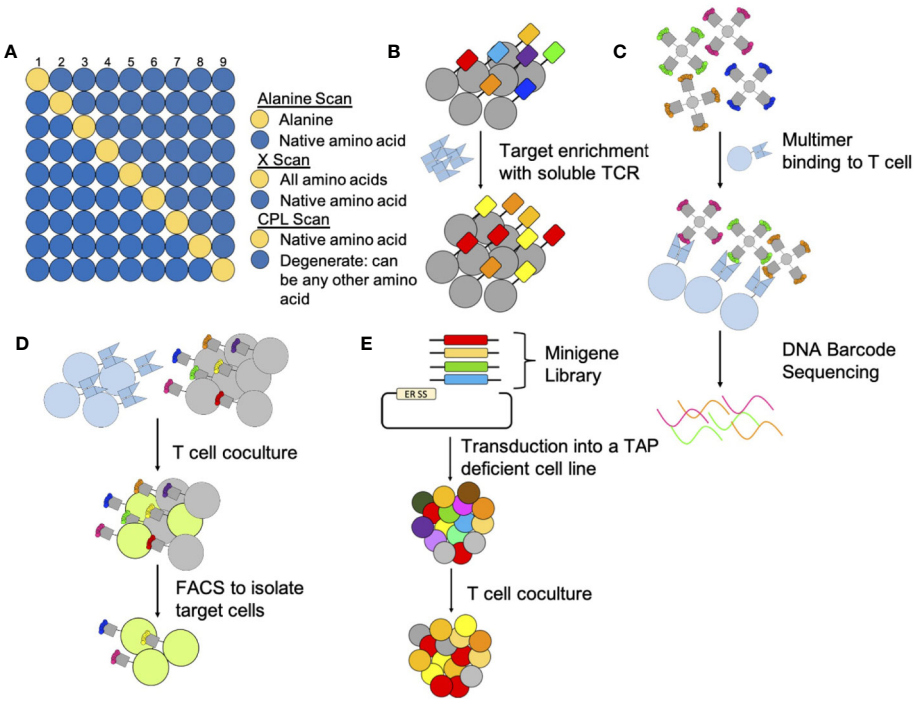

FIGURE 2 | Methods to determine targets and off targets of TCR-based therapeutics. **(A)** Peptide Scans: Peptide scanning techniques determine which residues are important and in which positions. Alanine scans and x-scans hold all positions in the native peptide (blue) except one (yellow); one position is switched to an alanine for alanine scans or to all other amino acids for X-scans. CPL scans hold one position constant (yellow) and all other positions (blue) can be any combination of amino acids. Peptides are then pulsed onto target cells to assay for TCR reactivity. **(B)** Yeast Display: Yeast display libraries genetically encode a peptide linked to an MHC. Multiple rounds of selection with soluble TCRs select for peptide-MHC complexes that are recognized by the TCR. **(C)** Multimers: Multimers can identify T cells that bind specific peptide-MHC complexes. To identify reactive peptide-MHC complexes in a pooled setting multimers are DNA barcoded prior to incubation with and binding to T cells. **(D)** T scan and SABR: The T Scan method genetically encodes for longer peptides that go through endogenous processing and presentation. Target cells that present peptide-MHC complexes targeted by T cells fluoresce through a granzyme reporter system and are subsequently sorted by FACS. For the SABR method, TCR expressing cells bind to target cells expressing the SABR receptor. After TCR binding, this receptor, which consists of an MHC linked to a CD3z and CD28 domain, signals to an NFAT report system causing target cells to fluoresce. Target cells can then be sorted by FACS. **(E)** PresentER: The PresentER system genetically encodes for peptides to be presented in MHC complexes on TAP-deficient cells. Target peptides are identified through coculture depletion assays with T cells.

are identified after coculture screens with T cells by assaying for peptide dropout *via* DNA sequencing (175). This system has the advantages of yielding actual peptide-MHC molecules in the context of human cell surface membranes on live cells for both *in vitro* and *in vivo* work, as well as allowing functional assays such as recognition and killing of targets to be measured, but is limited in the number of peptides that can be scanned in a single assay to a few tens of thousands, whereas the proteome may contain a million potential epitope sequences that bind to an individual MHC. An additional library screening technique uses signaling and antigen-presenting bifunctional receptors (SABR) (**Figure 2D**), where the target cell expresses peptides linked to MHC receptors fused to intracellular CD3ζ and CD28 domains. The target cells are identified through fluorescence, as these cells have an NFAT-GFP reporter system which is activated upon signaling from CD3ζ after TCR engagement. The presented target peptides are subsequently identified through sequencing. As with PresentER libraries, SABR libraries are limited in their size. However, SABR libraries can contain up to one million epitopes (176). The previously described libraries genetically encode for short antigenic peptides, the T-scan reporter system (**Figure 2D**) on the other hand encodes for larger amino acid sequences that need to undergo endogenous processing and presentation (177). Trogocytosis, which describes the facilitated exchange of membrane bound proteins after immune cells and target cells come into close contact, has also been used to identify TCR targets in library screens. This mechanism then allows for the identification of the recognized antigen presented on target cells, that were engaged by a T cell (178). Other library screening methods use DNA-barcoded MHC multimers (**Figure 2C**). The MHC multimer is screened for binding with a TCR followed by sequencing of the DNA barcode to determine which peptides were able to bind the TCR of interest or to develop recognition motifs to predict additional off targets (163). Such methods may prove useful in the preclinical characterization of TCR reactivity and could be paired with tissue expression data of off-target genes to predict site-specific toxicities.

CURRENT TCR-BASED CELLULAR AGENTS IN CLINICAL STUDY

The earliest studies in which TCR-T cells were infused into patients were reported in 2006 (143), an effort that was the culmination of decades of work by Rosenberg and colleagues to characterize the antitumor activity of TILs (179–181). In this early study, TCRs specific for the HLA-A*02:01 presented self-antigens MART-1, gp100, NY-ESO-1, and p53 were transduced into autologous peripheral blood mononuclear cells (PBMC) and infused into melanoma patients. TCRs specific for these antigens are among the most actively studied in TCR gene therapy clinical trials.

Notably, NY-ESO-1-reactive TCRs are being investigated by multiple academic and industry entities worldwide for the treatment of a range of solid and liquid tumors such as melanoma, sarcomas, lung cancers, and multiple myeloma (8, 121, 182, 183). GSK3377794, comprised of autologous T cells transduced with an affinity-matured NY-ESO-1–reactive TCR (184) has reached Phase Ib/II trials testing it in combination with checkpoint inhibitor, anti-PD1, therapy in NSCLC (NCT03709706).

WT1-directed TCRs have also shown promise for treatment of AML (185). WT1 has been designated a highly prioritized antigen (186), and the cytotoxicity of WT1-specific T cells against leukemic cells has been reported by multiple groups (6, 20, 187). WT1-specific T cells can be readily generated from most healthy donors; accordingly, a TCR isolated from a healthy donor could be used without enhancement of its native antigen-binding capability. Moreover, donor-derived Epstein-Barr virus-specific T cells were transduced with the TCR, rather than autologous T cells. This study is notable in several aspects as it utilizes a healthy donor-derived TCR, genetic modification of allogeneic T cells, and provides validation of the preclinical work involved in characterizing WT1 as a leukemia-associated antigen (188, 189). Results from two WT1 TCR Phase I/II trials utilizing autologous T cells are expected to clarify the relationship, if any, between graft versus host disease (GvHD), which has been seen in trials, and WT1-targeting (NCT02550535, NCT01621724, NCT02408016).

A class II-restricted TCR directed against an epitope of MAGE-A3 presented by HLA-DP*04:01/04:02 was shown to be well-tolerated and the CD4 autologous T cells persisted in 17 patients with metastatic cancer in a basket trial (190). Three partial responses in a variety of cancers correlated with T cell persistence of at least one month. Though this TCR was derived from a regulatory T cell clone (191), infused T cells did not appear to differentiate to regulatory T cells (Treg) on the basis of FOXP3 expression. The safety profile of this TCR contrasts that of previous MAGE-A3 TCRs (142, 192), and thus could prove to be an effective therapy with minimal toxicity in a wide range of tumors.

Of particular concern in TCR gene therapy is the safety of affinity enhanced self-antigen TCRs due to potential on and off-target toxicities (10, 123). The TCR targeting an HLA-A*02:01 presented epitope of CEA, described earlier, was found to induce severe colitis in all three colorectal cancer patients tested (7, 193) because of baseline CEA expression in colonic mucosa. An affinity enhanced TCR specific for MAGE-A3 (114) was found to cause severe neurotoxicity due to reactivity with a similar MAGE-A12 epitope (142). Another affinity enhanced TCR to MAGE-A3 caused lethal cardiotoxicity due to recognition of a titin-derived epitope (65, 192). These cases exemplify the critical need for characterizing TCR target recognition before clinical translation.

The safety of neoantigen reactive TCRs appears to be in stark contrast to that of the aforementioned self-antigen reactive TCRs because neoantigens derived from private somatic mutations are theoretically only presented by the tumor. In addition to tumor-selectivity, the potential for acquired resistance to TCR therapies targeting such mutations are expected to be lower in the case of targeting driver mutations. For example, T cells targeting mutant KRAS found in the endogenous TILs of a patient with KRAS-driven metastatic colorectal cancer showed minimal toxicity and all seven of the patient's lesions initially regressed. Interestingly, at 9 months a lesion escaped by means of downregulating the restricting HLA allele, while maintaining the same KRAS mutation (141). Hence, KRAS driver mutations are not easily mutated into escape variants, but downregulation of HLA can be an alternative mechanism of immune escape. To this end, KRAS mutant-specific TCRs have been generated in HLA transgenic mice and are currently being tested in phase I trials (42) (NCT03190941). Similarly, T cell responses to TP53 hotspot mutations have been found in TILs in different epithelial cancers with multiple HLA allele restrictions (84, 194). The recurrence of TP53 mutations encoding immunogenic neoantigens presents profound opportunities for TCR-based therapy across a variety of solid tumors (195).

Several TCR-transduced cells are being tested in ongoing or recently completed Phase I and II trials (**Table 2**). New antigens being targeted in these trials include HERV-E and TRAIL-DR4. New TCR modalities are being assessed as well, such as suicide gene-containing T cells that provide a kill-switch and Vγ9Vδ2 TCR-transduced T cells which recognize uncharacterized tumor antigens in an MHC-independent manner.

TCR-BASED, NON-CELLULAR THERAPIES

Current T cell therapies using either TILs or TCR transduced T cells are patient-specific and require TCR gene transduction or expansion of the patient's T cells *in vitro* before reinfusion into patients. Such processes have proven to be difficult to translate into a widely available therapy. Several therapeutic modalities (**Table 3**) have been developed to overcome such limitations and broaden therapeutic options to a wider range of patients. In particular, soluble T cell redirecting biologics based on either TCR or immunoglobulin molecules, in conjunction with redirection of powerful T cell cytotoxicity provides a promising alternative.

Immune Mobilizing Monoclonal TCRs Against Cancer (ImmTac)

A new class of bi-specific molecules, ImmTacs, are soluble T cell engagers (sTE), designed to use a TCR specific for a peptide-HLA complex, genetically linked to a single chain variable fragment

TABLE 2 | Selected TCRs and T cells in ongoing or recently completed clinical trials.

Trial ID	Target	HLA allele	Phase	Industry partner	Citations	Notes and Indication(s)
NCT01586403	Tyrosinase	A*02:01	I		(196)	Melanoma
NCT03399448	NY-ESO-1	A*02:01	I	Tmunity Therapeutics	(197)	CRISPR-edited to replace endogeneous TCR Myeloma, melanoma, sarcoma
NCT01343043	NY-ESO-1	A*02:01 A*02:05 A*02:06	I	GlaxoSmithKline	(198)	Affinity enhanced TCR Synovial Sarcoma
NTR6541	Unknown; CD277-mediated presentation	N/A	I	Gadeta	(199)	Vγ9Vδ2 TCR AML, MDS, MM
NCT03132922	MAGE-A4	A*02	I	Adaptimmune Therapeutics	(200)	Affinity enhanced TCR Various solid tumors
NCT04044768	MAGE-A4	A*02	II	Adaptimmune Therapeutics	(200)	Affinity enhanced TCR Synovial Sarcoma or Myxoid/Round Cell Liposarcoma
UMIN000002395	MAGE-A4	A*24:02	I	Takara Bio	(201, 202)	A24 transgenic mouse-derived TCR Esophageal cancer
UMIN000011519	WT1	A*24:02	I	Takara Bio	(6, 203)	AML, MDS
NCT02592577; NCT02989064	MAGE-A10	A*02:01 A*02:06	I	Adaptimmune Therapeutics	(204)	Affinity enhanced TCR NSCLC, melanoma, head & neck
NCT03503968	PRAME	A*02:01	I/II	Medigene		AML, MDS, MM
NCT02743611	PRAME	A*02:01	I/II	Bellicum Pharmaceuticals	(205)	incorporates inducible caspase-9 suicide gene AML, MDS, melanoma
NCT03686124	PRAME	Undisclosed	I	Immatics		Solid tumors
NCT03925896	EBV LMP2	A*02 A*11 A*24	I			Nasopharyngeal carcinoma
NCT03971747	AFP	A*02:01	I	Cellular Biomedicine Group		HCC
NCT03441100	MAGE-A1	Undisclosed	I/II	Immatics		NSCLC, HCC
NCT03326921	HA-1	A*02:01	I			incorporates inducible caspase-9 suicide gene Acute leukemias
NCT03354390	HERV-E	A*11:01	I		(206)	Renal cell carcinoma
NCT02988258	CMV pp65	A*02:01	I		(207)	post-HSCT CMV disease
NCT02719782, NCT02686372	HBV	Various	I	Lion TCR	(208, 209)	HCC
NCT00923390	TRAIL-DR4	N/A	I		(210)	MHC-independent TCR Renal cell carcinoma
NCT00991224	HIV SL9	A*02	I	Adaptimmune Therapeutics		Affinity enhanced TCR HIV/AIDS
NCT03132792	AFP	A*02	I	Adaptimmune Therapeutics		HCC

NY-ESO-1, New York esophageal squamous cell carcinoma 1; HCC, hepatocellular carcinoma; MDS, myelodysplastic syndrome; MM, multiple myeloma; MAGE, melanoma associated antigen; PRAME, preferentially expressed antigen in melanoma; EBV, Epstein–Barr virus; LMP2, latent membrane protein 2; AFP, alpha fetoprotein; HERV-E, human endogenous retrovirus group E; CMV, cytomegalovirus; HSCT, hematopoietic stem cell transplantation; HBV, hepatitis B virus; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR, death receptor; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome.

(scFv) of an anti-CD3 mAb. Structurally, an ImmTac begins with a human TCR or an affinity enhanced TCR. While recombinant soluble TCRs (lacking transmembrane and intracellular parts) should theoretically be an ideal therapeutic vehicle for targeting intracellular tumor antigens, TCRs are inherently unstable in soluble form and tend to form aggregates, which poses a significant technical challenge in developing such molecules as therapeutics (211). To address this issue, ImmTacs are designed to stabilize soluble TCRs through the incorporation of an interchain disulfide bond buried within the core of the TCR. Finally, an anti-CD3 scFv is encoded *via* a flexible linker to the beta chain of the TCR (212). Once TCRs engage their antigenic peptide-MHC complexes, the anti-CD3 effector arm mediates potent redirection of polyclonal T cells to the target. With this technology, cells expressing fewer than 100 copies of the targeted peptide-MHC complexes can be killed. Since natural TCRs have low, micromolar affinity, ImmTac technology allows increases in TCR affinity up to the subnanomolar range, allowing ImmTacs to target low density peptide-MHC complexes of intracytoplasmic tumor antigens (213). The most extensively studied ImmTac molecule, tebentafusp (IMCgp100), an affinity enhanced TCR specific for a gp100 peptide (a melanocyte differentiation antigen) presented on HLA-A*02 complexes, has demonstrated clinical efficacy as a monotherapy against the gp100-positive uveal melanoma (214, 215). In addition, three other molecules: GSK01 (directed to the cancer germline antigen NY-ESO-1), IMC-C103C (directed to cancer germline antigen MAGE-A4) and IMC-F106C (directed to a cancer testis antigen PRAME) are in phase I/II trials for treatment of multiple myeloma, melanoma and a range of other cancers (216). ImmTacs' high potency and drug-like soluble format make them easy agents to develop and distribute widely.

TABLE 3 | Characteristics of TCR, TCRm and traditional mAb.

Feature of Agent	TCR	TCR mimic mAb	mAb
Structure	Heterodimer which functions in a complex and forms a synapse upon activation.	Various soluble Ig formats or as transmembrane CAR in cells.	Various soluble Ig formats or as transmembrane CAR in cells.
Affinity (Typical)	Micromolar or modified to nanomolar.	Picomolar to nanomolar	Picomolar to nanomolar
Plasma kinetics	Soluble forms are unstable alone. ImmTac half-life is 6–8 h	Half-life may be from hours to weeks based on structure.	Half-life may be from hours to weeks based on structure.
Epitope targets	Peptide-MHC complex on cell surface. Peptides derived from total proteome or may be viral or microbial in origin.	Peptide-MHC complex on cell surface. Peptides derived from total proteome or may be viral or microbial in origin.	Protein or carbohydrate on cell surface. Soluble proteins or other molecules. Limited to extracellular and secreted proteome. May also be viral or microbial components.
Therapeutic formats available	Bispecific forms or transduced as receptor into cell.	Native or modified IgG; BiTE and Bispecific forms; CAR; ADC; radioconjugate.	Native or modified IgG; BiTE and Bispecific forms; CAR; ADC; radioconjugate.
Effector functions	Directs T cells to kill.	Redirects T cells to kill; ADCC; ADCP; CDC; recruits NK cells or macrophages to kill. Can serve as a vehicle for drug or isotope delivery.	Neutralizes or activates signaling. Redirects T cells to kill; ADCC; ADCP; CDC; recruits NK cells or macrophages to kill. Can serve as a vehicle for drug or isotope delivery.
Marketed agents	None.	None.	Numerous, for multiple diseases.
Minimal epitope number required	May be a dozen or less	Tens to hundreds	Hundreds to Thousands and much higher

BiTE, bi-specific T cell-engager; ADC, antibody-drug conjugate; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; NK, natural killer.

TCR-Mimic Monoclonal Antibody (TCRm)

MAb-based therapy has become one of the most successful and important strategies for the treatment of cancer and rheumatologic diseases (217, 218). MAbs are characterized by high target specificity, limited side effects and prolonged half-life *in vivo*. The intrinsic multifunctional cellular engagement of mAbs include antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), blocking of ligand-based signaling, and direct signaling or inhibition *via* various pathways. In addition to their intrinsic properties, mAbs can in an antigen-specific manner deliver potent cytotoxic agents such as toxins, drugs, or radionuclides to cancer cells (219). Finally, mAbs can be re-engineered to generate CARs or bi-specific antibodies to redirect the T cells or other effector cells for potent anticancer therapy (220). Commercial therapeutic mAbs are directed to extracellular or cell surface proteins; therefore, the vast majority of intracellular tumor-associated antigens (TAAs) are not addressed by FDA approved mAb therapy (221).

TCRms targeting peptide-MHC complexes, combine TCR recognition of peptide-MHC complexes, with the potency and versatility of mAb drugs. The well-characterized platform of TCRms provides a highly feasible method to target intracellular tumor antigens for a broad range of patients. Increasing advances in library screening technology allow rapid identification and selection of highly specific TCRms to intracellular tumor antigens. A number of mouse and human TCRm antibody fragments such as antigen binding fragments (Fab) or scFvs have been identified (222, 223), as well as several full-length human TCRms, and have been investigated as potential therapeutic agents. A murine hybridoma-generated TCRm (8F4) reactive with the myeloid leukemia antigen PR1-derived epitope (VLQELNVTV) was found to bind HLA-A*02:01 and was later humanized. This TCRm eliminated human AML in xenografts (224) and has now advanced to clinical trials. While active alone, to improve its potency 8F4 was engineered into a bi-specific T cell-engager (BiTE) to redirect polyclonal T cells to PR1-positive leukemias (225). The first fully human TCRm, ESK1, specific for a WT1-derived epitope/HLA-A*02:01 complex, was developed by our group (226). WT1 is expressed in a wide range of human cancers and has been an important target for TCR-based adoptive cell transfer of engineered T cells, as well as peptide, DNA, and dendritic cell vaccines (185, 227–231). WT1 expression encompasses both hematopoietic and solid tumors and therefore, a TCRm targeting this oncoprotein should have a broad application as a therapeutic agent against a variety of human leukemias, myeloma and solid tumors (226, 232).

Our group has described TCRms specific for a cancer germline antigen, PRAME, which is widely expressed in various cancers (recognizing ALYVDSLFFL/HLA-A*02:01) (46), and FOXP3, the hallmark protein for Tregs (recognizing TLIRWAILEA/HLA-A*02:01) (233). The TCRm specific for the FOXP3 epitope is particularly interesting because of the profoundly suppressive role of Tregs in the TME. Strategies of depleting Tregs by mAbs against surface proteins such as CD25, CCR4 and GITR, have not been successful thus far because these molecules are shared between Tregs and other immune effector cells (234–236). Other human TCRms to important cancer-associated and viral targets have been described (**Table 4**).

The antibody-based format for TCRm offers the opportunity for optimization *via* protein engineering strategies to address different needs:

A. Fragment crystallizable (Fc) region modification. The ADCC activity of mAbs can be enhanced 5-to 10-fold by Fc region protein engineering (247) or by modification of Fc-region glycosylation (248, 249).
B. Bispecific mAbs (BsAb). BsAbs are designed to recognize two different epitopes or antigens, and they comprise a large family of molecules, with a wide variety of formats (250). Such bispecific molecules function by recruiting and activating polyclonal T cells or other effector cells. BiTEs are a subtype of BsAb, composed of a scFv specific for tumor antigen on one arm, linked to a scFv for CD3 on the other arm. Such a BiTE molecule functions by recruiting and activating polyclonal T cells at tumor sites, thereby bypassing MHC restriction and co-stimulation, while retaining epitope specificity needed for traditional TCRs (251). The ESK1-BiTE was the first TCRm-based BiTE, which showed superior cytotoxicity than an immunoglobulin form against a wide range of tumor cells expressing WT1 *in vitro* and *in vivo* in mice. The ESK1-BiTE also induced robust secondary CD8 T cell responses against other epitopes *via* epitope spreading (232). Such a mechanism may be important for long-lasting anti-tumor immunity by controlling the outgrowth of tumor cells that have lost the target protein or that have downregulated the primary target during tumor evolution. In addition, as a small molecule, BiTEs may penetrate more easily than CAR T cells into the TME of solid tumors, where it can bridge tumor targets with TILs.
C. TCRm CAR T cells. CAR T cell constructs use immunoglobulin scFvs, recognizing extracellular cell surface protein antigens expressed by cancer cells. The scFv is linked to a transmembrane and intracellular signaling domain generally containing the CD3ζ chain of the TCR complex, as well as costimulatory domains of CD28 or 41BB. Following the clinical success of CD19 CAR T cell therapy in human leukemia (252), many CAR T cells have been developed targeting a variety of cell surface molecules (253). Using TCRms, described above, CAR T cells have been generated against the intracellular tumor antigen WT1, by use of the ESK1 TCRm scFv, thereby opening the door for CAR T cells to enter an entirely new universe of antigens (254). These studies show CAR T cells can be used to target intracellular tumor antigens, in contrast to CAR T cells using traditional scFVs that target extracellular proteins, and thus expand the utility of this platform to include a large majority of cancers. The first CAR T cells expressing a TCRm scFv for alpha fetoprotein (AFP)-HLA-A*02 recently advanced to human trials for the treatment of hepatocellular carcinoma. Currently, more CAR T cells are being developed from TCRms recognizing various epitopes from NY-ESO-1, gp100, and MAGE-A1, in the context of HLA molecules (240).
D. Affinity maturation. Cell surface protein targets of mAbs normally have a high density of typically 10,000 to 1,000,000 molecules per cell. In contrast, intracellular tumor antigens presented as peptide-HLA complexes on the cell surface typically have low densities, often far less than a few hundred molecules (46, 226, 255, 256). Therefore, WT1-specific TCR gene therapy (185) and ImmTac molecules use affinity enhanced TCRs (212). Similarly, *in vitro* affinity maturation of mAbs, often with phage library technology, has successfully been used to optimize specific mAbs with increased affinity for their targets (257). Most TCRms have been derived using phage display technology and have yielded relatively high affinity TCRms, thereby reducing the need for affinity maturation.

Challenges and Opportunities for Soluble TCR Constructs and TCRm

Soluble TCR-based agents represent novel classes of biologics that make immunotherapy accessible for some of the most

TABLE 4 | Human TCRm reported.

Antigen target	HLA restriction	Indications and uses	Citations
Proteinase 3	A*02:01	Myeloid Leukemias; formatted as IgG and CAR T cell	(224, 225)
WT1	A*02:01	Leukemias and various solid tumors; formatted as IgG, BiTE, and CAR T cell	(226)
PRAME	A*02:01	Leukemias and various solid tumors; formatted as IgG, BiTE, and CAR T cell	(46)
FOXP3	A*02:01	Tregs, FOXP3+ T cell malignancies and other types of cancers; formatted as IgG, BiTE	(233)
Ras G12V	A*02:01	Wide range of solid tumors: pancreatic, colon, ovarian and more; formatted as IgG	(237)
Epstein Barr Virus	A*02:01	B cell lymphoma and carcinoma; formatted as IgG	(238)
WT1	A*24:02	Leukemias and various solid tumors; formatted as CAR T cell	(239)
Minor HA-H1	A*02:01	Leukemias; formatted as CAR T cell	(240)
AFP	A*02:01	Hepatic carcinoma; formatted as CAR T cell	(241)
hCG-beta	A*02:01	Ovarian, colon, and breast cancer; formatted as hIgG1, mIgG2a	(242)
NY-ESO-1	A*02:01	Melanoma and solid tumors; formatted as Fab, CAR T cell	(243, 244)
MAGE-A1	A*01:01	Melanoma; formatted as CAR T cell	(240)
GP100	A*02:01	Melanoma; formatted as CAR T cell	(240)
MUC-1	A*02:01	Breast cancer; formatted as Fab	(245)
hTERT	A*02:01	Melanoma and prostate cancer; formatted as Fab	(246)

FOXP3, forkhead box P3; hCG, human chorionic gonadotropin; GP100, glycoprotein 100; MUC-1, mucin 1; hTERT, human telomerase reverse transcriptase.
TCRm mAbs for proteinase 3 and AFP are currently in clinical trials.

interesting and highly tumor specific intracellular antigens and offer pharmacological and manufacturing advantages (**Table 3**). However, fundamental questions remain to be studied in order to further advance these drugs. First, given the intrinsic nature of TCR recognition of a linear peptide bound to HLAs, cross reactivity to other similar complexes is an important issue in all TCR-based therapies. Second, unlike TCR, TCRm are not natural structures that evolved with thymic selection to recognize peptide-HLA complexes. As antibodies are generally selected on membrane bound soluble proteins or carbohydrate antigens during B cell development *in vivo*, selection methods using phage or other libraries may introduce unnatural biases and unstable structures in addition to cross-reactivity. TCRm may never completely mimic natural TCR recognition. For example, crystallography studies have shown that the ESK1 Fab primarily interacts with N-terminal residue of the peptide and HLA-A*02:01 (258). An alanine substitution study showed that the TCRm mAb specific for the PRAME peptide-HLA-A*02:01 mainly recognized the C-terminal residues of the peptide (46). In general, TCRs dock onto peptide-HLA complexes using a conserved canonical binding mode, forming a large binding interface between the TCR and peptide-HLA, enabling broader contacts across both peptide backbones and HLA heavy chain. Lessons learned from the early development of TCRm could help optimize screening strategies of phage libraries to select ideal phage clones that more closely mimic TCR recognition. These strategies include selection of mAbs that bind with optimal valency between HLA and peptide, or mAbs that bind to a broader range of amino acid residues in the center of the peptide in a fashion similar to TCRs (259). Additionally, native TCRs tend to have orders of magnitude lower affinities than TCRm or engineered TCRs. The impact this has on activity and specificity, and the importance of affinity in different formats is not well understood. For example, a TCR-T cell or TCRm CAR T cell may need less affinity than a soluble format such as a BiTE due to its multivalent avidity. However, an anti-Ep-CAM BiTE has been shown to form a synapse after engaging its target because of the proximate contacts between effector and target cells (260). A recent study directly compared a scFv specific for NY-ESO-1p157/HLA-A*02:01 complex in BiTE and CAR-T cell formats. The conclusion was that the BiTE and CAR T cells showed a similar functional avidity, assessed by cytokine production and killing activity (261). A study comparing a TCR specific for a WT1-derived peptide/HLA-A*02:01 complex and a TCRm specific for the same complex showed that while the native low-affinity alpha-beta TCR maintained potent cytotoxic activity and specificity, the high-affinity TCR-like antibody CAR T cells exhibited reduced activity and loss of specificity. This TCR-like mAb in a monovalent or bivalent context maintained high specificity, however, when the avidity of this mAb was increased through expression in a CAR T cell format, it exhibited loss of specificity (262). This study suggested that TCRm is less suitable for CAR T cell format than being used as mAb format coupling with more potent drugs. However, function of each mAb depends on the specificity of the particular TCRm used for construction of the CAR T cells. Although this study raised an interesting question, one pair of TCRm vs TCR may not generalize the function of these two formats. Interestingly, a number of TCRms have been converted to a CAR T cell format (**Table 4**). With a rapidly growing numbers of these new modalities, detailed studies are required to address these fundamental biological questions.

In addition, despite all the advantages that TCR-based, non-cellular therapies offer, they also have certain limitations. Both ImmTacs and BiTEs have a short half-life (4 to 8 h), which requires continued administration of the agents (216, 232). However, this can be overcome by a growing number of engineering technologies.

SUMMARY AND PERSPECTIVE

TCR-based therapies provide a number of unique advantages over other immunotherapies, but also present challenges associated with their structures and the methods used for their generation. On and off-target identifications and toxicity prediction remain problematic. TCR-based agents are a powerful modality on which to create immunotherapies as the TCR is able to target the vast repertoire of cancer associated and mutated proteins found in all subcellular locations (3). The TCR's unique and valuable recognition properties have been taken advantage of in adoptive cell therapies, where reactive T cells are enriched or T cells are modified to express reactive TCRs, and in non-cellular therapies, which bypass the extensive process of T cell enrichment, modification and expansion, while mimicking the peptide recognition properties of the TCR in the form of soluble TCRs or antibodies. In distinct contrast to traditional antibodies and CAR T cells, TCR-based therapies recognize a short peptide bound to an MHC found on the surface of cells (144). The TCR's unique ability to recognize intracellular proteins in these complexes both shapes and constrains their functions. Moreover, the origins of their antigenic specificity are dependent on a linear sequence of amino acids that may be shared by other proteins in the proteome. In addition, the complicated and highly regulated process of antigen presentation means that not all peptides are presented, and others are displayed are at insufficient densities to trigger TCR-based recognition or responses. This problem may be overcome in some cases with small molecule drugs or cytokines and such a strategy could be considered for combination therapies. Endogenous TCRs are able to discern between peptides with single amino acid changes (95), enabling precise differentiation between peptides; despite this, TCRs are inherently promiscuous to enable proper immune surveillance, resulting in potential liabilities for their use therapeutically. Compounding this issue is that in silico predictions of peptide presentation on MHC molecules are inaccurate. Ideally, selected epitopes should be validated by using mass spectrometry to verify peptide-MHC presentation, yet these methods are costly and tedious.

While manipulating TCR affinity has been shown to increase their effectiveness (65, 74, 123) and would provide agents with better pharmacologic properties, TCRs whose affinities are too

high risk cross reactivities (74, 115, 123). Additionally, affinity enhanced, modified and artificial TCR-based therapies do not necessarily adhere to the same binding rules as native TCRs, due to the absence of thymic selection. Therefore, toxicities from TCR-based therapies are of concern and methods to identify potential cross-reactive or off-target toxicities are imperative. Current preclinical strategies to predict toxicities by determining on-target/off-tumor and off-target antigens are largely empiric and are unable to cover the vast potential repertoire of epitopes in the proteome. These failures have led to clinical toxicity in early trials. To avoid toxicity, the targeting of neoantigens may be used, but identifying patient specific tumor antigens and reactive TCRs is challenging, costly and currently not feasible broadly. Alternatively, public tumor antigens offer broader applications, but are rare and may not be absolutely cancer specific.

Despite challenges to development, TCR-based therapies have shown great potential in clinical use, targeting seemly un-targetable intracellular proteins. In distinction to commercial therapeutic mAbs or CAR T cells, which are generally limited to a small number of extracellular or cell surface proteins; TCR-based agents allow, for the first time, access to the vast majority of intracellular tumor associated antigens (TAAs) that are not currently addressed by FDA approved therapies. It is expected that technologies to automate the identification of target and off-target epitopes, and rapid new methods to generate TCRs, as well as new soluble and cell bound structures that take advantage of the unique recognition properties of the TCR, will soon result in a great expansion in these agents to a broader population of patients with cancer and other diseases.

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Conflict of Interest: MSKCC has filed for patent protection on behalf of DAS and TD for inventions related to this paper. TD is an advisory board member for Eureka Therapeutics. DAS is an advisor for, or has equity in, Eureka Therapeutics, Oncopep, Sellas, Pfizer, and Iovance,

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Global Perspective on the Development of Genetically Modified Immune Cells for Cancer Therapy

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Since the first genetically-engineered clinical trial was posted to *clinicaltrials.gov* in 2003 (NCT00019136), chimeric antigen receptor (CAR) and T-cell receptor (TCR) therapies have exhibited unprecedented growth. USA, China, and Europe have emerged as major sites of investigation as many new biotechnology and established pharmaceutical companies invest in this rapidly evolving field. Although initial studies focused primarily on CD19 as a target antigen, many novel targets are now being evaluated. Next-generation genetic constructs, starting materials, and manufacturing strategies are also being applied to enhance efficacy and safety and to treat solid tumors as well as hematologic malignancies. Fueled by dramatic clinical efficacy and recent regulatory approvals of CD19-targeted CAR cell therapies, the field of engineered cell therapeutics continues to expand. Here, we review all 745 genetically modified CAR and TCR clinical trials with anticipated accrual of over 28,000 patients posted to *clinicaltrials.gov* until 31st of December 2019. We analyze projected patient enrollment, geographic distribution and phase of studies, target antigens and diseases, current strategies for optimizing efficacy and safety, and trials expected to yield important clinical data in the coming 6–12 months.

Keywords: chimeric antigen receptor T cells, T cell receptor, cell therapy, genetically modified cells, clinical trials

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INTRODUCTION

Genetic engineering of immune cells to express defined antibody-based chimeric antigen receptors (CAR) has led to highly effective cell-based therapeutics for cancer. Several groundbreaking preclinical and clinical efforts in the late 1980's and 1990's laid the groundwork for modern CAR and TCR clinical investigation. These include clinical studies of ex vivo expanded tumor infiltrating lymphocytes (1) and the first chimeric receptors developed by Kurosawa, Eshhar, and colleagues (2–4). When combined with advances in viral transduction of immune cells (5–7) and the incorporation of costimulatory domains with receptor constructs (8–10) these different streams of scientific discovery enabled the dramatic number of CAR and T cell receptor-based (TCR) therapies that are currently ongoing. This foundational work has been previously well-reviewed (11–13). Now, already in clinic for over a decade, anti-CD19 CAR-T cells have shown remission rates as high as 90% in pediatric ALL and 50%–90% in adult B-cell malignancies (14–16). Remarkable efficacy in patients with multiply relapsed or refractory disease led to approval of

tisagenlecleucel and axicabtagene ciloleucel anti-CD19 CAR-T therapies for relapsed B cell leukemia and lymphoma, in the United States, Europe and Japan (17–20). In July 2020, the US FDA granted accelerated approval to brexucabtagene autoleucel for relapsed adult mantle cell lymphoma (21). Additional anti-CD19 CAR-T constructs are in late stage development, and Phase III trials are now comparing CAR products with standard second-line therapies. The results of these studies may further extend the indications for anti-CD19 CAR-T therapy to patients prior to developing highly resistant tumor cells and complications from prior therapy.

The success of CAR-T therapies has sparked a worldwide surge of clinical trials seeking to improve safety and efficacy and identify new disease indications for genetically-engineered immune cell therapies. To quantitatively explore the evolution of this new treatment modality, we generated a database encompassing all CAR and TCR-based interventional clinical trials posted on *clinicaltrials.gov* from the first trial recorded in 2003 to the last one registered in 2019.

METHODS

Clinical trials involving TCR and CAR therapies were extracted from *clinicaltrials.gov*. Data were then sorted and analyzed manually for quality control purposes. Only interventional trials posted prior to December 31, 2019 were selected. Trials investigating lymphocyte-based therapies not featuring genetic modifications with CARs or TCRs, such as antigen-expanded cytotoxic T lymphocytes or tumor-infiltrating lymphocytes, were excluded from this report. Average patient numbers were rounded to the closest and smaller integer. Of note, reported patient enrollment indicates anticipated enrollment over the full course of each trial and does not reflect actual numbers of patients enrolled at any given time point. Country totals reflect the location of anticipated patient enrollment to the clinical trial and not the sponsor's country of origin. Clinical trial phases and sponsors were defined as described in **Supplementary Tables 1** and **2**. Trial status, e.g. active, recruiting, not yet recruiting, was not collected in this analysis. *Clinicaltrials.gov* data is limited by what end-users disclose in trial entries and not all trials are registered. If a parameter was omitted in a *clinicaltrials.gov* entry, the trial was excluded from reported totals for that variable, e.g. a CAR study with unspecified target was excluded from tables summarizing number of trials per target. Additional information regarding these trials may be publicly available but was not included in this analysis. Other databases such as EudraCT also catalogue clinical trials conducted with genetically modified immune cells. Since these databases largely overlap with *clinicaltrials.gov*, we limited our analysis to trials listed in *clinicaltrials.gov*.

SEVENTEEN-YEAR HISTORY OF CLINICAL INVESTIGATION

Since the first genetically-engineered T-cell trial was listed on *clinicaltrials.gov* in 2003, 745 total CAR and TCR clinical trials have been registered (**Figure 1A**). Between 2003 and 2007, only eight trials were listed. Studies increased in the next 5-year period 2008-2012, in which 72 new clinical trials were posted. Annual new trials rose dramatically beginning in 2014 (**Figure 1A**). In the most recent 5-year period, 608 new trials were registered on *clinicaltrials.gov*. Reported patient enrollment reflected trends observed in the number of new clinical trials (**Figure 1B**). (Of note, reported patient enrollment on *clinicaltrials.gov* may not reflect actual patient totals on trial). In the early period from 2003 to 2008, only 306 patients were anticipated to enroll in a limited number of clinical trials. From 2009 to 2014, expected eventual enrollment across all trials increased to 1,709 patients. This number rose dramatically in the last 5-year period in which 24,695 patients were anticipated in CAR and TCR trials.

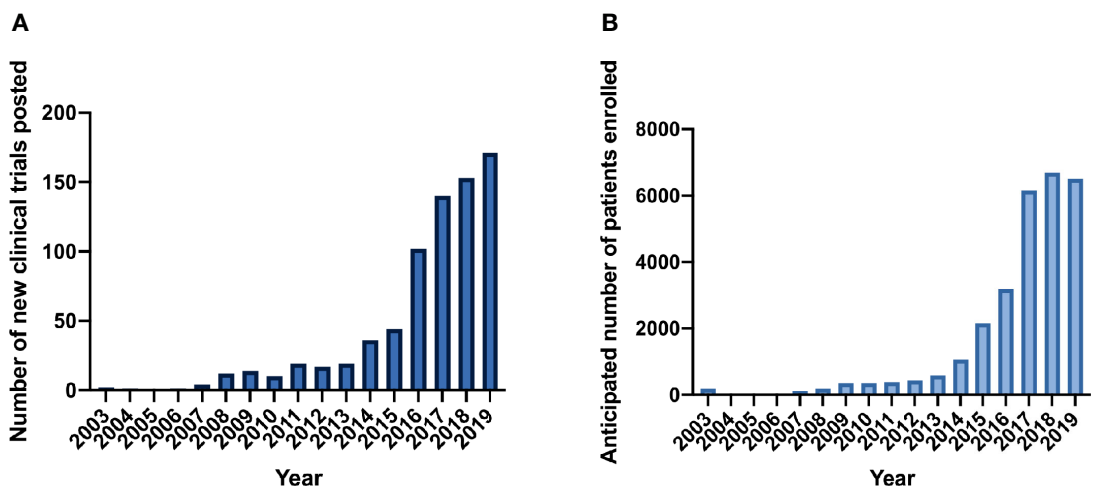

FIGURE 1 | CAR and TCR clinical trials on *clinicaltrials.gov*. **(A)** New clinical trials posted per year. **(B)** Anticipated patient enrollment per year.

Overall, 641 CAR clinical trials were listed compared to 102 TCR-based therapies, while two trials involved dual CAR and TCR therapies. Although genetically-engineered TCR trials predominated from 2003 to 2008, the sharp increase in clinical investigation that began in 2014 focused on CAR trials (**Figure 2A**). This trend continued in 2019, when 158 new CAR trials were listed while only 11 new TCR trials were started.

EVOLVING INTERNATIONAL LANDSCAPE OF GENETICALLY MODIFIED CELL THERAPIES

To better understand where CAR and TCR trials have taken place, we analyzed clinical trial postings by geographic area. Forty-seven percent of overall trials were based in China, 44% from USA, and 6% European (**Figure 2B**). Other countries including Japan, Australia, Canada, Malaysia, New Zealand, and Israel hosted few trials (n=14, collectively). While most CAR trials opened in China (n=330 of 641), overtaking USA in 2015, most TCR studies were conducted in the USA (n=69 of 102).

From 2003 to 2007 only eight trials were listed in *clinicaltrials.gov*, four CAR trials and four TCR-based studies, all conducted in the USA. The first CAR trial began in the USA in 2003. Registered CAR studies opened in Europe in 2010, and China entered the field in 2012 (**Figure 3A**). From 2012 to 2015, China hosted 23 CAR trials. In 2016 alone, 61 CAR trials occurring in China were registered. Since 2016, more CAR trials have opened in China than in USA (307 vs. 159, respectively). During this same period, 31 new CAR trials began in Europe. Following this trend, trials in China reported the highest annual patient enrollment since 2016, followed by USA and Europe (**Figure 3B**). Though most CAR clinical trials took place in China, average expected patient enrollment per trial was significantly lower than in USA trials (p-value = 0.0003, **Figure 3C**). This difference was especially striking for late phase trials (phase II/III and III). USA opened five late phase trials with average anticipated enrollment of 208 patients, while China had five late phase trials, averaging 18 reported patients per trial.

From 2003 until 2013 academic sponsors supported the vast majority of trials (91%). Subsequently, industry became more engaged in the field and commercial entities are now the predominant sponsors of both CAR and TCR-based clinical trials. In the last 5 years, 54% of studies had industry sponsors and 46% had academic sponsors. This trend is particularly evident in China, where 55 different companies funded CAR and TCR trials versus 34 in USA and 12 in Europe from 2003 to 2019. This may reflect differences in regulatory requirements between countries and Chinese initiatives such as "Made in China 2025" targeting investments in research and development (22, 23). Across all countries, industry sponsored nearly half of all CAR (n=301) and TCR trials (n=42) (**Supplementary Table 2**). International collaborations and mergers have supported the worldwide increase in genetically-modified immune therapies, largely in CAR products. In 2017, Johnson & Johnson invested $350 million in the Chinese Biotech Nanjing Legend Biotechnology Co. for the global rights to co-develop and market experimental CAR treatments (24) and Novartis invested $40 million into Cellular Biomedicine Group in USA (25). Major corporate buyouts of USA CAR manufacturers also occurred. Gilead bought Kite Pharma in August 2017 (26), and Celgene bought Juno Therapeutics in January 2018 (27). Bristol Myers Squibb then acquired Celgene in 2019 (28).

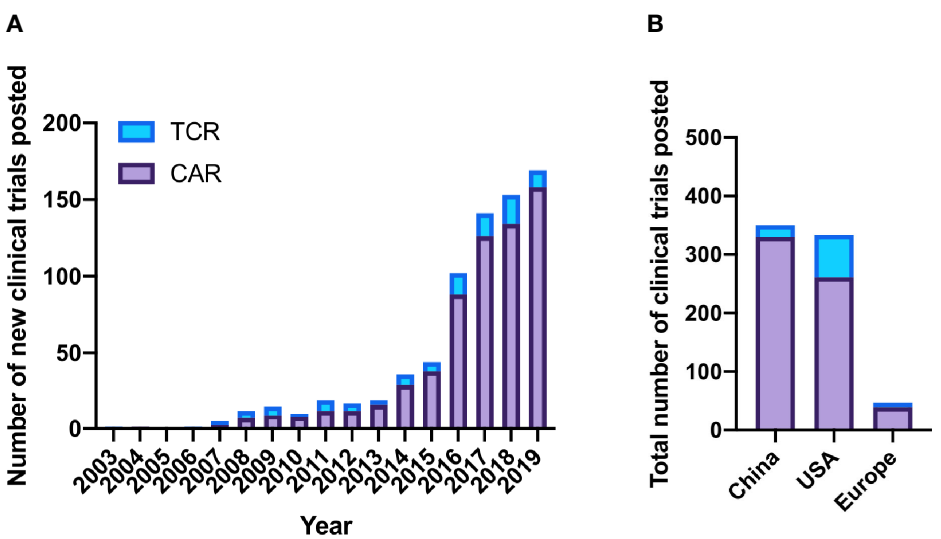

FIGURE 2 | CAR vs. TCR clinical trials posted on *clinicaltrials.gov*. **(A)** CAR and TCR clinical trials posted per year. **(B)** Total CAR and TCR trials by location 2003-2019.

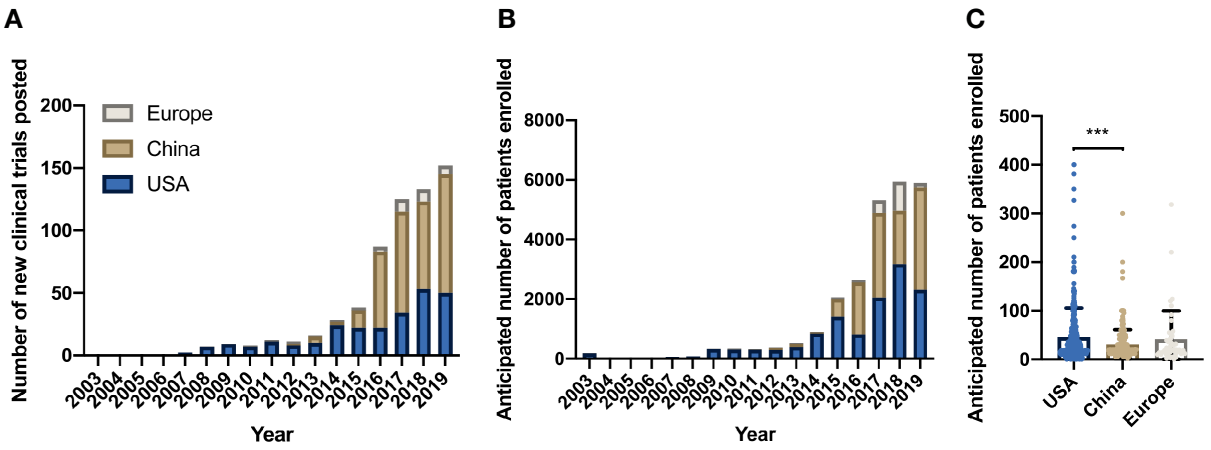

FIGURE 3 | Location of CAR trials on *clinicaltrials.gov*. **(A)** New CAR trials per year by location. **(B)** Anticipated patient enrollment in CAR trials per year by location. **(C)** Comparison of total anticipated patient enrollment by location 2003-2019 (p=0.0003, one-way ANOVA test). *** denotes p-value < 10^{-3}.

MOST STUDIES CONTINUE TO BE EARLY-PHASE CLINICAL TRIALS

CAR trials posted from 2003 to 2015 were all early-phase studies (phase I, I/II, or II), which focused on patients with relapsed/refractory disease with few treatment options and did not include comparator cohorts. While the dramatic efficacy of anti-CD19 CAR-T cells demonstrated in phase II trials was sufficient for regulatory approval, randomized trials were initiated in 2018 to extend their clinical indications. Late phase studies were distributed across USA, China, and Europe (n=5, 5, and 3, respectively). USA and Europe held the highest total anticipated patient enrollment in late phase trials (n=1041, 558 respectively vs. 90 in China). The first randomized phase III trial opened in January 2018, a study of Kite's axicabtagene ciloleucel anti-CD19 CAR-T cells in adult relapsed/refractory diffuse large B cell lymphoma vs. standard of care second-line salvage chemotherapy plus autologous stem cell transplant (ASCT) (NCT03391466, **Table 1**). In 2018, Novartis initiated phase III randomized trials of tisagenlecleucel anti-CD19 CAR-T versus standard of care second-line therapies (such as blinatumomab or inotuzumab ozogamicin) in adult patients with relapsed/refractory hematological malignancies (NCT03628053, NCT03570892). Celgene also launched phase III trials the same year to test its anti-CD19 CAR-T in Non-Hodgkin's Lymphoma (NCT03575351) and BCMA-CAR T in multiple myeloma, (NCT03651128) in comparison to standard therapies (salvage chemotherapy and ASCT or later-generation Imids/proteasome inhibitors and/or daratumumab, respectively). In 2019, Janssen initiated a phase III study of its anti-BCMA CAR-T vs. combination pomalidomide, bortezomib, dexamethasone or daratumumab, pomalidomide and dexamethasone in lenalidomide-refractory adult myeloma (NCT04181827). In July 2019, the first late phase trial involving CAR-T cells in solid tumors was listed, a phase IIb study of an anti-CEA CAR combined with either nanoparticle albumin-bound paclitaxel, 5-fluorouracil/folinic acid, or capecitabine vs. chemotherapy alone in CEA+ pancreatic adenocarcinoma liver metastases (NCT04037241). In contrast, all TCR trials continue to be early-phase, and none have demonstrated sufficient efficacy to garner fast-track approval to date. That the clear majority of clinical trials continue to be early phase studies indicates the field of genetically-engineered cellular therapeutics remains relatively young, with great emphasis on innovation and development of new cellular products. These products will require demonstration of safety and preliminary efficacy before they can expand to large multicenter trials that include comparisons with standard treatments.

Long-term clinical data is emerging for early anti-CD19 CAR-T constructs. Two-year follow-up of patients receiving axicabtagene ciloleucel in Zuma-1 (NCT02348216) indicates the drug can induce durable responses and median overall

TABLE 1 | CAR therapies compared to standard of care.

NCT#	Year posted	Phase	CAR-target	Company	Disease	Anticipated Patient Enrollment
NCT03391466	2018	3	CD19	Gilead/Kite	DLBCL	350
NCT03628053	2018	3	CD19	Novartis	ALL	220
NCT03570892	2018	3	CD19	Novartis	NHL	318
NCT03575351	2018	3	CD19	Celgene	NHL	182
NCT03651128	2018	3	BCMA	Celgene	MM	381
NCT04181827	2019	3	BCMA	Janssen	MM	95

survival greater than 2 years with manageable long-term safety in adult large B-cell lymphoma (29). Follow-up studies of patients receiving commercial axicabtagene ciloleucel and tisagenlecleucel confirm the remarkable efficacy and manageable safety profiles of these drugs beyond the clinical trial setting (30–33). Though not yet commercially approved at the time of this review, long-term trial data from 269 adult B-cell lymphoma patients receiving lisocabtagene maraleucel, an anti-CD19 CAR-T cell therapy, indicated 18.8 month median overall survival, 73% objective response rate, 53% complete response rate, and 12% occurrence of grade 3 or worse cytokine release syndrome or neurological events (NCT02631044) (34). However, across studies it appears there is still room for optimization of these therapies. Late phase randomized studies may help to refine treatment algorithms, perhaps allowing for patients to receive CAR therapies as earlier treatment options and further identifying prognostic indicators to help precisely identify patients who would most benefit from particular CAR therapies.

CAR AND TCR TARGETS

The first successful CAR-T cells targeted CD19 expressed on pre-B ALL, B-cell lymphomas, and normal B cells. Two-hundred fifty-five CAR clinical trials enrolling an anticipated 11,783 patients targeted CD19 (**Table 2A**). The next most frequent target in hematologic malignancies was BCMA in multiple myeloma, with 2,517 patients expected to enroll in 47 single-agent BCMA CAR trials. The clinical efficacy of BCMA CAR-T cells has been noted in several studies in relapsed/refractory myeloma, and FDA approval is expected for several such products in the near future (35–38). CD22 has emerged as a new CAR target in B-ALL and B-cell lymphoma (n=15) and CD30 in relapsed Hodgkin's disease and T-cell lymphomas (n=15). Overall, CARs targeting 23 different surface antigens expressed by hematologic malignancies have been evaluated in clinical trials (**Table 2A**). Many of these antigens are expressed selectively by normal B cells and B-cell malignancies (ALL, CLL, B-cell lymphoma, myeloma). In these cases, successful therapy also results in the elimination of normal B cells or plasma cells, and patients are supported by administration of normal human gamma globulin until normal B cells recover sufficiently to produce endogenous antibodies. Several recent trials also target antigens expressed on T-cell or myeloid malignancies. In these cases, CARs may also target normal T cells or normal hematopoietic stem cells, potentially resulting in severe immune deficiency or life-threatening pancytopenia. In many of these trials, treatment is planned as a bridge to allogeneic stem cell transplantation. In other cases, innovative approaches are designed to mitigate these toxicities.

Although most single-target CARs were specific to antigens expressed primarily on hematopoietic malignancies (n=390), many solid tumor CARs were also evaluated (n=156, **Table 2B**). The first solid tumor CAR trial was posted on

TABLE 2A | Single targets for CAR therapies in liquid tumor indications.

Target	Number of clinical trials	Expected patient enrollment
CD19	255	11783
BCMA	47	2517
CD22	15	472
CD30	15	391
CD123	14	535
CD20	11	378
CD33	6	136
CS1	3	102
CD7	4	155
NKG2DL	4	194
CD138	2	43
CD4	2	72
LeY	2	51
CD133	1	20
CD37	1	34
CD38	1	72
CD44v6	1	10
CD5	1	21
FLT3	1	35
KLC of Ig	1	54
PD-L1	1	20
ROR	1	0
TRBC1	1	55

TABLE 2B | Single targets for CAR therapies in solid tumor indications.

Target	Number of clinical trials	Expected patient enrollment
Mesothelin	22	654
GD2	17	528
EGFR	16	379
HER2	14	463
GPC3	13	330
CEA	12	260
MUC1	9	399
PSMA	7	140
EpCAM	5	174
IL13Rα2	5	201
NKG2DL	5	144
CLD18	3	60
ROBO1	3	42
B7-H3	2	80
c-Met	2	16
CD147	2	54
PD-L1	2	42
PSCA	2	150
ROR	2	148
AFP	1	18
CD171	1	40
CD20	1	18
CD70	1	113
Chlorotoxin	1	18
EphA2	1	60
FAP	1	4
Folate receptor	1	18
GP100	1	6
LeY	1	82
LMP	1	20
MUC16	1	30
VEGFR2	1	24

TABLE 2C | CAR therapies targeting multiple antigens.

Target	Number of clinical trials	Expected patient enrollment
CD19/CD22	17	598
CD19/CD20	13	275
CD19/BCMA	5	85
BCMA/CS1	1	84
BCMA/CD138	1	10
BCMA/CD38	1	80
CD20/CD22/CD10	1	30
CD19/CD20/CD22/CD30	1	10
CD20/CD3	1	12
CD123/CLL1	1	20
CD123/CD33	1	10
CD33/CLL1	1	70
c-MET/PDL1	1	50
Mesothelin/CD19	1	4
Muc1/CLL1/CD33/CD38/ CD56/CD123	1	10
Nectin4/FAP	1	50
PD-L1/CD80/CD86	1	10

clinicaltrials.gov in 2003 and utilized anti-L1-CAM (CD171) CAR CD8+T cells to treat patients with neuroblastoma (NCT00006480) (39). Other early solid tumor trials include an anti-GD2 CAR-T trial in neuroblastoma in 2004 (NCT00085930) (40) and an anti-CAIX CAR-T in renal cell carcinoma not registered on *clinicaltrials.gov* but published in 2006 (41). For all trials listed on *clinicaltrials.gov*, 32 different solid-tumor antigens have been evaluated as targets in early-phase clinical trials. Mesothelin has been the most frequently targeted antigen in solid-tumor CAR trials (n=22), followed by GD2 (n=17), and EGFR (n=16). Current and prospective solid-tumor targets have been previously well reviewed (42, 43). Identification of appropriate target antigens is particularly important in solid tumors. Target antigens expressed at high density on solid tumors are often also expressed on normal tissues and are likely to result in unacceptable toxicities when attacked by CAR-T cells *in vivo* (42, 43). One approach to address this limitation focuses on tumor-specific splice variants of proteins such as EGFRvIII, which has been evaluated in 8 different trials (44). Although CARs directed at many solid-tumor antigens have been evaluated and clinical responses have been observed, the clinical efficacy of these products has thus far been modest. Overall results in these trials are not comparable to the remission rates achieved in patients with B-cell malignancies (45–47).

In contrast to CARs specific for cell surface molecules, TCR-based therapies are directed against peptide epitopes presented in the context of the patient's individual HLA molecules. This approach has the advantage of targeting intracellular proteins, including unique tumor neoantigens and tumor-associated antigens that are not widely expressed on normal tissues. However, TCR specificity is HLA-restricted, and trial enrollment is therefore limited to patients (and tumors) that express specific HLA alleles. NY-ESO-1 has been the most frequent antigen targeted in TCR trials (n=34), followed by MAGE (n=9, **Table 3A**) (14). Eighty percent of TCR trials (79 of 102) are restricted to patients who are HLA-A02 positive. HLA-A11 and HLA-A24 were the next most prevalent eligible HLA alleles, included in 8 and 5 trials, respectively. Only 10 TCR studies targeted hematopoietic tumor antigens, mainly WT1 (n=3) and NY-ES0-1 (n=3). In December 2019, results of a phase I trial featuring a TCR specific to peptides shared by NY-ESO-1 and LAGE-1 yielded a 42% overall response rate 1-year post infusion in multiple myeloma (NCT01352286) (48). In efforts to reduce on-target off-tumor toxicities, TCRs have also been designed for cancer-specific mutations, like TGF-β receptor II polymorphisms in colorectal cancer (NCT03431311), EBV-LMP1 epitopes that are highly expressed in EBV-associated nasopharyngeal carcinoma (NCT03648697), and KRAS G12V in pancreatic cancer harboring that mutation (NCT04146298).

TABLE 3A | Single targets for TCR therapies.

Target	Number of clinical trials	Expected patient enrollment
NY-ESO-1	34	526
MAGE	9	313
HPV Ag	6	556
MART-1	4	100
WT1	4	65
HBV Ag	3	38
KRAS	3	191
CEA	2	17
CMV Ag	2	19
EBV Ag	2	45
gp100	2	24
HIV Ag	2	26
P53	2	15
AFP	1	9
Folate Receptor	1	0
HER2	1	33
HERV	1	24
MCP Ag	1	16
Mesothelin	1	10
TGFbRII	1	5
TRAILxDR4	1	5

TABLE 3B | Multiple targets for TCR therapies.

Target	Number of clinical trials	Expected patient enrollment
NY-ESO-1/LAGE-1a	4	132
AFP/HLA-A2	1	24
HER2/CD3	1	8
gp100/CD3	1	327
gp100/MART-1	1	4
Melan-A/MART1	1	12

Though anti-CD19 and anti-BCMA CARs frequently induced disease remission, some patients relapse after initial response. The most frequent reasons for tumor relapse have been either lack of persistence of genetically-modified effector cells *in vivo* or loss of epitope expression by the tumor cells (49). In a phase IIa single agent anti-CD19-CAR trial in adult NHL, post-infusion biopsies showed CD19 antigen loss in 20% of non-responding patients (NCT02030834) (15). Similarly, CD19 epitope loss occurred in 28% of pediatric patients who

received anti-CD19 CAR-T cells (14, 50). This demonstration has led to a series of new clinical trials using CARs targeting 2 different surface antigens as a strategy to prevent relapse due to antigen loss.

Eighteen different dual CAR constructs that target multiple antigens simultaneously have now entered clinical trials (**Table 2C**). Multiantigen-specific CARs may have three or more targets but typically have two. Anti-CD19/CD22 (n=17) and anti-CD19/CD20 (n=13) combinations were most frequently tested. Several multiantigen-specific TCRs have also been developed, mainly NY-ESO-1/LAGE-1a (n=4) (**Table 3B**). In a different approach, two trials explored a modulable receptor using a CD16v CAR co-administered with monoclonal antibodies (mAb). By enabling generic antibody binding through CD16, this strategy allowed targeting of different diseases by interchanging commercially available mAbs like anti-CD20 (NCT02776813) and anti-HER2 (NCT03680560).

BOOSTING CELL SURVIVAL AND EFFICACY THROUGH ADDITIONAL GENETIC MODIFICATIONS

Prolonged disease remission and survival are associated with immune effector cell persistence in the tumor microenvironment (51). Immune checkpoint signaling, hypoxia, and metabolic milieu of the tumor microenvironment all promote immune evasion and tumor survival (52). As summarized in **Table 4** and **Supplementary Table 3**, numerous strategies in the last 4 years have been used to enhance functionality of CARs and TCRs in the tumor microenvironment, including: (1) resistance to exhaustion/negative regulation, (2) secretion of cytokines, and (3) enhanced tumor homing.

The most frequent approaches entailed PD-1 knockout (n=6 CAR, n=1 TCR) and genetic modification to effect local secretion of anti-PD-1 (n=5 CAR, n=2 TCR). Five trials describing CARs with PD-L1 blockers featured constructs that rewired PD-1 extracellular domains to an internal activating domain (n=5) (53). Three trials studied CARs that could locally secrete anti-PD-1 and anti-CTLA-4 antibodies (n=3). Other modifications included a PD-1 Fc-receptor-like fusion protein (n=2), shRNA suppression of PD-1 expression (n=1), activated cytoplasmic PD-1 (n=1), conferring resistance to TGF-β (n=1), and CRISPR-mediated knockout of *HPK1* in anti-CD19 CAR-T (NCT04037566) (54, 55).

Inducing local cytokine secretion was another approach found in CAR trials (n=12) to modify the tumor microenvironment and enhance effector cell activity *in vivo*. Local cytokine secretion by activated effector cells further avoids potential toxicities of systemic cytokine infusion. More than half of these trials targeted solid tumors (n=7), but no TCR trials employed this strategy. Five trials leveraged IL-15 secretion to promote expansion and survival of memory T cells and enhance NK-cell mediated cytotoxicity, and one trial used both IL-15 and IL-21 (56, 57). Two trials explored the expression of IL7/CCL19 alone, while two others tested direct expression of IL-7 receptor to enhance cell survival. IL-12 secretion by CAR cells was used for local dendritic cell maturation and T-cell proliferation with or without IL-7/CCL19 (NCT03542799,

TABLE 4 | Novel genetic modifications in CAR and TCR therapies from 2016 to 2019.

	CAR		TCR	
RESISTANCE TO NEGATIVE REGULATION	PD-1 knock-out	n = 6	PD-1 knock-out	n = 1
	Anti-PD-1 expression	n = 5	Anti-PD-1 expression	n = 2
	PD-L1 blocker expression	n = 5		
	Anti-PD-1 and anti-CTLA-4 expression	n = 3		
	PD-1 Fc fusion protein secreted	n = 2		
	PD-1 shRNA expression	n = 1		
	Activated cytoplasmic PD-1	n = 1		
	TGF-beta resistance	n = 1		
	Endogenous HPK1 disruption	n = 1		
INTERLEUKIN EXPRESSION	IL15	n = 5		
	IL-7 and CCL19	n = 2		
	IL-7 receptor	n = 2		
	IL-7 and CCL19 or IL12	n = 1		
	IL-15 or both IL-15 and IL-21	n = 1		
	IL-12	n = 1		
TUMOR HOMING	CCR4 expression	n = 1		
	CXCR5 modified	n = 1		
FRATRICIDE RESISTANCE	CD7 knock-out CD7-targeting CAR	n = 1		
SAFETY SWITCH	iCasp9	n = 13	iCasp9	n = 1
	EGFRt	n = 13		
	EGFRt and HERt on two different CARs	n = 1		
	Herpes simplex virus thymidine kinase	n = 1		
	RQR8	n = 1		
VIRUS RESISTANCE	Resistant to HIV by CCR5 modification	n = 1		
IMMUNE RESISTANCE	β2m and TCR disruption	n = 1	TCRα β disruption	n = 1

NCT03932565). To enhance trafficking to tumors, CARs have been engineered to express chemokine receptors in two trials: CCR4 in anti-CD30 CAR-T for cutaneous lymphoma (NCT03602157) and CXCR5 co-expressed with anti-EGFR CAR-T in non-small cell lung cancer (NCT04153799).

The ability to use CAR-T cells to target T-cell tumors has been complicated by the fact that normal T cells also express the same target antigens. This results in fratricide and loss of CAR-T cells during manufacturing and *in vivo* after infusion (58). To avoid this cross-reactivity, one trial (NCT03690011) used CRISPR-based gene editing to knockout CD7 in T cells prior to CD7-CAR transduction of CAR-T for T-cell malignancies. While that trial was the only one to employ fratricide resistance modifications, recent results of a phase I CD5 CAR-T trial in r/r T-cell leukemia and lymphoma showed no sign of T-cell fratricide (NCT03081910) (59).

INCREASING SAFETY OF GENETICALLY-ENGINEERED CELLS

Along with remarkable efficacy, CAR-T cell therapies have also been associated with serious toxicities directly related to rapid expansion and activation of large numbers of activated effector cells *in vivo*. This has led to the development of different approaches to rapidly and selectively eliminate CAR-T cells *in vivo* in patients with life-threatening toxicities. One approach relies on genetic modifications such as "safety switches" that induce expression of drug-targetable molecules leading to rapid elimination of modified cells. Twenty-nine trials featured this strategy; 28 were CAR therapies, of which 22 were conducted in the USA. Safety switch triggers were based predominately on already approved drugs with known, manageable adverse effects and were, to date, studied in CARs and one TCR trial (**Table 4** and **Supplementary Table 3**). The most studied method required rimiducid, which prompts dimerization of caspase-9 (iCasp9) to induce apoptosis (n=13 CAR, 1 TCR). Remaining strategies were studied only in CAR trials. Another safety switch mechanism relied on forced expression of non-functional surface molecules that can be targeted by systemic administration of monoclonal antibodies, like truncated endothelial growth factor receptor (EGFRt) (n=13, 2 allogeneic trials), a target of cetuximab-driven antibody-dependent cell-mediated cytotoxicity (60). One trial studied EGFRt and the related truncated human epidermal growth factor receptor 2 (HERt) on two different CAR constructs. Cells edited to express herpes simplex virus-1 thymidine kinase (HSV-TK) were found in one allogeneic CAR trial and could be systemically killed upon ganciclovir administration. A construct called RQR8, which combines CD34 and CD20 target epitopes, enabling both a CD34 selection to enrich edited cell populations in manufacturing and a rituximab-induced safety-switch, was found in one trial (NCT03590574) (61). Select allogeneic CAR trials are also known to employ a rituximab-targetable CD20 mimic embedded in CAR constructs, though the trial entries did not specify use of this kill switch molecule on *clinicaltrials.gov* (NCT03203369, NCT03190278, NCT04142619, NCT04150497, NCT04106076, NCT03229876, NCT02746952) (62).

ALTERNATE CELLS OF ORIGIN

Prior to 2019, cells used for CAR and TCR manufacturing were typically T cells sourced from autologous leukapheresis products (96% of trials). Rarely, cord blood was used as a source of allogeneic immune cells (2 CAR-NK and 1 CAR-T cell trials). The T-cell subsets infused affects CAR-T efficiency and persistence (63). Very few trial listings specify detailed information on the composition of the CAR T cell products. Central memory cells are implicated in longer persistence and survival of adoptively-transferred immune cells and were selectively utilized in at least 4 CAR trials (64). γδT cells, which can target cells in an MHC-independent manner and have been implicated in anti-tumor responses with less cytokine release and functional exhaustion than in TCRαβ signaling, have been selectively utilized in allogeneic CAR trials (n=2) (47, 65, 66). Natural Killer (NK) cell CARs have also been investigated (n=21 since 2016). NK cells express multiple innate MHC-independent activating receptors (e.g. NKG2D, natural cytotoxicity receptors, and DNAM-1) that respond to commonly upregulated ligands on transformed cells (MICA, MICB, ULPB 1-6, CD112 and others). NK cells also mediate antibody-dependent cytotoxicity through expression of CD16 (67–70).

The limited lifespan of NK cells *in vivo* reduces their potential for long-term adverse effects such as B-cell aplasia, and NK cells are less likely to provoke GVHD than T cells in the allogeneic setting (71, 72). However, NK-mediated efficacy can be short-lived as these cells become functionally exhausted and senesce, requiring supportive cytokines like IL-2, IL-15, and/or IL-21 to survive beyond 2 weeks (73, 74). Manufacturing NK CARs can also be challenging but a recent report summarizing clinical outcomes after infusion of anti-CD19-CAR NK cells derived from umbilical cord blood demonstrated excellent results (75). In this study, anti-CD19-CAR NK cells also secreted IL-15 that prolonged persistence *in vivo*, and clinical responses were achieved with little systemic toxicity. These results will likely lead to many more NK-CAR trials in the next few years.

COMBINING AND MODIFYING CELL ADMINISTRATION

As an alternative approach to enhance safety and efficacy, a variety of trials combined CAR/TCR-modified cell infusion with other systemic agents or explored alternate routes of administration and infusion (**Table 5** and **Supplementary Table 4**). Drug-based strategies to mitigate severe inflammatory events like cytokine release syndrome (CRS) and associated neurotoxicity appeared initially in the setting of anti-CD19 CAR-T therapies (n= 7 including one CD19/CD20 dual receptor trial). Tocilizumab, which binds soluble and membrane-bound IL-6 receptor, was the earliest reported safety drug, first mentioned in 2016 and in 3

TABLE 5 | Novel combination and administration strategies in CAR and TCR therapies from 2016 to 2019.

		CAR		TCR	
Combination with other medicines	***SAFETY DRUGS***	Tocilizumab	n = 3		
		Anakinra	n = 3		
		Defibrotide	n = 1		
	EFFICACY DRUGS	Checkpoint inhibitor	n = 12	Checkpoint inhibitor	n = 9
		Interleukin-2	n = 6	Interleukin-2	n = 13
		Ibrutinib	n = 2		
		Inhibitor of Gamma Secretase	n = 1		
	CELLS OR VIRUS	Pulsed dendritic cells	n = 1	Pulsed dendritic cells	n = 1
		T cell antigen presenting cells expanding CAR-T	n = 1	TIL Expressing TGFbDNRII	n = 1
		Oncolytic virus	n = 1	Oncolytic virus	n = 1
Administration	***REGIONALLY DELIVERED***	Hepatic transarterial	n = 8		
		Intracerebral	n = 7		
		Intraperitoneal	n = 5	Intraperitoneal	n = 1
		Intra-tumoral	n = 3	Intra-tumoral	n = 1
	INTERVENTION DESIGN	Combined CAR injection	n = 5		
		Split dose	n = 2		
		Sequential administration	n = 1		

trials (76). Two studies have added safety management arms to their CAR studies, investigating tocilizumab prophylaxis for CRS and neurotoxic events (NCT03467256, NCT02348216) (77). Three late 2019 trials administered anakinra, an IL-1 receptor antagonist capable of crossing the blood brain barrier (78). NCT04148430 and NCT04150913 utilized prophylactic anakinra on day 0 or 2 post CAR infusion, respectively. NCT04205838 studied anakinra following onset of neurotoxicity and CRS. Registered in early 2019, one trial administered defibrotide, an oligonucleotide with protective effects on endothelial cells, from 5 days before lymphodepletion until 7 days post-CAR infusion (NCT03954106) (79).

Drugs were also employed to mitigate negative regulation of edited cells and promote immune activity. TCR trials utilized systemic administration of checkpoint inhibitors (n=9) and IL-2 (n=13). Checkpoint inhibitors were the most studied in CAR settings and have been previously well reviewed (n=12) (47, 80). CAR trials further explored coadministration with IL-2, ibrutinib, and gamma-secretase inhibitor (n=6, 2, and 1, respectively). CAR and TCR cells were co-administered with pulsed dendritic cells, T-cell antigen presenting cells, tumor-infiltrating lymphocytes, and oncolytic viruses (n=6). Targeted regional delivery of CARs was more widely tested than with TCRs (n=22 and 2, respectively). CARs were locally supplied *via* hepatic artery (n=8), intracerebrally (n=7), intraperitoneally (n=5), and intratumorally (n=3) (**Table 5** and **Supplementary Table 4**). Of trials specifying intracerebral CAR delivery, study arms included intracavitary and/or intraventricular (n=5), intratumoral (n=1), and intracranial (n=1) injections. TCRs were administered intraperitoneally (n=1) and intratumorally in non-small cell lung cancer (n=1) (**Table 5** and **Supplementary Table 4**). CAR trials also studied alternate infusion methods for modified cells, including sequential (n=1, NCT03407859), split-dose (NCT03152435, NCT03407859), or simultaneous administration (n=8, NCT02924753, NCT03152435, NCT03407859, NCT03497819, NCT03620058, NCT03207178, NCT03549442, NCT04194931).

UNIVERSAL ALLOGENEIC CARS

The high cost of approved autologous CAR therapies, i.e., $373,000 USD per patient for axicabtagene ciloleucel and tisagenlecleucel for adults with lymphoma and $475,000 USD for tisagenlecleucel for children and young adults with B-ALL, highlights the need for less expensive and more efficient manufacturing processes (81–83). Currently, autologous CAR cell production requires a lengthy production period ranging from 8 days when manufactured at local sites to ≥ 4 weeks when produced at commercial sites. Moreover, autologous products cannot be manufactured in time for all eligible patients, particularly individuals with rapidly progressive disease or those with insufficient peripheral immune cell numbers (84, 85). To address these issues, several groups have initiated allogeneic CAR and TCR-based trials (n=56 total), 50 of which employed CAR-T cells, 58% being anti-CD19-CARs.

Donor CARs and TCR-edited cells are promising off-the-shelf therapies if the challenges of graft versus host disease (GVHD) and short-term persistence can be mitigated by HLA-matching, specific gene knock-outs, or other cell modifications to prevent immunologic recognition and elimination of allogeneic cells in the recipient. Three allogeneic CAR trials specified HLA restrictions (NCT02050347, NCT01195480, NCT04107142). One trial initiated in 2017 described CRISPR-knockout of endogenous TCRα/β and β2-microglobulin to eliminate expression of MHC class I molecules (NCT03166878) (84). Two trials used infusion of an anti-CD52 antibody to ablate recipient lymphocytes and prevent rejection of allogeneic effector cells (NCT03939026, NCT04093596) (86). Six trials employed safety switch molecules iCasp9 (n=3, NCT01494103, NCT03056339, NCT03579927), EGFRt (n=2, NCT03114670, NCT02028455) or HSV-TK (NCT01082926) to eliminate allogeneic effector cells in the event that effector cell infusions caused severe graft versus host disease in recipients. Remaining allogeneic trials did not specify further cell modifications on *clinicaltrials.gov*.

Early results from allogeneic CAR studies are encouraging, but the number of patients treated on current trials is relatively low, and as with autologous CARs there continues to be room for further optimization. A 2019 report of anti-CD19/CD123 dual allogeneic CAR administered to three relapsed/refractory acute lymphoblastic leukemia adults achieved complete responses lasting 7–11 months (NCT03125577) (87). Cord blood-derived anti-CD19 CAR-NK cells achieved a 73% overall response in adult non-Hodgkin's Lymphoma and chronic lymphocytic leukemia patients (NCT03056339) (75). The current status and future prospects of allogeneic CARs have been extensively reviewed (88, 89). While long term follow-up data is emerging, larger studies will be needed to establish the role of allogeneic CARs in the treatment of patients with hematologic and non-hematologic cancers.

NON-ONCOLOGY INDICATIONS AND FUTURE APPLICATIONS

Thus far, relatively few CAR and no TCR trials have focused on non-oncology indications (n=7). Four trials focused on patients with HIV, including a CAR-T construct with zinc-finger-mediated CCR5 disruption to evade HIV infection (NCT03617198). CARs were also studied in patients with Neuromyelitis Optica, Lupus Erythematosus, and Myasthenia Gravis. Lessons learned from CAR trials have already been applied to SARS-CoV-2 research. One China-based CAR trial initiated in March 2020 employs dual-targeting anti-NKG2D-ACE2 CAR-NK cells, which secretes IL-15 superagonist for NK cell longevity and has a GM-CSF neutralizing receptor to prevent cytokine release syndrome, in COVID-19 patients (NCT04324996). Based on previous success against CAR-induced cytokine storm, trials are investigating tocilizumab in the context of systemic inflammation and/or pneumonia from COVID-19 (n>10) (90, 91).

While not currently in trials at time of this review, there is considerable preclinical interest in regulatory T cell (Treg) CARs as alternatives to chronic immunosuppression in solid organ transplant and prevention and/or mitigation of autoimmune diseases like Type I Diabetes, inflammatory bowel disease, autoimmune hepatitis, encephalitis, and arthritis (92, 93). However, CAR-Treg utilization remains challenged by relatively low abundance in circulation, lack of highly efficient GMP-compliant enrichment protocols and limited stability during *in vitro* expansion (92).

SUMMARY

CAR and TCR therapies have birthed a promising field where initial successes for patients with unmet clinical needs have inspired further research, investment, and technological advances. The exponential growth in this field includes expansion to new and multiantigen targets, additional genetic-engineering approaches to optimize efficacy and safety, novel combination strategies, administration techniques, and exploration of different starting cell types. Late-phase studies have begun to compare anti-CD19 and anti-BCMA CAR-T therapies against current standards of care. This review of clinical trials initiated over the past 17 years demonstrates less activity in the TCR and solid tumor spaces versus CAR-T cell utilization in hematologic malignancies, though the repertoire of targeted solid tumor antigens is expanding. If CARs targeting solid tumors or for TCR-based cellular therapies can achieve similar efficacy to that seen in CARs for hematologic malignancies, dramatic increases in numbers of clinical trials and patients anticipated on these trials will certainly follow. As evident from the current distribution of early phase clinical trials, there is a great deal of international participation in the development of novel cell therapies for patients with cancer. This is truly a global effort that is supported by well-established pharmaceutical companies as well as large numbers of new biotech companies and academic investigators. China is beginning to play an important role and will continue be a major contributor to new clinical trials. This review did not cover the massive pre-clinical pipeline upstream of current clinical trials. Additionally, we excluded cell therapy modalities such as cytotoxic T lymphocytes, tumor-infiltrating lymphocytes, NK cells, immunomodulatory dendritic cell- or tumor-based vaccines. We anticipate that a similar review of commercially-approved cell therapy products and trials registered on *clinicaltrials.gov* 2 years from now will show an exponential rise in commercial cell therapy approvals and expansion of European activities in this space. Following current trends, a skew towards CARs and hematologic malignancies may continue, albeit given earlier in treatment algorithms rather than the current relapsed/refractory setting. If advances in the engineering of CARs and TCRs for solid tumors can increase the clinical efficacy of these products, clinical activity in these areas will progress rapidly and change the practice of clinical oncology forever.

AUTHOR CONTRIBUTIONS

LP generated, analyzed the database, and reviewed the manuscript. LP and AC generated figures and tables. AC wrote the manuscript. HT-N, SN, and JR reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Perspectives for the Use of CAR-T Cells for the Treatment of Multiple Myeloma

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During recent years considerable progress has been made in the treatment of multiple myeloma. However, despite the current improvements in the prognosis of this malignancy, it always ends with relapse, and therefore new therapy approaches for destroying resistant cancer cells are needed. Presently, there is great hope being placed in the use of immunotherapy against refractory/relapsed multiple myeloma which is unresponsive to any other currently known drugs. The most promising one is CAR-T cell therapy which has already shown tremendous success in treating other malignancies such as acute lymphoblastic leukaemia (ALL) and could potentially be administered to multiple myeloma patients. CAR-T cells equipped with receptors against BCMA (B-cell maturation antigen), which is a surface antigen that is highly expressed on malignant cells, are now of great interest in this field with significant results in clinical trials. Furthermore, CAR-T cells with other receptors and combinations of different strategies are being intensively studied. However, even with CAR-T cell therapy, the majority of patients eventually relapse, which is the greatest limitation of this therapy. Serious adverse events such as cytokine release syndrome or neurotoxicity should also be considered as possible side effects of CAR-T cell therapy. Here, we discuss the results of CAR-T cell therapy in the treatment of multiple myeloma, where we describe its main advantages and disadvantages. Additionally, we also describe the current results that have been obtained on using combinations of CAR-T cell therapies with other drugs for the treatment of multiple myeloma.

Keywords: multiple myeloma, CAR-T cells, T lymphocyte, B-cell maturation antigen (BCMA), immunotherapy, cytokine release syndrome

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INTRODUCTION

Multiple myeloma (MM) remains an incurable haematological malignancy although substantial progress in the treatment has occurred in recent years (1). The introduction of immunomodulatory drugs (IMiDs) such as thalidomide, lenalidomide, and pomalidomide has resulted in significant improvements for the long-term survival and the quality of life of MM patients (2, 3). The other drugs, namely proteasome inhibitors (bortezomib, carfilzomib, ixazomib), immune checkpoint inhibitors, and monoclonal antibodies have contributed substantially to improve the prognosis of MM patients (2, 4). Apart from these agents, another way of treatment is autologous stem-cell

transplantation (ASCT), which is prescribed for patient populations with limited comorbidities and in good overall condition (5). Despite the progress made, MM generally progresses towards relapse/refractory to the available therapies (6). This creates an unmet need for new types of treatments, especially those which are based on a distinct mechanism of action. During recent years many new ideas have appeared and one of the most promising is chimeric antigen receptor T cell (CAR-T) therapy, which has already succeeded in the treatment of other haematological malignancies such as acute lymphoblastic leukaemia (ALL) and diffuse large B cell lymphoma and was approved by the FDA and EMA (7). In this review, our goal is to summarize the results, present challenges, and to describe new ideas for CAR-T cell treatment in refractory/relapsed multiple myeloma patients.

Multiple Myeloma

Multiple myeloma (MM) is one of the most common malignancies of the hematopoietic system (15%) and accounts for approximately 1% of all neoplasms. Multiple myeloma typically affects elderly patients with a median age of 70 and with 63% of them being older than 65 (8). Although the prognosis in this disease is now much better than a few years ago, there are individual differences in survival that range from progression-free survival of more than 15 years in some patients (9), while approximately 15% of patients reach median survival of less than 2 years (10). The differences in survival outcomes occur because MM is a molecularly very heterogeneous disease (11). Additionally, the heterogeneity of this disease makes it improbable to develop a single molecularly targeted therapy (12). While progress can be made by searching for such a therapy for each distinct subset of MM, another way to approach the problem is to focus on cell surface targets that are common to all or a majority of MM cases. However, the main problem is that only a small fraction of antigens are common to myeloma cells while not being simultaneously expressed in other healthy tissues. Immunotherapies that include monoclonal antibodies, bi-specific T-cell engagers (BiTE), and CAR-T cell therapy are examples of such an approach with outstanding results in some cases.

Chimeric Antigen Receptor T Cell Therapy

Chimeric antigen receptor T (CAR-T) cells are genetically engineered by the introduction of a construct coding for a receptor that is specific for target neoplastic cells. Hence, these cells are redirected to the selected target molecules on the surface of cancer cells. Specific CAR-T cell binding to targeted cancer cells triggers their death without major histocompatibility complex restriction (13). This approach provides the opportunity to overcome tumor escape mechanisms that can lead to cancer cell survival, such as i) the down-regulation of MHC molecules, ii) the processing of aberrant antigens, and iii) the creation of an immunosuppressive milieu which deactivates T cells (14). That being mentioned, it seems logical that this novel technique should find its application especially in tumors that rely on immunoregulatory mechanisms, and a good example of such malignancy is multiple myeloma.

CARs consist of four different elements: the recognition domain, the extracellular spacer, costimulatory elements, and the activating endodomain (15). The recognition domain [most common is a single-chain variable fragment—scFv (16)] binds to its ligand on the cell surface (signal 1), where the extracellular spacer provides the optimal space between the two cells to allow activation of the CAR-T cells and not suppression. Once binding to the ligand has occurred, the costimulatory molecule (CD28 or 4-1BB) becomes activated and initiates the response for signal 2; the onset of signal 2 is vital for optimal CAR-T cell activation and the prevention of anergy. The propagation of signals 1 and 2 are then transmitted to the activating domain (CD3-zeta) where both signals are forwarded to the CAR-T cell for activation (17).

The most important factor that determines the success of CAR-T therapy is the selection of target antigen. The first requirement in the process of designing CARs is to verify that the selected molecule is uniformly and sufficiently expressed on tumor cells. The second one is the absence or limited expression of the selected molecule on the normal cell surface. Otherwise, CAR-T cell therapy could lead to the debulking of the tumor but also to serious side effects with the deterioration of related normal cells (18). And that is exactly the main problem with multiple myeloma because it presents exceptional heterogeneity that effectively constricts the search for new antigens (19). However, some targets are now being used in clinical trials with promising results. One of them is CD19, with impressive results in the treatment of acute lymphoblastic leukaemia (20–23). Other antigens being under investigation are CD44v6, CD70, CD56, CD38, CD138, signaling lymphocyte-activating molecule F7 SLAMF7 (CS1), K light chain, NKG2D, and the most promising one about which we are particularly focused on in this review: B-cell maturation antigen (BCMA) (13, 15) (**Figure 1**).

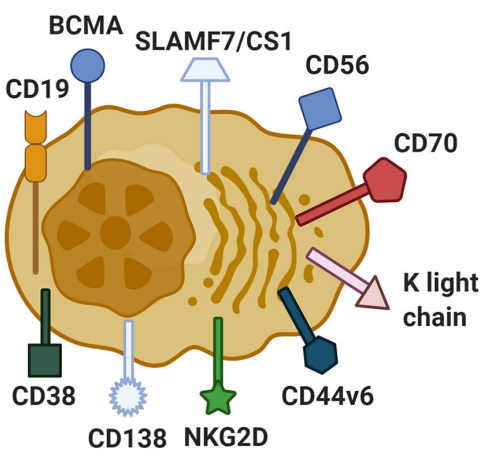

FIGURE 1 | Antigens on multiple myeloma cells used as a target in CAR-T cell treatment. In the process of producing CAR-T cells, the main aim is to find the appropriate antigen which could be characterized by high expression levels on tumor cells and no expression on other healthy cells. The most known antigens used in the refractory/relapsed multiple myeloma CAR-T cell treatment are CD138, CD70, NKG2D, kappa light chain, CD19, SLAMF7/CS1, CD44v6, CD56, CD38, and BCMA. Created with BioRender.com.

The manufacturing of CAR-T cells involves the collection of patients' T cells using apheresis as the first step. Once the T-cells are collected, they are further purified and activated with cytokines *in vitro*. Then they are transduced with viral vectors (retrovirus, lentivirus) containing a genetic construct coding for CAR. The efficiency of the transduction is in the range reaching up to 30% depending on the technique. After this, genetically engineered cells are further expanded, aliquoted, and stored frozen. In the meantime, the patient is receiving lymphocyte depleting chemotherapy to avoid rejection and enable the expansion of modified autologous CAR-T-cells. The procedure ends with the infusion of CAR-T cells to the patient in a specific dosing regimen (13).

FIRST-GENERATION B-CELL MATURATION ANTIGEN-TARGETED CHIMERIC ANTIGEN RECEPTOR T CELL

As mentioned, the crucial problem in designing functional CAR-T cells is the selection of the appropriate antigen. Its expression on target cells should be uniform and preferably it should not be present on healthy cells. The problem is that the majority of antigens present on neoplastic cells are also expressed on some other normal cells.

The best known and explored target for CAR-T cells so far is CD19. This is an antigen of normal B cells that is also present on B cell leukemias and lymphomas (24, 25). But when it comes to multiple myeloma, which is also B cell neoplasia, CD19 is present only on a subset of malignant cells (26). Therefore, there is a risk that anti CD19 CAR-T cells would not eliminate myeloma, although it is also hypothesized that CD19+ component has myeloma stem cell functionality (27). Also, attempts to use anti-CD19 CAR-T cells in MM gave inconsistent results and will be reviewed later. This is why the search for other, more commonly expressed antigens on myeloma cells was undertaken. Among a few such candidates, B-cell maturation antigen (BCMA) exhibited the most favorable characteristics. Carpenter et al. showed that BCMA is present almost on all multiple myeloma cells and on none of 34 different tissues as well as on CD34+ hematopoietic stem cells (28). On the other hand, its expression was also found in normal plasma cells and mRNA for its production was noticed in the trachea and gastrointestinal organs which was proven to be associated with B cells in lamina propria and Peyer's patches (28).

BCMA (CD269) is a member of the TNF receptor superfamily and is present on plasma cells and a small fraction of B cells (29). It binds B-cell activating factor (BAFF) or a proliferation-inducing ligand (APRIL) (30). Although it is particularly found on the cell surface, it can be also released into the serum when it is cleaved from the cell surface by γ-secretase. Patients with high infiltration of bone marrow with MM cells show elevated concentration levels of soluble BCMA in serum; this observation has proven to be particularly useful for the evaluation of CAR-T therapy (31).

Carpenter et al. conducted a few preclinical studies in which they have shown that transduced T cells demonstrated interesting features. CAR-T cells collected from patients could efficiently kill cells that possessed BCMA antigen on its surface even when soluble BCMA was added to the cells' environment. These *in vitro* conditions reflect the bone marrow environment where BCMA is present on the cell surface and in the soluble form (28).

Human clinical trials on CAR-T cells in MM were first conducted with the same anti-BCMA CAR-T cells that were reported in the previous paragraph (28). Ali et al. selected 12 patients with a median of seven lines of previous treatments and administered to them increasing numbers of CAR-T cells 0.3×10^6 cells/kg through 1×10^6, 3×10^6 up to the highest dose of 9×10^6 cells/kg. The highest dose (9×10^6 cells/kg) demonstrated the best clinical efficacy but also the most intensive adverse effects (32). All 12 patients experienced toxicities and each of them had cytopenias (mostly transient and attributed to conditioning therapy except in the case of two patients whose symptoms were protracted) that were easily managed. Another adverse event was cytokine release syndrome (CRS) with the greatest intensity in patients receiving the highest dose. CRS was characterized by fever, tachycardia, hypotension, hypoxemia, dyspnoea, and in one patient even with delirium. These symptoms were alleviated with the use of anti-IL 6 antibody tocilizumab and vasopressors. Some patients had elevated serum creatine phosphokinase levels with muscle weakness. Because of the BCMA presence on the normal plasma cells, the level of polyclonal serum antibodies dropped and some patients required intravenous immunoglobulin infusions to avoid infections (32). The best response achieved was with a patient whose bone marrow was 80% infiltrated with MM cells and for 28 weeks he had no detectable IgG lambda in the serum. Eventually, the patient relapsed which could be attributed to a small subset of MM cells which did not express BCMA on its surface and therefore were not selected by anti-BCMA CAR-T cell therapy (32). A few other interesting observations have been made after CAR-T cell infusion. Before CAR-T cell infusion the median CD8+/CD4+ T cell ratio was 1.1 but two weeks following the treatment CD8 positive cells became the main population of T cells. Moreover, these cytotoxic cells expressed new antigens such as PD-1 on their surface which are associated with senescence, exhaustion, and a decrease in proliferation ability. Summarizing, not only the loss or lack of antigen could contribute to eventual relapse but also loss of the efficient CAR-T cells (32).

In the continuation of this trial, Brudno et al. treated the next 24 patients with different dosages of CAR-T cells ranging from 0.3×10^6 cells/kg, 1×10^6, 3×10^6 to 9×10^6 cells/kg (33). Of the 10 patients who received $0.3–3 \times 10^6$ cells/kg, only two showed partial or better responses to the treatment. Among the other 16 patients (two of them were treated a second time after the first clinical trial (32)) treated with 9×10^6 cells/kg, the majority experienced partial or better responses but unfortunately the better the response was, the more intensive the adverse events were. Some of the responses exhibited symptoms of cytokine release syndrome (CRS) that was treated with tocilizumab, the

others showed cytopenias with the most frequent being thrombocytopenia which was managed with the thrombopoetin agonist eltrombopag and prednisone. What was particularly interesting was the fact that patients with grades 3 and 4 of CRS also had higher numbers of MM cells in the bone marrow (33).

Moreover, the level of soluble serum BCMA decreased substantially after treatment with CAR-T cells, while during progression its concentration started to increase. This observation leads to the conclusion that soluble BCMA could be used as a predictive biomarker for CAR-T cell therapy response and also for early progression (33).

SECOND-GENERATION B-CELL MATURATION ANTIGEN-TARGETED CHIMERIC ANTIGEN RECEPTOR T CELL (IDECABTAGENE VICLEUCEL)

After promising results in applying first-generation CAR-T cells, a search was initiated for determinants of better responses in MM patients. Several experiments have shown a significant positive correlation between peak CAR-T cell blood levels and clinical responses (34, 35). Furthermore, patients with better responses to treatment have been proven to have increased CAR-T cells that persist in the blood over a prolonged period of time (32, 33). Friedman et al. reasoned that the type of costimulatory molecule that is incorporated in the engineering of CAR-T cells could substantially affect both serum peak levels and persistence of CAR-T cells in blood. With that concept in mind, they designed T cells with chimeric antigen receptors but instead of CD28 as a costimulatory domain, as utilized in the previous preclinical and clinical experiments, they used another molecule—4-1BB (CD137) (36). Furthermore, an additional reason for changing the costimulatory domain was the high intensity and kinetics of CAR-T cell activation when CD28 is utilized. This high intensity and kinetics of CAR-T cell activation leads to the initiation of early-onset CRS. Moreover, T-cells with CD28 produced increased levels of Th-2 cytokines which mitigated their ability to kill cancer cells (37, 38) (**Figure 2**).

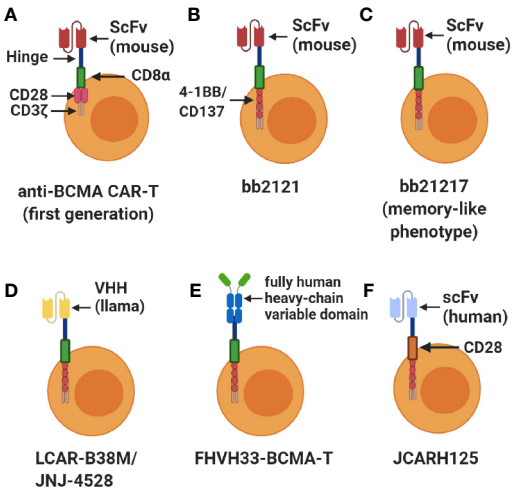

FIGURE 2 | Different constructs of CARs targeting BCMA. BCMA is an antigen that is the most studied in CAR-T cells for relapsed/refractory MM. In use are the following types of anti-BCMA CAR-T cells **(A)** first generation of anti-BCMA CAR-T cells which consists of scFv as a binding molecule, hinge, transmembrane molecule (CD8α), costimulatory molecule (CD28), and an activating domain (CD3ζ); **(B)** the main change in the second generation of anti-BCMA CAR-T cells named bb2121 (Indecabtagene Vicleucel) was a new costimulatory molecule (4-1BB/CD137); **(C)** bb21217 differed from bb2121 with a memory-like phenotype of the former; **(D)** LCAR38M/JNJ-4528 are identical cells possessing single variable fragment of heavy chains (llama) instead of scFv (mouse) as a binding domain which can bind two different epitopes of BCMA; **(E)** FHVH33-BCMA-T as a binding domain was used with a fully human heavy-chain variable domain; **(F)** in the JCAR125 (Orvacabtagene Autoleucel) binding domain consists of a human scFv and a transmembrane domain costimulatory molecule (CD28). Created with BioRender.com.

bb2121

Raje et al. conducted a multicenter, open-label phase I trial with newly designed anti-BCMA CAR-T cells known as bb2121 which exhibited desired characteristics in preclinical experiments (39). They selected 33 patients with refractory/relapsed MM with a median age of 60 years and divided treatment into two phases. The first phase was with a dose escalation (50, 150, 450, and 800 × 10^6 cells; 21 patients) in which the main aim was to evaluate the maximum tolerated dose (MTD). The second phase was with a dose expansion (150 to 450 × 10^6 cells; 12 patients) where patients were treated with the estimated MTD to collect additional data (40). Both groups of patients were pre-treated with lymphodepleting therapy using fludarabine 30 mg/m^2 and cyclophosphamide 300 mg/m^2 over three days: –5, –4, and –3 prior to cell infusion. According to this and previous experiments, it seems unlikely that chemotherapy contributes to the outcomes because there were no responses in patients administered with subtherapeutic doses of anti-BCMA CAR-T cells (32). All but one patient received autologous stem cell transplantation previously (39).

The most frequent side effect of CAR-T cell treatment was cytopenia, especially neutropenia which occurred in 85% of patients and lasting a median of 10.9 months. Additionally, 75% of patients showed symptoms of CRS which occurred typically on the second day after infusion and persisted for 5 days on the average. Analysis of the severity of CRS showed a correlation between CRS score and the dose of the cells, CRP serum level, TNF alfa, ferritin level, and BCMA expression on MM cells. What is more, the peak presence of CAR-T cells in the blood was significantly higher in patients with a score of 3 and 4 on the scale of CRS. Twenty-one percent of patients received tocilizumab, and 12% were given corticosteroid to alleviate the symptoms of CRS. Furthermore, what is important is that these treatments did not affect CAR-T efficacy. Another side effect of the therapy was infections (42%), but they were easily controlled with antimicrobial agents (39).

The objective response rate (ORR) in all patients was 85%, and median PFS was 11.8 months. Forty-five percent achieved a complete response, and there was a significant effect of the dose of infused cells on the duration and frequency of response. Very good partial response (VGPR) occurred only in patients

receiving at least 150×10^6 CAR-T cells. Persistence of CAR-T in the host was improved compared to previous trials with 96, 86, 57, and 20% CAR-T cells after 1, 3, 6, and 12 months after infusion, respectively. In this study, 4-1BB instead of CD28 was used as a costimulatory domain. Another interesting remark was that the level of BCMA expression did not influence the patient's response to CAR-T cell therapy (39). This could be because for anti-BCMA CAR-T cells only 222 BCMA molecules per cell were sufficient to induce cytotoxicity in *in vitro* experiments. Normally, MM cells exhibit far higher expression of this antigen on its surface (36).

While the main drawback of this trial was a small study group, it compared favourably to other salvage regimens for refractory and relapsed MM such as pomalidomide plus dexamethasone with a response of 31% (41) and BCMA directed antibody with a response of 60% (42).

During ASCO20 (American Society of Clinical Oncology Annual Meeting 2020), Munshi presented a multicenter, single-arm phase II trial with Indecabtagene Vicleucel for R/R MM. Enrolled patients (n = 140) were already pretreated with ≥3 prior regimens (Imbeds, PIs, anti-CD38 mAbs) and refractory to their last regimen per International Myeloma Working Group (IMWG) criteria (43). After leukapheresis, 88% of patients were administered with bridging therapy ≥14 days before lymphodepletion, which was conducted with cyclophosphamide 300 mg/m^2 and fludarabine 30 mg/m^2 on days −5, −4, and −3. Eventually, 128 patients received CAR-T cells and among them, four patients were treated with 150×10^6 cells, 70 patients with 300×10^6 cells, and 54 patients with 450×10^6 cells. At data cut-off (16 Oct 2019) ORR was 73% and median PFS 8.6 months. Additionally, these values were significantly higher as the dose of CAR-T cells increased. Cytopenias occurred in 97% and CRS in 84% of patients but were most frequent within a group of higher CAR-T cell doses. Persistence and peak exposure was higher in responders when compared to non-responders (44).

bb21217

There is evidence indicating that the persistence of CAR-T cells post-infusion is a determinant of the duration of the response. Other studies have shown that memory-like T cells represent a more potent and persistent phenotype of CAR-T cells compared to the unselected type. Therefore, enriching CAR-T cells in memory-like phenotype could lead to enhanced and prolonged responses. Such a change in phenotype of T cells may be reached by the utilization of phosphoinositide 3-kinase inhibitor bb007 during the *ex vivo* culture of CAR-T bb2121 cells. The product of this procedure named bb21217 after showing efficacy in preclinical studies was used in first-in-human study CRB-402 (45).

LCAR-B38M

Zhao et al. designed dual epitope-binding CAR-T cells and utilized them in the first-in-human study named LEGEND-2, which was a multicenter on-label study conducted in China. LCAR-B38M cells have been created to bind to two different epitopes on BCMA target antigens on MM cells. This was intended to confer increased avidity of the cell binding to the target. Consequently, the dose of CAR-T cells could be lowered which was anticipated to decrease the frequency of the adverse events. Fifty-seven patients with refractory/relapsed MM and a median age of 54 were enrolled. All of them received conditioning therapy on days −5, −4, and −3 before infusion which consisted only of one agent—cyclophosphamide in a dose of 300 mg/m^2 (the rationale for changing lymphodepletion protocol compared to other trials was safety). Also, the treatment regimen was distinct when compared to previous experiments. Patients having received a median dose of 0.5×10^6 cells/kg split into three doses (20% + 30% + 50% of total dose over 7 days). ORR in this study was 88% (50 out of 57 patients exhibited a clinical response), CR occurred in 68% of patients, VGPR in 9%, and PR in 14%. The median duration of response at cut-off day was 14 months and PFS was 15 months. BCMA expression did not correlate with the response to the therapy. Although decreased doses of cells were administered, the adverse events occurred in the majority of patients. Ninety-one percent of patients developed pyrexia, 90% CRS, 49% thrombocytopenia, and 47% leukopenia. Stage 1 CRS occurred in 47%, stage 2 in 35% and stage 3 in 7% of patients. One patient developed grade 1 neurotoxicity: aphasia, agitation, and seizure-like behavior (46).

At the ASCO20 Meeting, Berdeja presented the results of the CARTITUDE-1 study with a median follow-up of 9 months when phase 1b was complete with n = 29. The CAR-T cells that were used were identical to LCAR-B38M cells used in the Chinese study. The median dose of the CAR-T cells was 0.73×10^6 cells/kg administered in a singular infusion. The ORR for patients was 100%, where 76% had a stringent complete response. Further, 21% of the patients had VGPRs, and 3% had PR. This study showed that after 6 months 22/28 patients had a level of JNJ-4528 cells that was below the level of quantification, suggesting that the response did not correlate with CAR-T persistence in the peripheral blood (47, 48).

In another study, scientists investigated whether lymphodepletion therapy influences CAR-T cell function. They created anti-BCMA CAR-T cells with 4-1BB as a costimulatory domain and divided 25 patients into three cohorts as follows: cohort 1 with a range of infused cells: $1–5 \times 10^8$ without lymphodepleting agents, cohort 2 with a range $1–5 \times 10^7$ CART-BCMA cells preceded by cyclophosphamide 1.5 g/m^2 and cohort 3 with a range $1–5 \times 10^8$ CART-BCMA cells plus cyclophosphamide 1.5 g/m^2. Percentage of responders in groups 1, 2, and 3 were as follows: 44, 20, and 64%. The longest response to therapy lasted for 32 months. Notable was the fact that BCMA expression on MM cells was significantly lower after CAR-T cell therapy (49).

FHVH33-CD8BBZ

FHVH33-CD8BBZ designed by Lam et al. is a type of CAR-T cells which instead of a scFv possess a fully human heavy-chain variable domain (FHVH) which is connected to the 4-1BB costimulatory domain to increase the persistence of CAR-T cells in the host. The fully human heavy-chain variable domain is less immunogenic than scFv and the use of this receptor should lead to less severe adverse events. This could be especially advantageous in the case of a second transplantation. Moreover, genes used for FHVH creation are smaller than scFv which results in better expression of CARs on T cells.

This feature could be particularly useful when it comes to designing CAR-T cells that target two antigens simultaneously (50). Mikkilineni et al. were conducting the first clinical trial of FHVH33-CD8BBZ with encouraging results. Twelve patients were divided into 3 groups with a different dosing regimen of 0.75, 1.5, and 3×10^6 cells/kg. Ten patients out of 12 reached objective responses and CRS occurred in 11 patients. These results, while based on 12 patients only, have shown that the efficacy of CAR-T cells with FHVH could be reached at a far lower dosing regimen in comparison to products based on scFv (51).

JCARH125

EVOLVE is an open-label phase I/II study in which a CAR-T cell product named Orvacabtagene Autoleucel (JCARH125) was administered to 62 patients. These CAR-T cells differed from other types of anti-BCMA CAR-T cells in that their binding domain is fully human with low affinity for soluble BCMA. In this study, patients with at least ≥3 prior therapies including Imbeds, PIs, ASCT, and anti-CD38 mAbs with lack of response to the last administered regimen were infused with 300×10^6, 450×10^6, or 600×10^6 CAR-T cells. ORR was 92%, CR occurred in 36%, VGPR in 32% and PR in 24% of patients (52).

OTHER PROMISING TARGET ANTIGENS IN CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY AGAINST MULTIPLE MYELOMA

Taking into consideration that the main cause of relapse MM after CAR-T cell therapy is the loss of BCMA antigen, it is the basis of rationale to search for other antigens that could overcome this problem (53).

SLAMF7/CS1

Signaling Lymphocytic Activation Family Member 7 (SLAMF7) is a robust marker of premalignant cells of MGUS and malignant cells in MM. The healthy cells that exhibit expression of SLAMF7 are: plasma cells, NK, and some CD8+ T cells, activated monocytes, and dendritic cells, and activated B cells (54, 55). This relative lack of specificity compared to BCMA requires changes in the treatment procedure. Therefore, SLAMF7 could be potentially used as a target of CAR-T cells only when associated with suicidal gene neighbouring CAR-T construct whose expression could be initiated on demand. An example of such a gene is inducible caspase 9 which is activated with rimiducid. This strategy aims to protect the patient from cytopenias in the case of severe adverse events (56). A functional antibody against SLAMF7 known as elotuzumab is already used in the treatment of MM, which confirms that it is a valuable target (57, 58). In a preclinical study, Wang et al. designed two CAR-T cell models. One targeted BCMA molecule whereas the second one was binding with SLAMF7. The effector function of both cells was similar although in a mouse model anti-SLAMF7 CAR-T cells proved to have better antitumor efficacy (59). Several SLAMF7-based CAR-T cells have entered clinical trial phases.

CD19

Garfall et al. presented a study in which another type of CAR-T cell was used that uses CD19 as target molecule instead of BCMA. These cells are currently utilized in the management of acute lymphoblastic leukaemia with success (7, 21). The main difference between BCMA and CD19 is its profile of expression—CD19 was proposed to be present on the surface of myeloma stem cells whereas BCMA is present both on myeloma cells and normal plasma cells (60). The authors decided to infuse patients with a history of previous autologous stem cell transplantation (ASCT) with CAR-T cells expressing receptor targeting CD19 (CTL019) following high-dose melphalan conditioning therapy and second ASCT. Two out of 10 patients after ASCT + CTL019 compared with the first ASCT showed significantly longer PFS. The authors of this study indicate two possible reasons for such low efficacy of the therapy. First, due to safety reasons, the combination of CTL019 and ASCT required a 10-fold dose reduction of the former compared to other studies of CTL019. Therefore, the CAR-T cell engraftment within the patient's bone marrow was impaired and consequently CD19+ cells were not sufficiently targeted. The second possible explanation is the inter-patient heterogeneity in the case of CD19+ expression on myeloma stem cells. Authors suspect that the immunophenotype of this subset of myeloma cells not can only vary between patients but also change over time in individuals (61) (**Table 1**).

BI-SPECIFIC CHIMERIC ANTIGEN RECEPTOR T CELLS

In vitro experiments in which a mixture of two populations of CAR-T cells was administered (one population consisted of CD19 CAR-T cells and the second one of CD20 CAR-T cells) ended with unsatisfactory results. This is probably because of the different proliferation rates in these two populations which led to selective expansion of one population at the expense of the second one (62). One way to avoid competition between two populations of CAR-T cells is bi-specific CAR-T cells which express two receptors for two different target antigens on one T cell. In theory, it should allow CAR-T cells to debulk tumors containing both or one of the targeted molecules.

B-Cell Maturation Antigen + Transmembrane Activator and Calcium-Modulator and Cyclophilin Ligand

A proliferation-inducing ligand (APRIL) is a natural ligand for both BCMA and transmembrane activator and calcium-modulator and cyclophilin ligand (TACI). According to Lee et al., TACI expression on human MM is limited to 78%, whereas BCMA was found on cells from all 50 tested patients. They have created bi-specific CAR-T cells which apart from BCMA targeted also TACI on MM cells. Reported results showed the efficacy of this approach in an *in vivo* model of tumor escape where BCMA+TACI− and BCMA−TACI+ cells were equally killed by CAR-T cells, whereas scFv bearing CAR-T cells against

TABLE 1 | Results of clinical trials of CAR-T Cells in Multiple Myeloma.

Target antigen	Authors of the study	CAR	Number of patients	Conditioning therapy	Adverse events of CAR-T therapy	Effects of treatment
BCMA	Brudno (33)	CAR-T-BCMA	16	CXC 300 mg/m2 + FD 30 mg/m^2	* Severe CRS (2) * Hypotension (6) * Adrenal insufficiency (4) * Encephalopathy (1) * Pancytopenia (2)	ORR 81% CR 13% VGPR 50%
BCMA	Raje (39)	B2121	33	CXC 300 mg/m^2 + FD 30 mg/m^2	* CRS (76%) * Neurologic toxicities (42%)	ORR 85% CR 45%
BCMA	Bardeja (45)	B21217	22	CXC 300 mg/m^2 + FD 30 mg/m^2	* CRS (59%) * Neurotoxicity (23%)	ORR 83%
BCMA	Zhao (46)	LCAR-B38M	57	CXC 300 mg/m^2	* Pyrexia (91%) * CRS (90%) * Thrombocytopenia (49%) * Leukopenia (47%) * Neurotoxicity (2%) * AST increased 39%)	ORR 88% CR 68% VGPR 5%
BCMA	Madduri (48)	JNJ-4528	25	CXC 300 mg/m^2 + FD 30 mg/m^2	* CRS (88%) * Neutropenia (80%) * Anemia (76%) * Thrombocytopenia (72%)	ORR 91%* CR 29% VGPR 33%
BCMA	Cohen (49)	CAR-T-BCMA (human scFv)	25	CXC 1,5 g/m^2 or without conditioning	* CRS (88%) * Neurotoxicity (32%)	Different ORR in 3 cohorts ranging 20-64%
BCMA	Mikkilineni (51)	FHVH-BCMA-T	12	CXC 300 mg/m^2 + FD 30 mg/m^2	* CRS (92%)	ORR 83% CR 17% VGPR 25%
CD19	Garfal (61)	CTL019 +ASCT	10	MEL 140 mg/m^2 or 200 mg/m^2	* CRS (10%) * Autologous GVHD (10%) * Most of AEs are due to high-dose melphalan	ORR 80%

**21 patients evaluable for response.*

CAR, chimeric antigen receptor; BCMA, B-cell maturation antigen; CRS, cytokine release syndrome; CXC, cyclophosphamide; FD, fludarabine; MEL, melphalan, ORR, overall response rate; CR, complete remission; VGPR, very good partial remission; AST, aspartate aminotransferase; GVHD, graft-versus-host disease; AE, adverse events; FHVH, fully-human heavy-chain-only; ASCT, autologous stem-cell transplantation.

BCMA only was insufficient to prevent the outgrowth of BCMA-negative tumor (63).

B-Cell Maturation Antigen + G Protein-Coupled Receptor Class C Group 5 Member D

G protein-coupled receptor class C group 5 member D (GPRC5D) was another antigen used for the creation of bi-specific CAR-T cells with BCMA. De Larrea et al. showed that in mice not only parallel infusion of anti-BCMA and anti-GPRC5D CAR-T cells could result in preventing tumor escape but also a single bicistronic vector encoding two 4-1BB-containing CARs has a significant efficacy and additionally avoids some problems with a parallel production of both CAR-T containing cells (64).

CHIMERIC ANTIGEN RECEPTOR T CELL WITH OTHER AGENTS

γ-Secretases Inhibitors

As mentioned before, the main obstacle that makes patients vulnerable to tumor relapse is the loss of the target antigen. A possible strategy to avoid at least some of the relapses could be the administration of inhibitors of γ-secretases, the enzymes responsible for BCMA loss from MM cell surface. Treatment of MM in NOD/SCID/gamma c−/− mice has a notable impact on the antitumor efficacy of anti-BCMA CAR-T cells alone. Furthermore, the antigen cleavage from the cell surface is decreased leading to less soluble antigen accumulation in the bone marrow which could improve antigen recognition by CAR-T cells and consequently MM cells being killed. The FDA-approved clinical trial (NCT03502577) has been initiated where gamma-secretase inhibitors are combined with anti-BCMA CAR-T cells (65).

Lenalidomide

Wang et al. showed increased *in vitro* and *in vivo* effect of lenalidomide addition to genetically engineered T cells. Lenalidomide added during *in vitro* transduction and expansion of CAR-T cells increased cytotoxicity, memory maintenance, Th1 cytokine production, and immune synapse formation. Moreover, the addition of *in vivo* lenalidomide improved the persistence of anti-CS1 CAR-T cells in the host. These observations led to the design of a clinical trial combining lenalidomide and anti-CS1 CAR-T cells (59).

TABLE 2 | Advantages and disadvantages of CAR-T Cells therapy in Multiple Myeloma.

Advantages	Disadvantages
Novel therapy for refractory/relapsed patients	Potentially can cause life-threatening complications
Recognize cells without HLA expression	Relapses after treatment (antigen escape)
Eliminates only cells with targeted antigen	Immunogenicity of CARs
One-time treatment with long therapy-free intervals providing patients with a high quality of life	High costs of the therapy

PD-1 Inhibitor

Bernabei et al. demonstrated the efficacy of PD-1 inhibitor combination with anti-BCMA CAR-T cells. In the clinical study, they administered pembrolizumab, PD-1 inhibitor to five patients with relapsed MM after treatment with CAR-T cells. This combination proved to be efficient in inducing re-expansion of T cells in relapsed patients. However, re-expansion occurred infrequently and serious adverse events were evident (66).

SUMMARY AND FUTURE DIRECTIONS

Previous studies have shown great efficacy of CAR-T cell therapy in MM in the context of remissions, even in a population of refractory/relapsed MM patients after many lines of treatment. It seems that the results of treatment are improving with the development of the new CAR constructs. Still, the main problem constitutes relapses of disease and shortness of remissions. Bearing in mind that relapses are frequently developed by MM cells possessing target antigens, it appears that future studies should be focused on improvements in the durability of response by prolonging the efficacy of CAR-T cells in the patient. That could be reached by modification of CAR constructs (especially in the costimulatory domain), use of different growth factors, and by avoiding CAR-T cell exhaustion (lower strength of binding to the antigen or decreasing intensiveness of activation signal; a combination of CAR-T cells with checkpoint inhibitors). Studies of CAR-T cells should be accompanied by studies of the biology of the cells that are responsible for relapses after CAR-T cell treatment. The study of the cells causing relapses can play a critical role in the search for new target antigens. Similar to transplantations, it seems that the combination of CAR-T cells with other drugs currently used in MM (new immunomodulatory drugs, proteasome inhibitors, or monoclonal antibodies) is the future of the field. Additionally, the conception of conditioning—CAR-T cell administration-consolidation/maintenance with new drugs or even a combination of CAR-T cell therapy with autologous/allogeneic transplantation requires deep consideration.

CONCLUSIONS

Given the fact that MM is the second most frequent cancer in haematological practice, the need for new treatment strategies is considerable. The most promising trials include CAR-T cells targeted against BCMA antigen but use of other antigens like CS1 or CD19 could also lead to a better prognosis for refractory/relapsed MM patients. Although a few CAR-T cell products are currently registered by the FDA in the US for the treatment of other haematological malignancies, we are still waiting for the next phases of clinical trials regarding the use of CAR-T cells in multiple myeloma refractory/relapsed patients. Observations of the use of CAR-T cells in other malignancies have shown that a similar strategy will be probably common in the future in a matter of a few years. However, we should not forget about the potentially serious side effects of this therapy. Only selected MM patients can benefit from CAR-T treatment, and therefore, studies are needed to identify these groups. Furthermore, the majority of patients relapse after a relatively short time, so also studies regarding methods of blocking mechanisms of tumor escape are necessary (61) (**Table 2**).

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Chimeric Antigen Receptor T Cells Targeting Integrin αvβ6 Expressed on Cholangiocarcinoma Cells

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Cholangiocarcinoma (CCA) is a lethal bile duct cancer that responds poorly to current standard treatments. A new therapeutic approach is, therefore, urgently needed. Adoptive T cell transfer using chimeric antigen receptor (CAR) T cells is a new therapeutic modality with demonstrated efficacy in hematologic malignancies. However, its efficacy against solid tumors is modest, and further intensive investigation continues. An important factor that influences the success of CAR T cell therapy is the selection of a target antigen that is highly expressed on cancer cells, but markedly less so in normal cells. Integrin αvβ6 is upregulated in several solid tumors, but is minimally expressed in normal epithelial cells, which suggests integrin αvβ6 as an attractive target antigen for CAR T cell immunotherapy in CCA. We investigated integrin αvβ6 expression in pathological tissue samples from patients with liver fluke-associated CCA. We then created CAR T cells targeting integrin αvβ6 and evaluated their anti-tumor activities against CCA cells. We found overexpression of the integrin αvβ6 protein in 23 of 30 (73.3%) CCA patient tissue samples. Significant association between high integrin αvβ6 expression and short survival time ($p = 0.043$) was also observed. Lentiviral constructs were engineered to encode CARs containing an integrin αvβ6-binding peptide (A20) derived from foot-and-mouth disease virus fused with a second-generation CD28/CD3ζ signaling domain (A20-2G CAR) or with a fourth-generation CD28/4-1BB/CD27/CD3ζ signaling domain (A20-4G CAR). The A20-2G and A20-4G CARs were highly expressed in primary human T cells transduced with the engineered lentiviruses, and they exhibited high levels of cytotoxicity against integrin αvβ6-positive CCA cells ($p < 0.05$). Interestingly, the A20-2G and A20-4G CAR T cells displayed anti-tumor function against integrin αvβ6-positive CCA tumor spheroids ($p < 0.05$). Upon specific antigen recognition, A20-4G CAR T cells produced a slightly lower level of IFN-γ, but exhibited higher proliferation than A20-2G CAR T cells. Thus, the A20-4G CAR T cells with lower level of cytokine production, but with higher proliferation represents a promising potential adoptive T cell therapy for integrin αvβ6-positive CCA.

Keywords: adoptive T cell therapy, chimeric antigen receptor, integrin αvβ6, immunotherapy, cholangiocarcinoma

INTRODUCTION

Cholangiocarcinoma (CCA) arises from the cancerous transformation of epithelial cells lining the bile duct. It is a relatively rare cancer, but its rates of both incidence and mortality are increasing worldwide (1). The highest incidence of CCA was found in the Northeast of Thailand where infection with an oncogenic liver fluke [*Opisthorchis viverrini* (OV)] is known to be a strong risk factor for CCA (2). Surgical resection is a curative treatment for CCA; however, only 20-40% of tumors are resectable, and the recurrence rate after surgery is high (3). For non-resectable patients, the standard first-line therapy is gemcitabine in combination with cisplatin. However, this therapeutic regimen achieves a 5-year overall survival rate of <5%, and the median overall survival is <1 year (4). Immunotherapy and targeted therapy for this difficult-to-treat disease have been reported (5, 6); however, the limited efficacy of these therapies highlights the need for an alternative treatment approach.

Generally, CCA and other cancers develop when transformed cells escape immune surveillance. Downregulation of MHC molecules that conceal cancerous cells from T cell recognition is one of many cancer immune escape mechanisms (7, 8). To overcome this problem in cancer treatment, adoptive transfer of T cells expressing a chimeric antigen receptor (CAR) has been developed as a promising therapeutic approach. CARs are synthetic receptors that mimic natural T cell receptor function by combining a cancer antigen-binding domain with a T cell activating signaling domain. CAR T cells recognize cancer antigen in a direct, antibody-like fashion, which leads to the activation of intracellular signaling. As a result, CAR T cells kill cancerous cells in an MHC-independent manner.

Different generations of CAR T cells have been developed by combining the intracellular part of T cell receptor (CD3ζ) and one or more co-stimulatory domains. Recently, three second-generation CAR (2G-CAR) T cells targeting CD19 for hematologic malignancies were approved by the U.S. Food and Drug Administration (USFDA), namely Kymriah (4-1BB/CD3), Yescarta, and Tecartus (CD28/CD3ζ). However, clinically successful CAR T cell therapies in patients with solid tumors have been limited, and studies to improve the efficacies of these therapies are intensively ongoing. Several research groups have designed other generations of CAR T cells by adding more co-stimulatory domains into the CAR molecule (9, 10). Third-generation CAR (3G CAR) T cells consisting of CD28/CD137/CD3ζ (11, 12) or CD28/CD27/CD3ζ (13) were created and tested. Fourth-generation CAR (4G CAR) T cells containing CD28/CD137/CD27/CD3ζ have also been produced and proven effective in the treatment of B cell leukemias (10, 14, 15).

An essential factor that influences the success of CAR T cell immunotherapy is the selection of a target antigen that is highly expressed on the surface of cancerous cells, but that is only minimally expressed on normal cells. Binding between the target antigen on cancerous cells and the extracellular antigen-binding domain of the CAR molecule leads to activation of CAR T cells to kill cancerous cells. An attractive potential target antigen in solid tumors is integrin αvβ6 because it is overexpressed in multiple epithelial malignancies, including pancreatic ductal adenocarcinoma (16), ovarian cancer (17), head and neck squamous cell carcinoma (18, 19), breast cancer (20), and CCA (21, 22). Integrin αvβ6 is also a target for diagnostic imaging and anti-cancer therapies (19). Several integrin αvβ6-specific therapeutic agents have been studied in clinical trials (23–25). Notably, the specificity of integrin αvβ6 immunohistochemistry for CCA (100%) surpassed all other tested markers, and the sensitivity was very similar to that of cytokeratin 7 (CK7) (86 vs. 90%) (26). Integrin αvβ6 is, thus, a potential target antigen for development of CAR T cell therapy for CCA.

A single-chain variable fragment (scFv) derived from a monoclonal antibody is most commonly used as the extracellular cancer antigen-binding domain within tumor-specific CAR molecules. However, antigens on cancer cells can also be specifically bound by the peptide with high specificity for the desired target (27). Second-generation (2G) of CAR T cells targeting integrin αvβ6 were recently tested in multiple solid tumors, including pancreatic, breast, and ovarian cancers (28). The antigen-binding domain of this 2G CAR was a peptide containing 20-mers of amino acids (A20) that was derived from the viral capsid protein 1 (VP1) of foot-and-mouth disease virus (FMDV), and it bound explicitly with high affinity (<1 nM) only to integrin αvβ6 (29). However, the effect of CAR T cells targeting integrin αvβ6 in the killing of CCA cells is still unknown.

Expression of integrin αvβ6 has not been previously characterized in CCA tumors or cell lines from patients with liver fluke-associated CCA. To evaluate its potential as a therapeutic target antigen in CCA, we analyzed integrin αvβ6 expression in pathological tissue samples and CCA tumor cell lines. We then compared the anti-tumor activity of A20-2G CAR T cells and A20-4G CAR T cells, containing the CD28/CD3ζ and CD28/4-1BB/CD27/CD3ζ signaling domains, respectively. Both CARs demonstrated integrin αvβ6-dependent anti-tumor activity in models of CCA, but the same anti-tumor activity was not observed in non-transduced (NT) T cells. The results of this study provide proof-of-principle for the use of integrin αvβ6-specific CAR T cells in adoptive T cell immunotherapy of CCA.

MATERIALS AND METHODS

Ethical Approval

In this study, paraffin-embedded tissues from patients with CCA were collected at Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand. The protocols for collection of the tissue samples and the experimental studies were approved by the Ethics Committee for Human Research, Khon Kaen University (No. HE591063). Written informed consent was obtained from each patient before the study. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers who had provided written informed consent in accordance with a protocol approved by the Siriraj Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (COA No. *Si* 829/2020).

Immunohistochemistry

Integrin αvβ6 was detected on the formalin-fixed, paraffin-embedded tissue sections using immunohistochemistry (IHC). Specifically, 4-μm-thick paraffin sections were deparaffinized in xylene, and then rehydrated in gradient ethanol. These tissue sections were antigen-retrieved in 0.1% trypsin, and endogenous peroxidases were blocked in 3% H_2O_2. After blocking of non-specific binding with 5% skim milk, the tissue sections were incubated overnight with an anti-αvβ6 monoclonal antibody (dilution 1:100, Clone 6.2A1; Biogen Inc., Cambridge, MA, USA) that was generously provided by Dr. Shelia M. Violette. The treated tissue sections were then reacted with EnVision Kit/Horseradish Peroxidase (HRP)^{TM} (Agilent Technologies, Santa Clara, CA, USA) at room temperature (RT) for 1 h. Immunoreactive integrin αvβ6 from those tissue sections were developed using 3, 3′-diaminobenzidine (DAB) solution. The tissue sections were subsequently counterstained with Mayer's Hematoxylin (Sigma-Aldrich Corporation, St. Louis, MO. USA), mounted, and observed under a light microscope (Nikon Eclipse Ti2; Nikon Instruments, Tokyo, Japan).

The IHC-stained tissues were evaluated independently by two investigators who had no prior knowledge of patient clinical or survival data. The frequency of integrin αvβ6 was semi-quantitatively scored according to the percentage of positive cells, as follows: 0%, negative; 1-25%, +1; 26-50%, +2; and, 50%, +3. The intensity of protein staining was scored, as follows: weak, 1; moderate, 2; and, strong, 3. The expression of integrin αvβ6 was evaluated using H-score by multiplying the frequencies and intensities. The patients were then categorized into two groups according to the median IHC score. Log-rank test was used to analyze the difference between these two groups relative to overall survival.

Cell Culture

Human CCA cell lines, KKU-055 (JCRB1551), KKU-100 (JCRB1568) (29), and KKU-213A (JCRB1557) (30), were established from patients with CCA in opisthorchiasis endemic areas located in the Northeastern region of Thailand. The Lenti-X 293T cell line is a highly transfectable subclone of the transformed human embryonic kidney cell line HEK 293, which supports high levels of viral production (Takara Bio, Kusatsu, Shiga, Japan). A375.puro (αvβ6-negative) and A375.β6 (αvβ6-positive) cell lines were used a negative and positive controls, respectively (28, 31). Tumor cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) or in DMEM/F12 (Gibco; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37°C with 5% CO_2.

Immunofluorescence Staining

Cells were cultured on glass coverslips and fixed with 4% paraformaldehyde for 15 min on ice. The cells were then washed with phosphate-buffered saline (PBS) before incubation with 5% bovine serum albumin (BSA) blocking solution for 30 min. The cells were then incubated with a 1:100 dilution of anti-β6 polyclonal antibody (clone ITBG6; Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight. After washing, the cells were incubated with anti-mouse Alexa Fluor® 488-labeled (clone A21206; Thermo Fisher Scientific), and their nuclei were counterstained with Hoechst 33342 dye for 1 h at RT. The coverslips were mounted on a glass slide and immunofluorescence signals were visualized using a Zeiss LSM 800 confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

Flow Cytometry

Integrin αvβ6 expression on the surface of CCA cells was detected using mouse anti-integrin αvβ6 antibody (Clone 10D5; Merck Millipore, Burlington, MA, USA) at a dilution of 1:100. The cells were then washed and incubated with Alexa Fluor® 488-tagged secondary antibody (Thermo Fisher Scientific). CAR expression on transduced T cells was detected using anti-cMyc FIT-C-tagged antibody (clone ab1394; Abcam, Cambridge, UK). Phenotypic analysis of T cells was performed by using anti-CD3-FITC (Clone UCHT-1), anti-CD4-APC (Clone MEM-241), anti-CD8-APC (Clone UCHT-4), anti-CD16-APC (Clone 3G8), and anti-CD56-PE (Clone AB_2563925). All of these antibodies were purchased from BioLegend (San Diego, CA, USA). Flow cytometry was performed using a BD Accuri C6 Plus Flow Cytometer (BD Biosciences, San Jose, CA, USA), and the data was analyzed by using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Construction of Chimeric Antigen Receptor

Second-generation CAR T cells containing A20 peptide for targeting integrin αvβ6 (A20-2G) CAR T cells were created as previously described (28). Briefly, A20 peptide derived from FMDV was placed downstream of a CD124 signal sequence, followed by human *c-myc* peptide tag (EQKLISEEDL), as shown in **Figure 3A**. The DNA fragment encoding the required parts was synthesized by Integrated DNA Technologies (Coralville, IA, USA). The A20 codon-optimized gene was sub-cloned into self-inactivating lentivirus vectors (pCDH) containing expression cassettes encoding CD8 short hinge, a CD28 transmembrane domain, and the CD28/CD3ζ (A20-2G) or CD28/4-1BB/CD27/CD3ζ (A20-4G) signaling domains. Transgene expression is driven by the elongation factor-1α (EF-1α) promoter. Plasmid DNA was isolated using a Midiprep Kit (Qiagen, Hilden, Germany) and sequences were verified by DNA sequencing.

Lentivirus Production and T Cell Transduction

Lenti-X 293T cells were transfected with the A20-2G or A20-4G plasmid and two packaging plasmids (psPAX2 and pMD.2G) at a ratio of 5:3:1 using calcium phosphate transfection method. The supernatant was collected at 48 and 72 h post-transfection and filtered through a 0.45 μm filter unit (Merck Millipore) to remove cell debris, followed by concentration at 20,000 × g for 2 h at 4°C (Sorvall RC-6 Plus Centrifuge; Thermo Fisher Scientific). Virus titer was determined using a qPCR Lentiviral Titration Kit (ABM, Richmond, BC, Canada) according to the manufacturer's instructions.

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of healthy donors using density gradient

centrifugation, and activated by culturing with 5 μg/ml phytohemagglutinin-L (PHA-L) (Roche Applied Science, Penzberg, Germany) in AIM-V medium supplemented with 5% human serum, IL-2 (20 ng/ml), IL-7 (10 ng/ml), and IL-15 (40 ng/ml) (Immunotools, Friesoythe, Germany). On day 3 after activation, T cells were transduced with lentiviral particles using 10 μg/ml protamine sulfate (Sigma-Aldrich). The cells were centrifuged at 1200 × g at 32°C for 90 min, followed by incubation at 37°C and 5% CO_2 overnight.

Immunoblot Analysis

Immunoblotting was used to detect the expression of A20-2G or A20-4G CAR construct following transfection in Lenti-X 293T cells. In brief, the cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. Cell lysate was then resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim-milk in Tris-buffered saline (TBS) and 0.1% Tween-20 (TBS-T), and then detected with anti-CD3ζ (clone sc-166435) and anti-GAPDH (clone sc-32233) from Santa Cruz Biotechnology (Dallas, TX, USA). The membrane was incubated with HRP-conjugated secondary antibody (Invitrogen) and the immunoreaction was developed using chemiluminescence reagents (SuperSignal® West Pico Substrate; Thermo Fisher Scientific). The signal from the reaction was captured on X-ray film and quantified using ImageJ program (National Institutes of Health, Bethesda, MD, USA). The expression level of GAPDH was used as loading control.

Cytotoxicity Assays

The monolayer of target cells (1×10^4) was co-cultured with A20-2G CAR T cells or A20-4G CAR T cells or NT T cells at three different effector to target (E:T) ratios (5:1, 2.5:1, and 1.25:1) for 24-48 h. After removal of the CAR T cells, 100 μl of crystal violet fixing/staining solution was added to each well and incubated for 20 min. The plates were washed and the cell-bound dye was dissolved in methanol. The absorbance was measured at a wavelength of 595 nm using a Sunrise™ Absorbance Microplate Reader (Magellan™ data analysis software version 6.6.0.1; Tecan, Männedorf, Switzerland). Cytotoxicity was calculated using the following formula: [1-(absorbance of monolayer culture with T cells/absorbance of monolayer culture alone)] × 100%.

In addition, cytotoxicity assay was conducted by using three-dimensional (3D) spheroid model. In brief, a total number of 2×10^3 cancer cells were firstly stained with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate) Dye (Thermo Fisher Scientific, Waltham, MA) and then seeded into an ultra-low attachment 96-well round-bottomed plate (Corning, NY, USA) containing 2.5% Corning matrigel matrix (Corning, NY, USA). To generate a single spheroid, the plate was centrifuged at 1,000 × g at 4°C for 10 min and then cultured for 48 h. T cells in culture medium containing 1 μg/ml propidium iodide (PI) were added to the spheroid at E:T ratio of 5:1. After co-culturing for 3 days, dead cancer cells, which were stained with PI, were analyzed by a confocal microscope (Nikon Instruments Inc., Melville, NY, USA). Quantification of mean fluorescence intensity (MFI) was conducted by using NIS-Elements software. Cytotoxicity was calculated by following formula: [(experimental MFI-spontaneous MFI)/(maximum MFI-spontaneous MFI)] × 100. Experimental and spontaneous MFIs were MFI of sample spheroids when co-cultured with or without CAR T cells, respectively. Maximum MFI was MFI of spheroid treated with 0.1% Triton-X 100.

Intracellular Cytokine Staining and T Cell Proliferation Assay

CAR T cells were cultured with target cells in media containing Brefeldin A (BioLegend) at an E:T ratio of 5:1 for 6 h in 5% CO_2 at 37°C. The CAR T cells were harvested and the cell surface markers were stained with anti-CD3-FITC and anti-CD8-APC antibodies (Immunotools). The cells were then fixed with 4% paraformaldehyde for 15 min. Intracellular cytokine was stained by incubation with anti-IFN-γ-PE (Immunotools) antibody in the presence of 0.5% saponin permeabilization agent on ice for 30 min. The cells were then subjected to flow cytometry to analyze cytokine production levels.

T cell proliferation was assessed by tracking cells labeled with carboxyfluorescein succinimidyl ester (CFSE). Briefly, 1×10^5 of T cells were stained with 1 μM CFSE for 10 min at 37°C. After washing twice with culture media, CFSE-labeled T cells were co-cultured with target cell monolayers at an E:T ratio of 5:1 in AIM-V medium supplemented with 5% human serum. On day 3 after co-culturing, CFSE dilution was measured by flow cytometry to estimate proliferation of total CAR T cells. Note that no exogenous cytokines were added during the proliferation assay.

Statistical Analysis

GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM). For comparison between two groups, a two-tailed *t*-test was used. For comparisons among three or more groups, one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was used. A p-value <0.05 was considered statistically significant.

RESULTS

Expression of Integrin αvβ6 in Human CCA Tissues and Cell Lines

Immunohistochemistry (IHC) staining was performed to examine the expression of integrin αvβ6 in liver fluke-associated CCA tumors and cell lines, including KKU055, KKU100 and KKU213A. The results revealed the presence of integrin αvβ6 in human CCA tissues, whereas the protein was virtually non-existent all non-tumorous tissues (**Figure 1**). Low to high intensities of staining signals were observed in CCA tissues (**Figures 1B,C**). The stained protein was located at both the cell membrane and within the cytoplasm of CCA cells. Twenty-three out of 30 (73.3%) human CCA tissue samples showed positive staining, of which 13 tumors (43.3%) showed high level expression. The expression levels of the protein were calculated as H-scores (**Figure 1D**). Cumulative survival of patients with low/negative and high expression levels of integrin αvβ6 was compared. All patients with CCA had died by the end of the

follow-up period. Analysis of survival times showed a significant difference between the CCA patients who had low/negative and those who had high integrin αvβ6 expression levels. The median survival time was 308.0 days (95% confidence interval [CI]: 178.9–437.1) in the patients with low/negative integrin αvβ6 expression level, and 155.0 days (95% CI: 77.5–232.5) in those with high integrin αvβ6 expression level (log-rank test; $p = 0.043$) (**Figure 1E**).

The expression and localization of integrin αvβ6 was also examined in CCA cell lines. The results showed integrin αvβ6 to be expressed at the cell surface of 58.5 ± 6.2%, 73.2 ± 12.5%, and 87.7 ± 2.6% of KKU055, KKU100, and KKU213A cell lines, respectively (**Figures 2A–C**). Two known cell lines with negative and positive expression of integrin αvβ6, β6-negative A375.puro cells (4.3±4.7%) and β6-positive A375.β6 cells (87.6±7.0%) (32), were also stained and used as negative and positive controls in further studies.

Expression of Chimeric Antigen Receptor Targeting Integrin αvβ6 in Lenti-X 293T and Human Primary T Cells

Second- and fourth-generation CAR targeted against integrin αvβ6 (A20-2G CAR and A20-4G CAR) (**Figure 3A**) were expressed using lentiviral vector. The expression of A20-2G CAR and A20-4G CAR in Lenti-X 293T cells was examined by immunoblotting under reducing conditions using anti- CD3ζ antibody. The results showed that A20-2G CAR and A20-4G CAR were expressed at the predicted sizes of 32 and 43 kDa, respectively (**Figure 3B**). To generate A20-2G CAR and A20-4G CAR T cells, primary human lymphocytes isolated from a healthy donor were transduced with lentiviruses carrying either A20-2G CAR or A20-4G CAR construct. A representative flow cytometry profile after lentiviral transduction of the lymphocytes is shown in **Figure 3C**. The median CAR expression of A20-2G CAR was 71.5±17.5%, and A20-4G CAR was 69.6±19.1%, as examined on day five post-transduction (**Figure 3D**). The phenotypes of CAR T cells were analyzed by flow cytometry, which showed that within the $CD3^+$ population, there were significantly more cytotoxic $CD8^+$ T cells than helper $CD4^+$ T cells in the groups of NT T cells (61.8±11.28% and 27.7±6.3%, p=0.038), A20-2G CAR T cells (72.7±7.0% and 25.3±8.0%, p=0.004), and A20-4G CAR T cells (73.7±7.0% and 24.6±7.5%, p=0.003) (**Figure 3E**). Furthermore, the generated CAR T cells were enriched in $CD45RA^-CD62L^+$ central memory (T_{CM}) and $CD45RA^+CD62L^+$ naïve T cells (**Figure 3F**). The expression of these markers did not significantly differ between the NT T cells and the A20-2G or A20-4G CAR T cells.

Anti-tumor Activities of A20-2G and A20-4G CAR T Cells Against Integrin αvβ6-Expressing Cells

The anti-tumor activity of A20-2G and A20-4G CAR T cells against integrin αvβ6-expressing cells was tested by co-culturing these CAR T cells with target cell lines expressing different levels of integrin αvβ6 at the indicated E:T ratio (**Figure 4**), and using NT cells as control T cells. After co-culture and removal of effector cells, crystal violet solution was added to stain the remaining target cells (**Figure 4A**). The results revealed that both A20-2G and A20-4G CAR T cells exhibited strong cytotoxic effects in a dose-dependent manner, and both had higher cytotoxic effects than NT T cells. At an E:T ratio of 5:1 and an incubation time of 24 h, the killing activity of A20-2G and A20-4G CAR T cells on integrin αvβ6-negative A375.puro cells was very low (**Figure 4B**), and the killing activity of A20-2G and A20-4G CAR T cells on integrin αvβ6-positive A375.β6 cells was as high as 65.9±5.06% and 69.4±10.1%, respectively, compared to the killing activity of NT T cells on A375.β6 cells, which was 25.7±6.9% (p<0.05) (**Figure 4C**). Killing effects were also observed when A20-2G and A20-4G CAR T cells were co-cultured with KKU055 cells (34.6±2.9% and 52.8±7.5%, respectively) compared to the killing activity of NT T cells on KKU055 cells, which was 21.3±0.8% (p<0.05) (**Figure 4D**). At an E:T ratio of 5:1 and an incubation time of 24 h, no significant cytotoxicity was observed in assays with the KKU100 and KKU213A cells (data not shown). However, after co-culturing for 48 h, the killing activity of A20-2G and A20-4G CAR T cells on KKU100 cells was 62.4±4.7% and 59.4±3.0%, respectively, compared to the killing activity of NT T cells on KKU100 cells, which was 35.1±0.4% (p<0.05) (**Figure 4E**). Lastly, the killing activity of A20-2G and A20-4G CAR T cells on KKU213A cells was 50.9±6.1% and 54.5±6.3%, respectively, compared to the killing activity of NT T cells on KKU213 cells, which was 25.6±4.5% (p<0.05) (**Figure 4F**).

Anti-tumor Activities of A20-2G and A20-4G CAR T Cells Against Three Dimensional CCA Spheroids Expressing Integrin αvβ6

Anti-tumor activities of A20-2G and A20-4G CAR T cells against three-dimensional CCA spheroids expressing integrin αvβ6, which appeared like solid tumor in the human body, were also examined. The A20-2G or A20-4G CAR T cells were co-cultured with tumor spheroids for 3 days, and then dead tumor cells were stained by propidium iodide (PI). Confocal microscopy and computerized image processing were employed to locate area of tumor spheroid, and cancer cell death was quantified by integrating PI fluorescence intensity in the spheroid area (**Figure 5A**). The results revealed that both A20-2G and A20-4G CAR T cells specifically killed the αvβ6-positive A375.β6 and CCA spheroids (KKU055, KKU100, and KKU213A) (**Figures 5B–G**). While A20-2G and A20-4G CAR T cells showed very weak cytotoxicity on αvβ6-negative A375.puro spheroids, they exhibited more potent cytotoxicity against A375.β6 spheroids, compared to NT T cells (55.6±21.4% or 52.4±14.1% vs. 0.3±0.2%; p<0.05). Killing effects were observed when A20-2G and A20-4G CAR T cells were co-cultured with KKU055 spheroids (86.4±1.0% and 86.64±12.7%, respectively), compared to that of NT T cells on KKU055 spheroids, which was 14.0±7.7% (p<0.01). The killing activity of A20-2G and A20-4G CAR T cells on KKU100 spheroids was 60.9±15.2% and 53.1±19.1%, respectively, compared to that of NT T cells on KKU100 spheroids, which was 6.0±3.8% (p<0.05). Killing effects were also observed when A20-2G and A20-4G CAR T

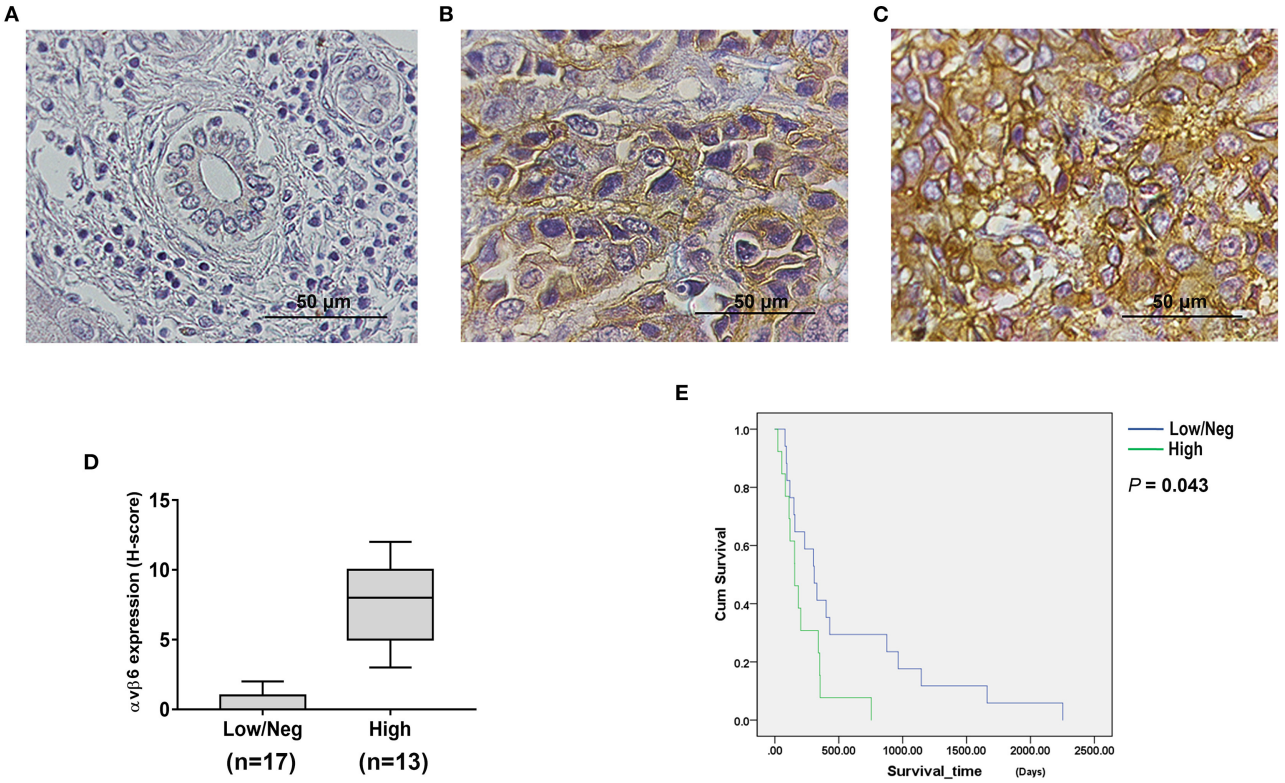

FIGURE 1 | Integrin αvβ6 was upregulated in cholangiocarcinoma (CCA) tissues. Immunohistochemical (IHC) staining was performed to examine integrin αvβ6 expression in CCA tissues obtained from liver fluke-associated CCA patients. **(A)** Negative staining of normal bile duct epithelial cells and hepatocytes surrounding the tumor area was observed. Positive staining of the integrin αvβ6 protein was detected in the cytoplasm and membrane of tumor cells with **(B)** low and **(C)** high expression levels. Scale bars represent 50 μm. **(D)** Expression levels of integrin αvβ6 in CCA tissues were summarized as H-scores. **(E)** Cumulative overall survival curves of CCA patients with different expression levels of integrin αvβ6. The cumulative survival time of patients who had high expression levels of integrin αvβ6 was significantly shorter than that of the patients who had low/negative expression levels of integrin αvβ6 (p=0.043).

cells were co-cultured with KKU213A spheroids (85.9±20.7% and 55.6±13.0%, respectively), compared to that of NT T cells on KKU213A spheroids, which was 6.4±1.4% (p<0.05). These data indicated that CAR T cells targeting integrin αvβ6 could infiltrate into three-dimensional spheroids and kill cancer cells expressing integrin αvβ6.

Production of Interferon-γ in and Proliferation of A20-2G and A20-4G CAR T Cells After Co-culturing With Integrin αvβ6-Expressing Target Cells

Production of interferon-γ (IFN-γ) in A20-2G and A20-4G CAR T cells was examined after these CAR T cells were co-cultured with integrin αvβ6-negative A375.puro or integrin αvβ6-positive A375.β6 cells at an E:T ratio of 5:1 and an incubation time of 6 h. As positive control, the CAR T cells were activated using phorbol-12-myristate-13-acetate (PMA) and ionomycin (IONO) in the presence of brefeldin A. In all cases, production of IFN-γ in $CD8^+$ T cell population was examined by intracellular cytokine staining and analyzed by flow cytometry (**Figure 6A**). When NT T cells, A20-2G CAR T cells, and A20-4G CAR T cells were co-cultured with integrin αvβ6-negative A375.puro cells, the production of IFN-γ was minimal (**Figure 6B**). When these cells were co-cultured with integrin αvβ6-positive A375.β6 cells, the production of IFN-γ in A20-2G CAR T cells was clearly increased compared to those co-cultured with NT T cells (4.5±1.9 % vs. 1.1±0.7%; p=0.0156). However, when A20-4G CAR T cells were co-cultured with integrin αvβ6-positive A375.β6 cells, the production of IFN-γ was not significantly increased (1.3±9.5% vs. 1.1±0.7%; p>0.99), (**Figure 6B**).

The proliferation of NT cells, A20-2G CAR T cells, and A20-4G CAR T cells was examined after co-culturing for 3 days with integrin αvβ6-negative A375.puro or integrin αvβ6-positive A375.β6 cells at an E:T ratio of 5:1 without addition of exogenous cytokine. Carboxyfluorescein succinimidyl ester (CFSE) dilution of proliferating NT and CAR T cells was analyzed on day 3. The results showed that, after co-culturing with integrin αvβ6-negative A375.puro cells, neither A20-2G CAR T cells nor A20-4G CAR T cells showed significant difference of proliferation, compared with NT T cells (16.2±2.2% or 23.6±7.5% vs. 8.3±2.3%, p>0.05). After co-culturing with integrin αvβ6-positive A375.β6 cells, A20-2G CAR T cells proliferated to a significantly greater extent than NT T cells

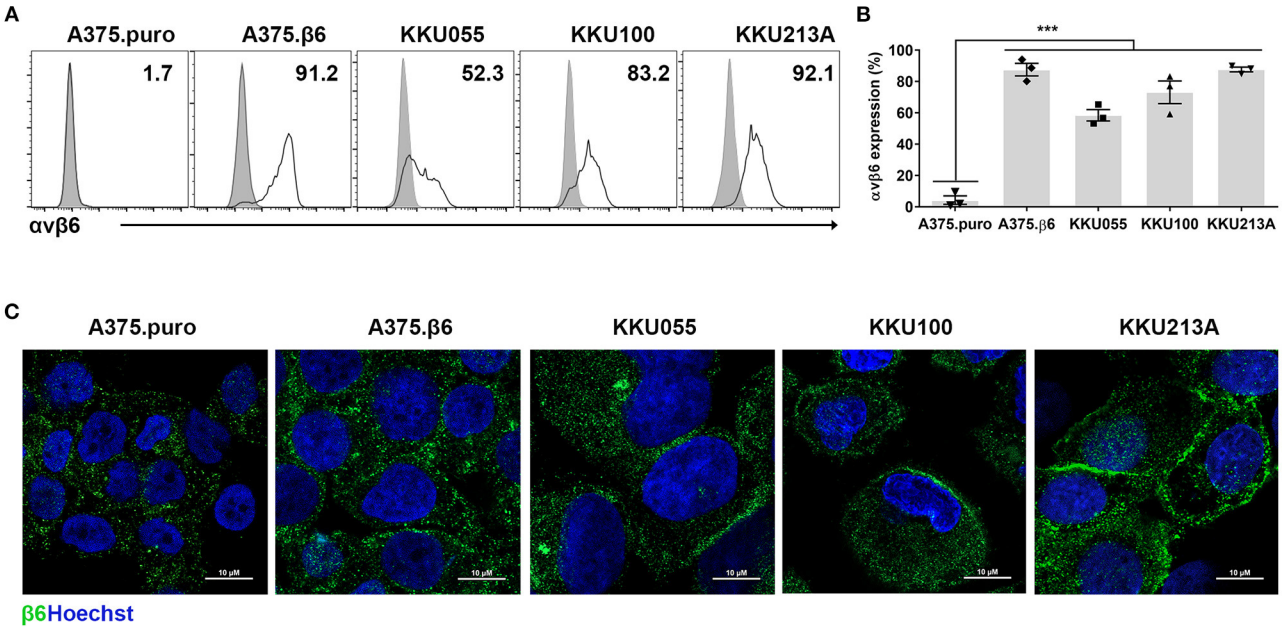

FIGURE 2 | Expression of integrin αvβ6 on cholangiocarcinoma (CCA) cell lines. **(A)** Integrin αvβ6 was stained with anti-integrin αvβ6 mAb in the indicated CCA cell lines and analyzed by flow cytometry. A representative result of flow cytometry analysis demonstrates CCA cell lines stained with isotype-control antibody (gray peaks), and CCA cell lines stained with anti-integrin αvβ6 mAb (white peaks). **(B)** A quantification bar-graph summarizes the data from 3 independent experiments (***$p<0.001$). The data with error bar represent mean ± standard error of mean (SEM). Differences between the group of β6-negative A375.puro cells and those of cell lines with positive expression of integrin αvβ6 were analyzed by one-way analysis of variance (ANOVA). **(C)** Immunofluorescence images of CCA cell lines show membranous and granular expression of integrin β6 stained with anti-β6 polyclonal antibody (green) and nuclei staining (Hoechst 33342; blue), and A375.β6 was used as a positive control. Scale bars represent 10 μm.

(56.5±5.5% vs. 9.6±2.6%, respectively; $p<0.001$). Surprisingly, after co-culturing with integrin αvβ6-positive A375.β6 cells, A20-4G CAR T cells proliferated to an even greater extent than NT T cells (74.6±5.9% vs. 9.6±2.6%, respectively; $p<0.001$) (**Figures 6C,D**).

DISCUSSION

A more effective treatment for patients with advanced unresectable/metastatic CCA is urgently needed. Adoptive T cell therapy using CAR T cells has provided promising outcomes against hematological malignancies (33), and its potential for treatment of solid cancers is being extensively investigated. The results of the present study provide evidence that A20-2G and A20-4G CAR T cells targeting integrin αvβ6 protein are efficient in killing CCA cell lines, which indicates their potential for treatment of CCA.

A few candidate target antigens are currently being investigated in clinical trials of CCA (34). The integrin αvβ6 protein is overexpressed in several solid tumors, but it is only minimally expressed in normal tissues (29). Here, we show that integrin αvβ6 represents a novel therapeutic target antigen for CAR T cell immunotherapy in patients with CCA. We initially examined the expression of the integrin αvβ6 protein in liver fluke-associated CCA tissues by IHC staining. We found that 73.3% of tumor samples from the Thai patients with this type of CCA had increased expression of integrin αvβ6 (**Figure 1**). Expression was highly specific to CCA cells compared to adjacent non-malignant biliary epithelia that had an undetectable level of integrin αvβ6. These results are consistent with those observed in CCA tissues from other ethnic groups, including Swiss (26), Japanese (21), and Chinese populations (22). Notably, the survival time of CCA patients who had low/negative integrin αvβ6 protein expression was significantly longer than the survival time of those who had high integrin αvβ6 expression (**Figures 1D,E**). Integrin αvβ6 has been reported to promote resistance of CCA cells to cisplatin-induced apoptosis (22), which indicates that it should be targeted using other therapeutic approaches, such as immunotherapy. In this study, we also reported the expression of integrin αvβ6 on the surface of patient-derived CCA cell lines, including KKU055, KKU100, and KKU213A cells (**Figure 2**). We then generated CAR T cells targeting integrin αvβ6 and tested their anti-tumor function in these cell lines. The expression level of the target antigen was reported to affect CAR T cell functionality (35). Thus, we selected a panel of CCA cell lines with different expression levels of integrin αvβ6 to demonstrate the effectiveness of CAR T cells specific to this target antigen.

In previous clinical trials involving patients with B cell malignancy, CAR T cells containing either CD28 or 4-1BB presented different properties, but they showed a similar antitumor response. CD28-based CAR T cells were rapidly

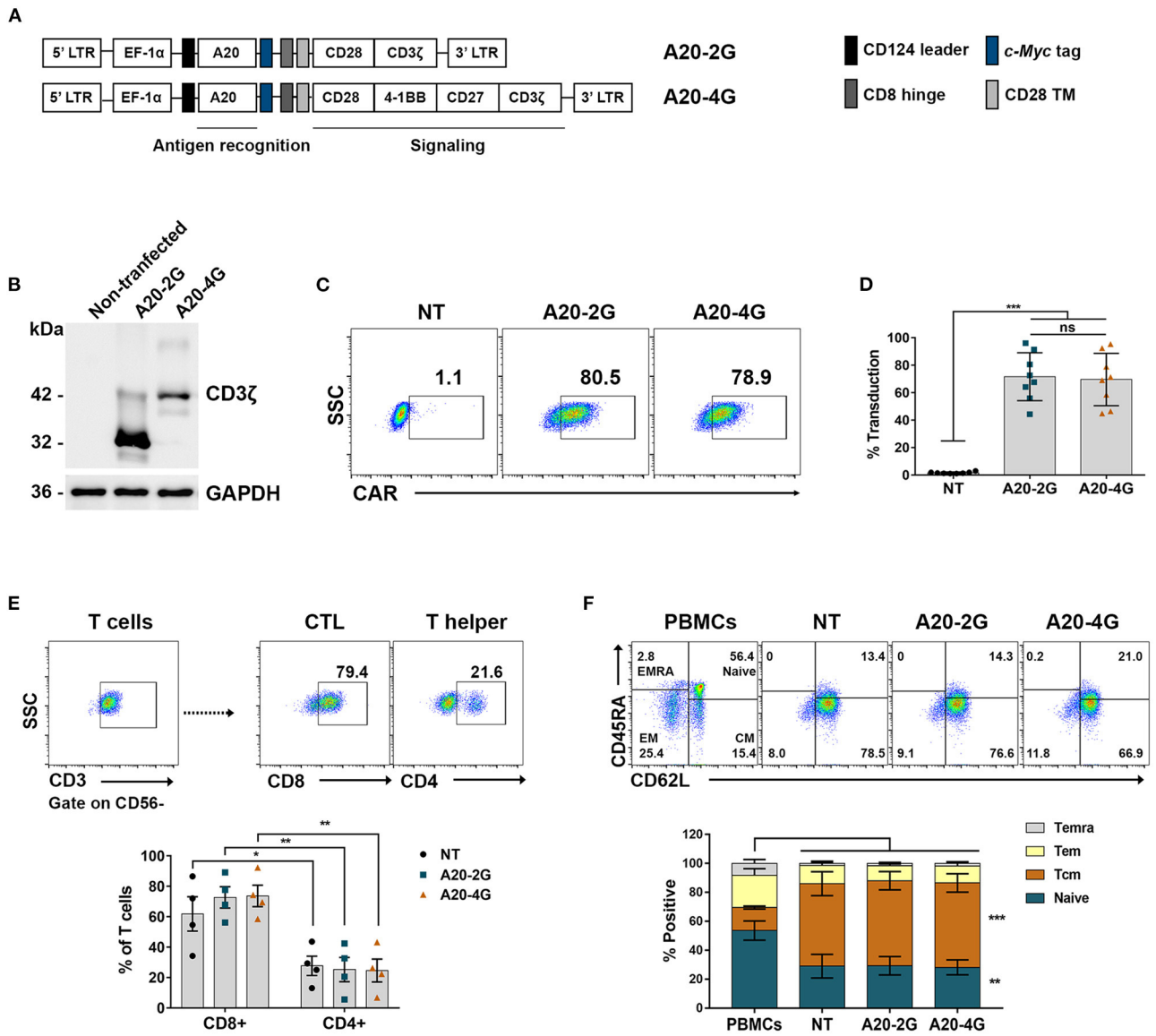

FIGURE 3 | Generation of chimeric antigen receptor (CAR) T cells targeting integrin αvβ6. **(A)** The schematic map demonstrates the A20-2G and A20-4G CAR constructs. The sequence of A20 (integrin αvβ6-targeting ligand) was cloned in-frame into lentiviral vectors linked with the sequences of CD8 hinge, CD28 transmembrane domain (TM), costimulatory domain of CD28/CD3ζ (A20-2G), or CD28/4-1BB/CD27/CD3ζ (A20-4G). **(B)** Expression of A20-2G CAR and A20-4G CAR proteins in Lenti-X 293T cells was detected by immunoblotting analysis using anti-CD3ζ antibody. The data shown represent the mean of three experiments, all of which showed similar results. **(C)** Representative histogram shows expression of A20-2G and A20-4G CAR on the surface of T cells as examined by anti-cMyc FITC-conjugated antibody and flow cytometric analysis. **(D)** Expression of A20-2G or A20-4G CAR on transduced T cells was examined and summarized from eight healthy donors as mean ± standard deviation (SD). Non-transduced (NT) T cells were used as control. **(E)** Representative flow cytometric gating of $CD3^+CD56^-$ T cell population (upper) and summarized data (lower). The phenotypes of CAR T cells were cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells. Flow cytometric gating was based on cells stained with isotype-match control antibody. **(F)** The subgroups of A20-2G or A20-4G CAR T cells consisted of $CD45RA^-CD62L^+$ central memory (T_{CM}) and $CD45RA^+CD62L^+$ naïve T cells. These data were derived from four healthy donors (ns, non-significant, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

activated and their cytolytic activities were enhanced; however, their persistence was short (<3 months) (36). In contrast, 4-1BB-based CAR T cells demonstrated slow response and exhaustion, but their survival was longer (>1 year) (37). The findings of previous studies suggested that complete treatment response required persistence of CAR T cells (37, 38). Accordingly, a CD27 signaling domain was combined in our 4G CAR design to further support the activation, proliferation, and survival of CAR T cells *in vitro* and *in vivo* (39). In our study, we generated the A20-2G CAR construct containing A20/CD28/CD3ζ to be a control, while the A20-4G CAR construct comprising A20/CD28/4-1BB/CD27/CD3ζ (**Figure 3A**) was designed to combine the

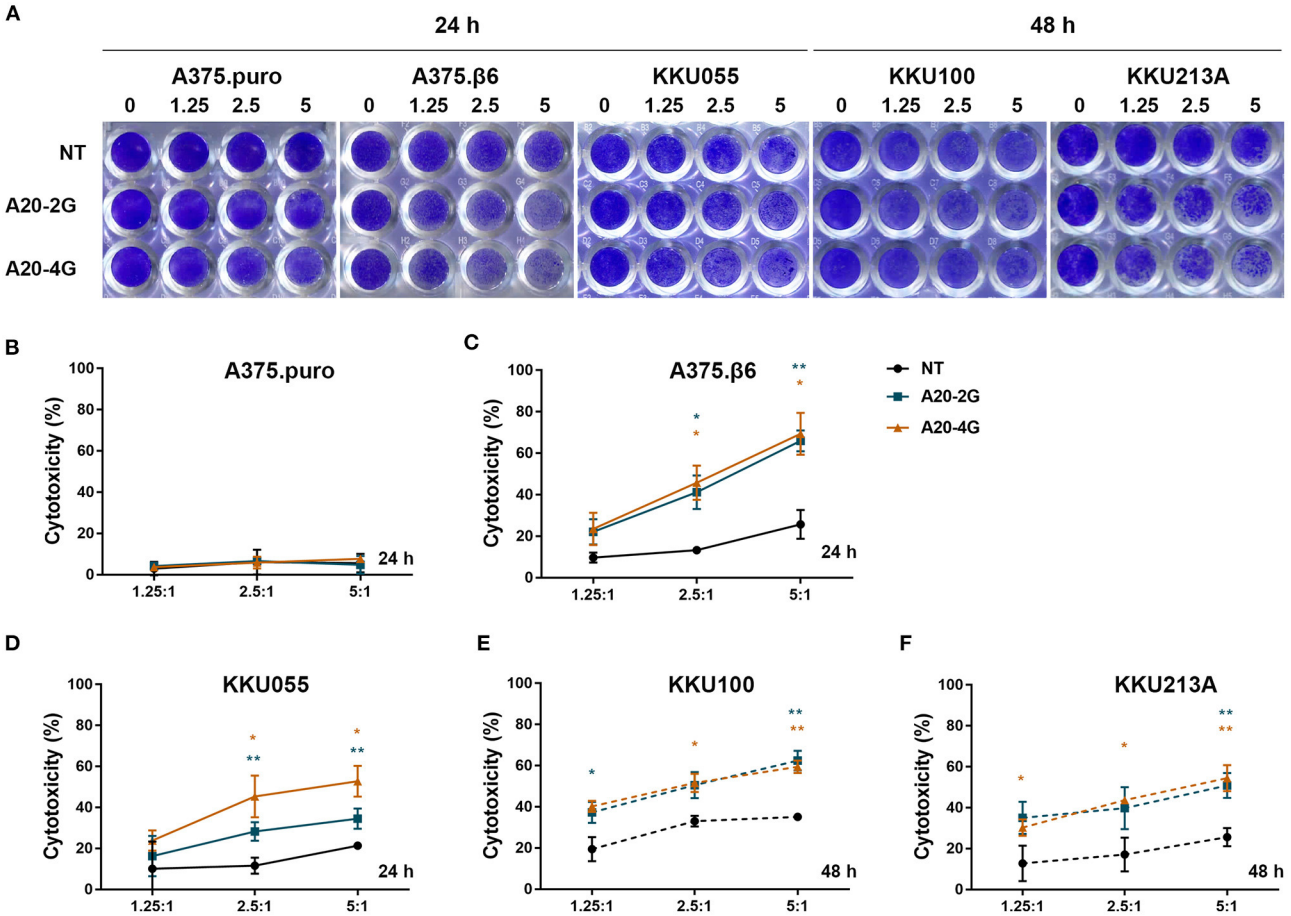

FIGURE 4 | Cytotoxic effects of A20-2G and A20-4G chimeric antigen receptor (CAR)-T cells on integrin αvβ6-negative A375.puro cells, integrin αvβ6-positive A375.β6 cells, and three integrin αvβ6-positive cholangiocarcinoma (CCA) cell lines, including KKU055, KKU100, and KKU213A. The effector CAR T cells were co-cultured with target cells at the indicated effector to target (E:T) ratios (1.25:1, 2.5:1, and 5:1) for 24 h (*solid line*) or 48 h (*dashed line*). **(A)** Cytotoxicity of target cells after co-culturing with effector cells quantified by crystal violet staining assay. Integrin αvβ6-negative A375.puro cell line served as negative control while integrin αvβ6-positive A375.β6 cells was served as positive control. **(B)** Cytotoxic effects of A20-2G and A20-4G CAR T cells on integrin αvβ6-negative A375.puro cells with non-transduced (NT) T cells used as a control for comparison. **(C)** Cytotoxic effects of A20-2G and A20-4G CAR T cells on integrin αvβ6-positive A375.β6 cells with NT T cells used as a control for comparison. Cytotoxic effects of A20-2G and A20-4G CAR T cells on integrin αvβ6-positive KKU055 **(D)**, KKU100 **(E)**, and KKU213A **(F)** cells as compared to the killing activity of NT T cells on the same target cells. Percentage of cytotoxicity relative to the total number of tumor cells alone (set at 0% cytotoxicity) from three independent experiments presented as mean ± standard error of the mean (SEM) (*$p<0.05$, **$p<0.01$).

favorable properties of CD28, 4-1BB, and CD27. These two constructs were expressed using a self-inactivating lentiviral vector system. The A20-2G and A20-4G CAR proteins of the predicted size could be detected in Lenti-X 293T cells (**Figure 2B**). Moreover, A20-2G and A20-4G CAR T cells were successfully generated using T cells isolated from eight healthy donors. The expressions of the A20-2G and A20-4G CARs were 71.5±17.5% and 69.6±19.1%, respectively (**Figures 3C,D**). The final products contained cytotoxic T cells ($CD3^+CD56^-CD8^+$) with a $CD45RA^-CD62L^+$ phenotype as major populations (**Figures 3E,F**). This phenotype was reported to support cancer immune surveillance, long-term expansion, and persistence *in vivo* (40). However, the cell phenotypes did not differ between A20-2G and A20-4G CAR T cells compared to NT T cell control, which suggests that their phenotypes may depend on the manufacturing process that we undertook using PHA-L activation and T cell culture in media containing IL-2, IL-7, and IL-15, which were reported to promote cytotoxic T cell memory phenotype (40, 41).

Our data demonstrates that while the A20-2G and A20-4G CAR T cells had minimal cytolytic activity against αvβ6-negative A375.puro cells, the two CAR T cell populations were clearly able to kill αvβ6-positive A375.β6 cells and CCA cells in an E:T ratio-dependent manner (**Figure 4**), which indicates their specific killing ability. Additionally, both A20-2G and A20-4G CAR T cells killed KKU055 cells within 24 h, which was faster than the time it took for them to kill KKU100 and KKU213A cells within 48 h. It should be noted that high levels of integrin αvβ6 expression on CCA cells did not correlate with high levels of effector activities of the two CAR T cells. The most likely

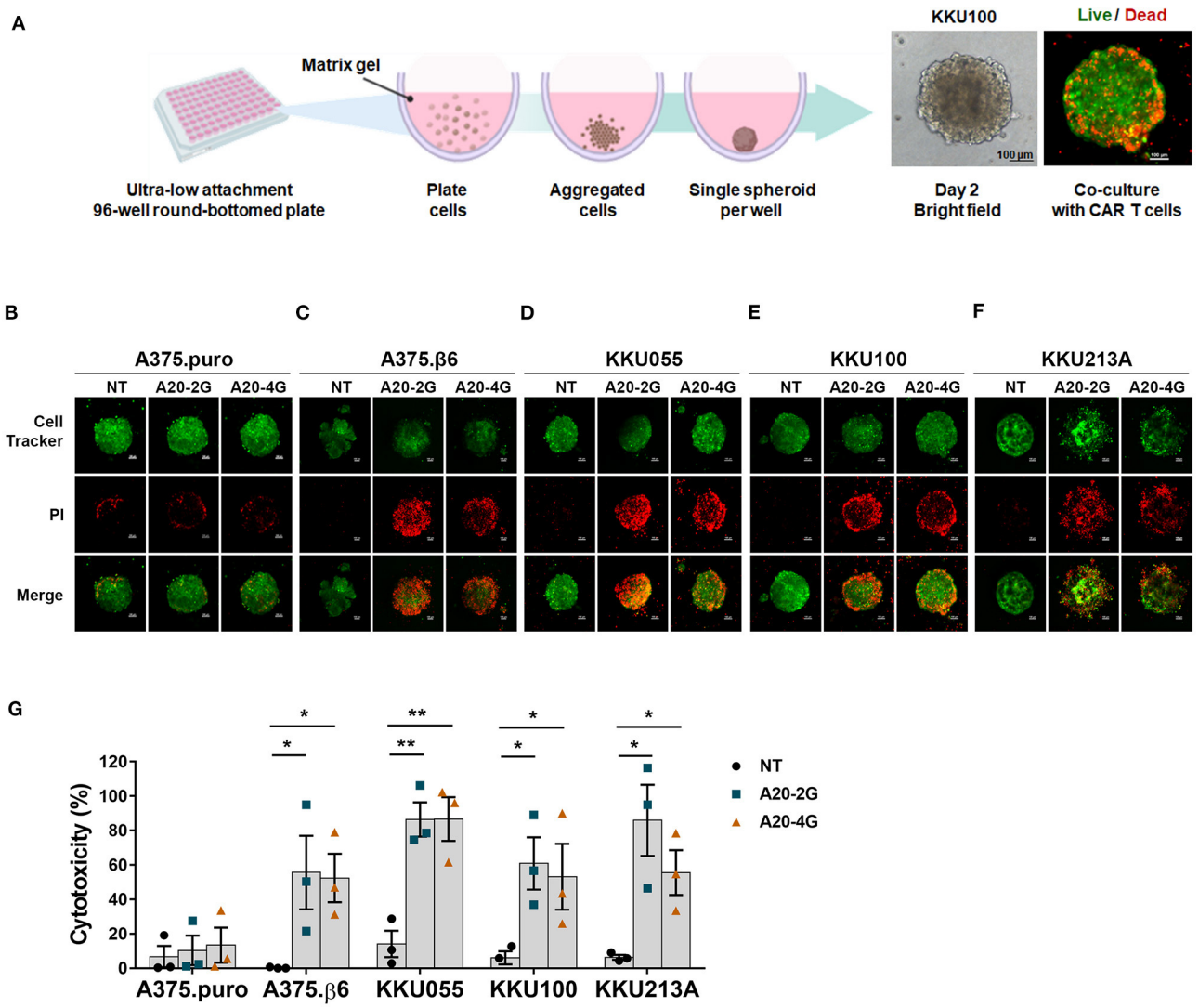

FIGURE 5 | A20-2G and A20-4G CAR T cells mediated killing of CCA tumor spheroids. **(A)** Schematic diagram of spheroid forming in a round-bottomed plate. Representative images on the right show bright filed of a spheroid formed by 2 × 10^3 cells for 48 h and a fluorescence image after co-culturing CAR T cells with the spheroid. **(B–F)** Representative images showing cytolytic activities of A20-2G or A20-4G CAR T cells compared to NT cells at an effector to target (E:T) ratio of 5:1 on the spheroids of A375.puro, A375.β6, and CCA (KKU055, KKU100, KKU213A) at 72 h of co-culturing. Dead of green fluorescence-labeled cells were visualized by PI uptake (Red). **(G)** Histogram shows cytotoxic activities of NT cells, A20-2G CAR T cells, and A20-4G CAR T cells on tumor cells determined by PI incorporation of dead cells within spheroids. Note that lower level of the percentage of cytotoxicity of A20-4G CAR T cells for KKU213A resulted from more CCA cell lysis. The bar graphs represent mean ± standard error of the mean (SEM) (n = 3; *p<0.05, **p<0.01).

explanation is that CCA cells expressing the integrin αvβ6 protein may also express immune checkpoint molecules to suppress CAR T cell function. The upregulation of PD-L1 in CCA cells has been reported (42), and it can induce T cell exhaustion via the engagement of PD-1 on $CD8^+$ T cells (43, 44). A combination of CAR T cells and immune checkpoint inhibitor may improve effector functions of CAR T cells for treatment of CCA (45).

The traditional two-dimensional (2D) culture model based on the growth and proliferation of monolayer cells might not represent the condition with the presence of cell-cell and cell-extracellular matrix interactions. Thus, three-dimensional (3D) CCA spheroids that appeared like solid tumor were generated to evaluate anti-tumor activities of A20-2G and A20-4G CAR T cells. The results showed that the two CAR T cells could infiltrate into the spheroid and displayed potent anti-tumor activities, as demonstrated by dead cancer cells in the spheroids stained by propidium iodide (PI) (**Figure 5**). In the CCA patients, T cell infiltration in the CCA tissue is a positive outcome predictor (46). A study using 3D culture system revealed that gene expression in this culture system was much closer to clinical expression profiles than those observed in the 2D culture system (47), indicating the suitability of the 3D culture system for preclinical studies.

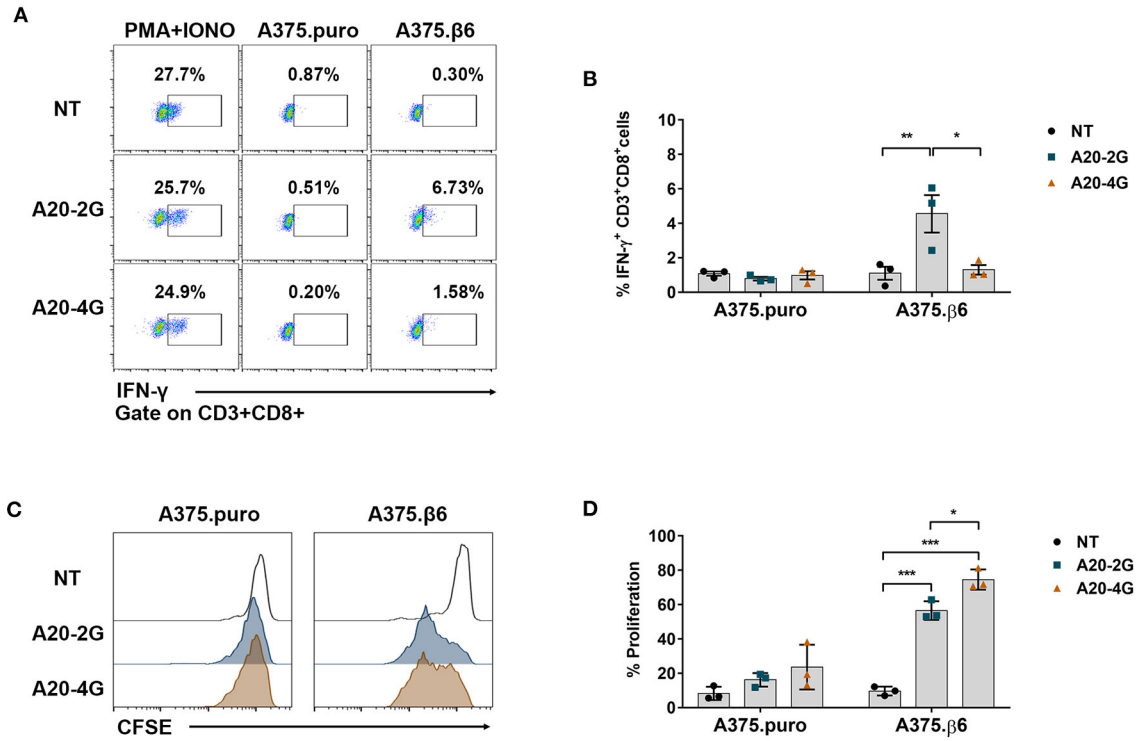

FIGURE 6 | Production of interferon-γ (IFN-γ) and cell proliferation non-transduced (NT) T cells, A20-2G CAR T cells, and A20-4G CAR T cells after co-culture with integrin αvβ6-expressing target cells. **(A)** IFN-γ production was examined by intracellular cytokine staining and flow cytometry analysis in NT T cells, A20-2G CAR T cells, and A20-4G CAR T cells after co-culturing with integrin αvβ6-negative A375.puro or integrin αvβ6-positive A375.β6 cells. **(B)** IFN-γ expression from three individual healthy donors. Data are presented as mean ± standard error of the mean (SEM). **(C)** Proliferation of NT cells, A20-2G CAR T cells, and A20-4G CAR T cells was examined after co-culturing for 3 days with integrin αvβ6-negative A375.puro or integrin αvβ6-positive A375.β6 cells at an effector to target (E:T) ratio of 5:1 without addition of exogenous cytokine. **(D)** Summary data of T cell proliferation derived from 3 individual healthy donors. (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

After co-culturing with αvβ6-positive cells, A20-2G CAR T cells produced greater levels of IFN-γ than A20-4G CAR T cells (4.5±1.9% vs. 1.1±0.7%; $p=0.0156$) (**Figures 6A,B**). This may be an advantage of A20-4G CAR T cells since clinical studies of the 4G CAR T cells targeting CD19 found that low levels of IFN-γ might be beneficial to limit CAR T cell-mediated cytokine release syndrome (CRS) (10, 15), which was often observed in patients who received CAR T cell therapy (48, 49). Furthermore, A20-4G CAR T cells showed a higher proliferation rate than A20-2G CAR T cells (74.6±5.9% vs. 56.5±5.5%, $p=0.0175$) (**Figures 6C,D**). This higher proliferation rate may result from the incorporation of CD27 into the A20-4G CAR construct because its signaling is known to typically promoting T cell proliferation (38). Our data may also suggest the addition of 4-1BB/CD27 into the A20-4G CAR construct to be more effective than the addition of only CD28 into the A20-2G CAR construct. Thus, A20-4G CAR T cells possibly offer better benefits than A20-2G CAR T cells for CCA treatment because they may cause less severe CRS response and they have a higher proliferation rate.

A previous study reported the use of CAR T cell immunotherapy to treat a patient with advanced unresectable/metastatic CCA that proved resistant to chemotherapy and radiotherapy (34). Sequential infusions of CAR T cell therapies targeted against EGFR and CD133 induced partial response (PR) for 8.5 months and 4.5 months, respectively. That study showed that CAR T cell therapy targeting two or more antigens is feasible for resolving the problem of tumor heterogeneity in CCA (34). Thus, the generation of CAR T cells specific to integrin αvβ6 and other tumor-associated antigens for treatment of CCA warrants further study. Moreover, since CCA is characterized as having desmoplastic stroma and an immune suppressive tumor microenvironment (TME), the combination of CAR T cell therapy with other treatment modalities, such as chemotherapy using gemcitabine/cisplatin drugs (5), immune checkpoint blockade (45), and/or FGFR inhibitor (6), may overcome immune escape mechanisms of CCA.

Here, we report integrin αvβ6, which is upregulated in CCA tissues, to be a promising target antigen for adoptive T cell therapy of CCA. A20-2G and A20-4G CAR T cells targeting integrin αvβ6 were successfully generated, and both were found to effectively kill αvβ6-positive CCA cells in both monolayer cell and spheroid culture systems. The A20-4G CAR T cells were found to be superior to the A20-2G CAR T cells concerning their higher proliferative potential

and lower cytokine production. Thus, the A20-4G CAR T cells warrant further study for their therapeutic potential against CCA.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Siriraj Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NP designed and performed experiments, analyzed data, interpreted results, and prepared manuscript. CS and KS partly performed experiments and analyzed data. MJ and PY conceptualized, managed, and supervised the study. NP, CS, KS, TC, JS, SW, JM, MJ, and PY provided materials and reagents, designed experiments, interpreted results, and edited the manuscript. All authors read and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The CD28-Transmembrane Domain Mediates Chimeric Antigen Receptor Heterodimerization With CD28

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Anti-CD19 chimeric antigen receptor (CD19-CAR)-engineered T cells are approved therapeutics for malignancies. The impact of the hinge domain (HD) and the transmembrane domain (TMD) between the extracellular antigen-targeting CARs and the intracellular signaling modalities of CARs has not been systemically studied. In this study, a series of 19-CARs differing only by their HD (CD8, CD28, or IgG_4) and TMD (CD8 or CD28) was generated. CARs containing a CD28-TMD, but not a CD8-TMD, formed heterodimers with the endogenous CD28 in human T cells, as shown by co-immunoprecipitation and CAR-dependent proliferation of anti-CD28 stimulation. This dimerization was dependent on polar amino acids in the CD28-TMD and was more efficient with CARs containing CD28 or CD8 HD than IgG_4-HD. The CD28-CAR heterodimers did not respond to CD80 and CD86 stimulation but had a significantly reduced CD28 cell-surface expression. These data unveiled a fundamental difference between CD28-TMD and CD8-TMD and indicated that CD28-TMD can modulate CAR T-cell activities by engaging endogenous partners.

Keywords: chimeric antigen receptor, CAR T cell, CD28, transmembrane domain, hinge domain, heterodimerization, dimer, CAR

INTRODUCTION

Chimeric antigen receptor (CAR)-engineered T cells are emerging as promising therapies for otherwise untreatable diseases (1). The United States Food and Drug Administration (FDA) has approved two anti-CD19 CAR (19-CAR) T-cell products, namely tisagenlecleucel (CTL-019, KYMRIAH, Novartis Pharmaceuticals Corp.) and axicabtagene ciloleucel (KTE-19, YESCARTA, Kite Pharma, Inc.), for the treatment of acute lymphocytic leukemia and relapsed/refractory large B-cell lymphoma. A third CAR-T product, lisocabtagene maraleucel (JCAR-17, LISO-CEL, Bristol-Myers Squibb), is currently under review by the FDA for adults with relapsed/refractory large B-cell lymphoma. The success of these CAR-T products can be attributed to their antigen specificity, all conferred by the single-chain variable fragment (scFv) of the anti-CD19 antibody clone FMC63

and their intracellular signaling domains (ICDs), namely 28ζ for KTE-19 (2–4) and 4-1BBζ for CTL-019 (5–8), and JCAR-17 (9). It is worth noting that these products differ in their hinge domain (HD) and transmembrane domain (TMD), that is, CD28-HD/TMD for KTE-19, CD8-HD/TMD for CTL-019, and IgG_4-HD/CD28-TMD for JCAR-17.

In earlier iterations of CAR designs, CD28- and CD8-TMDs were chosen because they are considered to be inert when compared to the CD3z-derived TMD that mediated the association of the CAR with the endogenous T-cell receptor (TCR)/CD3 complexes (10). Emerging evidence, however, suggests potential contributions of the HD and the TMD to the function of CAR-T cells. The group of June first observed unexpectedly sustained proliferation after a single *in-vitro* stimulation of CD28-HD/TMD-based-CAR T cells, but not CD8-HD/TMD-based-CAR T cells, directed against mesothelin (11). Majzner et al. (12) demonstrated that replacing a CD8-HD/TMD with a CD28-HD/TMD lowers the threshold for CAR activation to CD19 in an ICD-independent fashion. These results corroborate the findings reported by Kochenderfer et al. and show that T cells with CD28-HD/TMD-containing CARs secrete higher levels of interferon-γ upon CAR stimulation (13, 14).

The mechanisms underlying the differences between CD8-HD/TMD and CD28-HD/TMD domains remain to be defined (15). In the present study, the impact of CD28-TMD on 19-CARs in human T cells was investigated, and it was discovered that CD28-TMD mediated a transmembrane domain-dependent heterodimeric association of the CAR with the endogenous CD28 receptor.

MATERIALS AND METHODS

Human T-Cell Isolation

Human blood from deidentified normal donors was purchased from STEMCELL Technologies (Vancouver, Canada), which collected and distributed de-identified human blood products with consent forms, and according to the protocols, approved by the Institutional Review Board (IRB). Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation, and T cells were further enriched using the EasySep Human T Cell Isolation Kit (STEMCELL Technologies) as per the instructions of the manufacturer. Enriched T cells or $CD4^+CD127^+CD25^{low}$ conventional T cells purified by fluorescence-activated cell sorting (FACS) were used for experiments. Cells were either used fresh or cryopreserved in fetal calf serum (FCS) with 10% DMSO. Frozen cells, when used, were thawed and cultured overnight in 300 IU/ml of IL-2 before editing and cell activation.

Genome Editing Using Ribonucleoprotein Complex

Ribonucleoprotein complexes (RNPs) were made by mixing CRISPR RNAs (crRNAs) and trans-activating crRNAs (tracrRNA, Integrated DNA Technologies, Coralville, IA) with recombinant Cas9 protein (QB3 Macrolab, UC Berkley, CA) as previously described in a study by Roth et al. (16). Guide RNA sequences used for gene editing were as follows: (1) T-cell receptor β chain constant region (TRBC): CCCACC AGCTCAGCTCCACG; (2) CD19: CGAGGAACCTCTAGT GGTGA; and (3) CD28: TTCAGGTTTACTCAAAAACG. Lyophilized RNAs were resuspended at 160 μM in 10 mM Tris-HCl with 150 mM KCl and stored in aliquots at −80°C. On the day of electroporation, crRNA and tracrRNA aliquots were thawed and mixed at a 1:1 volume and annealed for 30 min at 37°C. The resulting 80 μM guide RNA complex was mixed at 37°C with Cas9 Nucleic Localization Signal (NLS) at a 2:1 gRNA to Cas9 molar ratio for another 15 min. The resulting RNP was used for genome editing. About 1×10^6 T cells were mixed with appropriate RNP and electroporated using a Lonza 4D 96-well electroporation system (pulse code EH115) to delete *TCR* or *CD28* genes. For generating $CD19^-$ variants of Raji cells, Raji cells (ATCC® CCL-86™, Manassas, VA) were electroporated (pulse code EH140) with RNP targeting *CD19*, and the CD19- negative fraction was purified by FACS after culturing the cells for more than 1 week.

Gene Editing of Human T Cells

$CD4^+$ T cells were gene-edited before stimulation with anti-CD3/CD28 beads (Dynabeads Human T-Activator CD3/CD28, Thermo Fisher Scientific, Waltham, MA). Cells were cultured in RPMI supplemented with 10% FCS and 300 IU/ml of IL-2 (Prometheus laboratories, Nestle Health Science, Lausanne Switzerland) for the first 2 days, and then, the concentration of IL-2 was reduced to 30 IU/ml for $CD4^+$ T cells and to 100 IU/ml for bulk T cells. Lentiviruses encoding anti-CD19-I_4-28-4-1BBζ-T2A-EGFRt and anti-CD19-I_4-28-28ζ-T2A-EGFRt were provided by Juno Therapeutics (Bristol-Myers Squibb, New York, NY). Other lentiviral constructs, present in **Figure 2A**, were cloned into the pCDH-EF1-FHC vector (Addgene plasmid #64874, Watertown, MA) as previously described by Hill et al. (17). Later, genes encoding CAR constructs were purchased from gBlocks™ Gene Fragments (Integrated DNA Technologies) (17, 18) and amplified by PCR and cloned into the pCDH vector using In-Fusion Cloning Tools (Takara Bio, Kusatsu, Japan). Sequences for all clones used in subsequent experiments were confirmed by sequencing. Transduction was performed on day 2 after $CD4^+$ T cell activation at a multiplicity of infection of one by spinoculation (1,200 g, 30 min, 30°C) in a medium supplemented with 10% FCS and 0.1 mg/ml of protamine. For AAV production, 30 mg of helper plasmid pDGM6 (a kind gift from YY Chen, University of California, Los Angeles), 40 mg of pAAV helper, and 15 nmol PEI were utilized. AAV6 vector production was carried out by iodixanol gradient purification. After ultracentrifugation, the AAVs were extracted by puncture and further concentrated using a 50 ml Amicon column (Millipore Sigma Burlington, MA) and directly titrated on primary human T cells.

Abbreviations: CAR, Chimeric antigen receptor; HD, Hinge domain; ICD, Intracellular signaling domain; scFV, Single chain variable fragment; TMD, Transmembrane domain.

In vitro Activation of Gene-Edited CAR T Cells

For some experiments, cells were restimulated on day 9 after primary stimulation without separating edited and transduced cells. For proliferation assays, the cell mixtures were stained with 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFDA SE, ThermoFisher, referred to as CFSE) before restimulation with anti-CD3/CD28 beads. For other experiments, cells were

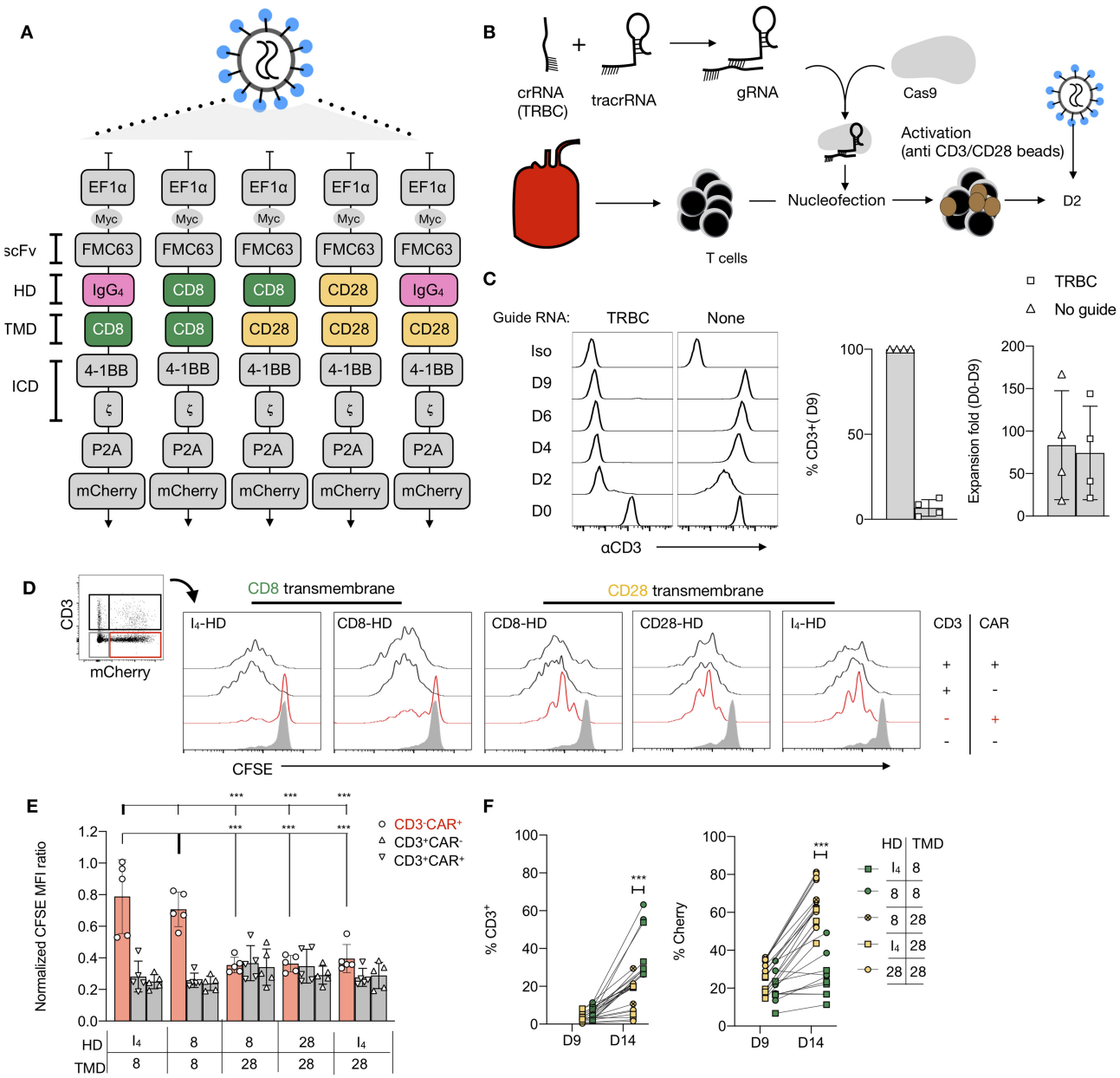

FIGURE 1 | Anti-CD28 stimulation of CD19-chimeric antigen receptors (CAR) T cells is TMD dependent. **(A)** Designs of five CAR against CD19 bearing a 4-1BB costimulatory domain and differing by their hinge domain (HD) and their transmembrane domain (TMD). **(B)** Fluorescence-activated cell sorting (FACS) -sorted $CD4^+CD127^+CD25^{low}$ T cells were electroporated with a CRISPR-Cas9 ribonucleoprotein complex (RNP) targeting the constant region of the TCR β chain gene (TRBC), followed by stimulation with anti-CD3/CD28 beads (1:1 ratio). **(C)** Representative results of flow cytometric analysis of the CD3 expression over time of cells electroporated with or without RNP. Percentages of residual $CD3^+$ population and fold-expansion after 9 days of culture of $CD4^+$ T cells electroporated with or without RNPs targeting TRBC are shown. Results from four independent experiments. **(D)** A representative example of carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution of a mixed population of $CD3^{+/-}CAR^{+/-}$ T restimulated with anti-CD3/28 beads. **(E)** The normalized CFSE mean fluorescence intensity (MFI) ratio for $CD3^-mCherry^+$, $CD3^+mCherry^-$, and $CD3^+mCherry^+$ cells was calculated by dividing CFSE MFI of these populations with the MFI of the $CD3^-mCherry^-$ cells in the same culture. Two-way ANOVA was used for statistical analysis (bold line set as reference). **(F)** Percentages of $CD3^+$ and $mCherry^+$ cells before and 5 days after restimulation of edited T cells with anti-CD3/CD28 beads. The Unpaired *t*-test was performed by comparing CD8-TMD and CD28-TMD-containing CARs on D14. For **(E,F)**, the results shown are a summary of two independent experiments using T cells from five unrelated donors for each construct. $^{***}p < 0.001$.

separated by FACS on day 9 to purify $CD3^+$ and $CD3^-$ T cells with or without CAR. For assessing $CD25^+CD71^+$ upregulation, purified CAR T cells were stimulated with parental $CD19^+$ Raji cells or $CD19^{deficient}$ Raji cells for 2 days. In some cultures, CTLA-4 Ig (provided by Dr. Vincenti, UCSF) was added at a concentration of 13.5 μg/ml. For measurements of proliferation, purified cells were stimulated with soluble anti-CD28 (clone CD28.2, 1 μg/ml, BD Pharmigen), plate-bound anti-CD28 (clone CD28.2, 10 μg/ml), or soluble anti-CD3 (clone HIT3α 2 μg/mL. BD Pharmigen). After 48 h, a portion of the supernatant was collected and analyzed for cytokine secretion using multiplexed Luminex (Eve Technologies, Calgary, Canada). The cells were then pulsed with 0.5 μCi of ^3H thymidine and cultured for another 16–18 h before harvesting to determine the level of ^3H thymidine incorporation using a scintillation counter.

Flow Cytometry

The following antibodies were used for phenotyping and proliferation assays: anti-CD3-PE/Cy7 (clone SK7, BioLegend, San Diego, CA), anti-CD4-PerCP (clone SK3, BD Pharmigen, San Jose, CA), anti-CD4 A700 (clone RPA T4, BioLegend), anti-CD19 APC (clone HIB19, BD Pharmigen), anti-CD25 APC (clone 2A3, BD Pharmigen), anti-CD71 FITC (clone CY1G4, BioLegend), anti-Myc FITC or APC (clone 9B11, Cell Signaling, Danvers, MA), anti-FMC19 idiotype APC (Juno Therapeutics), anti-EGFRt PE (Juno Therapeutics), anti-CD28 APC (clone 28.2, Biolegend), and CD8 APC-Cy7 (clone SK1, BioLegend). DAPI (ThermoFisher, Waltham, MA) was used to stain dead cells for exclusion during analysis. Flow cytometric analyses were performed on an LSR II Flow Cytometer System (BD Biosciences). Fluorescence-activated cell sorting was performed on an FACSAria III Cell Sorter (BD Biosciences). All flow cytometry data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

Immunoprecipitation

FACS-purified $CD3^-CAR^+$ or $CD3^-CAR^-$ $CD4^+$ T cells (8×10^6 each) were lysed in Pierce™ IP Lysis Buffer (ThermoFisher) supplemented with cOmplete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) for 30 min using a vertical rotator. Cell lysis was completed by briefly sonicating cells using a Q500 sonicator (QSonica, Newtown, CT). Pierce™ anti-c-Myc magnetic beads (clone 9E10, ThermoFisher) were used for immunoprecipitation of the CAR. Alternatively, rabbit anti-human CD28 (clone D2Z4E, Cell Signaling) followed by anti-rabbit IgG Pierce™ protein A/G magnetic beads (ThermoFisher Scientific) were used for CD28 immunoprecipitation of the cell lysate according to the instructions of the manufacturer.

Western Blotting

Equal masses of protein lysate or equal volumes of immunoprecipitation eluents were loaded into NuPAGE 4–12% Bis-Tris, 1.0 mm gels (ThermoFisher Scientific). After electrophoresis, proteins were transferred onto PVDF membranes (ThermoFisher Scientific) using an iBlot 2 Dry Blotting System. After blocking with Tris-buffered saline with 0.1% Tween-20 and 5% bovine serum albumin (TBSTB), membranes were stained with primary and secondary antibodies diluted in TBSTB. The following antibodies used were mouse anti-Myc (clone 9B11, Cell Signaling), rabbit anti-CD28 (clone D2Z4E, Cell Signaling), HRP-conjugated anti-mouse IgG (Cell Signaling), and HRP-conjugated anti-rabbit IgG (Cell Signaling).

Three-Dimensional Model Prediction and Validation

Structural modeling of the different CARs was performed using Iterative Threading ASSembly Refinement (I-TASSER) software (19). Amino acid corresponding to the scFv was modeled on the UCHT1 scFv template (PDB ID code 1XIW) (20). The HD coordinates were recovered from the crystal structure of the pembrolizumab template (PDB ID code 5DK3) (21) and the crystal structure of human CD28 (PDB ID code 1YJD) (22) for IgG4 and CD28, respectively. Modeling of the CD8-HD was performed using the Rosetta protein modeling suite (23). Structures were assembled with PyMOL (*S*chrodinger, LLC). Models were further evaluated with MolProbity software (24).

RESULTS

Generation of 19-CAR T Cells With Various HDs and TMDs

To investigate the role of CAR TMD, we first generated a panel of 19-CARs differing only by their HD (CD8, CD28, or IgG_4) and their TMD (CD8 vs. CD28), all of which have been used to engineer CAR T cells for clinical applications (**Figure 1A**, **Supplementary Figure 1**). Each CAR was designed with an MYC tag on the N-terminus of the scFv and a mCherry reporter (**Figure 1A**). For most experiments, we selected 4-1BB as a costimulatory domain in the ICD to avoid potential interactions with the endogenous CD28. Furthermore, we disrupted the *TRBC* locus using CRISPR/Cas9 to prevent any potential confounding influence by the endogenous TCR (**Figure 1B**). The *TRBC* gene-disrupted human T cells retained the cell surface expression of TCR/CD3 proteins for a few days after editing and could thus be activated with anti-CD3/CD28 beads. Edited $CD4^+$ T cells were transduced with various lentiviral CAR constructs by spinoculation 2 days after activation. On day 9, after stimulation, 87–98% of the cells were found to CD3-negative, demonstrating successful TCR deletion in the majority of the cells (**Figure 1C**). Comparable transduction efficiencies were observed across the different CAR constructs, as assessed by the mCherry expression and all CAR T cells responded to CD19 restimulation (**Supplementary Figures 2A–C**).

CAR T Cell Proliferation in Response to Anti-CD28 Stimulation

Restimulation of TCR-edited CAR-transduced T cells, containing a mixed population of $CD3^{+/-}$ and $CAR^{+/-}$ cells, with anti-CD3/CD28 beads on day 9, resulted in the expansion of $CD3^+$ T cells that escaped TCR deletion (**Figures 1D,E**). However, TCR-deficient $CD3^-CAR^+$ T cells with CARs containing a CD28-TMD, but not CD8-TMD, also proliferated. Consequently, CAR^+ T cells with a CD28-TMD, but not a CD8-TMD, were enriched

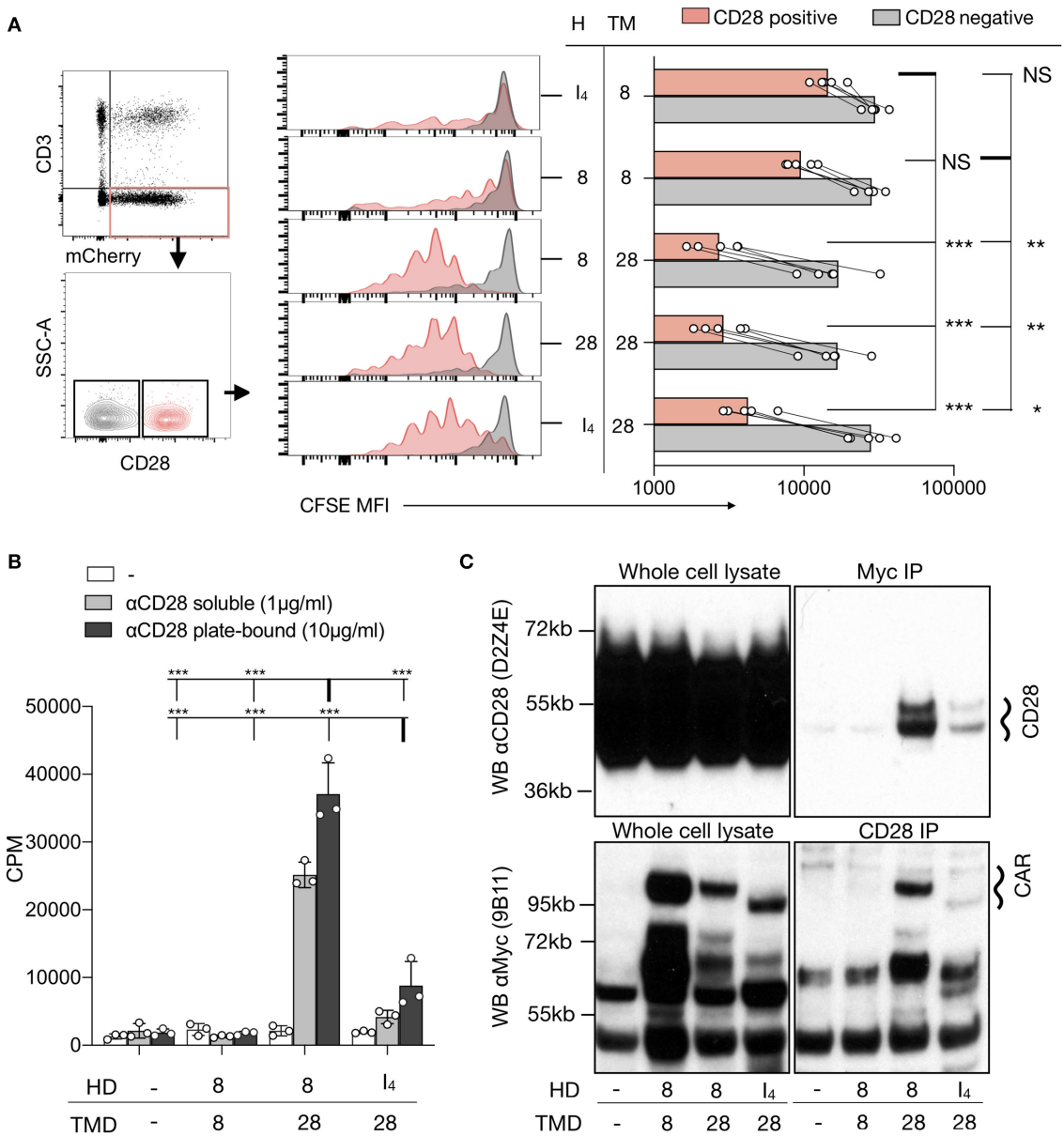

FIGURE 2 | CD28-TMD-containing CARs interact with CD28. **(A)** A mixture of CFSE-labeled CD4$^+$ T cells with or without CD3, CD28, and CAR expression. CFSE MFI of five independent donors in two independent experiments is reported. The one-way ANOVA was used for statistical analysis. **(B)** Proliferation of purified CD3$^-$CAR$^+$ CD4$^+$ T cells in response to plate-bound or soluble anti-CD28 stimulation. Results are representative of three independent experiments. The two-way ANOVA was used for statistical analysis. **(C)** CD28 or the Myc-tag of CD3$^-$CAR$^+$ T cells were immunoprecipitated. Western blot analysis of the input (5% of the whole cell lysate) as well as of the precipitated cells was performed using anti-CD28 (clone D2Z4E) and anti-Myc (clone 9B11). Results are representative of two to three independent experiments for each condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Counts per minute (CPM). Not Statistically Significant (NS).

at the end of the 5-day restimulation (**Figure 1F**). The lack of proliferation of CD8-TMD-containing CAR T cells showed that expansion was not a consequence of bystander effects, such as IL-2 production by the CD3$^+$CAR$^+$ T cells in the same culture. To determine if this is unique to CARs with 4-1BB-ICD, the experiment was repeated using CARs with a CD28-ICD, and a similar pattern of proliferation and enrichment of CD3$^-$CAR$^+$ T cells after anti-CD3/28 bead restimulation was observed (**Supplementary Figures 3A–D**).

Since the CD3$^-$CAR$^+$ T cells had no TCR expression on the cell surface, the proliferation was likely stimulated by the anti-CD28 component of the anti-CD3/28 beads. To verify the need for the endogenous CD28 receptor for proliferation in response to anti-CD3/CD28 beads, both the *CD28* and *TRBC* genes were deleted in T cells before activation and lentiviral CAR transduction (**Figure 2A**). CD3$^-$CAR$^+$CD28$^+$ T cells expressing CARs containing a CD28-TMD, but not a CD8-TMD, proliferated in response to anti-CD3/28 beads. The

deletion of CD28 abrogated the ability of CD28-TMD-containing CAR T cells to proliferate in response to anti-CD3/CD28 beads, demonstrating that anti-CD28-induced activation was dependent on endogenous CD28 (**Figure 2A**). These results excluded the possibility that anti-CD3/CD28 beads directly bind to the CAR.

To further confirm that $CD3^-$ T cells with a CD28-TMD-containing CAR could respond to anti-CD28 stimulation in the absence of other cells in the culture, we FACS-purified $CD3^-CAR^+$ cells before restimulation with plate-bound or soluble anti-CD28 antibodies (clone CD28.2). For these experiments, we excluded CD28-HD containing CARs to avoid potential interaction mediated by the CD28-HD. The results confirmed that CAR T cells engineered with a CD28-TMD, but not a CD8-TMD, proliferated in response to anti-CD28 alone (**Figure 2B**). The proliferative response induced by anti-CD28 alone in $CD3^-CAR^+$ T cells was similar in CAR T cells with a 4-1BB or a CD28 costimulatory domain in their ICD (**Supplementary Figure 4A**). Moreover, anti-CD28 induced the secretion of multiple cytokines by $CD3^-CAR^+$ T cells but not by $CD3^+CAR^-$ or $CD3^-CAR^-$ control cells (**Supplementary Figure 4B**). Collectively, these results show that CD28-TMD containing CARs can be activated by anti-CD28 without antigen recognition by the CAR or the TCR.

CD28 and CAR Interaction

The results discussed earlier, together with recent reports of phosphorylation of endogenous CD28 upon CAR stimulation (all with a CD28-TMD domain) (25, 26), suggest interactions between CD28 and CD28-TMD-containing CARs. To directly determine if CD28-TMD-containing CAR and CD28 can physically interact, we performed co-immunoprecipitation experiments. CD28-TMD-containing, but not CD8-TMD-containing, CARs co-immunoprecipitated with endogenous CD28. Conversely, endogenous CD28 co-immunoprecipitated with CD28-TMD-containing, but not CD8-TMD-containing, CARs demonstrated that the CD28-TMD of the CAR interacted with the endogenous CD28 receptor (**Figure 2C**). CD8-HD/CD28-TMD CARs and CD28 co-immunoprecipitated more efficiently when compared to the IgG_4-HD-CD28-TMD construct, which is consistent with improved proliferation observed with CD8-HD/CD28-TMD CAR upon anti-CD28 stimulation (**Figure 2B**). Because of the difficulties in expanding $CD3^-CD28^-$ CAR T cells and the unlikelihood that anti-CD28 mAb directly binds to the CD28 TMD, we did not perform the purified CAR T-cell proliferation and immunoprecipitation studies with $CD28^-$ T cells.

Residues in CD28 TMD Involved in CD28-CAR Heterodimerization

Next, we generated a series of CD28-TMD CAR mutants to determine the molecular basis of the CAR–CD28 interaction. We first mutated the two glycines, G160L and G161L (M1), that may function as part of a glycine-zipper motif, a process known to control TMD dimerization (27). The second mutation replaced the C165 cysteine with alanine, as cysteine can form disulfide bonds (M2). The third (M3) mutations were made on two bulky hydrophobic tryptophans at the border of the TMD (W154L and W179L), and the fourth mutation (M4) targeted four amino-acid residues (C165L, Y166L, S167L, and T171L) present at the core of the TMD, as cysteine could form a disulfide bond and others may form hydrogen bond (**Figure 3A**). All CARs with TMD mutants were readily expressed on the cell surface (**Figure 3A**). The various $CD3^-CAR^+$ cells with mutated CD28-TMD were examined for their ability to proliferate to anti-CD28 stimulation. For the analysis of these experiments, $CD3^-CAR^+$ cells were further defined as low, intermediate, or high CAR expression based on the level of the mCherry expression. CAR T cells with the wild-type CD28-TMD ($CD28\text{-}TMD^{WT}$) proliferated to anti-CD3/CD28 stimulation, regardless of the level of CAR expression (**Figures 3B,C**). The $CD28\text{-}TMD^{M4}$, but not the other TMD-mutants, abrogated the proliferation of $CD3^-CAR^{low}$ cells and significantly reduced the proliferation of $CD3^-CAR^{int}$ cells with either CD8-HD or IgG_4-HD (**Figures 3B,C**). Interestingly, $CD3^-CAR^{high}$ T-cell proliferation was only weakly affected by M4 mutations. The $CD3^-CAR^{high}$ T cells did not undergo proliferation when restimulation was not carried out, demonstrating that the activation was dependent on anti-CD28 stimulation and was not a result of autonomous CAR tonic signaling (**Figure 3B**).

To confirm that the $CD28\text{-}TMD^{M4}$ disrupted the interaction between CD28 and the CAR, we sorted CAR T-cells based on the Cherry expression, engineered either with CD8-HD/$CD28\text{-}TMD^{WT}$ or with CD8-HD/$CD28\text{-}TMD^{M4}$, and rechallenged them with a plate-bound anti-CD28 (**Figure 3D**). In this assay, only CAR T cells with a $CD28\text{-}TMD^{WT}$ showed significant proliferation, as measured by radiolabeled-thymidine incorporation. Importantly, co-immunoprecipitation of the endogenous CD28 and the CD28-TMD-containing CAR was abrogated by the M4-mutant, demonstrating that the four amino acids present at the core of the CD28-TMD are necessary for CAR-CD28 heterodimerization (**Figure 3E**).

CD28-CAR Heterodimers Response to CD80 and CD86

To determine if the natural ligands of CD28, CD80, and CD86 can activate CARs by engaging CD28-CAR heterodimers, we stimulated CAR T cells with different HD and TMD with CD19-deficient Raji cells that express high levels of CD80 and CD86 (**Supplementary Figure 5A**). $CD19^{deficient}$ Raji induced CAR T-cell activation, although at a lower intensity than that induced by the $CD19^+$ Raji cells (**Supplementary Figure 5B**, **Figure 4A**). This "off-target" activation was mostly seen in T cells with a high CAR expression (**Figures 4A,B**). Moreover, CAR T-cell activation by $CD19^{deficient}$ Raji was significantly reduced by binding CTLA-4 Ig, a high-affinity competitive inhibitor of CD28, to CD80 and CD86 (**Figures 4A,B**), demonstrating that the off-target activation of CAR T cells is predominantly driven by the CD28 interaction between CD80 and CD86. Importantly, $CD28\text{-}TMD^{M4}$ did not markedly change the off-target activation of either IgG_4-HD/$CD28^{WT}$ or CD8-HD/$CD28^{WT}$ CARs, demonstrating the inability of CD28-CAR heterodimers to respond to natural CD28 ligands.

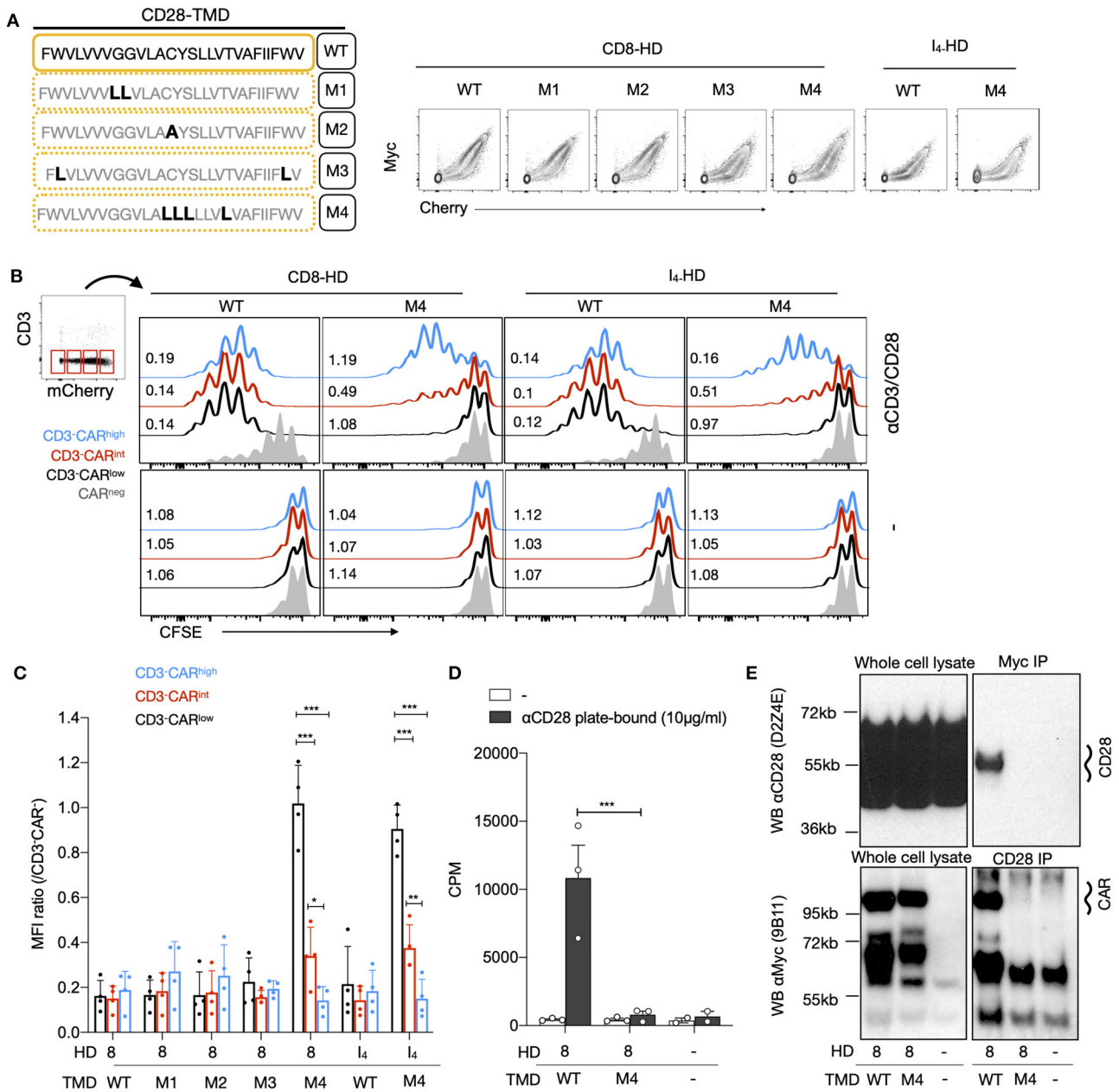

FIGURE 3 | The dimerization of the CD28-TMD depends on a core of four amino acids. **(A)** Diagram representing the amino acid sequence of the wild type and four mutants of the CD28-TMD. A representative example of MYC and the mCherry expression for each mutant is shown. **(B)** A representative example of CFSE dilution of a mixed population of $CD3^{+/-}CAR^{+/-}$ T cells restimulated with anti-CD3/CD28 beads and unstimulated conditions. Representative CFSE MFI ratios (/CAR negative CD4 T cells) for low, intermediate, and high CAR expression with or without stimulation are shown **(C)** The normalized CFSE MFI ratio for CD3-$CAR^{low/int/high}$ was calculated by dividing the MFI of each of these population with the MFI of $CD3^{-}$mCherry^{-} cells within the same culture. A summary of results using T cells from four unrelated donors in two independent experiments is shown. **(D)** Proliferation of purified $CD3^{-}CAR^{+}$ T cells in response to plate-bound anti-CD28 stimulation. Results represent the mean of three independent experiments. **(E)** CD28 or the Myc-tag of $CD3^{-}CAR^{+}$ T cells were immunoprecipitated. Western blot analysis of the input (5% of the whole cell lysate) as well as of the precipitated was performed using anti-CD28 (clone D2Z4E) and anti-Myc (clone 9B11). Results are representative of two independent experiments for each condition. The two-way ANOVA was used for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Regulation of CD28 Expression by CD28-CAR Heterodimers

We next examined the impact of CAR-CD28 heterodimerization on CD28 expression. Since lentiviral transduction resulted in a wide range of CAR expression levels that influenced on- and off-target T-cell activation, we expressed various CARs by knocking them into the TCR alpha constant (*TRAC*) gene locus using homology-directed repair that provided more homogenous

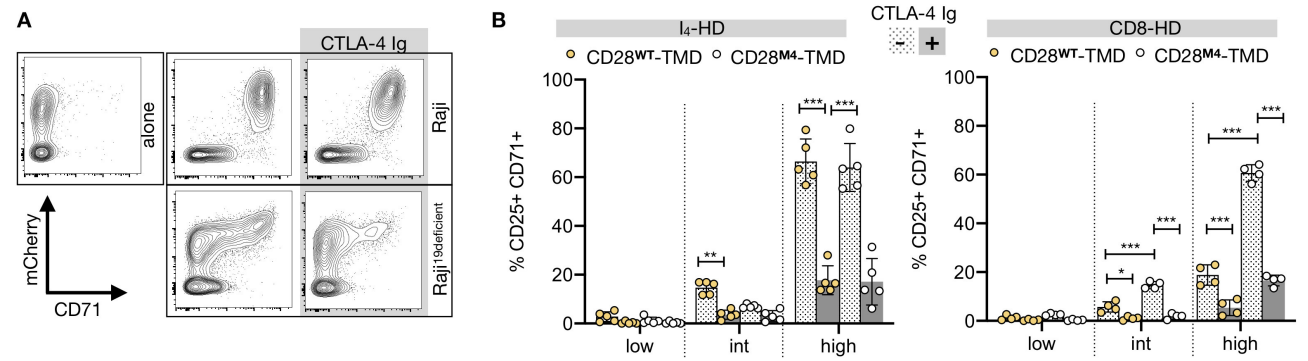

FIGURE 4 | CAR-CD28 heterodimers are B7-unresponsive. **(A)** A representative example showing CD71 upregulation in CAR T cells containing an IgG_4-HD/CD28-TMD co-cultured for 48 h with irradiated (4,000 Rad) CD19-wild type or deficient Raji cells with or without CTLA-4 Ig. **(B)** $CD25^+CD71^+$ T cells were analyzed in low, intermediate (int), or high mCherry-expressing CAR T cells using the gating strategy described in **Supplementary Figure 5**. Data were pooled from four independent experiments using T cells from four to five unrelated donors. The two-way ANOVA was used for statistical analysis. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expression of the CAR (**Figure 5A**) (28). Knock-in efficiencies ranged between 17 and 72% across the various CAR constructs, but the levels of the CAR expression were similar regardless of the differences in editing efficiency (**Supplementary Figure 6A**). In addition, all CAR T cells proliferated upon stimulation with $CD19^+$ NALM-6 target cells (**Supplementary Figure 6B**), demonstrating that CARs containing M4 mutations remained functional. Six days after CAR knock-in prior to exposing the cells to target cells, we observed a 26–51% reduction in the CD28 mean fluorescence intensity on CAR^+ T cells containing a wild-type CD28-TMD, but not an M4 CD28-TMD with either a CD8-HD or CD28-HD (**Figures 5B,C**). This reduction was seen in both $CD4^+$ and $CD8^+$ T cells with CARs containing either 28ζ or 4-1BBζ ICD. The downregulation of CD28 in CAR T cells engineered with an IgG_4-HD/$CD28^{WT}$-TMD was minimal, echoing the earlier result of inefficient CAR-CD28 heterodimerization in the context of IgG_4-HD.

Modeling of Hinge-Hinge Interactions

Given the impact of the HD on CAR-CD28 heterodimerization, we next modeled the hinge-hinge interactions to better understand how the HD might influence the interaction of CD28 with CARs. As few TMD templates are available for modeling and their structures difficult to solve by nuclear magnetic resonance spectroscopy, we limited the modeling only to the extracellular domain of the CAR and CD28 receptors. The cysteine residue in the HD of the CD28 receptor C123 was aligned with the cysteine in the HD of CD28-HD-containing and CD8-HD-containing CARs (**Figure 6**). However, for IgG_4-HD-containing CARs, the cysteines in the HD could not be aligned with C123 of the CD28 (**Figure 6**). The modeling presented in the study demonstrated that the presence of a disulfide bound with endogenous CD28-HD is possible for the CD28-HD- and CD8-HD-containing CARs (**Figure 6**). However, as seen in **Figures 5B,C**, the lack of CD28 downregulation in CAR T cells with a CD28-HD and a M4-CD28-TMD suggests the CD28-HD alone was not sufficient to mediate the heterodimerization. Moreover, when various $CD3^-CAR^+$ T cells were stimulated with anti-CD3/CD28 beads, we did not observe enhanced CFSE dilution of T cells engineered with the CD28-HD/M4-CD28-TMD CAR construct when compared to CAR T cells with the CD8-HD/M4-CD28-TMD constructs (**Supplementary Figures 7A,B**). These results further support the notion that the cysteine bridge in the CD28-HD is insufficient to mediate CD28-CAR heterodimerization without interactions in the CD28-TMD. Taken together, these data suggest that cysteines and inter-molecular disulfide bonds in HDs are not the drivers of CAR-CD28 heterodimerization but can be involved in the stabilization of the CAR-CD28 heterodimers.

DISCUSSION

In this study, we discovered that the CD28-TMD mediates CAR and CD28 heterodimerization *via* a core of up to four polar amino acids. The efficiency of CAR-CD28 heterodimerization depends on the HD but not the ICD. While the heterodimers are unresponsive to CD28 ligands, namely CD80 and CD86, they lead to the downregulation of CD28 on the surface of CAR T cells. These data unveil a new attribute of CD28-TMD that may impact the function of CD28 TMD-containing CAR T cells.

Our results demonstrate that CAR-CD28 heterodimers can be expressed at the cell surface as a consequence of CD28 TMD dimerization. While concluding this study, Leddon et al. reported that CD28 homodimerization was dependent on the YxxxxT motif of the CD28-TMD (29). This motif is shared with the CTLA-4 receptor and is also structurally related to CD3ζ dimerization (30). The CD28 homodimer is covalently linked by a disulfide bond (C123) in the HD (22, 31). Interestingly, CD28 dimerization and its subsequent cell surface expression could be efficiently prevented only upon combined mutations of C123S (in the CD28-HD) and YT/LL (in the CD28-TMD) (29, 31). This demonstrates that the HD is also a critical aspect to consider in the formation of receptor dimers. The results in the study demonstrated the role of HD in CAR-CD28

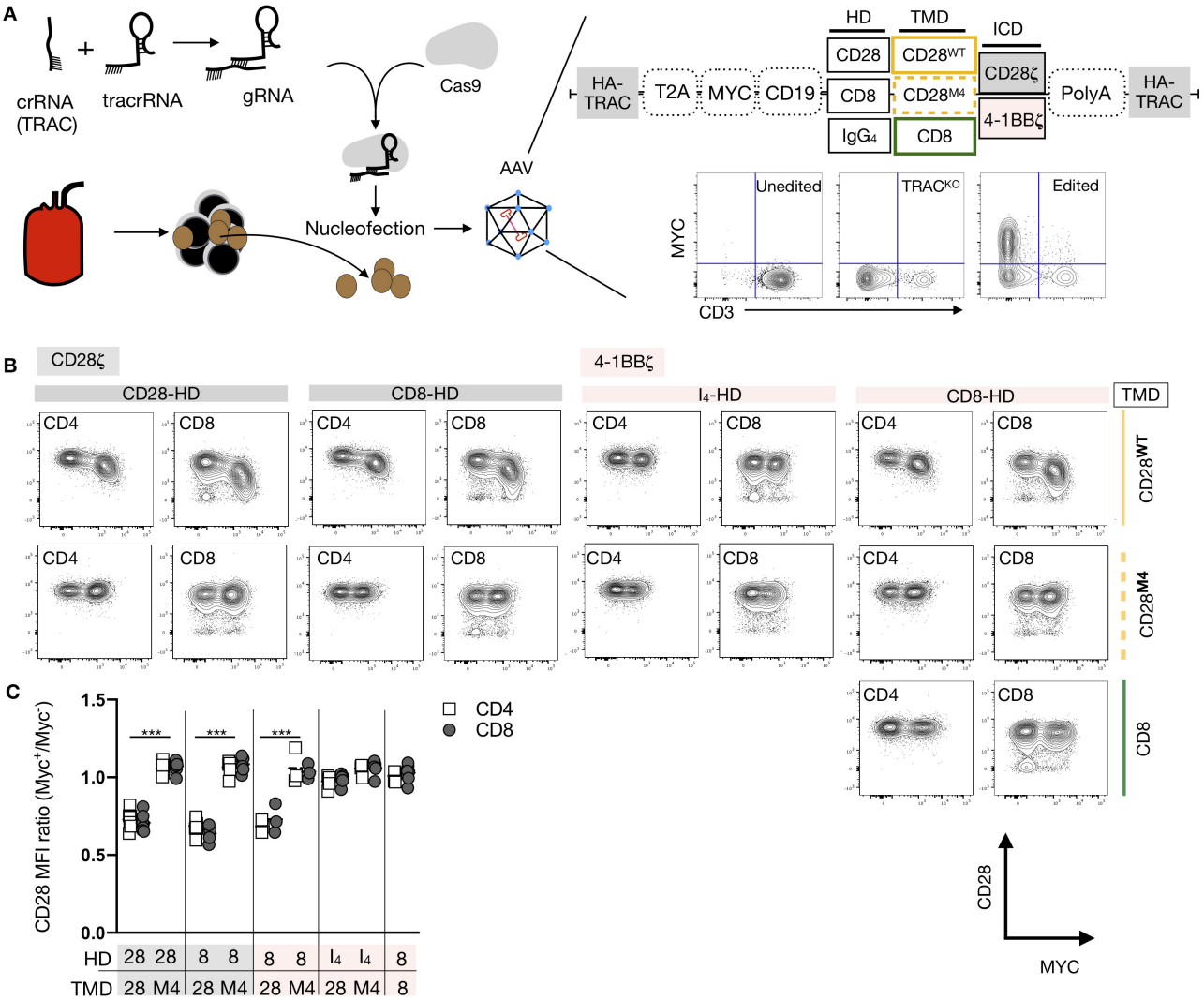

FIGURE 5 | CAR-CD28 heterodimers reduce CD28 expression. **(A)** Editing strategy and homology-directed repair-mediated integration into the TRAC locus of various CD19 CARs using an AAV-6 transduction protocol. **(B)** The expression of Myc and CD28 in a representative example was analyzed 6 days after editing and the removal of beads. **(C)** The CD28 MFI ratio was calculated by dividing CD28 MFI of Myc^+ cells by Myc^- cells in the same culture. Pooled data from three to four independent experiments across five unrelated donors are shown. Each dot represents one independent editing condition. The two-way ANOVA was used for statistical analysis. ***$p < 0.001$.

heterodimerization, with IgG_4 HD being less efficient than HDs from CD28 and CD8 (32). The hinge-hinge modeling suggests that the membrane proximity of the cysteine in the IgG_4-HD may not readily form disulfide bonds with the cysteine in the CD28-HD of the endogenous CD28 receptor and therefore leads to preferential CAR-homodimerization (31). This observation may also be linked to inflexibility of the short IgG_4-HD, leading to steric hindrance by the globular scFv domain (32).

The modeling data suggest that the CD28 extracellular hinge can form a cysteine bridge with the endogenous CD28 receptor. However, the cysteine bridge by itself is likely insufficient to mediate CD28 dimerization. This finding is reported due to high CD28 expression (**Figures 5B,C**) and the lack of preferential proliferation of T cells engineered with the CD28-HD/M4-CD28-TMD CAR construct (**Supplementary Figure 7**). These results are consistent with the observation of Leddon et al. (29), who demonstrated that mutations in the CD28-TMD alone strongly reduce the expression of the CD28 receptor. Asimilar reduction in the CD28 expression was found when mutating the C123S in the CD28-HD, further suggesting that the hinge is necessary for CD28 homodimerization, possibly through stabilization of the dimer rather than its formation (29).

The findings that anti-CD28 can activate CAR T cells through the CAR-CD28 heterodimers raised the concern that the natural ligand of CD28 may also induce off-target activation of CAR T cells. The results of the study showed that CAR T cells can indeed be activated in the absence of target antigen in a CD80- and

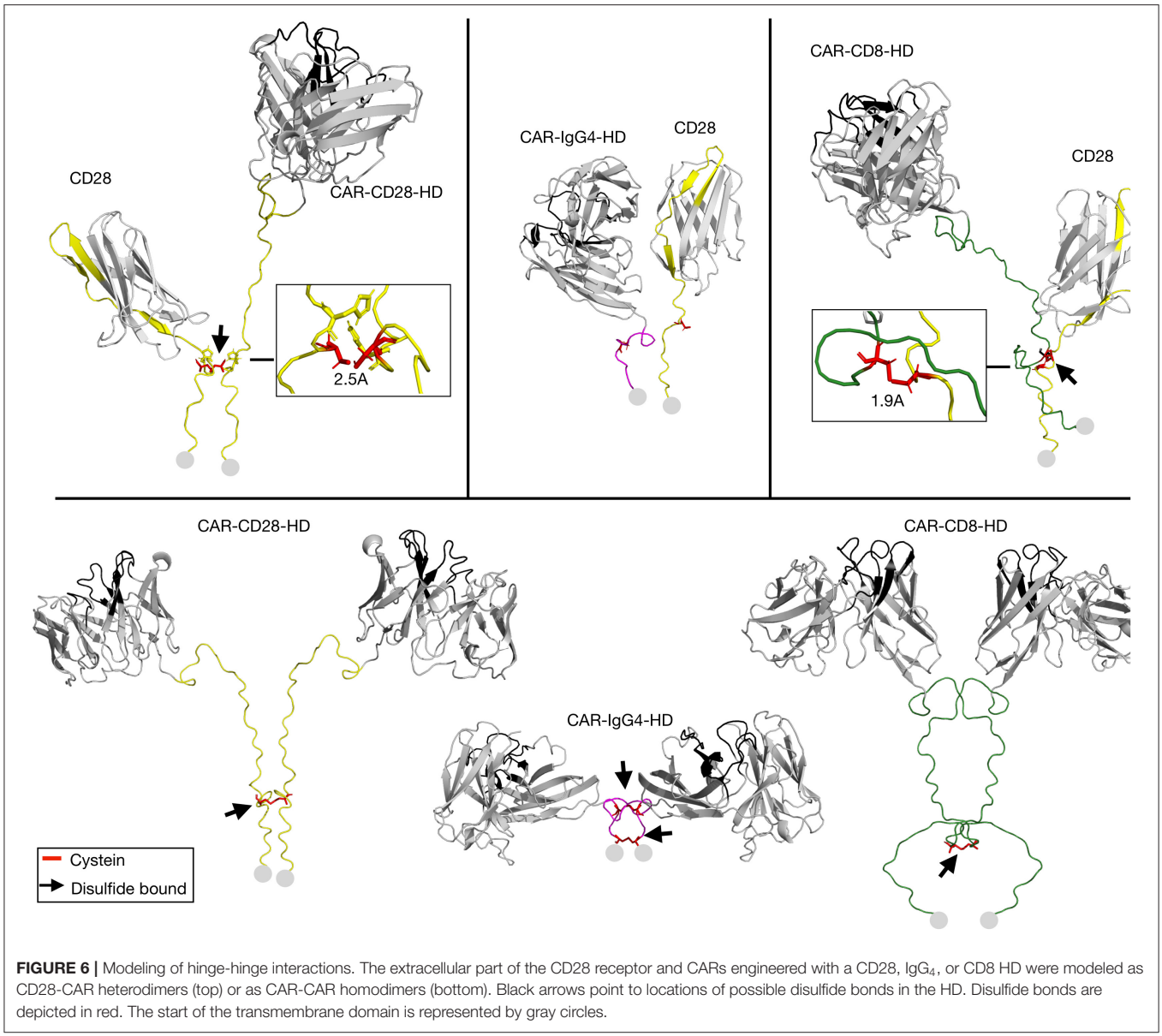

FIGURE 6 | Modeling of hinge-hinge interactions. The extracellular part of the CD28 receptor and CARs engineered with a CD28, IgG_4, or CD8 HD were modeled as CD28-CAR heterodimers (top) or as CAR-CAR homodimers (bottom). Black arrows point to locations of possible disulfide bonds in the HD. Disulfide bonds are depicted in red. The start of the transmembrane domain is represented by gray circles.

CD86-dependent manner in T cells expressing a high amount of CAR, but this off-target activation was independent of CD28-CAR heterodimers. This may be explained by the fact that CD80 and CD86 are engaging CD28 homodimers (31). It is also possible that the extracellular conformation of the CD28 monomers is not stable enough to interact with CD80/CD86. Nonetheless, these data suggest that the off-target activation was induced by CD28 homodimers. In T cells with a high level of CAR expression, endogenous CD28 homodimers may induce CAR clustering through membrane compartmentalization. Thus, it has been reported that CD28-mediated costimulation can induce coalescence of membrane microdomains that were enriched for signaling molecules, resulting in an enhanced T-cell activation (33). These results encourage further investigations to explore the utility of costimulation blockade as a new approach to prevent off-target CAR toxicities as antigen-presenting cells are present in virtually all organs and that the $CD80^+/CD86^+$ expression is upregulated during inflammation.

We found that upon efficient CD28-CAR heterodimerization, the level of CD28 expression was significantly reduced in both $CD4^+$ and $CD8^+$ CAR T cells, possibly because of the recruitment of CD28 into CAR heterodimers. This demonstrates that a CD28-TMD-containing CAR may bind away a substantial fraction of CD28 at the cell surface. It is worth noting that since CD28 in the heterodimers with CAR can bind to anti-CD28 in our functional and immunoprecipitations assays, the reduced MFI is likely due to the loss of expression on the cell surface and not a lack of detection. The functional significance of the loss in the CD28 expression remains to be determined experimentally. A recent study has shown that the CD28 expression in 19-CAR T cells engineered with a CD28-TMD was indeed significantly lower when compared to 19-CAR T cells engineered with

a CD8-TMD (34). This correlated with a significantly lower number of CD28-TMD-containing CAR T cells in the peripheral blood 1 month after infusion, suggesting that the reduced CD28 expression might have impaired CAR T-cell persistence. Thus, one could speculate that reduced CD28 expression increases the sensitivity of CAR T cells' to exhaustion or alter their differentiation to effector/memory programs (35).

The present study showed a major biochemical difference between CD28-TMD and CD8-TMD CARS, although the exact functional consequences of this difference remain to be investigated. Several studies demonstrated that 19-CAR T cells engineered with a CD28-HD/TMD have increased sensitivity to low abundant antigens as compared to 19-CAR T cells with a CD8-HD/TMD (11–13). CD28-HD/TMD-containing CARs seem also to be associated with an increased risk of neurotoxicity (4, 36–38) as compared to CD8-HD/TMD-containing CARs (6, 8, 39). A recent report suggested that neurotoxic events can be significantly reduced when replacing the CD28-HD/TMD with a CD8-HD/TMD, independent of the signaling domain (34). Another recent report suggested that severe neurotoxicity observed in clinical trials with 19-CAR T-cell may be due to the presence of $CD19^{+}$ murals cells in the vasculature of the brain (40). With these findings, we hypothesize that the CD28-CAR association may increase CAR sensitivity for ectopically expressed low abundant antigens, such as the CD19 expressed on mural cells, thus demonstrating higher off-tumor activation of CARs. The lack of functional analysis on the consequences of CAR-CD28 heterodimerization remains an important limitation of the present study.

In conclusion, this study shows that the CD28-TMD is not inert and can lead to the formation of CD28-CAR heterodimers. This suggests that, in general, TMDs of CARs can impact CAR association with endogenous proteins, which is a function of the CAR T cell. Thus, optimization of CAR designs should consider TMD-mediated receptor interactions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YDM and QT: conceptualization and manuscript writing. YDM: formal analysis. QT: funding acquisition. YDM, DPN, LMRF, CR, ZC-W, RBV, and LP: investigation. YDM, DPN, TR, PH, JE, and LP: methodology. QT, JAB, AM, and JAW: resources. QT and JAB: supervision. YDM, QT, DPN, LMRF, CR, AM, JE, TR, FVG, ZC-W, RBV, JAW, and JAB: writing-review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.639818/full#supplementary-material

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Conflict of Interest: A provisional patent on CAR-CD28 heterodimerization has been submitted. JAB and QT are co-founders of Sonoma Biotherapeutics. AM and TR are co-founders of Arsenal Biosciences. AM is also a co-founder of Spotlight Therapeutics. JAB and AM have served as advisors to Juno Therapeutics. AM was a member of the scientific advisory board at PACT Pharma and was an advisor to Trizell. QT, JAB, and AM have received sponsored research support from Juno Therapeutics. AM is cofounder, member of the Boards of Directors and member of Scientific Advisory Boards of Spotlight Therapeutics and Arsenal Biosciences. AM has served as an advisor to Juno Therapeutics, was a member of the scientific advisory board at PACT Pharma and was an advisor to Trizell. AM has received honoraria from Merck, a consulting fee from AlphaSights, and is an investor in and informal advisor to Offline Ventures. AM owns stock in Arsenal Biosciences, Spotlight Therapeutics and PACT Pharma. AM has received research support from Epinomics, Sanofi, GlaxoSmithKline, and gifts from Gilead and Anthem. JAW is co-Founder of Soteria Biotherapeutics developing small molecule switchable biologics, on the SAB of Spotlight, and recipient of sponsored research from Bristol Myers Squibb. JE is an advisor for Mnemo Thérapeutics and Cytovia and received research support from Cytovia. TLR is a co-founder, member of Scientific Advisory Board, and founding Chief Scientific Officer of Arsenal Biosciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Infection Temperature Affects the Phenotype and Function of Chimeric Antigen Receptor T Cells Produced *via* Lentiviral Technology

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Chimeric antigen receptor (CAR)-T cell therapy has become an important method for the treatment of hematological tumors. Lentiviruses are commonly used gene transfer vectors for preparing CAR-T cells, and the conditions for preparing CAR-T cells vary greatly. This study reported for the first time the influence of differences in infection temperature on the phenotype and function of produced CAR-T cells. Our results show that infection at 4 degrees produces the highest CAR-positive rate of T cells, infection at 37 degrees produces the fastest proliferation in CAR-T cells, and infection at 32 degrees produces CAR-T cells with the greatest proportion of naive cells and the lowest expression of immune checkpoints. Therefore, infection at 32 degrees is recommended to prepare CAR-T cells. CAR-T cells derived from infection at 32 degrees seem to have a balance between function and phenotype. Importantly, they have increased oncolytic ability. This research will help optimize the generation of CAR-T cells and improve the quality of CAR-T cell products.

Keywords: Lentivirus, Infection temperature, chimeric antigen receptor (CAR), naive T cells, immune checkpoints

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INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapy is a kind of adoptive immunotherapy that uses genetic engineering to express one or more specific chimeric antigen receptors (CARs) on T cells (1–3). CARs are artificial fusion proteins consisting of an antigen-recognition region connected to a signal element through a hinge and a transmembrane region (4). Antigen recognition regions are usually single-chain variable fragments (scFvs) derived from antibodies. The signal domain includes the costimulatory domain from proteins such as CD28 and 4-1BB, and the activation domain is usually from CD3ζ. CAR-T cells can perform non-MHC-dependent antigen recognition to effectively bypass the tumor's main immune escape mechanism, the downregulation of MHC molecules, thereby specifically killing tumor cells.

The introduction of CAR-T cell immunotherapy has been a milestone in tumor immunotherapy in recent years, especially in the immunotherapy of hematological tumors (5–7). CAR-T cells targeting CD19 and BCMA have shown significant antitumor effects in the treatment of relapsed/

refractory (r/r) B cell tumors and multiple myeloma. Four anti-CD19 CAR-T cell products (tisagenlecleucel, axicabtagene ciloleucel, brexucabtagene autoleucel and breyanzi) have been approved by the FDA as drugs since 2017 (5). One anti-BCMA CAR T-cell product (idecabtagene vicleucel, bb2121, ide-cel) is likely to be approved by FDA (6, 7).

Virus-mediated gene transfer is widely used in the preparation of CAR-T cells because viral vectors can effectively transfer genes to a variety of cell types and can stably integrate into their genomes, leading to long-term gene expression, which persists in progeny cells (8). However, the infection conditions for preparing CAR-T cells from viruses vary greatly (9–20). Tisagenlecleucel uses a 37-degree infection (16), breyanzi and axicabtagene ciloleucel use a 32-degree infection (15, 21), and some studies have used room temperature or a 4-degree infection to produce CAR-T cells with viral vectors (12, 13, 17, 20). As seen above, temperature is a very important infection condition. Studies with retroviruses have shown that because the virus has a longer half-life at 32 degrees, 32 degrees is more efficient than 37 degrees for infecting cells (14, 18). Other studies have shown that the culture temperature has an impact on the productivity of mammalian cells and the stability of the virus (22–24). However, the optimal infection temperature in lentiviruses, which are widely used for gene delivery in the preparation of CAR-T cells, has not yet been studied. Here, we used different temperatures to observe the influence of the temperature used during lentiviral infection of T cells on the preparation of CAR-T cells; these experiments will help to determine the optimal infection temperature to improve the quality of CAR-T cell products.

MATERIALS AND METHOD

Cell Lines and Primary Cells

HEK-293T (ATCC) cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Biological Industries). NALM-6 [peripheral blood B cell precursor leukemia cells (acute lymphocytic leukemia (ALL)), CD19+] and MOLM-13 [human acute myeloid leukemia cells (AML), CD123+] cells were grown in RPMI 1640 medium supplemented with 10% FCS (Gibco). All cell lines were cultured at 37 degrees, 5% CO2 and 95% humidity for up to 1 month. The cells were divided every 2 to 3 days, and the number of passages did not exceed 20. Peripheral blood samples were obtained from healthy donors (n = 3) in The Tianjin First Central Hospital after informed consent was obtained according to the institutional guidelines. Peripheral blood mononuclear cells (PBMNCs) were enriched through a Ficoll Hypaque gradient.

Transgene Constructs

The scFv targeting CD19 originated from the FMC63 clone. The scFv targeting CD123 originated from the 7G3 clone. The CAR vectors contained the scFv and human 4-1BB and CD3ζ signaling domains, which were subcloned into the pCDH-MND-MCS-T2A-Puro lentiviral plasmid vector. The CAR sequence was preceded by the RQR8 tag separated by a short T2A peptide for detection (25).

Lentivirus Production

The preparation of the lentivirus was performed according to the manufacturer's instructions (GeneCopoeia). Briefly, two days before transfection, plate HEK-293T lentiviral packaging cells in a 10-cm dish in 10 ml of DMEM supplemented with 10% heat-inactivated fetal bovine serum so that the cells are 70–80% confluent at the moment of transfection. In a sterile polypropylene tube, dilute 2.5 μg of lentiviral expression plasmid and 5.0 μl (0.5 μg/μl) of Lenti-Pac HIV mix into 200 μl of Opti-MEM® I (Invitrogen). In a separate tube, dilute 15 μl of EndoFectin Lenti into 200 μl of Opti-MEM I. Add diluted EndoFectin Lenti reagent drop-wise to the DNA solution while gently vortexing the DNA-containing tube. Incubate the mixture for 10–25 minutes at room temperature to allow the DNA-EndoFectin complex to form. Add the complex directly to each dish. Replace the overnight culture medium with fresh DMEM medium supplemented with 2–5% heat-inactivated fetal bovine serum. Add 1/500 volume of the TiterBoost reagent to the culture medium. Collect the pseudovirus-containing culture medium in sterile capped tubes 48 hours post transfection and centrifuge the tubes at 500g for 10 minutes to get rid of cell debris. Following centrifugation, filter the supernatant through 0.45 μm polyethersulfone low protein-binding filters. Viral supernatants were concentrated using ultracentrifugation at 50,000 for 2 hr 30 min. Virus-containing pellets were resuspended in complete X-Vivo15 media and stored at −80°C until use.

Lentivirus Titration

The number of transducing units (TU/mL) was determined by the limiting dilution method. Briefly, HEK-293T cells were seeded 12 hr before transduction. Then, 1:10 dilutions of the viral supernatant were prepared and added on top of the cells in complete DMEM + 5 μg/mL Polybrene. Cells were trypsinyzed 72 hr later and labeled with the QBEND-10 monoclonal CD34 antibody (Abcam) before being analyzed by flow cytometry. A dilution corresponding to 2%–20% of positive cells was used to calculate viral titer.

Production of CAR-T Cells

CD3+ T cells were separated from PBMCs using CD3 immunomagnetic beads (#130-097-043, Miltenyi Biotec, Germany) on day 1. T cells were amplified using CD3/CD28 stimulation beads (#11131D, Thermo Fisher Scientific) and IL-2 (100 IU/mL; Miltenyi Biotec) in X-VIVO 15 Cell Medium (Lonza). Cells were then activated and expanded for 48 hours were transduced 2 hr later with the lentivirus (multiplicity of infection is 10) by different temperatures incubation in the presence of polybrene (Sigma) at 8 μg/mL. Then, the cells continue to expand at 37 degrees at an appropriate concentration (0.5-1×106 cells/ml). The transduction efficiency was determined 3 days after transduction. Generally, the T cells were engineered *via* 9-12 days of manufacturing to express a CD19-specific CAR or CD123-specific CAR.

Detection of CAR Expression by Transduced T Cells

For each analyzed T cell culture, one sample of cells was stained with Alexa-Fluor 647-labeled polyclonal goat anti-mouse IgG (H+L) antibodies (Affinity) to detect CAR-T cells. In addition, we also detected the expression of the CAR with the QBEND-10 monoclonal CD34 antibody (Abcam) labeled with an RQR8 tag. Subsequently, all cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD3 antibodies (Abcam).

Immunophenotyping

Anti-human monoclonal antibodies against CD3 (Biolegend), CD4 (Biolegend), CD8 (Biolegend), CD34 (Abcam), CD45RO (Biolegend), CD62L (Biolegend), PD1 (Biolegend), LAG3 (Biolegend), and TIM3 (Biolegend) were used for immunophenotypic analysis. All flow cytometry analyses of stained cells were performed with a Coulter Altra flow cytometer equipped with CytExpert software (Beckman Coulter).

Assessment of Cytokines

Toxicities were evaluated relative to a baseline assessment conducted before CAR-T cell infusion. The concentrations of serum inflammatory markers, including IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and GM-CSF were evaluated by Luminex assay according to the manufacturer's instructions.

Cytotoxicity Determination

CD19+ NALM-6 cells and CD123+ MOLM-13 cell lines were used to determine the cytotoxic activity of CD19 CAR-T and CD123 CAR-T cells, respectively. CAR expression was detected 72 hours after transduction by flow cytometry, and CAR-T cell cytotoxic activity was evaluated the next day. NALM-6 CAR-T cells were previously labeled with CellTrace CFSE (Invitrogen) according to the manufacturer's instructions. To compensate for the change in transduction efficiency, the effector cell population was normalized to the absolute number of T cells by adding untransduced T cells. NALM-6 cells without effector cells were used as a control. After 24 hours of incubation, the cell mixture was stained to visualize dead cells using the fixable viability dye eFluor 780 (Thermo Fisher Scientific) according to the manufacturer's instructions and analyzed by flow cytometry. The percentage of dead target cells was determined using the CFSE-positive and viability dye-positive cell population (19).

In Vivo Leukemia Xenograft Study

Male NSG mice (Sipeifu) aged 5-6 weeks were injected intravenously with 2×10^6 luciferase-expressing NALM-6 cells cultured in our laboratory. Three days later, 5×10^6 CD19 CAR-T cells or uninfected T cells were injected into the mice through the tail vein. To monitor tumor growth, each mouse was injected intraperitoneally with 3 mg of D-luciferin (Sigma, US) at the designated time point, and the mice were imaged using an IVIS Imager 10 minutes later.

Statistical Analysis

The results were analyzed with the GraphPad Prism program (GraphPad Software). Data that obeyed a normal distribution are presented as the mean ± standard deviation (SD), and multiple group comparisons were performed by using one-way analysis of variance (ANOVA), whereas data with a nonnormal distribution are shown as the median and quartiles and were compared by the Kruskal-Wallis test. The survival curves were analyzed using the Kaplan-Meier method with the log-rank test. $P<0.05$ was considered statistically significant.

RESULT

Successful Preparation of CAR-T Cells

CD19 is expressed on the surface of B cell tumors. The three CAR-T cell drugs currently on the market all target CD19. Therefore, research on CD19 CAR-T cells is very important. Our CAR structure contains the FMC63 single-chain antibody, the CD8 hinge region and transmembrane region, the 41-BB costimulatory signal and the CD3ζ signal domain. The structure is similar to the structures of Novartis products (**Figure 1A**). To obtain more reliable results, we also constructed a CAR vector targeting the CD123 molecule (**Figure 1B**). CD123 is mainly expressed on the surface of myeloid cells and targets AML cells. We also added the RQR8 tag sequence to detect the expression of the CAR. The tag can be labeled with the QBEND-10 monoclonal CD34 antibody. Through lentivirus-mediated gene transfer, the CAR fusion protein was successfully expressed on the surface of activated T cells, and CD19 CAR-T and CD123 CAR-T cells with high infection rates were obtained (**Figures 1C, D**). The produced CD19 CAR-T cells and CD19-positive NALM-6 cells were coincubated at different ratios. Compared with uninfected T cells, CD19 CAR-T cells could significantly lyse NALM-6 cells (**Figure 1E**). Detecting the cytokines in the supernatant showed that the levels of IFN-γ, IL-6, IL-2, TNFα and other cytokines in the culture medium after CD19 CAR-T cells and target cells were coincubated were significantly increased (**Figure 1F**). We coincubated CD123 CAR-T cells with the CD123-positive MOLM-13 AML cell line, detected cytotoxicity and cytokines, and obtained similar results to those for the CD19 CAR-T cells (**Figures 1G, H**). The above data prove that our CAR-T cells targeting CD19 and CD123 were successfully produced, and these CAR-T cells could kill specific target cells and secrete abundant cytokines.

Infection Temperature Can Affect the Proliferation and Infection Efficiency of Produced CAR-T Cells

Many studies have found that the temperature during virus infection affects the activity and proliferation of host cells. To explore the impact on CAR-T cells, we selected several commonly used temperatures in previous studies, namely, 4 degrees, 25 degrees, 32 degrees, and 37 degrees, for our experiments. Two hours after T cells were activated by lentivirus infection, they were placed in an incubator for normal culture, and the cells were counted on the 3rd, 6th, and 9th days of culture. Compared with uninfected T cells, CD19 CAR-T cells and CD123 CAR-T cells infected with virus had

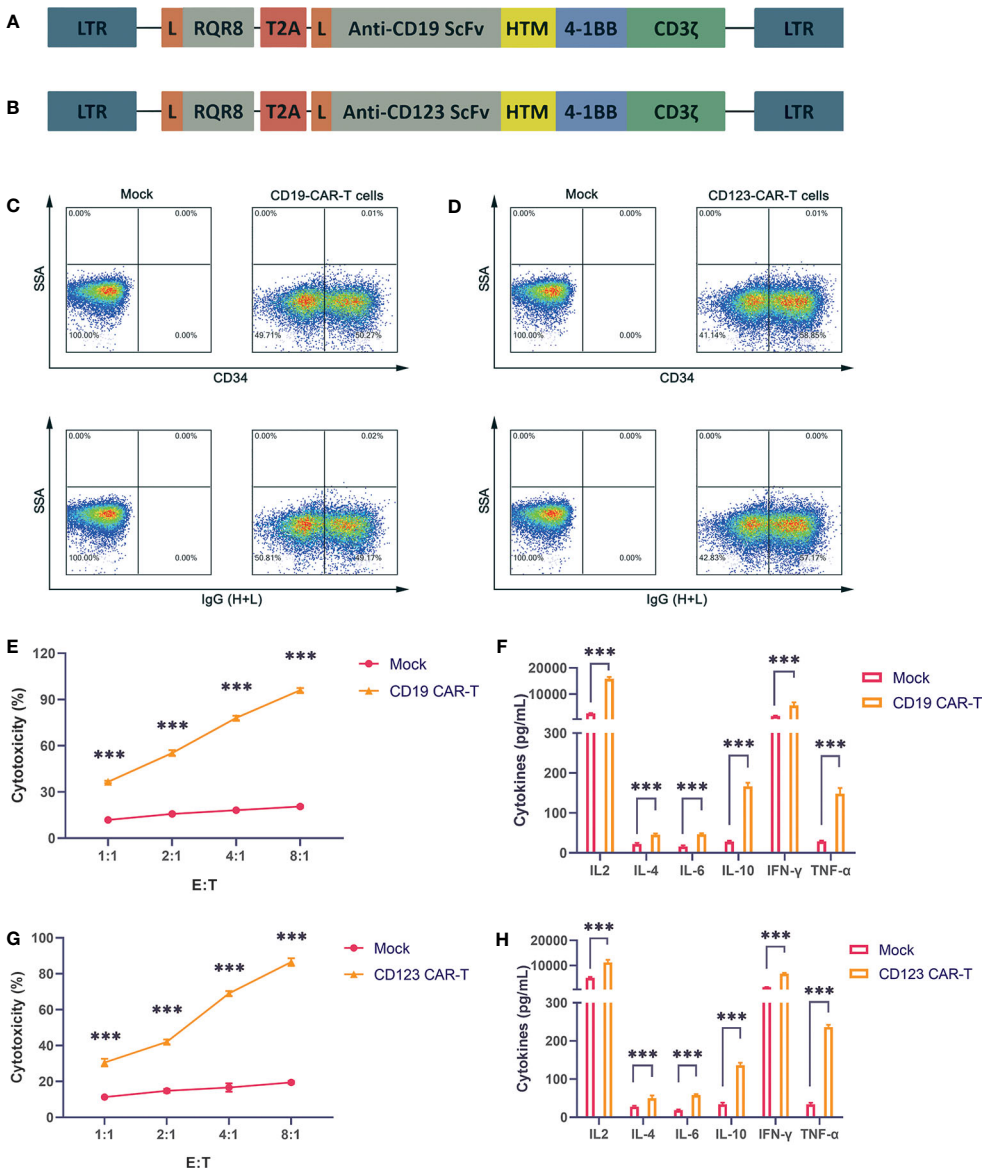

FIGURE 1 | Successful production of well-functioning CAR-T cells **(A)** CD19 CAR vector schematic diagram. HTM is the CD8 hinge and transmembrane region. **(B)** CD123 CAR vector schematic diagram. **(C)** Anti-CD34 antibody and anti-IgG (H+L) antibody labeling of CAR-T cells, and detection of the infection rate of CD19 CAR-T cells by flow cytometry. **(D)** Anti-CD34 antibody and anti-IgG (H+L) antibody labeling of CAR-T cells, and detection of the infection efficiency of CD123 CAR-T cells by flow cytometry. **(E)** Cytotoxicity of CD19 CAR-T cells and CD19-positive NALM-6 cells coincubated at different effector:target (E:T) ratios for 24 hours. **(F)** Coincubation of CD19 CAR-T cells and CD19-positive NALM-6 cells at a 1:1 ratio, and detection of the secretion levels of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNFα in the supernatant. **(G)** Cytotoxicity of CD123 CAR-T cells and CD123-positive MOLM-13 cells coincubated at different E:T ratios for 24 hours. **(H)** Coincubation of CD123 CAR-T cells and CD123-positive MOLM-13 cells at a 1:1 ratio, and detection of the secretion levels of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNFα in the supernatant. Three independent experiments were conducted. Mean ± SD. ***$p < 0.001$.

obviously lower cell viability and proliferation rates (**Figures 2A–D**). The proliferation rate of the 37-degree-infection group was significantly higher than the proliferation rates of the infection groups employing other temperatures, and the 4-degree-infection group had the lowest proliferation rate. Compared with uninfected T cells, CAR-T cells have lower cell activity. There is no significant difference in cell viability among the other temperature groups. Infection efficiency is a very important point in the preparation of CAR-T cells. High infection efficiency can save costs and time for cell culture *in vitro* and improve the success rate of treatment. We used flow cytometry to detect the CD19 CAR-T cell infection efficiency. Our results showed that the proportion of CAR-positive T cells in the 4-degree-infection group was higher than those in the

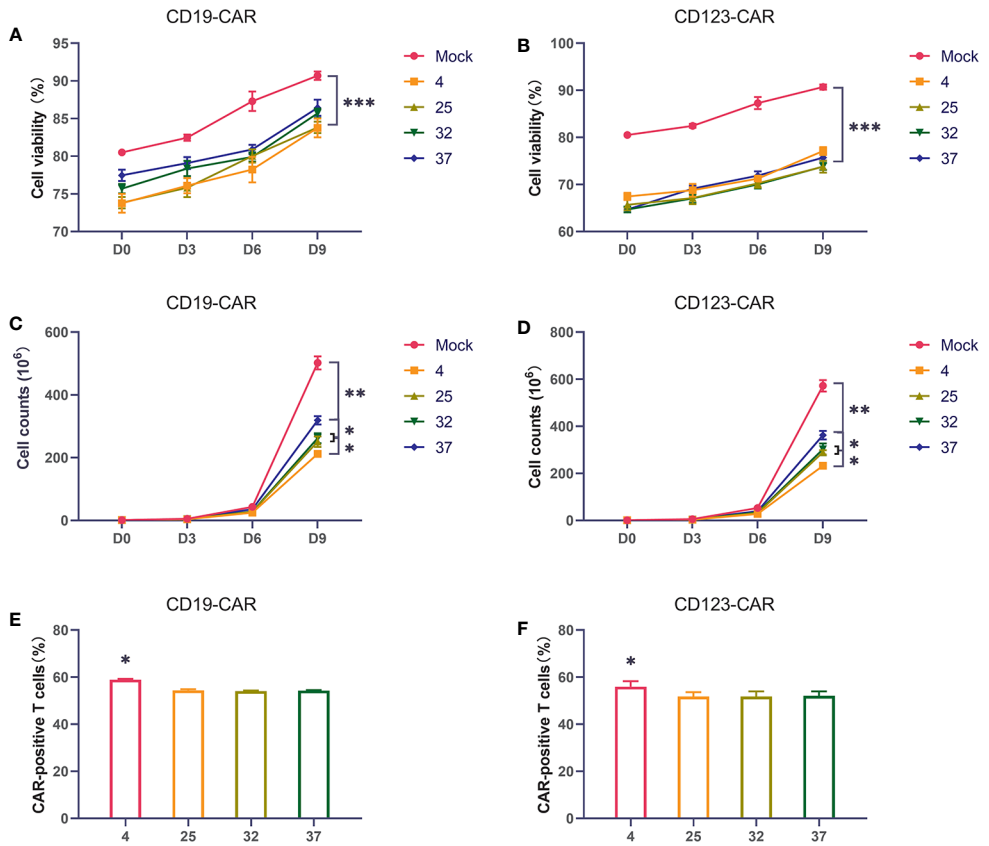

FIGURE 2 | Infection temperature can affect the proliferation and infection efficiency of CAR-T cells **(A, B)** The proportion of living CD19 and CD123 CAR-T cells produced at different infection temperatures on the first day, the third day, the sixth day, and the ninth day after infection. **(C, D)** The number of living CD19 and CD123 CAR-T cells produced at different infection temperatures on the first day, the third day, the sixth day, and the ninth day after infection. **(E, F)** The infection efficiency of CD19 and CD123 CAR-T cells produced at different infection temperatures. Three independent experiments were conducted. Mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

other groups, which showed similar proportions (**Figure 2E**). The same result was also verified in CD123 CAR-T cells (**Figure 2F**). In addition, we also detected the secretion of cytokines in the culture medium on the 6th day of culture. Compared with that of uninfected T cells, CD19 CAR-T cell culture media has significantly higher levels of secreted cytokines. There was no significant difference in cytokine levels among the other temperature groups (**Supplement 1A–F**).

Infection Temperature Can Affect CAR-T Cell Phenotype

Previous reports have shown that the subpopulation distribution of CAR-T cells can impact the therapeutic effect of CAR-T cells. CAR-T cell products with a high proportion of naive T cells have better therapeutic effects after infusion than those with a low proportion of naïve T cells (26–28). We used flow cytometry to detect the proportion of naive cells (CD62L+CD45RO-) in the CAR-T cells. Compared with uninfected T cells, T cells infected with virus had an obviously lower proportion of naive T cells. The 32-degree- and 37-degree-infection groups had higher proportions of naive T cells, in terms of both CD8-positive T cells or CD4-positive cells, than the other infection groups (**Figures 3A–C**). Immune checkpoint expression is an important indicator for evaluating the quality of CAR-T cell products. CAR-T cells with lower expression of immune checkpoints have better therapeutic effects than those with higher expression of immune checkpoints. We detected the expression of PD1, TIM3 and LAG3 on the surface of CAR-T cells (29–32). Our results showed that compared with uninfected T cells, T cells infected with virus had a significantly higher percentage of immune checkpoint expression. There was no significant difference in the expression of immune checkpoints between different temperature groups, but a lower expression trend was observed in the 23-degree group and the 32-degree group (**Figures 3D–F**). These results were also reproduced in CD123 CAR-T cells (**Supplement 2A–F**).

32-Degree Infection Generates CAR-T Cells With the Best Killing Activity and Cytokine Secretion

The ultimate function of CAR-T cells lies in their oncolytic ability. Therefore, we coincubated CAR-T cells from different

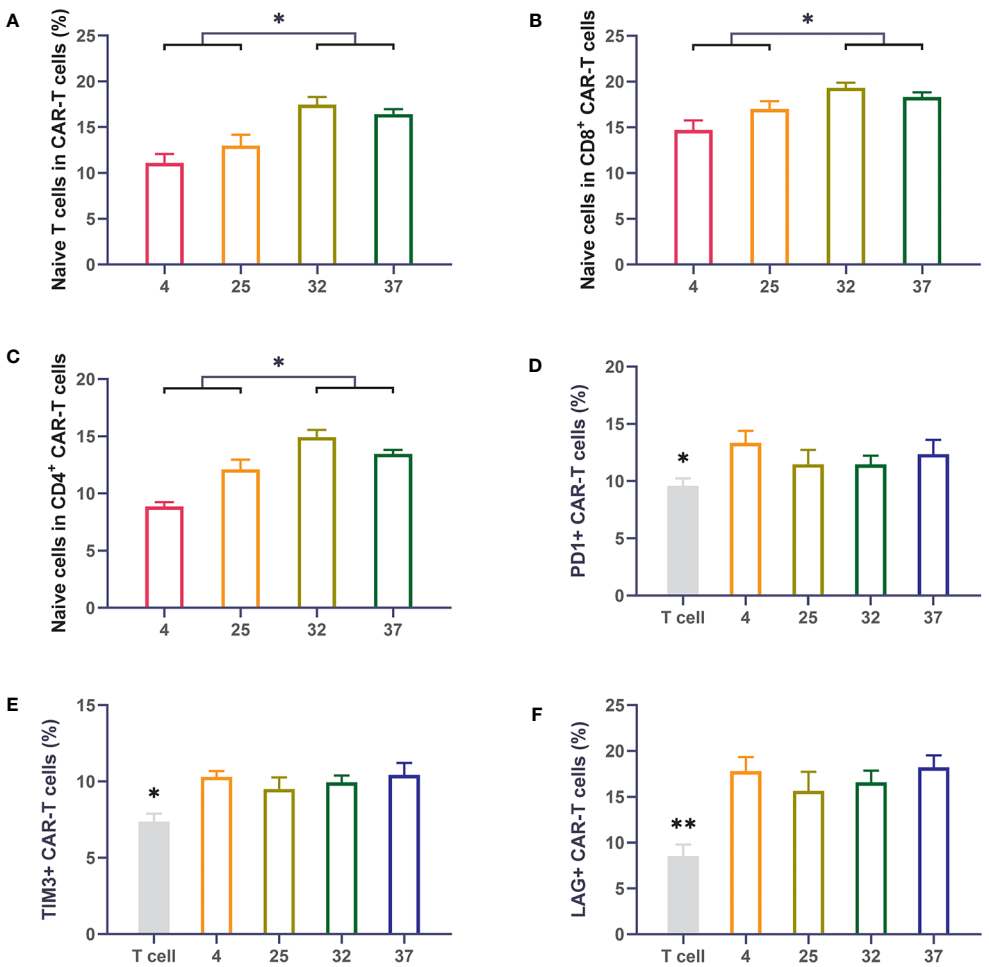

FIGURE 3 | Infection temperature can affect the proportions of CD19 CAR-T cell subsets and the expression of immune checkpoints **(A–C)** The proportion of naive T cells among CD3-positive, CD8-positive and CD4-positive CD19 CAR-T cells generated at different infection temperatures. **(D–F)** The expression of PD1, TIM3, and LAG3 on the surface of CD19 CAR-T cells generated at different infection temperatures. Three independent experiments were conducted. Mean ± SD. *$p < 0.05$, **$p < 0.01$.

temperature groups and target tumor cells at a ratio of 1:1 and then used flow cytometry to detect cytotoxicity. Compared with uninfected T cells, all CAR-T cells killed tumor cells significantly. The results showed that the CAR-T cells infected at 32 degrees had the strongest killing activity of the CAR-T cell groups infected at the 4 different temperatures (**Figure 4A**). The same result was also verified in CD123 CAR-T cells (**Figure 4E**). Cytokine storm is one of the problems faced by CAR-T cell therapy. To detect the ability of the CAR-T cells to induce cytokine release syndrome (CRS)-related toxicity, we used flow cytometry to detect the expression levels of related cytokines. Our results showed that CD19 CAR-T cells had significantly higher levels of cytokine secretion than uninfected T cells. Compared with the CAR-T cells generated at other temperatures, the CAR-T cells infected at 32 degrees secreted more cytokines, especially IFN-γ (**Figures 4B–D** and **Supplement 3A–D**). The same result was also verified in CD123 CAR-T cells (**Figures 4F–H** and **Supplement 3E–H**).

The Effect of Infection Temperature on CAR-T Cells Is Not Clearly Reflected in the Mouse Tumor Model

To further verify the results of our *in vitro* experiments, we injected mice with luciferase-expressing NALM-6 cells through the tail vein. On the third day after the tumor cells were injected, we injected the produced CAR-T cells into the mice through the tail vein. In vivo imaging technology was used to detect tumor burden in mice at specific time points. Our results show that compared with the uninfected T cell group, the group of mice injected with CAR-T cells had a significantly lower tumor burden and longer survival time (**Figures 5A–C**). The lack of obvious differences between mice injected with CAR-T cells from each temperature group may be due to the considerable heterogeneity between mice.

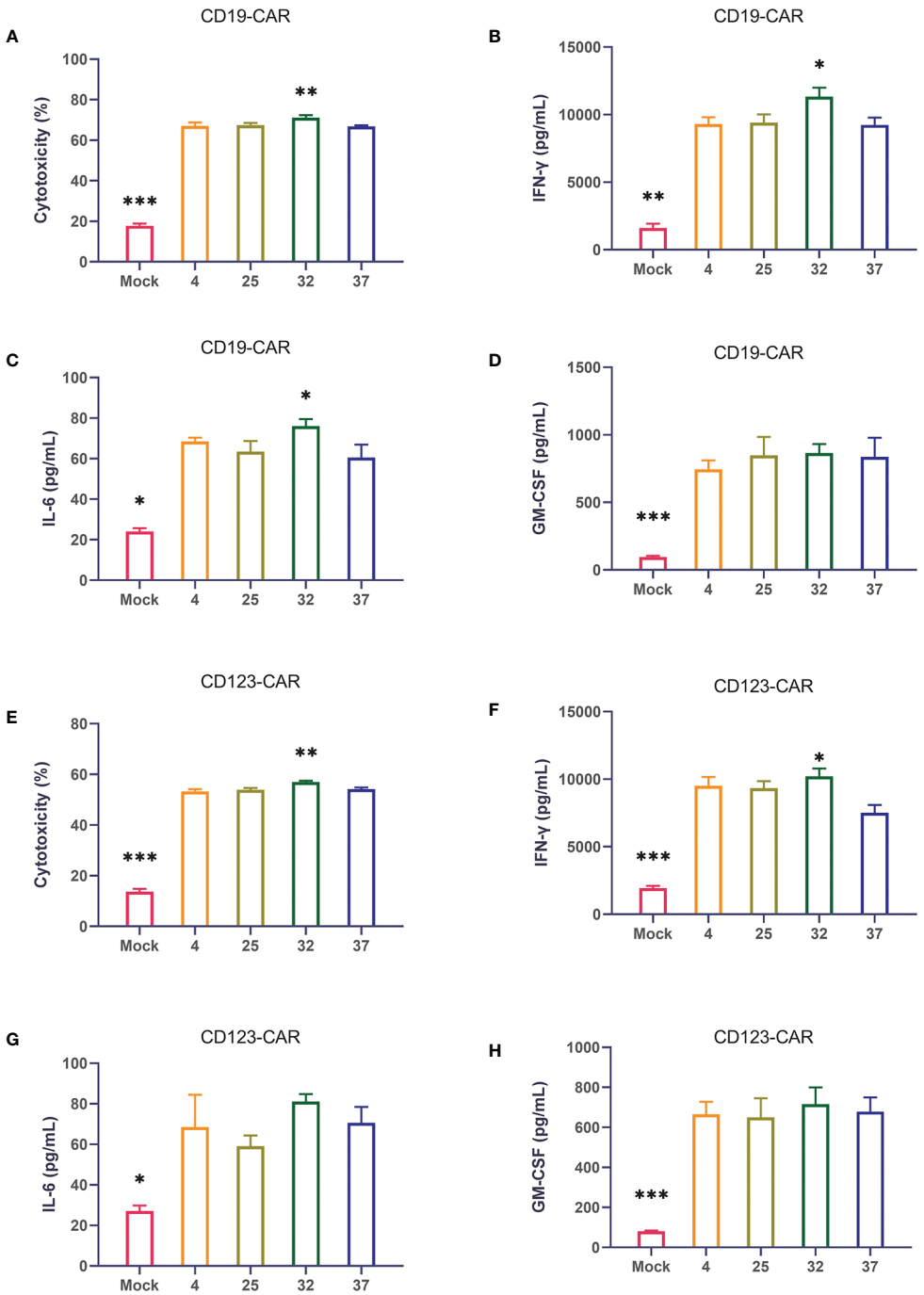

FIGURE 4 | 32-Degree infection generates CAR-T cells with optimal cytotoxicity and cytokine secretion **(A–D)** Determination of the cytotoxicity of CD19 CAR-T cells generated at different infection temperatures. CD19 CAR-T cells were coincubated with CD19-positive NALM-6 cells for 24 hours, and the concentrations of IFN-γ, IL-6 and GM-CSF in the culture supernatant were detected. **(E–H)** Determination of the cytotoxicity of CD123 CAR-T cells generated at different infection temperatures. CD19 CAR-T cells were coincubated with CD123-positive MOLM-13 cells for 24 hours, and the concentrations of IFN-γ, IL-6 and GM-CSF in the culture supernatant were detected. Three independent experiments were conducted. Mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

DISCUSSION

Recently, CAR-T cell therapy has become an important method for the treatment of hematological malignancies. There are a variety of methods available for gene delivery in CAR-T cell preparation, including lentivirus, retrovirus, mRNA, transposon, and sleeping beauty methods. Lentiviruses have a wide host range, infect both dividing and nondividing cells, and can

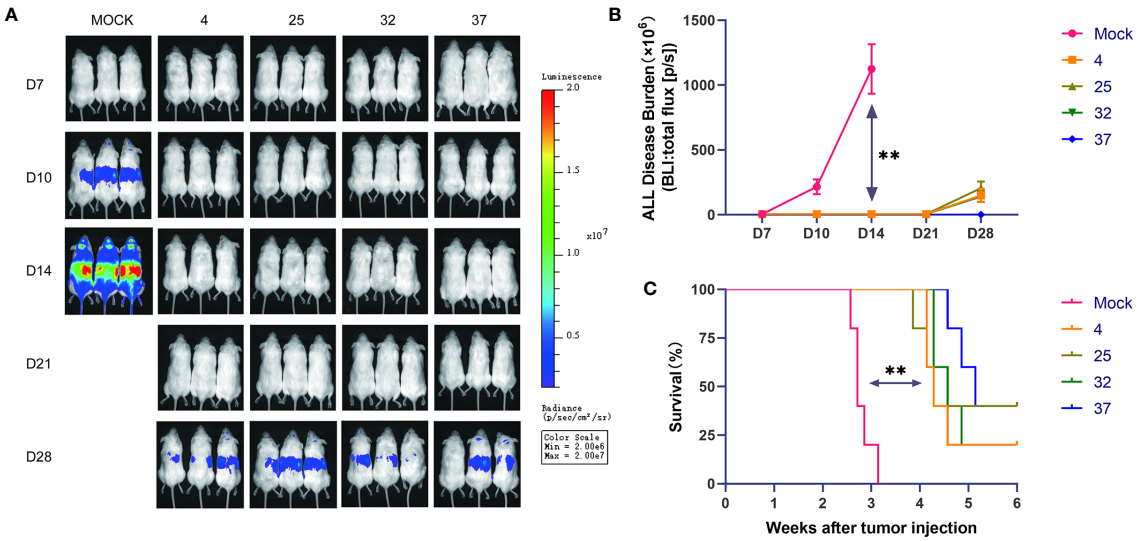

FIGURE 5 | Effect of infection temperature on CAR-T cells in a mouse tumor model **(A)** Mice were injected with luciferase-containing NALM-6 cells through the tail vein. On the third day, the prepared CAR-T cells were injected into the mice through the tail vein. The tumor load in the mice was detected by *in vivo* imaging technology on the 7th, 10th, 14th, 21st and 28th days. (n=5, each group gives a representative picture of 3 mice). **(B)** Quantitative graphs of tumor burden in each group of mice at different time points are shown. **(C)** Survival of mice injected with uninfected T cells or CD19 CAR-T cells generated at different infection temperatures. Mean ± SD. **p < 0.01.

persistently express the delivered genes. Therefore, lentiviruses are widely used as gene transfer vectors in the production of CAR-T cells. The conditions under which the lentivirus infect T cells in the production of CAR-T cells are a key factor. Here, we studied the effects of different infection temperatures on the preparation of CAR-T cells. Our results recommend using 32-degree infection because the CAR-T cells produced by 32-degree infection seem to have a balance between function and phenotype.

To confirm our results, we constructed two CAR structures that target different antigens. First, we successfully produced CAR-T cells by infecting activated T cells with lentivirus. Cell function verification was then carried out, confirming that both types of CAR-T cells could lyse tumor cells and secrete sufficient cytokines. It is worth noting that our CD19 CAR gene sequence was similar to the sequence used in tisagenlecleucel (16), which was approved by the FDA in 2017, suggesting that our CD19 CAR-T cell may be suitable for practical applications.

Cell viability and proliferation capacity are important quality parameters for CAR-T cell products. Our test results show that cells infected at 37 degrees had the highest proliferation capacity, which may be because the 37-degree environment is more conducive to cell growth. However, CAR-T cells infected at 4 degrees had the highest infection efficiency, which may be related to the fact that a low temperature is beneficial to the survival of the virus.

The proportions of cell subsets within CAR-T cell populations and checkpoint expression levels have now become important indicators for measuring the function of CAR-T cells. Previous studies have confirmed that a large proportion of naive T cells and low expression of immune checkpoints are related to improved prognosis of CAR-T cell therapy (26–32). We used flow cytometry to detect the distribution of cell subsets within CAR-T cells and checkpoint expression levels. In the 32-degree- and 37-degree-infection groups, the proportions of naive cells were significantly higher than those in the 4-degree- and 23-degree-infection groups. The checkpoint expression level in the 25-degree- and 32-degree-infection groups was relatively low, but there was no statistical difference. The reason why the 4-degree-infection group had the lowest proportion of naive cells remains to be further explored. Next, we further verified the functional differences of CAR-T cells generated at different infection temperatures through cytotoxicity and cytokine secretion analyses. Although CAR-T cells generated at 4 degrees had a higher infection rate than CAR-T cells generated at different temperatures, they did not show the strongest cytotoxicity or secretion of cytokines when they were incubated with target cells *in vitro*. This may be related to the higher expression of immune checkpoints and the lower proportion of naive cells in the CAR-T generated at degrees. In addition, the CAR-T cells generated at 32 degrees had the strongest cytotoxicity and greater cytokine secretion than those generated at 4 degrees. Later, we conducted an *in vivo* model study, and the survival time and *in vivo* tumor burden of mice injected with CAR-T cells from different infection temperature groups were not significantly different. Reducing the heterogeneity in tumor burden in the mouse model and expanding the mouse sample size may help to detect differences.

Novartis and Bristol Myers use lentiviruses to infect at 37 degrees and 32 degrees respectively to produce CAR-T cells (16,

21), and Kite Pharma uses a 32-degree infection with gamma-retrovirus to infect T cells (15). This article only studied the infection temperature used for lentiviruses. Whether the optimal temperature for T cell infection is similar between γ-retroviruses and lentiviruses, which belong to the Retroviridae family, needs further research. Development of semi-automated devices that can reduce the hands-on time and standardize the production of clinical-grade CAR T-cells, such as CliniMACS Prodigy from Miltenyi, is key to facilitate the development of CAR T-cell therapies (33, 34). Our research is done in well plates or culture flasks. Whether this view is consistent in semi-automatic equipment remains to be studied. In summary, our results report for the first time that temperature can affect the function and phenotype of CAR-T cells produced by lentivirus infection. We recommend using lentivirus to infect T cells at 32 degrees to produce CAR T cells. This research may provide an important reference for the production of CAR-T cell products.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Tianjin First Central Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of Tianjin First Central Hospital.

AUTHOR CONTRIBUTIONS

MFZ designed the research. XJ, WYL, MZ, XX, RS, and YXW performed the research. XJ and MFZ analyzed the data. XJ, WYL, MZ, and MFZ wrote the manuscript. XJ, WYL, and MFZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.638907/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Reactions Related to CAR-T Cell Therapy

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The application of chimeric antigen receptor (CAR) T-cell therapy as a tumor immunotherapy has received great interest in recent years. This therapeutic approach has been used to treat hematological malignancies solid tumors. However, it is associated with adverse reactions such as, cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), off-target effects, anaphylaxis, infections associated with CAR-T-cell infusion (CTI), tumor lysis syndrome (TLS), B-cell dysplasia, hemophagocytic lymphohistiocytosis (HLH)/macrophage activation syndrome (MAS) and coagulation disorders. These adverse reactions can be life-threatening, and thus they should be identified early and treated effectively. In this paper, we review the adverse reactions associated with CAR-T cells, the mechanisms driving such adverse reactions, and strategies to subvert them. This review will provide important reference data to guide clinical application of CAR-T cell therapy.

Keywords: chimeric antigen receptor T cell, immunotherapy, adverse reactions, mechanism, coping strategies

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INTRODUCTION

Several studies have explored various methods for treatment of malignant tumors. In 2008, American immunologists James P Alison and Japanese immunologists Tasuku Honjo won the 2018 Nobel Prize in physiology or medicine for their contributions to the field of tumor immunity. The findings of the study provide basis for development of novel treatment methods for malignant tumors, and indicate that tumor immunotherapy has a huge potential for treatment of various tumors. CAR-T cell therapy, a type of tumor immunotherapy has been widely explored over the past few years, and is widely used in treatment of malignant tumors. CARs can target any cell surface molecules. CAR does not require antigen processing or human lymphocyte antigen (HLA) presentation unlike T cell receptor (TCR)-modified T cells. Therefore, it is broadly applicable to patient populations with different HLAs (1, 2).

Although advances in CAR-T cell therapy for treatment of hematological malignancies have been reported, it is associated with severe adverse reactions some of which are life-threatening. This paper reviews adverse reactions that occur during treatment with CAR-T cells, their formation mechanisms, and strategies for alleviating them. The findings of this study provide a basis for clinicians to improve management of adverse reactions related to CAR-T cell therapy.

ADVERSE REACTIONS RELATED TO CAR-T CELL THERAPY

Cytokine Release Syndrome (CRS)

CRS, also known as "cytokine storm", refers to the systemic inflammatory response syndrome caused by infection or administration of various drugs. CRS was first reported in 1990 in clinical trials exploring monoclonal antibody OKT3 as an immunosuppressant for kidney transplant patients (3). Advances in development of CAR-T cell therapy in recent years, has resulted in many studies exploring CRS which is the most common adverse reaction for this therapy. Studies report that incidence of CRS in CAR-T cell experiments targeting CD19 and BCMA is significantly high (**Table 1**). Previous meta-analysis reported that the incidence of CRS in patients with hematological malignancies receiving CAR-T cell therapy is approximately 55.3% (16), and the incidence of severe cytokine release syndrome (sCRS) is approximately 18.5% (17). In addition, use of immune-targeted drugs such as Nivolumab (18, 19) and Brentuximab Vedotin (20) is associated with severe CRS. In 2018, the American Society for Blood and Marrow Transplantation (ASBMT) stated that if after receiving any immunotherapy, the patient's endogenous or induced immune effector cells are activated in large numbers, then symptoms for the resulting superphysiological response must include fever and may also include hypotension, capillary leakage (hypoxia), and end-organ dysfunction for the response to be referred as CRS (21). The new definition expands application of the term as it is not limited to CAR-T cell therapy. Mild clinical manifestations of CRS include fever (mainly the first symptom), fatigue, headache, joint pain, and myalgia. Notably, severe cases are characterized by hypotension and high fever. Further exacerbations may cause shock, vascular leakage, disseminated intravascular coagulation (DIC), and multiple organ dysfunction syndrome (MODS) (22).

Pathogenesis of CRS associated with CAR-T cell therapy has not been fully explored. Previous experimental studies report that pathogenesis may be linked to the following mechanisms: (a) CAR-T cells release numerous cytokines after being activated, such as IL-6, IL-10, TNF-α, GM-CSF, and IFN-γ. Out of these cells levels of IL-6 are significantly higher compared with the levels of other cytokines. These cytokines thus induce CRS (23, 24). (b) Lysed tumor cells release a high amounts of cytokines, such as TNF-α (22). (c) IFN-γ induces activation of immune cells, especially macrophages. Activated macrophages release several cytokines, such as IL-6, IL-1, IL-10, TNF-α, and NO (22, 24–26). Norelli et al. performed a study using mouse animal models and reported that IL-1 and IL-6 derived from monocytes are cytokines necessary for production of CRS and neurotoxicity (26). (d) IL-6 can induce a strong immune response and play a key role in production of CRS (26). (e) Release of high amounts of cytokines, such as IL-6, TNF-α, and IFN-γ, can induce activation of endothelial cells. The activated endothelial cells play a significant role in the pathological process of CRS. Activated endothelial cells secrete large quantities of IL-6, resulting in a vicious circle (27, 28). (f) Incidence and severity of CRS are positively correlated with the patient's tumor burden

TABLE 1 | Summary of the incidence of CRS and ICANS in patients with hematological malignant tumors (partial data).

Time	Disease	CAR-T Cell Therapy	Phase	Case	Age (years)	Any Grade		Gade 3/4		Trial registration
						CRS	ICANS	CRS	ICANS	
2019	relapsed / refractory MM	BCMA-CAR-T cells	I	33	37–75	25 (76%)	14 (42%)	2 (6%)	1 (3%)	NCT02658929 (4)
2019	relapsed/refractory B-ALL	CD19-CAR-T cells	I	25	1–22.5	20 (80%)	18 (72%)	4 (16%)	7 (28%)	NCT01860937 (5)
2019	relapsed / refractory MM	BCMA-CAR-T cells	I	25	44–75	22 (88%)	8 (32%)	8 (32%)	3 (12%)	NCT02546167 (6)
2019	relapsed / refractory MM	BCMA-CAR-T cells	I	17	35–73	17 (100%)	–	6 (35%)	–	NCT02435849 (7)
2019	relapsed/refractory B-ALL	CD19-CAR-T cells	I/II	53	0–65	53 (100%)	8 (15%)	19 (36%)	–	NCT02965092 (8)
2019	relapsed / refractory MM	BCMA-CAR-T cells + CD19-CAR-T cells	II	21	49.5–61	16 (91)	2 (10%)	1 (5%)	–	ChiCTR-OIC-17011272 (9)
2019	relapsed or refractory diffuse large B-cell lymphomas	CD19-CAR-T cells	IIa	111	22–76	64 (58%)	23 (21%)	24 (22%)	13 (12%)	NCT02445248 (10)
2020	relapsed/refractory B-ALL	CD19-CAR-T cells	I	23	10–67	18 (78.3%)	3 (13%)	5 (21.7%)	1 (4)	ChiCTR-ONN-16009862, ChiCTR-1800019622 (11)
2020	relapsed or refractory large B-cell lymphomas	CD19-CAR-T cells	I	269	54–70	113 (42%)	80 (30%)	6 (2%)	27 (10%)	NCT02631044 (12)
2020	relapsed / refractory mantle-cell lymphoma	CD19-CAR-T cells	II	68	38–79	62 (91%)	43 (63%)	10 (15%)	21 (31%)	NCT02601313 (13)
2021	relapsed / refractory MM	BCMA-CAR-T cells	II	128	33–78	107 (84%)	23 (18%)	7 (5%)	4 (3%)	NCT03361748 (14)
2021	relapsed / refractory DLBCL	BCMA-CAR-T cells	I	31	24–82	23 (74%)	14 (45%)	0	10 (32%)	– (15)

MM, multiple myeloma; B-ALL, B-cell acute lymphoblastic leukemia; DLBCL, diffuse large B-cell lymphoma; BCMA, B-cell maturation antigen.

–: Relevant data are not mentioned in the experiment.

(29, 30). This can be attributed to activation of several CAR-T cells or destruction of high number of tumor cells being in the body (**Figure 1**).

Clinically, different treatment plans are used for treatment of CRS based on the CRS grade. Symptomatic treatment is adopted for patients with grade 1 CRS; supportive and symptomatic treatment are adopted for patients with grade 2 CRS, and patients who are elderly or have serious complications should receive immunosuppressive therapy. Supportive treatment and immunosuppressants are adopted for patients with grade 3 and 4 CRS (31). Most commonly used immunosuppressants include Tocilizumab and glucocorticoids. The latest ASTCT consensus recommends use of Tocilizumab for CRS ≥2. The dosage regimen is as follows: patients with body weight ≥ 30kg are administered with 8mg/kg; patients with body weight < 30kg with 12mg/kg and the maximum dose should be ≤ 800mg/dose (32). Furthermore, a slow intravenous administration (>1h) should be adopted and the administration frequency should be ≤ 4 times. Tocilizumab blocks membrane-bound and soluble IL-6 receptors, and prevents IL-6 from binding to its receptors through competitive inhibition. It then neutralizes the activity of IL-6 by classic signaling, trans-signaling and trans-presentation (33, 34). Therefore, tocilizumab is an antagonist of IL-6 receptor, and can block the vicious circle of IL-6 in CRS through the above-mentioned mechanisms. Tocilizumab rapidly relieves the clinical symptoms of CRS without affecting proliferation and anti-tumor activity of CAR-T cells (29, 35). It has been approved by the United States Food and Drug Administration (FDA) as a first-line drug for management of CRS caused by CAR-T cell therapy (36). If the symptoms cannot be alleviated or relief is not evident, tocilizumab should be replaced with glucocorticoids or a combinatory therapy of glucocorticoids and tocilizumab used. The high immunosuppressive effect of glucocorticoids plays an important role in treatment of ≥3 grade CRS. Glucocorticoid administration schedule is as follows: patients with grade 3 CRS are administered with methylprednisolone (2mg/kg/day) or dexamethasone (10mg/6h) whereas patients with grade 4 CRS are given methylprednisolone (1000mg/day for 3 consecutive days) (37, 38): Glucocorticoids affect proliferation and activity of CAR-T cells in the body (39, 40). However, several studies have reported contradicting results recently. Some studies report that glucocorticoids do not affect activity and efficacy of CAR-T cells (41, 42). However, glucocorticoids have significant immunosuppressive effect, therefore, the dosage and the treatment course of glucocorticoids should be designed carefully. Sachdeva et al. (43) reported that granulocyte-macrophage colony-stimulating factor (GM-CSF) is a key CRS-promoting protein. In addition, the findings showed that the incidence rate of CRS in GM-CSF-knockout CAR-T cells is significantly lower compared with the rate of CRS in GM-CSF-intact CAR-T cells. These findings imply that inhibition of GM-CSF can prevent occurrence of CRS. Furthermore, therapeutic plasma exchange (TPE) and hemofiltration are potential therapies for some patients with severe CRS (44, 45).

Currently, diagnosis of CRS mainly depends on clinical manifestations of patients. Cytokines or other biomarkers related to occurrence and development of CRS are screened, and targeted intervention and treatment is administered on time based on changes in levels of these biomarkers, to effectively prevent, block and treat CRS. However, it is challenging to find key cytokines of CRS and their associated treatment time points. This is mainly because CRS is caused by a variety of cytokines

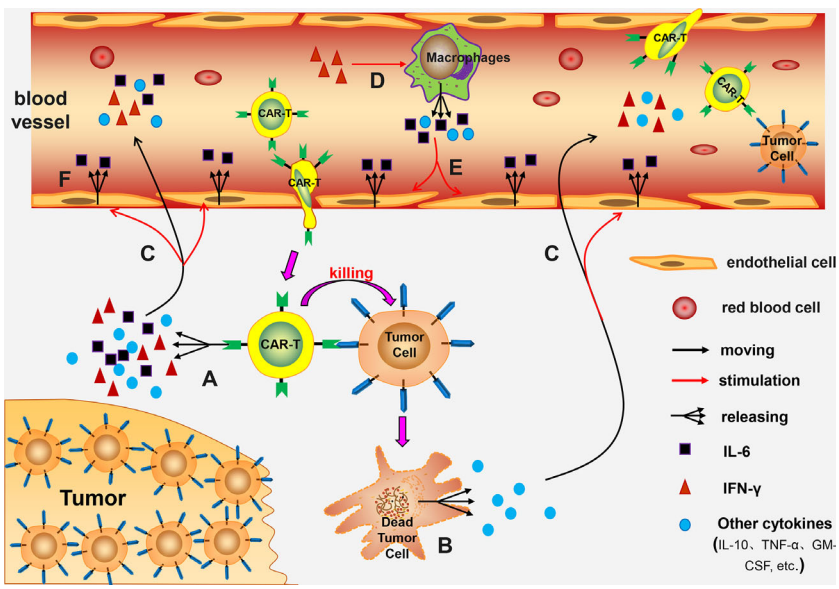

FIGURE 1 | The mechanism of CRS. **(A)** Activated CAR-T cells release numerous cytokines; **(B)** The lysed tumor cells release a large number of cytokines; **(C)** These cytokines enter the blood circulation and activate endothelial cells; **(D)** IFN-γ further induces macrophages activation; **(E)** The activated macrophages release many cytokines into the blood circulation which activate endothelial cells; **(F)** The activated endothelial cells release large amounts of IL-6, forming a vicious circle.

and multiple factors. Furthermore, the onset time of CRS is different in different patients. Some patients may develop CRS on the first or second day after receiving CAR-T cells, and some patients may develop CRS several days later or even later, therefore the timing of detecting cytokine changes is not definite. In addition, several types of related cytokines are present during the onset of CRS, and it is expensive to detect CRS-related cytokines at regular intervals (a few hours or every day). The optimum time point for anti-cytokine therapy for CRS is still being investigated. Freyer et al. (46) reports that detection of CRS is done through clinical diagnosis, and inflammatory cytokine profiles are useful in confirmatory tests; however, these data are not used to determine the grade or treatment of CRS. C-reactive protein (CRP) and ferritin are correlated with the severity of CRS and can be used as biomarkers to determine the grade of CRS (27, 47). In addition, Hay et al. (27) reported that monocyte chemoattractant protein-1 (MCP-1) has higher specificity and sensitivity in predicting and diagnosing grade 4 CRS. The level of MCP-1 in blood increased (>1343.5pg/mL) accompanied by fever (≥ 38.9C) within 36 hours after receiving CAR-T cells. Sensitivity and specificity of predicting grade 4 CRS were 100% and 95%, respectively.

Immune Effector Cell-Associated Neurotoxicity Syndrome (ICANS)

ICANS also known as neurotoxicity, is the second most frequent adverse event of CAR-T cell therapy. It can occur simultaneously with or after presentation of CRS. ICANS is activation or participation of T cells (autologous or exogenous) and/or other immune cells after receiving any immunotherapy, resulting in neurotoxic symptoms (21). The term of CAR-T-cell-related encephalopathy syndrome (CRES) is relatively limited. CRES only refers to neurotoxic symptoms caused by CAR-T cell therapy, therefore the term ICANS is commonly used. CAR-T cell experiments targeting CD19 and BCMA, report significantly high incidence of ICANS (**Table 1**). A previous meta-analysis reported that the incidence of ICANS in patients with hematological malignancies receiving CAR-T cell therapy was approximately 37.2% (16), whereas a different meta-analysis reported that the incidence of ICANS was approximately 21.7% (17). The main manifestation of CRES is toxic encephalopathy. Early symptoms include reduced attention, and language, and writing disorders. Other symptoms and signs include confusion, lethargy, and tremor. In severe cases, seizures, motor weakness, elevated intracranial pressure (ICP), and cerebral edema are reported (48–51).

Pathogenesis of ICANS has not been fully explored. Studies report that ICANS pathogenesis may be related to the following factors: (a) Levels of IL-1, IL-6, IL-15, TNF-α, and IFN-γ in blood are elevated, and are positively correlated with severity of ICANS (31, 38, 52, 53). These cytokines facilitate development and progression of CRES; (b) Activation of endothelial cells of the central nervous system (CNS) results in an increase in permeability of blood-brain barrier (BBB), which allows cytokines in the blood to enter the cerebrospinal fluid (CSF) and promotes development of ICANS (54, 55). The protein content of cerebrospinal fluid in CRES patients is high, implying that the BBB is destroyed (38). (c) CAR-T cells can enter the CSF and damage the CNS (52, 56). Studies report that the number of CAR-T cells in CSF of ICANS patients is significantly higher compared with that of patients without CRES (29, 52, 56). (d) Incidence of ICANS is positively correlated with the tumor burden and severity of CRS (31, 53). Early prevention and intervention of CRS may reduce occurrence of ICANS to a certain extent. Although tocilizumab can treat CRS, it has little or no effect on ICANS, primarily because it cannot penetrate BBB (29, 31).

The therapeutic regimens of ICANS include (38) fasting and intake of water, nutritional support treatment, and improved neurological examination (i.e., electroencephalogram, 30 minutes per day)for grade 1 ICANS whereas tocilizumab (8mg/kg, intravenous administration> 1 hour) or siltuximab (11mg/kg, intravenous administration> 1 hour) can be administered for grade 1 ICANS with CRS. In addition, tocilizumab (8mg/kg, intravenous administration>1 hour) or siltuximab (11mg/kg, intravenous administration>1 hour) is administered for grade 2 ICANS. If the above drugs are ineffective or poorly effective or a comorbidity of ICANS with CRS is reported, glucocorticoids (dexamethasone 10mg/kg/6h or methylprednisolone 1mg/kg/12h) should be administered. For grade 3 ICANS, it is recommended to transfer patients to ICU for further treatment and administration of glucocorticoid therapy (the dose is the same as above, until the patient's condition improves to grade 1 ICANS, and then the dose is gradually reduced). Patients with grade 4 ICANS are given high-dose glucocorticoid (methylprednisolone 1g/day for 3 days, then the dose is gradually reduced; the whole course of treatment is 9 days). Further, ICANS patients with CRES-related seizures are treated with glucocorticoids combined with levetiracetam (500-1000mg/12h) whereas ICANS patients with raised intracranial pressure should be treated with glucocorticoids combined with acetazolamide. ICANS patients with cerebral edema should receive high-dose glucocorticoids, and hyperventilation and hypertonic therapy (for example, mannitol: initial dose 0.5-1g/kg/6h, Maintenance dose 0.25–1 g/kg/6 h) should be administered concurrently. Unlike tocilizumab, siltuximab is a monoclonal antibody binding to IL-6. It has a high affinity to IL-6 and can prevent binding of IL-6 to its receptor. Gust et al. speculated that tocilizumab may increase the level of IL-6 in CSF and aggravate neurotoxicity; however, administration of siltuximab (IL-6 antagonist) does not increase the level of IL-6 in CSF; therefore, siltuximab can be used as the first choice drug for treatment of ICANS (55). In addition, Norelli et al. (26) reported that IL-1 and IL-6 derived from monocytes played an important role in occurrence and development of CRS and ICNAS in mouse models. Early use of anakinra (IL-1 receptor inhibitor) can effectively prevent CRS and ICANS.

Currently, diagnosis of ICANS mainly depends on clinical manifestations of patients. However, similar to CRS, it is challenging to find key cytokines associated with ICANS pathogenesis and the time points of treatment related to them. CRP and ferritin can be used in prediction and diagnosis of

ICANS. Karschnia et al. (57) analyzed the acute-phase protein levels in serum of 25 patients who developed ICANS after CAR-T cell therapy. The findings showed increase in the levels of CRP and ferritin in most patients after receiving CAR-T cells. Notably, the CRP reached a maximum level before appearance of neurological symptoms. On the other hand, the level of ferritin peaked after the onset of neurological symptoms. Moreover, ferritin level of patients with high-grade ICANS reached a peak (4533 ± 930 ng/mL, normal value: 1646 ± 472 ng/mL) 2 days after onset of neurological symptoms.

Off-Target Effects

For effective targeted therapy, tumor antigens to be targeted should only be expressed on tumor cells, and not expressed or expressed in very low on normal cells. These tumor antigens are known as tumor-specific antigens (TSAs). However, TSAs are few, and tumor-associated antigens (TAAs) are mainly used for targeted therapy [For example in digestive tumors, some TAAs are targeted by CAR-T cells (**Table 2**)]. CAR-T cells injected into the body kill tumor cells expressing the target antigens, and normal cells expressing the target antigens. This phenomenon is known as on-target-of-tissue effects. These effects sometimes can cause severe side effects and even death. Morgan et al. (89) developed CAR-T cells targeting ERBB2 (HER-2/neu) for treatment of cancer patients with ERBB2 overexpression. One patient with colon cancer that had metastasized to the lungs and liver received this treatment. Within 15 minutes of infusion with CAR-T cells, the patient developed respiratory distress. Chest X-rays showed pulmonary infiltration, and despite aggressive medical intervention, the patient died 5 days later. The researchers speculate that CAR-T cells entered the lungs after infusion. In this case, CAR-T cells targeted lung epithelial cells with low ERBB2 expression and a large number of cytokines were released, resulting in CRS. Use of TSAs to develop the corresponding CAR-T cells is an effective method to eliminate off-target effects. However, finding new TSAs is challenging and expensive. Therefore, studies should aim at increasing the specificity of CAR-T cells by optimizing the structure of CAR. Commonly used methods of increasing efficacy include synNotch receptor (90) and inhibitory CAR (iCAR) (91).

Anaphylaxis

Most CAR-T cells currently used in clinical trials contain antigen-recognition domains derived from murine monoclonal antibody (mAb) (92), which may be the major cause of anaphylaxis. In a previous clinical trial (93), four patients received multiple injections of MSLN (mesothelin)-CAR-T cells, and one of them developed cardiorespiratory failure at the end of the third infusion. Analysis showed presence of human anti-mouse antibodies and elevated trypsin antibodies in the patient's serum, implying that it was an IgE-mediated anaphylactic event. The adverse effects observed during the trial

TABLE 2 | Some TAAs targeted by CAR-T cells (taking gastrointestinal tumors as an example).

Associated Malignancy	Target Antigens	Co-stimulating domain	Generation of CAR-T	Authors	Reference
esophageal cancer	EphA2	4-1BB	2nd	Shi, et al.	(58)
liver	NKG2DL	4-1BB	2nd	Sun, et al.	(59)
cancer	GPC3	CD28	2nd	Wu, et al.	(60)
		CD28,4-1BB	3rd	Jiang, et al.	(61)
		CD28, ICOSL	3rd	Hu, et al.	(62)
		CD28	4th (IL12)	Liu, et al.	(63)
		CD28,4-1BB	4th (IL15,IL21)	Batra, et al.	(64)
	CD147	4-1BB	2nd	Zhang, et al.	(65)
	AFP	CD28	2nd	Liu, et al.	(66)
gastric	Trop2	CD28,4-1BB	3rd	Zhao, et al.	(67)
cancer	PD-L1	CD28,4-1BB	3rd	Zhao, et al.	(67)
	CLDN18.2	CD28 or 4-1BB	2nd	Jiang, et al.	(68)
	FOLR1	CD28	2nd	Kim, et al.	(69)
	HER2	4-1BB	2nd	Han, et al. ;Song, et al.	(70, 71)
	MSLN	CD28	2nd	LV, et al.	(72)
	NKG2DL	4-1BB	2nd	Tao, et al.	(73)
pancreatic	CEA	CD28	4th (IL18)	Chmielewski, et al.	(74)
cancer		CD28	2nd	Chmielewski, et al.	(75)
	HER2	4-1BB	2nd	Raj, et al.	(76)
	FAP	4-1BB	2nd	Lo, et al.	(77)
	CD47	CD28	2nd	Golubovskaya, et al.	(78)
	tMUC1	CD28	2nd	Yazdanifar, et al.	(79)
	B7-H3	CD28 or 4-1BB	2nd	Du, et al.	(80)
	MSLN	4-1BB	2rd	Zhang, et al.	(81)
	PD-L1	CD28,4-1BB	3rd	Yang, et al.	(82)
colorectal	GUCY2C	CD28,4-1BB	3rd	Magee, et al.	(83, 84)
cancer	NKG2DL	CD28,4-1BB	3rd	Deng, et al.	(85)
	CEA	CD28	2nd	Zhang, et al.	(86)
	DCLK1	CD28	2nd	Sureban, et al.	(87)
	EpCAM	CD28,4-1BB	3rd	Zhang, et al.	(88)

Taking gastrointestinal tumors as an example, the above-mentioned commonly used CAR-T cell targets are almost all TAAs.

may have caused by isotype switching to IgE (meaning that the specificity of the antibody remains the same, but its effects change). In addition, inappropriate timing of treatment also contributes to this situation, including treatment intervals. In another study (94), the antigen-recognition region of CAR was designed to contain only one human heavy-chain variable domain without a light-chain region or a linker. These CARs showed reduced immunogenicity and significantly reduced the size of the CAR-binding domains compared with the traditional CARs. Notably, these CARs did not show decrease in proliferation and tumor-killing effects of these CAR-T cells. Two major strategies are used in reducing immunogenicity of CAR-T cells including: (a) use of complete human sequences rather than murine sequences when constructing CAR; (b) Simplifying the structure of CAR (94–97). For patients receiving CAR-T cell therapy, the most important thing is to closely monitor changes in the condition and treatment of anaphylaxis in time. The treatment can be suspended or even terminated in case the effects are alleviated or if no response is observed.

Infections Associated With CAR-T-Cell Infusion (CTI)

Infections associated with CTI are relatively common in CAR-T cell therapy. In a clinical trial using CD19-CAR-T cells to treat relapsed B-cell acute lymphoblastic leukemia (B-ALL), about 42% of 53 patients developed infections during the first 30 days after CTI. The infections were mainly bacterial, with bloodstream infections (BSIs) showing the highest incidence (98). From 31st to 180th day after CTI, 31% of the 32 patient survivors in complete remission developed infections, mainly viral infection with respiratory viruses being more frequent.

Currently, the mechanism of CTI occurrence is unclear, and there is no unified treatment plan for prevention and treatment of CTI. The most commonly used CD19-CAR-T cell therapy and causes of infection after CTI may include (a) Severe CRS and/or CRES as a result of CAR-T cell therapy. These patients mostly undergo treatment in intensive care unit (ICU), which may increase the risk of nosocomial infections (31, 99); (b) Long-term and high-dose use of glucocorticoids for treatment of severe CRS and/or CRES can reduce the patient's immunity (31, 38); (c) CD19-CAR-T cells can cause B-cell dysplasia and hypogammaglobulinemia, increasing risks of infection (29, 100, 101); (d) Patients receiving stronger anti-tumor drugs and high-dose CAR-T cells may have a higher risk of infection (102); (e) High-grade CRS is significantly positively correlated with risk of infection (98) (**Figure 2**).

The main prevention and treatment measures for CTI related infections include (a) Paying attention to protection and avoiding cross-infections; (b) Administration of antibiotics and immunoglobulins to prevent and treat infections (102); (c) Reduction of the duration for glucocorticoids administration; (d) Active treatment of CRS; (e) Reduction of the input dose of CAR-T cells. Notably, patients receiving CD19-CAR-T cells rarely develop lethal infections (98, 102).

Tumor Lysis Syndrome (TLS)

A large number of tumor cells appear necrosis in a short time, resulting in the release of high amounts of intracellular substances and metabolites into the blood. Kidneys do not completely eliminate these substances, resulting in a range of serious metabolic disorders and clinical symptoms. This condition is known as TLS. Main clinical manifestations of TLS include hyperkalemia, hyperphosphatemia, hyperuricemia,

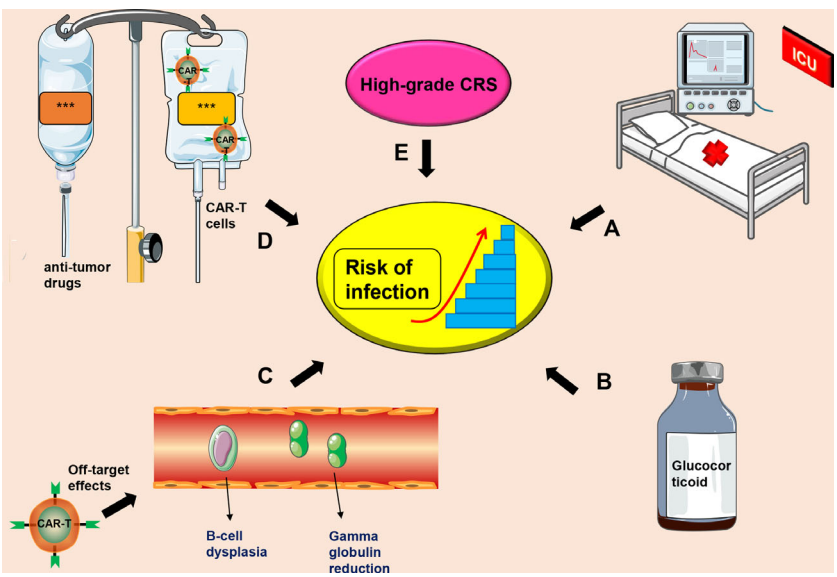

FIGURE 2 | Factors that induce CIT. **(A)** Severe CRS patients receiving further treatment in the ICU, which increases the risk of infection; **(B)** Long-term administration of high-dose use of glucocorticoids; **(C)** Off-target effects of CAR-T cell therapy may cause B-cell dysplasia and hypogammaglobulinemia; **(D)** A combination of anti-tumor drugs and CAR-T cells; **(E)** High-grade CRS. This symbol *** only represents drug information or patient information.

and hypocalcemia. In severe cases, patients may develop acute renal failure and severe arrhythmia. Incidence of TLS in hematological malignancies is significantly higher compared with that of solid tumors, especially large volume tumors and tumors characterized by vigorous metabolism, such as B-cell lymphoma, which has the highest risk of TLS (103). Use of CAR-T cell therapy as an anti-tumor therapy may also cause TLS.

Principles of treatment of TLS are similar despite the cause. Prevention and treatment principles of TLS include (a) Adequate hydration (however, for elderly patients with chronic heart or kidney disease, attention should be paid to the input); (b) Urine alkalization is no longer recommended due poor efficacy and can also precipitate calcium phosphate in the renal tubules; (c) Hypouricemic agents can be used. For patients with low or moderate risk, allopurinol can be used as a first preventive drug (104, 105). Rasburicase is the preferred preventive drug for patients at high risk of TLS, and the preferred treatment drug for TLS patients (106). Rasburicase is a recombinant urate oxidase that can convert uric acid into highly soluble allantoin, and should not be administered in patients with glucose-6-phosphate dehydrogenase deficiency. (d) Diuretics are used to maintain urine volume, thus promoting the excretion of metabolites and potassium ions; (e) Correcting electrolyte disturbance. However, asymptomatic hypocalcemia should not be corrected, to prevent occurrence of nephrocalcinosis. (f) Use of continuous renal replacement therapy (CRRT).

B-Cell Dysplasia

Currently, CD19 is the most frequently used target for CAR-T cells in treatment of hematological malignancies. Other targets include CD20, CD22, CD23, CD33, and CD123. CD19 is highly expressed on benign and most malignant B cells (non-B cells are characterized by low expression levels) (107). In addition to targeting tumor cells expressing target antigens, CAR-T cells attack normal B cells expressing target antigens, causing damage to normal B cells and ultimately leading to B-cell dysplasia (29, 99, 100, 108). B-cell dysplasia is common in CAR-T cell therapy targeting CD19 and is reported in all patients responding to treatment (100). B-cell dysplasia can last for a year (29) or even longer (4 years) (99) after disappearance of CAR-T cells in the body. The patient presents with hypogammaglobulinemia and is susceptible to infections (99). Treatment measures include gamma globulin infusion (99, 100) and prevention of infections.

Hemophagocytic Lymphohistiocytosis (HLH)/Macrophage Activation Syndrome (MAS)

HLH is a clinical syndrome characterized by excessive inflammation. It is caused by abnormal proliferation of lymphocytes and tissue cells, resulting in release of high levels of inflammatory cytokines. The main clinical manifestations of HLH include fever, hepatosplenomegaly, abnormal liver function, decreased blood cells, increased triglycerides, increased serum ferritin [≥500μg/L (109)] and decreased fibrinogen levels (110). Studies on etiology report that HLH may be caused by congenital inheritance or secondary to autoimmunity and malignancy or infection (111). MAS is a secondary HLH (sHLH). MAS is a clinical syndrome caused by excessive activation and proliferation of T cells and macrophages, resulting in release of large quantities of inflammatory cytokines. Clinical manifestations of MAS are similar to those of HLH; however, it is characterized by high incidence of central nervous system symptoms and bleeding tendency, elevated serum ferritin [≥684μg/L (112)]. Notably, MAS may not be characterized by blood cell reduction (113).

HLH/MAS is a relatively rare disease with high mortality [up to about 80% (114, 115)] and a poor prognosis. Incidence of HLH/MAS in CAR-T cell therapy is approximately 3.48% (116). Clinical manifestations of HLH/MAS and CRS are similar; therefore, it is difficult to distinguish diagnosis of the two. Some studies report that HLH/MAS is a severe manifestation of CRS.

Mechanisms of CAR-T-related HLH/MAS include (a) Lysis of tumor cells result in release of large quantities of inflammatory cytokines and pro-inflammatory cytokines (22, 117); (b) Induction of CD8+ T-cells by pro-inflammatory factors results in production of high amounts of Th1 cytokines, such as IFN-γ, TNF-α, and IL-6, which forms a positive feedback loop of inflammation (117); (c) Activated CAR-T cells can release numerous cytokines (23, 24); (d) IFN-γ is correlated with MAS, high levels of IFN-γ are correlated with severe MAS (118). IFN-γ activates macrophages, and the activated macrophages release more inflammatory cytokines (22, 24–26); (e) Serum ferritin in these patients is significantly elevated (>10,000μg/L) (47), and ferritin itself is an inflammatory mediator. Furthermore, high levels of serum ferritin can promote release of inflammatory factors by activating NF-κB signaling pathway (119); (f) Some viral infections such as Epstein-Barr virus (*EBV*) induce occurrence of sHLH (120).

Currently, there is no targeted treatment approaches available for HLH/MAS patients. In principle, more aggressive immunosuppressive therapies should be given in early stages. Glucocorticoids are the main mode of treatment. This includes intravenous injection of methylprednisolone (1g/day, continuous 3-5 days), in combination with gamma globulin (1g/kg, continuous for 2 days), the regimen can be repeated on day 14 (121). If clinical deterioration occurs after treatment or existence of sHLH is confirmed, anakinra (IL-1 receptor antagonist) should be administered (115, 122). Further, etoposide is administered to refractory patients (115, 122). Etoposide (111) or rituximab (123) is given for sHLH caused by *EBV* infection. Cyclosporine, a second line drug causes neurotoxicity (124) and should be avoided for patients with central nervous system symptoms and can be substituted with anakinra (122). Moreover, combined medication can be used.

Coagulation Disorders

Coagulation dysfunction often occurs during treatment with CAR-T cell therapy. Approximately 51%-56.6% of patients with hematological malignancies develop coagulation disorders after receiving CAR-T cell therapy (8, 125). Coagulation disorders occur within 6-20 days after infusion of CAR-T cells (125). Coagulation disorders associated with CAR-T cell therapy mainly include increased D-dimer, increased fibrinogen

degradation products, prolonged prothrombin time, decreased fibrinogen, and thrombocytopenia. Further exacerbation of coagulation dysfunction can cause disseminated intravascular coagulation (DIC). Currently, only a few studies report on the incidence of DIC related to CAR-T cell therapy. A previous study reports about 7% incidence (125), whereas a different one reports that the incidence is about 28.3% (8). Notably, studies report that the incidence of coagulation disorders and DIC is higher in patients with severe CRS (8). In addition, the severity of coagulation disorders is positively correlated with the grade of CRS (125).

Mechanisms of CAR-T-related coagulation disorders are not fully known, and may be linked to the following mechanisms: (a) Blood from patients with malignant tumors is in a hypercoagulable state (126); (b) High levels of cytokines like IL-6 and TNF-α in the blood cause activation and lesions of vascular endothelial cells, resulting in increased release of tissue factor (TF) (127, 128). Coagulation factor VII (FVII) combines with TF to form FVII/TF complex. A series of reactions activates the extrinsic coagulation pathway; (c) Damage to endothelial cells affects their integrity, and collagen fibers below the endothelial cells are exposed. Subsequently, coagulation factor XII (FXII) combines with exposed collagen fibers and are activated to form FXIIa. Activation of several factors by FXIIa activate the intrinsic coagulation pathway; (d) When a patient has severe CRS, levels of cytokines in the body are significantly increased, and these cytokines induce activation of vascular endothelial cells (27, 28). Activated endothelial cells release von Willebrand factor (vWF) (27, 55), which promotes blood coagulation (129); (e) Both high-mobility group box-1 (HMGB1) and histones promote blood coagulation (130, 131). Certain malignant tumor cells, such as leukemia cells, release HMGB1 and histone H3 after rupture, which promote coagulation dysfunction or DIC (132); (f) Histones injure endothelial cell (133, 134) thus indirectly activating intrinsic or extrinsic coagulation pathway; (g) Excess histones in the blood can also cause liver damage (135, 136), and serious liver damage affects production of coagulation factors; (h) Severe damage of liver cells caused by off-target effects of CAR-T cells affects production of coagulation factors (**Figure 3**).

Patients with coagulation disorders can be treated with conventional treatment approaches. Most patients with coagulation disorders can recover without intervention (125). When CRS is under control and the levels of multiple cytokines decrease, the coagulation disorders recover gradually, and progression of DIC is effectively inhibited (8). However, once DIC occurs, timely and effective intervention and treatment must be administered.

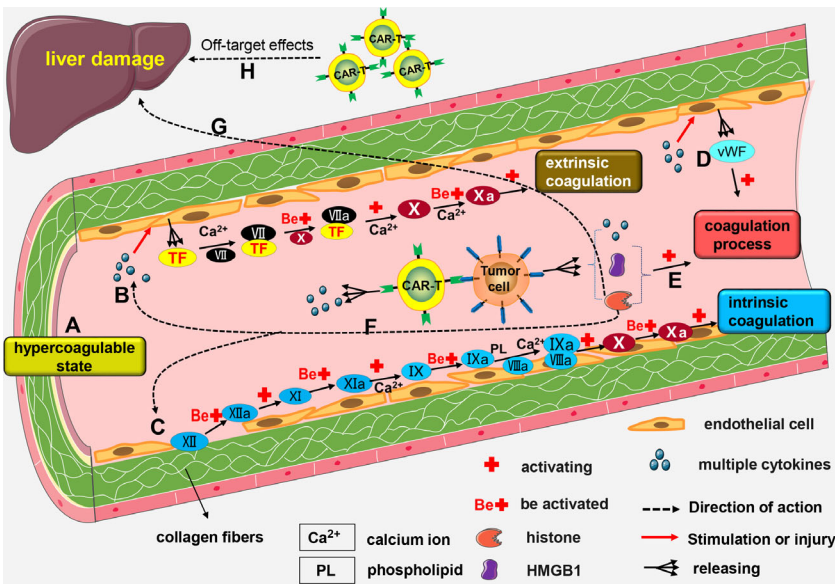

FIGURE 3 | The mechanism of CAR-T-related coagulation dysfunction. **(A)** Blood from patients with malignant tumors is in a hypercoagulable state; **(B)** High number of cytokines can trigger the formation of lesions by vascular endothelial cells, thereby promoting the release of TF. Calcium (Ca2+) facilitates the combination of FVII with TF to form the FVII/TF complex. Activated FX enhances the activation of FVII/TF complex to form FVIIa/TF complex. Calcium (Ca2+) mediates the activation of FX by VIIa/TF complex to form FXa. This results in the activation of the extrinsic coagulation pathway. **(C)** Damage to endothelial cells exposes the underlying collagen fibers. Next, FXII combines with the exposed collagen fibers to form FXIIa. FXI is activated by FXIIa and transformed into FXIa. FIX is then activated by FXIa and transformed into FIXa; a process driven by Ca2+. FIXa and FVIIIa combine to form a IXa/VIIIa complex under the regulation of Ca2+ and PL. The IXa/VIIIa complex complex activates FX and transforms it into FXa leading to the activation of intrinsic coagulation pathway. **(D)** Activated endothelial cells release vWF factor which contributes to blood coagulation. **(E)** Rupture of some malignant tumor cells results in the release of HMGB1 and histones, both of which cause coagulation dysfunction. **(F)** Histones can indirectly activate the intrinsic or extrinsic coagulation pathway. **(G)** Excessive production of histones into the blood stream may cause liver damage and impair the production of coagulation factors. **(H)** Severe damage to the liver cells cause by the off-target effects of CAR-T cells may also impair the production of coagulation factors.

Cytopenias

Cytopenia is a common adverse reaction in CAR-T cell therapy which is characterized by neutropenia, thrombocytopenia and anemia. Its incidence is not consistent, which can be attributed to different types of diseases and treatment options. Studies from the last three years (**Table 3**), report that incidence of hemocytopenia is high in CAR-T cell therapy. Furthermore, studies have reported that cytopenia is the most prevalent among all adverse reactions reported with ≥ grade 3 (13, 14).

To improve the efficacy of CAR-T cells, patients should be given the lymphodepleting chemotherapy regimen before CAR-T cell therapy. Currently, the most commonly used regimen is the combination of fludarabine and cyclophosphamide. Patients often develop cytopenias after receiving lymphocyte clearance therapy. Early cytopenias may be as a result of lymphatic failure chemotherapy (137).

It has been shown that cytopenias developing after CAR-T cell therapy may last for a long time, exceeding 30 days (10, 13, 15, 138, 139). This phenomenon is termed as prolonged hematologic toxicity (PHT), and it is characterized with ≥ grade 3 neutropenia or thrombocytopenia following CAR-T cells infusion(exceeding 30 days) (15). In a phase I clinical trial that tested the efficacy of CD19-CAR-T cells in the treatment of patients with relapsed/refractory diffuse large B-cell lymphoma (R/R DLBCL), it was found that 18 of 31 (58%) patients developed PHT (15). Moreover, the 1-year overall survival (OS) (36%) of patients with PHT was significantly lower than the 1-year OS (81%) of patients without PHT.

Although the development of PHT is not well understood, it may be triggered by the following factors: (a) PHT can be caused by a previous administration of higher-intensity chemotherapy (137); which may deteriorate hematopoietic function, and hence decrease the production of blood cells in the long run.(b)Patients with a history of hematopoietic stem cell transplantation (HSCT) and CRS are likely to develop PHT (137, 140). Notably, patients with high-grade CRS may have severe cytopenias and require longer recovery time compared to those with low-grade CRS (27). Usually, patients are treated with HSCT, followed by CAR-T cell therapy. The interval between these two treatments usually exceeds 1 year. HSCT can cause damage to the patient's hematopoietic function. The impaired hematopoietic function may not have fully recovered, and then the patient suffers a second blow (receiving CAR-T cell therapy). This mode of treatment may cause or even worsen PHT.

The treatment of cytopenia: (a) Patients with early and mild cytopenias, symptomatic and nutritional support are recommended. For such patients with neutropenia, active prevention or anti-infective treatments can also be given. (b) For patients with long-term neutropenia, granulocyte-colony stimulating factor (G-CSF) treatment is recommended. Currently, the U.S. Food and Drug Administration has approved the use of G-CSF (filgrastim) in the treatment of congenital and acquired neutropenia (141). The recommended dose of G-CSF is 5 mcg/kg/day (141). Other dosage forms of G-CSF include pegfilgrastim and lenograstim. (c) Patients with long-term neutropenia and thrombocytopenia may benefit from GM-CSF treatment at a dose of 250 mcg/m2/day (141), but it should not be administered in the first 3 weeks after injection of

TABLE 3 | Summary of the incidence of cytopenias associated with CAR-T cell therapy in patients with hematological malignant tumors (partial data).

Time	Disease	CAR-T Cell Therapy	Phase	Case	Age (years)	Any Grade			Gade 3/4			Trial registration
						Neutropenia	anemia	thrombocytopenia	Neutropenia	anemia	thrombocytopenia	
2019	relapsed / refractory MM	BCMA-CAR-T cells	I	33	37~75	28 (85%)	19 (58%)	19 (58%)	28 (85%)	15 (45%)	15 (45%)	NCT02658929 (4)
2019	relapsed/ refractory B-ALL	CD19-CAR-T cells	I	25	1~22.5	3 (12%)	–	4 (16%)	3 (12%)	–	4 (16%)	NCT01860937 (5)
2019	relapsed / refractory MM	BCMA-CAR-T cells	I	25	44~75	–	–	–	11 (44%)	5 (20%)	7 (28%)	NCT02546167 (6)
2019	relapsed / refractory MM	BCMA-CAR-T cells CD19-CAR-T cells	II	21	18~69	20 (95%)	20 (95%)	16 (76%)	18 (85%)	13 (62%)	13 (62%)	ChiCTR-OIC-17011272 (9)
2019	relapsed or refractory diffuse large B-cell lymphomas	CD19-CAR-T cells	IIa	111	22~76	22 (20%)	53 (48%)	14 (13%)	22 (20%)	43 (39%)	13 (12%)	NCT02445248 (10)
2020	relapsed/ refractory B-ALL	CD19-CAR-T cells	I	23	10~67	–	–	–	17 (74.9%)	7 (30.4%)	9 (39.1%)	ChiCTR-ONN-16009862, ChiCTR-1800019622 (11)
2020	relapsed or refractory large B-cell lymphomas	CD19-CAR-T cells	I	269	54~70	169 (63%)	129 (48%)	84 (31%)	161 (60%)	101 (37%)	72 (27%)	NCT02631044 (12)
2020	relapsed / refractory mantle-cell lymphoma	CD19-CAR-T cells	II	68	38~79	59 (87%)	46 (68%)	50 (74%)	58 (85%)	34 (50%)	35 (51%)	NCT02601313 (13)
2021	relapsed / refractory MM	BCMA-CAR-T cells	II	128	33~78	117 (91%)	89 (70%)	81 (63%)	114 (89%)	77 (60%)	67 (52%)	NCT03361748 (14)

MM, multiple myeloma; B-ALL, B-cell acute lymphoblastic leukemia; BCMA, B-cell maturation antigen.

–: Relevant data are not mentioned in the experiment.

KYMRIAH (a CD19-CAR-T cell therapy) or before CRS is resolved (142). The dosage forms of GM-CSF include sargramostim and molgramostim. (d) For patients with long-term and severe anemia and thrombocytopenia, red blood cell and platelet transfusion is recommended (143). (e) In cases of severe and prolonged cytopenias, eltrombopag (Thrombopoietin-receptor agonists, 50-150mg/day) has been used previously (15). This dose produced a median time for blood system recovery of 123 days (range: 41 to 145 days) in four patients. (f) Autologous or allogeneic stem cell transplantation is proposed as a potential treatment for cytopenia (140, 144), but the efficacy of this treatment has not been clarified through clinical trials.

FUTURE PROSPECTS OF CAR-T CELL THERAPY

As an emerging anti-tumor therapy, CAR-T cell therapy has made great achievements in the treatment of hematological malignancies. Currently, this therapy has begun to be applied to the research and treatment of solid tumors. However, it is associated with adverse reactions which limits its clinical application to a certain extent. Only by minimizing the incidence and impact of these adverse reactions can the safety of CAR-T cell therapy be effectively enhanced. By effectively preventing and treating these adverse reactions, more and more tumor patients will benefit from CAR-T cell therapy.

AUTHOR CONTRIBUTIONS

LM: Writing-Original draft preparation, Investigation, table and figure preparation. ZZ: Investigation, table and figure preparation. ZR: Investigation. YL: Conceptualization, Methodology, Supervision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Naturally Occurring Genetic Alterations in Proximal TCR Signaling and Implications for Cancer Immunotherapy

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T cell-based immunotherapies including genetically engineered T cells, adoptive transfer of tumor-infiltrating lymphocytes, and immune checkpoint blockade highlight the impressive anti-tumor effects of T cells. These successes have provided new hope to many cancer patients with otherwise poor prognoses. However, only a fraction of patients demonstrates durable responses to these forms of therapies and many develop significant immune-mediated toxicity. These heterogeneous clinical responses suggest that underlying nuances in T cell genetics, phenotypes, and activation states likely modulate the therapeutic impact of these approaches. To better characterize known genetic variations that may impact T cell function, we 1) review the function of early T cell receptor-specific signaling mediators, 2) offer a synopsis of known mutations and genetic alterations within the associated molecules, 3) discuss the link between these mutations and human disease and 4) review therapeutic strategies under development or in clinical testing that target each of these molecules for enhancing anti-tumor T cell activity. Finally, we discuss novel engineering approaches that could be designed based on our understanding of the function of these molecules in health and disease.

Keywords: T cell (antigen) receptor, cellular immunotherapy, cytokines, T cell signaling, cellular engineering

INTRODUCTION

T cell activation, differentiation, and effector functions are tightly controlled by highly specialized and interconnected signaling pathways. Major early mediators of T cell activation include: (1) activation *via* the α and β or γ and δ chains of the T cell receptor (TCR) molecules (1); (2) CD3 signal-transduction molecules (2); (3) CD4 and CD8 co-receptors that help stabilize TCR-peptide-MHC interactions (3, 4); (4) early signaling mediators such as LCK, FYN, and ZAP70 (5, 6); and (5) the LAT-signalosome that leads to activation of a myriad of downstream signaling intermediates and pathways (**Figure 1**) (7). These proximal signaling prote13ins interact with myriad of

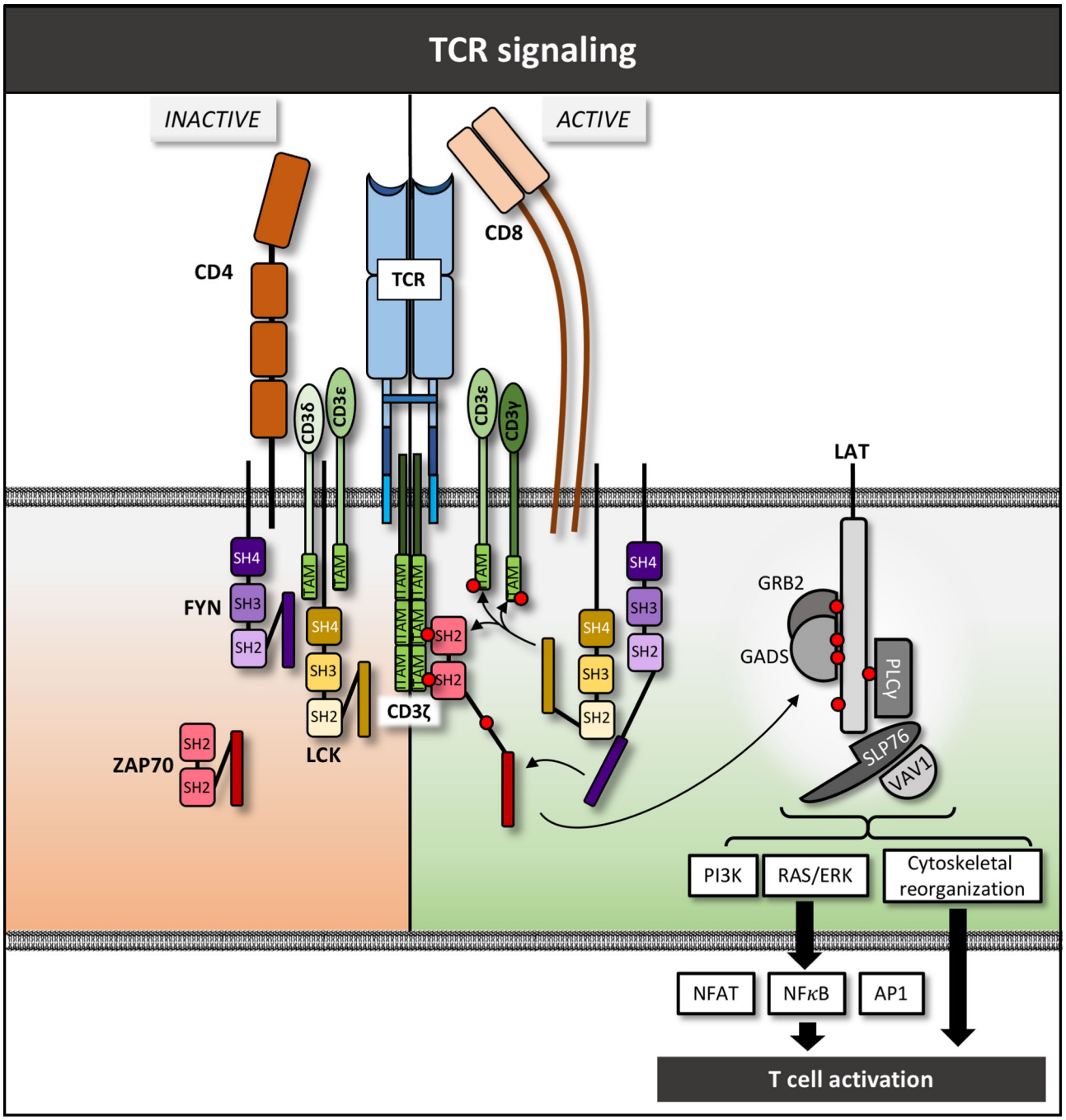

FIGURE 1 | TCR complex and downstream signaling pathway – schematic overview. Red circles denote phosphate groups.

intermediate molecules to ultimately initiate various multiple cellular processes including differentiation and effector function (7). We review the molecules involved in early TCR signaling and the receptors in T cells and consider how mutations or alterations in these molecules contribute to human disease, particularly immunity to cancer. We highlight therapeutic strategies designed to utilize this fundamental knowledge of molecular function for cancer treatment, with an emphasis on novel strategies that are showing early clinical potential. A discussion on T cell based immune therapy would be remiss without mentioning immune checkpoint inhibitors (ICIs) including monoclonal antibodies targeting PD-1, PD-L1 and CTLA4, as well as chimeric antigen receptor (CAR) T cells and tumor-infiltrating lymphocyte (TIL) therapy. These agents have revolutionized cancer therapy, but due to the wealth of literature on these topics, we will not address them directly in this review

except as they relate to specific molecules discussed in each section below.

T CELL RECEPTOR COMPLEX

TCR: Structure and Function

Unique from all other cell types, T cells express antigen-specific TCRs. The TCR complex is a heterodimer composed of either α/β or γ/δ chains, defining two major "flavors" of T cells. In humans, 95% of T cells are α/β heterodimers while only 5% are γ/δ homodimers (**Figure 2**). Within these heterodimers, each chain is comprised of extracellular variable and constant domains, a short linker peptide, and a transmembrane domain (8). The variable domains undergo somatic rearrangement during T cell development and contact the antigenic peptide presented by major-histocompatibility complex (pMHC). The interaction forms the physical basis by which T cells can recognize a myriad of targets (9, 10). The linker and transmembrane domains allow association with additional molecules such as the CD3 chains as well as CD4 and CD8 in their respective T cell types.

TCR Variable Domain Mutations: Connection to Human Disease and Immunotherapy

As briefly stated above, the variable domain of the TCR genes undergoes somatic recombination early in T cell development, thereby forming the basis for TCR diversity (11). This highly controlled process creates an array of T cells each expressing a unique TCR that can bind varied pMHC complexes. It is estimated that the human T cell repertoire can target on the order of 10 (12) unique specifities (13). Upon recognition of a unique pMHC expressed on target cells, various extracellular and intracellular accessory molecules are recruited to mediate a massive transcriptional shift towards T cell effector functions that allow effective killing of infected, malignant, or altered-self targets (14). Genetic variations in these TCR genes between individuals affect the clonality and diversity of that individual's T cell repertoire and can lead to disease. Numerous studies have linked intratumoral and peripheral blood TCR clonality and diversity with cancer prognosis and response to various treatment modalities. In cervical cancer fewer TCR clonotypes in sentinel lymph nodes correlated with worse outcomes (15), and in colorectal cancer, patients with

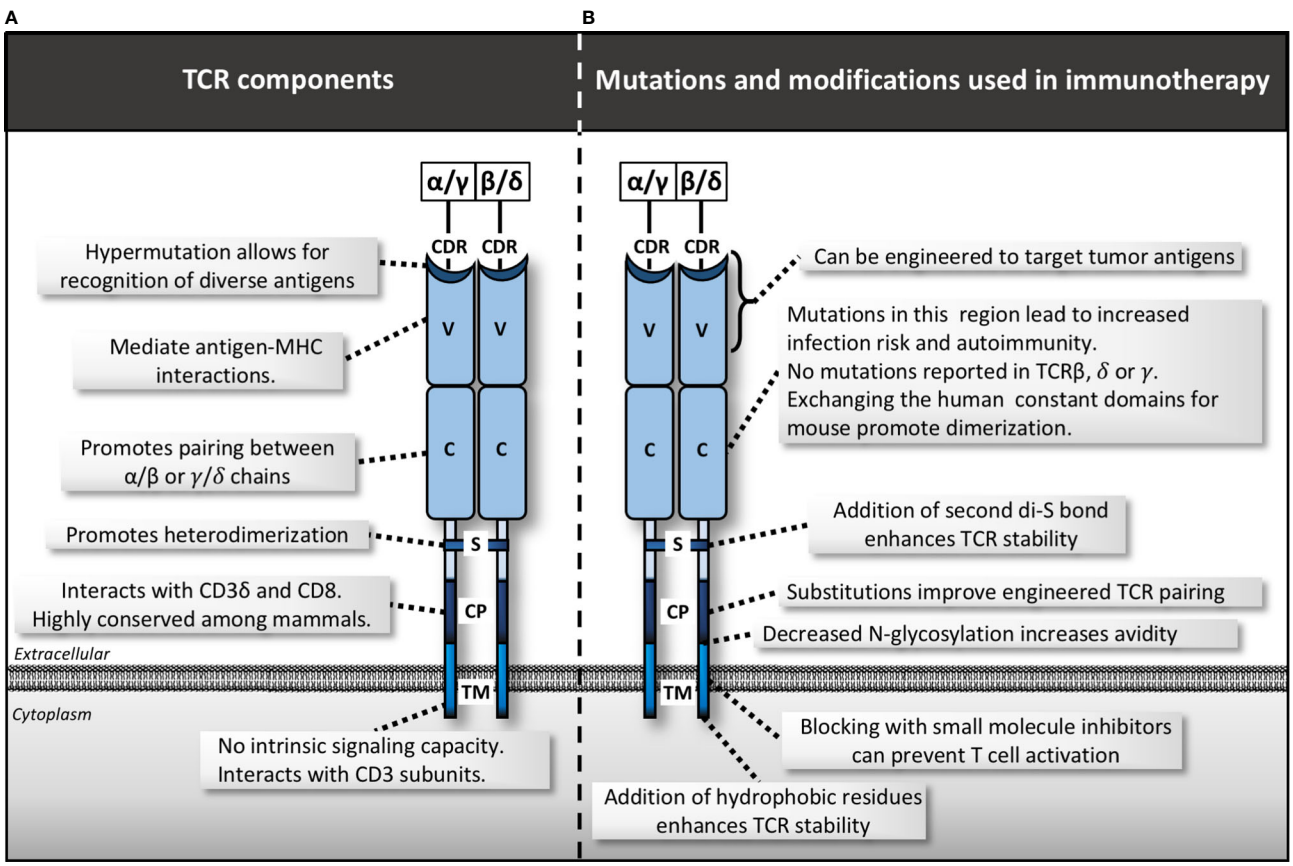

FIGURE 2 | TCR complex structure, mutations and manipulations for immunotherapy. **(A)** Various domains within the T cell receptor (TCR) including the complementarity-determining region (CDR), variable domain (V), constant domain (C), disulfide bond (S), membrane-proximal connecting peptides (CP) and transmembrane (TM). **(B)** Mutations and modifications used in immunotherapy by region.

metastatic disease harbored less TCR diversity in tumor draining lymph nodes (12). Comparing healthy individuals to a variety of cancer patients, Simnica et al. found that as people age, their TCR diversity diminishes and that cancer patients have reduced TCR diversity relative to healthy age-matched controls (16). Additional studies in melanoma and pancreatic cancer have shown that increased TCR diversity correlates with improved outcomes after immune checkpoint inhibition (ICI) (17–19), suggesting this treatment modality is reliant on an individual's T cell repertoire and ability to recognize tumor antigens for its beneficial effect. These data highlight the ability of T cells to recognize and fight cancer and suggest that therapeutic efficacy relies on a diverse TCR pool.

While most anti-tumor studies have manipulated α/β T cells, γ/δ T cells have unique features that could be exploited for cancer treatment. In pre-clinical studies, mice lacking γ/δ T cells have increased incidence of various cancer types (20–23). This anti-tumor effect is mediated *via* γ/δ T cell recognition of stress-associated molecules often upregulated in cancer such as heat-shock proteins, non-classical MHC molecules, and various phospho-antigens, a process encapsulated under the term the "lymphoid stress-surveillance response." (24) Furthermore, unique from α/β T cells, γ/δ T cells have potent activation responses prior to expansion, and express various NK cell receptors such as NKG2D that further enhance their ability to recognize altered or damaged self-cells (24). The proportion of γ/δ T cells infiltrating tumors is predictive of favorable prognosis (25). Among the various tumor types studied, melanoma tumors harbored the highest proportion of γ/δ T cells and the presence of these cells correlated with a lower risk of metastasis (26). Interestingly, γ/δ T cells are often over-represented in the heterogeneous adoptively transferred cell populations of successful tumor infiltrating lymphocyte (TIL) therapies, again suggesting potent anti-tumor activity (27). However, *in vivo* γ/δ T cell stimulating approaches using IL-2 and bisphosphonates has had underwhelming results (28, 29). Further studies are needed to define optimal *ex vivo* expansion strategies of γ/δ T cells, and to piece apart the differential benefit of γ/δ and α/β T cell subsets in adoptive transfer approaches.

TCR Transmembrane and Constant Region Mutations: Connection to Human Disease and Immunotherapy

Aside from the extracellular variable region of the TCR, subtle changes in the extracellular constant and transmembrane (TM) domains can affect the ability of the entire TCR complex to assemble and function (**Figure 2**). The only in-human mutation linked to the TCR α constant (TRAC) domain, is a G to A substitution at the C-terminus of exon 3. This mutation results in a complete lack of α/β T cells, implicating a key role in TRAC in regulating development of this cell type (30). No identified human diseases so far have been linked to mutations in the TM domain, or the TCR β, γ, or δ constant domains (TRBC, TRGC, or TRDC), suggesting they are all highly evolutionarily conserved. Indeed, numerous mutational and structural studies have confirmed the essential functions of both the TM and constant domains in assembly of the TCR complex and signal transduction largely via CD3 subunit recruitment and activation (31). More recent studies have only just begun to piece apart the nuanced mechanisms of these interactions (32–34).

New strategies are emerging to manipulate the TM and constant domains of the TCR for therapeutic benefit. For example, the TCR constant domain harbors a disulfide bond that promotes heterodimerization. Adding a second disulfide bond within this region has been shown to enhance TCR stability, signaling and T cell mediated tumor killing in cancer models (35). Adding additional hydrophobic residues to the TM domain enhanced surface expression and T cell avidity, leading to increased anti-tumor T cell activity *in vitro* (36). In mouse and human models, removal of conserved N-glycosylation sites in the TCR variable and constant domains improves T cell avidity and tumor cell recognition (37). Other studies have improved engineered-TCR technology *via* manipulation of constant domains. Exchanging the human constant-region for the murine equivalent (38), or just a 9 amino acid fragment thereof (39), prevents native-non-native heterodimerization. This results in improved pairing of TCR subunits with the desired specificity, enhanced CD3/TCR stability, and increased anti-tumor activity. Introduction of additional cysteine residues in the constant region achieved similar results (40). Finally, new approaches to target transmembrane domains with novel peptides, such as core peptide (CP) targeting of the TM domain of the TCR molecules, has shown early promise in various diseases such as autoimmune disease (41). Similar strategies could be extrapolated to anti-cancer applications. These insights highlight that modulation of anti-tumor immunity should be approached cautiously to maximize the effect on the malignant cells, while preventing detrimental side effects on the host.

CD3 Subunits: Structure and Function

Signaling through the TCR requires interaction with several CD3 subunits, as the TCR chains themselves do not contain intracellular signaling domains (42). There are four CD3 types: CD3ε and CD3δ form a heterodimer that binds to TCRα, CD3ε and CD3γ form another heterodimer that binds to the TCRβ chain, and two CD3ζ chains form a homodimer that associates with both TCR α and β chains (**Figure 3**) (31). In the case of the γ/δ TCR, two CD3ε/γ homodimers are involved in lieu of CD3δ (43). Despite extensive study, the exact geometry and binding sites between these CD3 molecules and the TCR have not been fully elucidated, but likely involve a combination of residues in the constant regions as well as ionizable and hydrophobic residues in the transmembrane regions (44). Signaling itself is mediated through phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the CD3 and ζ chains (45).

CD3 Mutations and Connection to Human Disease

Mutations in the CD3 molecules are associated with an array of human diseases ranging from severe-combined immunodeficiency (SCID) (46, 47) to autoimmune disorders (48). Regarding immunodeficiency, frameshift, nonsense, and splice variant mutations disrupt the ability of the CD3 molecules to be expressed or, if successfully translated, to bind to the TCR

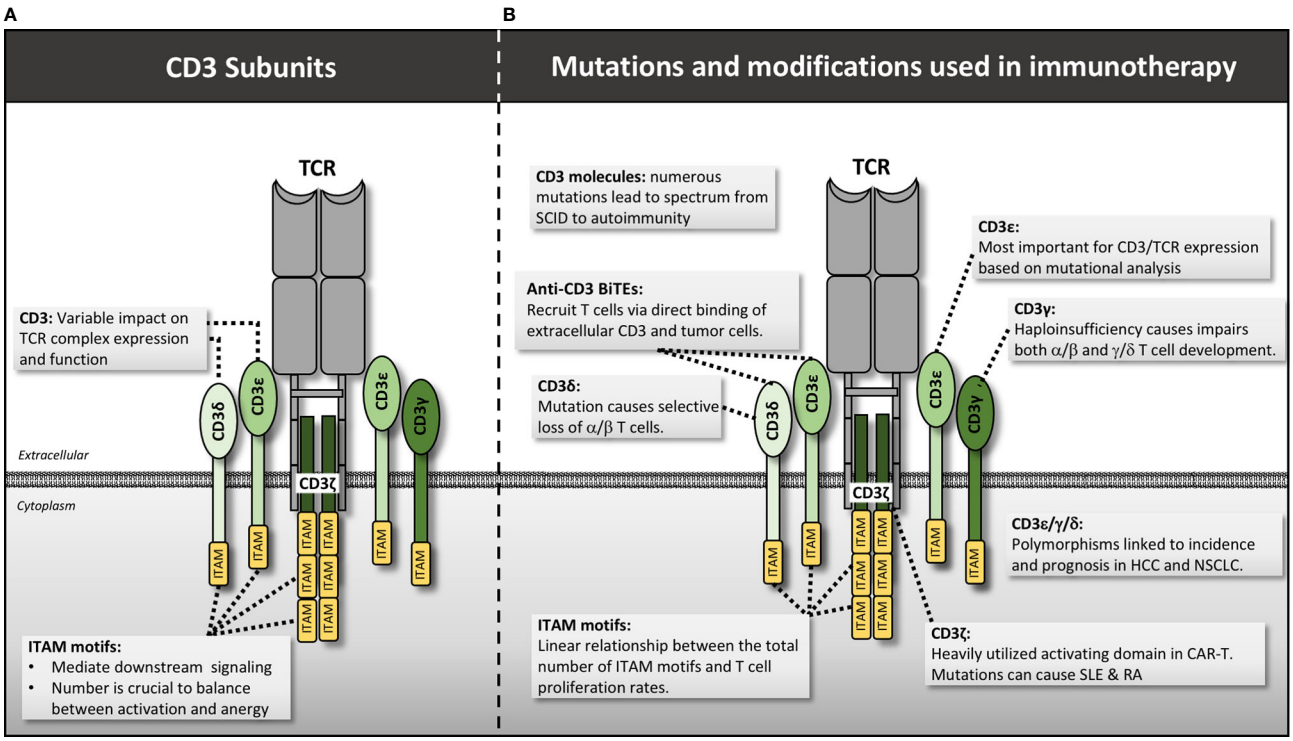

FIGURE 3 | CD3 structure, mutations and manipulations for immunotherapy. **(A)** CD3 molecules interact with the TCR in CD3δ/ε and CD3ε/γ heterodimers while CD3ζ forms a single homodimer. Each CD3 molecule contains one or more ITAM motif that is essential for downstream signaling. **(B)** Various mutations and genetic engineering approaches alter or manipulate CD3 function thereby impacting T cell activity. HCC, hepatocellular carcinoma; NSCLC, non-small cell lung cancer.

molecules to form a functional TCR complex (49). Without functional TCR signaling, T cells fail selection in the thymus, resulting in a complete lack of T cells and severe disease (46, 47, 50–52). More mild immunodeficiencies result in patients where TCR/CD3 is expressed but at much lower levels than normal, impairing T cell activation and function (53–55). The CD3 molecule affected determines the type of deficiency. For example, the CD3δ mutations C202T (that results in a premature stop codon at residue 68), and G to A substitution at position +5 of intron 2, were both found to cause a selective lack of α/β T cells but preserve the γ/δ T cell pool because the CD3δ molecule is not included within the γ/δ TCR complex (56–58). In contrast, CD3γ haploinsufficiency had a larger effect on the γ/δ T cell pool, suggesting this molecule is more important in γ/δ T cell development in humans (59).

At the opposite end of the spectrum are CD3 mutations that pre-dispose patients to autoimmunity. Of all the CD3 subtypes, mutations in CD3ζ and CD3γ harbor the strongest link to autoimmune and inflammatory conditions. Numerous SNPs and splice variants, affecting primarily the 3'UTR, intron 1, and exon 7 of CD3ζ have been associated with pathogenesis in systemic lupus erythematosus (SLE) (48, 60–64) and rheumatoid arthritis (RA) (65). A single SNP in intron 1 (rs858554) has a strong association with both diseases, as well as immune thrombocytopenia (ITP), suggesting a common underlying mechanism (48, 66) possibly via down-regulation of CD3ζ (67). Aside from germline mutations themselves, hypermethylation of the CD3ζ gene has been associated with severe SLE phenotypes and correlates with reduced CD3ζ expression (60). One might predict down-regulation of CD3ζ would decrease T cell activation. However, decreased CD3ζ expression in SLE patients' T cells was found to result in aberrant recruitment of FcRγ to the TCR complex in lieu of CD3ζ (68). Aside from mutations in CD3ζ, CD3γ mutations can also result in a range of phenotypes including mild immunodeficiency to autoimmunity. For example, Recio et al. found a common A to T mutation in nucleotide 205 of exon 3 in multiple patients from two different families in Turkey (69). This mutation results in a premature stop codon at residue 69 of the CD3γ protein. Despite sharing the exact same mutation, the patients from the first family had severe SCID and died in infancy, while the second patient was largely asymptomatic despite having similarly decreased levels of TCR/CD3 expression. A second study identified a mutation in another Turkish family at position -1 of exon 3 resulting in a premature stop codon at the protein level. This mutation disrupted CD3γ expression, resulting in a variety of autoimmune diseases (70). Thus CD3γ deficiency results in disparate phenotypes that may be augmented by the larger genetic and environmental context of each individual. To elucidate this further, Rowe et al. studied the function and clonality of T cells from CD3γ deficient patients predisposed to autoimmunity. They found that these individuals harbor decreased Treg function, and enrichment of hydrophobic

residues at positions 6 and 7 of the CDR3 chain within the variable domain of the TCR, a feature previously linked to auto-reactivity (71). Another detailed analysis of T cell subsets in CD3γ-deficient patients revealed impairment specifically in CD8 T cell development, but not CD4 development (72). This shift in the balance of T cell subsets could be responsible for the concurrent immunodeficiency and autoimmunity observed. In general, the heterogeneity of phenotypes resulting from CD3 mutations, particularly CD3γ, suggests complex underlying biology that is not fully encapsulated within our current mechanistic models. It will be interesting to apply this foundational knowledge of CD3-chain function to the improved design of engineered TCR's and CAR's, and their application in cancer treatment.

The number of ITAM motifs in the signaling domain appears to be key to regulating signal strength and thereby T cell activity. For example, Holst et al. conducted preclinical mouse studies using T cells with different numbers of ITAMs (73). They reconstituted *Rag*$^{-/-}$ mice with T cells in which almost every possible permutation of ITAM expression on all CD3 chains (a total of 25 recombinant conditions) was tested for its effect on T cell development and function. Their main conclusions include a quantitative linear relationship between the cumulative number of ITAM motifs in the CD3 complex and T cell proliferation rates. Despite decreased proliferation rates with fewer ITAM's, they observed that fewer than 7 total ITAMs results in severe autoimmune disease (73), thought due to failed negative selection in the thymus of highly self-reactive T cell clones. An important qualitative difference was observed in mice harboring 6 total ITAMs: autoimmune disease occurred in those with mutated CDζa, the proximal ITAM motif, and CDζc, the distal ITAM motif, but not with mutated intermediate CDζb. No autoimmune disease was observed in mice with wildtype CDζ that lacked ITAMs from CD3δ, γ, and ε. It is not known if these phenotypes recapitulate human biology but does imply that individual ITAM's perform specific roles and that the total number has important implications for T cell function. These types of detailed studies of CD3-subtype roles within the TCR complex will lead to better predictors of disease outcomes and means to genetically target and intervene for clinical benefit.

Notably, a few germline alterations in the CD3 subunits have been linked to cancer. An insertion/deletion polymorphism in the CD3γ promoter was linked with increased hepatocellular carcinoma incidence (74), and another (rs3181259T>C) in CD3δ was linked to recurrence in non-small cell lung cancer (NSCLC) (75). SNP rs967591G>A in CD3ε correlated with lower CD3ε expression and shorter survival in NSCLC suggesting a functional consequence could be impaired TCR signaling (76). It is surprising that such little data exists linking CD3 dysfunction with cancer given its essential role in T cell function and thereby adaptive immunosurveillance. Other disease factors in the heterogeneity of human cancers may cloud identification of a CD3 mutation signature in studies based on genetic analysis alone.

CD3: Connections to Immunotherapy

A variety of therapies have been designed to exploit CD3 subtype functions for cancer treatment. Non-specific stimulation of T cell pools with anti-CD3 antibodies has been used to enhance anti-tumor T cell responses. In TIL therapy, *ex vivo* culture with activating anti-CD3 antibodies along with IL-2 is the preferred method for activation and expansion (77). Compared to α/β T cell activation, CD3 conformational changes do not play as prominent a role in conventional γ/δ T cell activation. Nevertheless, binding of anti-CD3 antibodies (78) or Fab fragments (79) to γ/δ T cells enhanced tumor killing *in vitro*, suggesting exploiting the CD3 signaling pathway could augment novel anti-tumor properties of this unique cell type.

In CAR-T engineering, derivatives of CD3ζ chains are the favored intracellular signaling moieties incorporated into most CARs (80). Next generation CARs also include intracellular signaling components of costimulatory molecules such as 41BB or CD28 fused to the CD3ζ ITAM domains to further enhance CAR T activation (81). Engineered CAR proteins have been shown to interact with and signal *via* endogenously expressed TCR components (82). Therefore, fine-tuning the intracellular and transmembrane components may result in varied and potentially desirable enhancements of CAR T function. For instance the number and type of ITAM's impacts the risk of autoimmune disease development in mice (73). With this knowledge in mind, Feucht et al. selectively mutated 1 or 2 ITAM's of the CD3ζ within a CD19-CD28-CD3ζ CAR and tested the resultant impact on T cell function (83). CAR's with mutations (X) in the second and third ITAMs (denoted 1XX), were the most efficacious and induced long-term remission in a pre-B acute lymphoblastic leukemia mouse model. Based on the identification of aberrant FcRγ recruitment in autoimmune disease when CD3ζ was mutationally defective (84), one might predict that incorporation of the intracellular portion of FcRγ into CARs in lieu of CD3ζ domains would result in increased CAR T activation. However, FcRγ ITAM domains showed no benefit over CD3ζ when utilized in CAR technology (85, 86), suggesting this synthetic biology does not fully mimic mutation-driven phenotypes observed in nature. Other strategies to enhance CAR T signaling could be inspired by the hyper-activated T cell states observed in autoimmune diseases caused by mutations in other CD3 subunits, and by experimenting with ITAM number and CD3 subunit of origin. However, consideration into the potential risk of chronic activation would have to be investigated.

Another approach that exploits CD3 activity in T cell signaling is to use bi-specific T cell engagers (BITEs). These constructs are comprised of one antibody moiety binding to an antigen of choice and an opposing antibody moiety binding CD3 subunits on T cells (87). The BiTE could be thought of as a soluble CAR, bridging a T cell and target tumor cell. However, in contrast to CARs, BiTEs are still reliant on endogenous CD3 expression in the T cells they recruit. BiTEs have been shown to induce responses in polyclonal populations of CD4 and CD8 T cells (88), and do not have to be custom made for each patient. Currently most bind to the extracellular component of CD3ε (88). Based on our above appreciation for the heterogenous involvement of various CD3 subunits in T cell function and disease, targeting other CD3 subunits could be used to fine-tune desired T cell recruitment with BiTEs. BiTE efficacy is in part limited by specific tumor cell phenotypes, such as the expression

of sialophorin which limits T cell to tumor cell adhesion (89), and by similar side effects of CRS and neurotoxicity observed in CAR Therapy (90, 91). One study also identified a polymorphism in the CD3ζ chain (SNP rs2949655) that correlated with reduced cytotoxicity in response to BiTE treatment (92), demonstrating how CD3 mutational profiling could be used to help guide personalized treatment approaches targeting this pathway. Further improving our understanding of CD3 subunit function and signaling will help elucidate additional strategies for therapeutic intervention.

CD4 and CD8: Structure and Function

The co-receptors CD4 and CD8 are essential to T cell development and formation of a functional TCR-MHC synapse (93). They are considered co-receptors because they stabilize MHC-antigen-TCR complexes and contribute to the functions of CD4+ helper T cells and CD8+ cytotoxic T cells without direct antigen binding (94, 95). CD4 is comprised of four extracellular Ig domains that bind to MHC-II. CD8 is a dimer comprised of either a CD8α homodimer or a CD8α and CD8β heterodimer (96, 97). These extracellular domains are attached to a long extracellular stalk domain that, by means of differential sialylation/glycosylation, helps regulate CD8-MHC-I binding affinity (98). CD8αα is found on γδT cells, intestinal and dermal intra-epithelial T cells, as well as NK cells (99). CD8αβ, on the other hand, is primarily expressed on conventional cytotoxic CD8 T cells (100). Crystal structures have shown CD4 binds at membrane-proximal α2 and β2 domains of the MHC-II molecules at residues conserved between the different MHC-II types (101), while CD8 binds mainly at the α3 domain of MHC-I (102). MHC-II/CD4 or MHC-I/CD8 binding occurs at much lower affinity than MHC/TCR, presumably to help calibrate appropriate T cell selection during thymic development and avoid autoimmunity (100). Both CD4 and CD8 have transmembrane domains that contribute to the formation of their respective TCR complexes, as well as intracellular domains that associate with LCK to facilitate intracellular signaling.

CD4 and CD8 Mutations: Connections to Human Disease and Immunotherapy

The only known polymorphisms in CD8α (p.Gly111Ser) associated with human disease affect functional expression of the CD8α molecule, resulting in compete lack of CD8 T cells (103). No other mutations or even polymorphisms in the CD4 molecule or CD8β have thus far been linked to human disease, likely because each has such refined and essential functional requirements for T cell function. In mice, studies have shown that missense or non-functional mutations of CD4 and CD8 result in lack of either CD4 or CD8 T cell subtypes due to failure of thymic selection (104, 105).

The use of CD4 and CD8 as markers for specific T cell subsets and their functions has profound implications to tumor immunology and immunotherapy. Within the tumor microenvironment, increased T cell infiltration, specifically of CD8 cytotoxic T cells, and high CD8/FoxP3 ratios correlate with better overall survival in multiple cancer types (106). Th1 phenotypes of TILs correlate with improved outcomes (107). In adoptive cell therapy, it was thought that the CD8 component of TILs was the most important due to the known cytotoxic capability of CD8 T cells, with CD4 T cells playing a merely supportive role (108, 109). Indeed, in melanoma patients a higher frequency of CD8 T cells amongst the infused cells correlated with better responses (110). However, CD4 T cells have been shown to exert anti-tumor effects through largely unknown and likely multivalent mechanisms (111). Additionally, recent evidence has shown CD4 T cells can acquire cytotoxic capabilities in the presence of IL-2 and mediate direct tumor cell killing (112). For CAR T cell therapy, a combination of CD4 and CD8 T cells in a defined 1:1 ratio appears to be most efficacious (113). Current TIL therapy regimens use *ex vivo* IL-2 expansion, but do not select for T cell subsets prior to re-infusion (77). More detailed studies of CD4 and CD8 TIL subsets and means to expand and enhance their function *ex vivo*, as well as the optimal balance of CD4 and CD8 cell types in adoptive cell therapies, are required.

Various approaches to target CD4 have been applied or are in development for immunotherapy. CARs targeting CD4 have shown promise in pre-clinical models of peripheral T cell lymphomas (PTCLs) (114). Anti-CD4 antibodies have been used in patients with PTCLs as well as cutaneous T cell lymphomas with some early clinical benefit (115–118). Anti-CD4 antibodies have also shown benefit in non-hematologic malignancies *via* depletion of anti-inflammatory CD4 T cell subsets including Tregs, thereby allowing for enhanced proliferation of anti-tumor CD8 cytotoxic T cells (119). This approach is further bolstered with the addition of immune-checkpoint blockade (120) and is now being applied in early clinical trials (121).

Aside from using CD4 and CD8 as cell-specific markers, genetic manipulation of these molecules offers therapeutic potential. In our lab, a synthetic construct that fused the CD8α extracellular domain to a MyD88 intracellular domain, normally downstream of innate immune receptors, resulted in enhanced anti-tumor CD8 T cell function in mouse models (122). Another group demonstrated increased MHC-I binding affinity when sialylation of core 1 O-glycans on the CD8 stalk region was reduced either through neuraminidase treatment or mutation of ST3Gal-I sialyltransferase mutation (123). Through a phage display approach, Wang et al. found the substitutions Gln40Tyr and Thr45Trp in CD4 resulted in almost 500 fold increase in MHC-II binding affinity (101). Future studies are need to determine if other alterations in CD8 or CD4 extracellular domains could be used to further stabilize the TCR's interaction with low affinity tumor antigens and thereby improve anti-tumor CD8 and CD4 effects respectively. Furthermore, docking topology of self-reactive TCR-MHC-II complexes in autoimmune T cell types is different than that in non-self-reactive T cells, and is likely influenced by CD4 molecule binding (13). One could envision that modulating CD4-TCR-MHC topologies could in a similar manner enhance T cell responses to rare self-like tumor-associated antigens (TAA). In conclusion, the CD4 and CD8 molecules are well-established markers for T cell subsets, their potential as

therapeutic targets show early promise, and alteration in their function and/or binding activity warrants continued exploration.

EARLY DOWNSTREAM SIGNALING INTERMEDIATES: LCK, FYN, ZAP70

LCK: Structure and Function

Besides the molecules within the TCR signaling complex, many additional proximal signaling molecules also contribute to overall T cell function and hold potential as therapeutic targets. The precise events that occur following TCR activation remain controversial, but current data suggests that early proximal signaling events are largely mediated by the Src family kinases LCK and FYN (124). Upon antigenic stimulation of a T cell, pre-activated LCK is recruited first to the TCR, initiating phosphorylation of ITAMs within the CD3 intracellular domains (**Figure 4**). Subsequently, additional LCK bound to the CD8 and CD4 co-receptors localizes to the immune synapse, enhancing TCR-pMHC binding and enabling ZAP70 recruitment and activation (125–128). Additionally, LCK deficient mice have severely impaired T cell development (129–132) and LCK contributes to a T cells' ability to titrate its activation level based upon the affinity of the TCR-pMHC interaction. This graded signaling response is largely regulated via distinct patterns of ITAM phosphorylation executed predominantly by LCK (133). LCK activity also modulates T cell differentiation (134, 135), CD28 costimulatory signaling (136), and even cell death (137). Given its critical role in a variety of T cell functions, it is not surprising that LCK activity is tightly regulated to maintain immune homeostasis. Consequently, genetic variations and mutations that alter the function of LCK have profound implications for the development of cancer and immune-based therapies.

LCK Mutations and Connections to Human Disease

A homozygous missense mutation in a hydrophobic region of the catalytic domain (c.T1022C) has been identified in children presenting with severe recurrent infections, autoimmune manifestations and panniculitis. This mutation is associated with reduced CD4 and CD8 expression, impaired TCR activation, decreased Treg levels and expansion of an oligoclonal γ/δ T cell population (138). Additional splice mutation variants of LCK have been reported and correlate with impaired LCK function and immune dysregulation. Patients with these variants present with a range of clinical syndromes including epidermodysplasia verruciformis and recurrent bacterial infections (mutation: c.188-2A>G) (139), common variable immunodeficiency (CVID, mutation: lack of exon 7) (140), and severe combined immunodeficiency (SCID, mutation: lack of exon 7) (141). Mutations that delete a C-terminal regulatory tyrosine within LCK increase LCK activity resulting in sustained T cell activation and oncogenesis in mice (142), as well as increased IL-2 production independent of antigenic signaling in humans (143). In addition, a chromosomal translocation t(1;7)(p34;q34) has been identified in patients with T cell acute lymphoblastic leukemia (ALL) (144). This translocation also increases LCK activity and the degree of elevated activity is correlated with breakpoint location and disease severity (144). These findings are intriguing from a T cell engineering perspective given that modulating LCK function, even *via* changes in single residues, has significant impact on T cell function. However, based upon the stark clinical phenotypes observed among patients, it is clear that extreme caution is warranted when considering genetic manipulation of this crucial protein.

LCK: Connections to Immunotherapy

Understanding LCK activity and genetically altering this gene still holds promise for several therapeutic approaches in oncology. In particular, LCK modulation is an important area of study for CAR T cell treatment of solid tumors. For example, CAR T cells have been engineered with a deleted LCK-binding motif within CD28 (ΔCD28). In mice, these second generation ΔCD28/CD3ζ (145) and third generation ΔCD28-4-1BBζ CAR T cells (146) show reduced IL-2 production and improved tumor control in the presence of Tregs. Clinically, a patient with malignant pleural mesothelioma was treated with anti-FAP ΔCD28/CD3ζ CAR T cells and experienced stable disease for 1 year, suggesting that ΔCD28/CD3ζ CAR T cells may have contributed in controlling his disease (147). However, this finding is currently anecdotal and other studies suggest that the effect of ΔCD28/CD3ζ on CAR T cell function may depend upon the immunosuppressive mechanism within the tumor microenvironment (TME) (148). For instance, tumor models with high TGFβ within tumor tissue require an intact LCK motif within the CD28/CD3ζ CAR receptor in order to overcome TGFβ-mediated suppression (148). Therefore, patient stratification by TME immune profiles could identify patients who would benefit from ΔCD28/CD3ζ CAR T cells. Synthetic LCK modulation in CAR T cells has other therapeutic implications as well given that LCK deficient cells are resistant to activation induced cell death (149) and LCK is involved in PD-1 induced inhibition (150).

It is also important to consider the impact of LCK mutations in the context of immune checkpoint inhibition (ICI). For example, the LCK SNP rs10914542 G allele impairs TCR activation (151), suggesting that patients harboring this allele may be less likely to mount a robust response following ICI therapy. Conversely, a single amino acid variant in LCK (p.G85W of exon 4) was associated with autoimmune diseases including Sjogren syndrome, SLE and RA, suggesting that patients with this variant could be at increased risk of developing autoimmune complications in the setting of ICI (152). All together, these data suggest that artificial regulation of LCK or screening for LCK variants could inform immune-based therapeutic strategies for cancer.

FYN: Structure and Function

FYN is another Src family tyrosine kinase involved in proximal TCR signaling, however, the precise roles of this protein are less understood than LCK. FYN also associates with the TCR and is

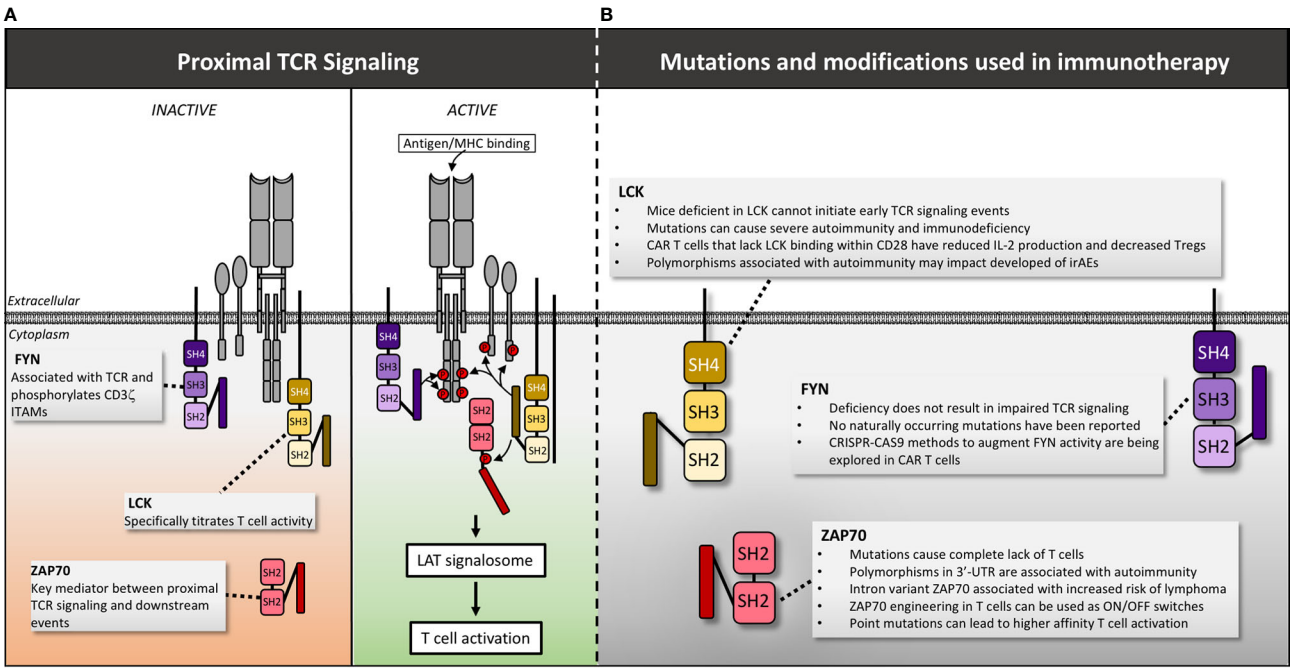

FIGURE 4 | LCK, FYN and Zap70 structure, mutations and manipulations for immunotherapy. **(A)** Following antigen/MHC binding, LCK is recruited first to the TCR, initiating phosphorylation of ITAMs within the CD3 intracellular domains. Subsequently FYN is recruited and assists in phosphorylation of CD3ζ ITAMs. LCK then phosphorylates the linker sequence of ZAP70 causing a conformational change that actives the kinase activity of ZAP70 permitting additional downstream signaling. **(B)** The impact of mutations and genetic manipulation of LCK, FYN and ZAP70 are detailed. Red circles denote phosphate groups.

involved in phosphorylation of CD3ζ ITAMs. However, LCK deficiency causes a much more dramatic phenotype than FYN deficiency, suggesting that FYN is not required for T cell activation (126). FYN interacts with many additional binding partners including PI3K (153, 154), lymphocyte-specific scaffold protein adhesion and degranulation-promoting adaptor protein (ADAP) (155), phosphoprotein associated with glycolipid-enriched membranes (PAG) (156), signaling lymphocyte activation molecule (SLAM) and others (157). These interactions collectively enable a diverse breadth of functions ranging from T cell activation to anergy (158). It is therefore reasonable to hypothesize that alterations in FYN function could modulate T cell activity in a variety of ways that could be exploited for therapeutic purposes.

FYN: Mutations and Connections to Immunotherapy

To date, no naturally occurring mutations in FYN have been linked to disease, suggesting that either existing genetic variants are relatively benign, or they cause lethality. Despite this ambiguity, modulating FYN activity remains an active area of interest in T cell engineering. For example, in a recent review by Thakar et al., the authors propose that inhibition of the FYN-ADAP pathway using CRISPR-CAS9 could provide a unique means of selectively downregulating cytokine production by CAR T cells without impairing cytotoxicity (159). This approach could be used to reduce the severity of cytokine release syndrome, a dangerous complication of CAR T cell therapy (159). Inhibiting FYN activity may also enhance T cell migration. A study by Schaeuble et al. reported that inhibition of FYN with the small molecule SU6656 promoted enhanced CCR7-driven migratory function of nonactivated T cells *in vitro* (160). CCR7 is expressed by both central memory T cells (T_{CM}) and T memory stem cells (T_{SCM}), both of which are promising substrates for both CAR T cell (161, 162), and transgenic T cell therapy (163). Therefore, inhibiting FYN activity in genetically engineered T cells could enhance their migratory capacity prior to activation within either a tumor-draining lymph node or the TME.

Alternatively, activating some functions of FYN could improve cellular based immunotherapy. One study found that deletion of FYN in a mouse model promotes differentiation of CD4+ T cells towards a Treg phenotype and away from a Th17 phenotype (164). While speculative, this suggests that activating Fyn could promote a Th17 phenotype. Some data suggest that Th17 CD4+ T cells have superior anti-tumor function and improved persistence as compared to Th1 cells in adoptive cell therapy settings (165–167). Therefore, FYN modulation in CD4+ T cell engineering approaches may not only reduce Treg induction but could also promote a Th17 phenotype. Increasing FYN activity has other potential beneficial implications. The SH2 domain of FYN binds Tim-3, promoting T cell activation and increasing cytokine production (168), a surprising finding as Tim-3 is classically associated with

T cell exhaustion. Increased FYN-Tim-3 binding *via* genetic modification could shift the balance of Tim-3 activity towards T cell activation and away from exhaustion (168). In contrast to the T cell activating function of FYN, FYN association with PAG and c-cbl can disrupt canonical TCR signaling and promote T cell anergy under certain conditions (156, 169–173). These findings indicate that FYN has highly varied and even paradoxical effects on T cell activity depending upon its binding partners. Importantly, the binding site for these various partners are not all known. Therefore, further characterization of the specific binding locations could enable genetic alterations to precisely tailor FYN activity and improve active immunotherapeutic approaches. Regarding passive immunotherapeutic strategies such as ICI, FYN-activating signatures have been associated with lupus nephritis, an autoimmune condition (174), loosely suggesting that increased FYN activity might increase the risk of autoimmune complications following ICI. However, no polymorphisms or genetic variants have been associated with autoimmunity thus it is unlikely that mutational profiling of FYN would predict development of immune related Adverse Events (irAE). Overall, further characterization of FYN function, binding sites and binding partners is warranted and may provide opportunities for various T cell engineering strategies in the future.

ZAP70: Structure and Function

Once LCK and FYN phosphorylate ITAMs on the CD3 molecules, the next step in TCR signal transduction involves binding of ζ-chain-associated protein kinase of 70 kDa (ZAP70) (45). Distinct from the Src family kinases described above, ZAP70 along with SYK are the two prominent members of the Syk family of kinases (175). ZAP70 is comprised of an auto-inhibited kinase domain and two amino-terminal SH2 domains that bind doubly-phosphorylated ITAMs (176) Upon ITAM binding, ZAP70 undergoes a conformational change that results in additional phosphorylation of residues in the second linker sequence by LCK. This relieves inhibition of the kinase domain and results in downstream signal propagation (177).

ZAP70 Mutations and Connections to Human Disease

Various ZAP70 mutations have been shown to cause a severe form of immunodeficiency characterized by complete lack of functional T cells (178). Most of these mutations affect the kinase domain (179–182), or lead to loss or destabilization of the protein transcript altogether (183–185). Syc can take the place of ZAP70 in T cell signaling when the latter is impaired, allowing for some CD4 cells to survive thymic selection, but these Syc+ZAP70- T cells are defective in IL-2 production and proliferation and provide aberrant help to B cells for antibody class switching (182, 185). In contrast, polymorphisms in the ZAP70 coding region or 3'-UTR have conversely been associated with autoimmune disorders including psoriasis and type 1 diabetes (rs17695937) (186, 187), inflammatory bowel disease (IBD, rs13420683) (188), and RA (rs2278699) (189). Finally, an intron variant of ZAP70 (rs7425883) is associated with a decreased risk of developing non-Hodgkin lymphoma (190), and aberrant elevated expression of ZAP70 in B cell CLL cells correlates with enhanced BCR signaling in the leukemic cells and poorer prognosis (191, 192). Mouse studies recapitulating some of these autoimmune or immunodeficient phenotypes have demonstrated point-mutations in the second SH2 domain, interdomain B, or paired mutations in the kinase region lead to aberrant thymic selection more permissive of higher affinity self-reactive clones, and quantitative differences in TCR signaling (193–195). Of particular interest, the tyrosine residues at positions 292, 315, and 492 have been shown to play a negative regulatory role when phosphorylated, and their mutation to phenyalanine (which prevents phosphorylation), allowed for T cell hyperactivation (196). These findings suggest that ZAP70 could be used not only as a prognostic marker of disease but also a therapeutic target.

ZAP70: Connections to Immunotherapy

Due to its upstream role in TCR signaling, ZAP70 has been gaining interest in the world of cancer-immunotherapy. Of greatest potential, engineered ZAP70 constructs have been designed to function as on/off switches to control T cell responses. In one approach, a larger analog of the kinase inhibitor PP1 was used to selectively inhibit an engineered ZAP70 with altered inhibitor binding affinity, resulting in impaired catalytic activity (197). Interestingly, Treg function was not affected by this approach, suggesting unique non-catalytic activity of ZAP70 is functioning in Tregs (198), and that therapeutic application of this design would not affect tolerance and protective roles of adaptive immune cell types. This analog sensitive ZAP70 could be employed to turn off unwarranted activation of adoptively transferred T cell therapies. Other investigators have developed a tetracycline-inducible ZAP70 gene promoter allowing for selective turning on of ZAP70 transcription (199). This has so far only been used in studies designed to assess the role of ZAP70 in thymic selection, and clinical application may be impaired by the need for tetracycline infusion and difficulty regulating expression levels once turned on. More recently, a dual small-molecule gated ZAP70 has been created *via* fusion of the analog-sensitive ZAP70 to the ligand binding domain of the estrogen receptor (200), allowing for both on and off signals. *In vitro*, this new ZAP70 construct could be controlled on a minute-by-minute timeframe and regulated calcium flux and CD69 expression levels. However, cytokine production was impaired in the "on" configuration, thus more refinement is required before clinical application (200). Future studies could examine the role of specific ZAP70 point mutants, such as those identified above that result in autoimmune phenotypes, in regulating adoptive T cell therapy efficacy. Additionally, the differential role of Syk and ZAP70 with regards to TCR signaling is still an area of inquiry with some conflicting evidence regarding the potency of the two molecules for T cell activation (182, 185, 201, 202). Although early studies of CAR design have favored CD3ζ and ZAP70 dependent designs, recruiting the Syk tyrosine kinase in lieu of

ZAP70 may have some benefit in specific contexts. Finally, CXCR3-mediated T cell chemotaxis was shown to be dependent on ZAP70 and was impaired by TCR signaling (203). Modulating ZAP70 crosstalk between these two important pathways could affect the ability of T cells to infiltrate the tumor microenvironment and maintain TCR signals and resultant activation states. Collectively, this early evidence demonstrates artificially controlling ZAP70, or variants of ZAP70 with different signaling thresholds, could be powerful tools for cancer immunotherapy.

THE LAT SIGNALOSOME: LAT, BINDING PARTNERS AND DOWNSTREAM PATHWAYS

LAT: Structure and Function

LAT serves as a major junction point in TCR signaling, forming a nexus between the early antigen-recognition machinery and a multitude of downstream pathways (**Figures 1** and **5**) (204). Phosphorylation of LAT at multiple intracellular tyrosine residues by ZAP70 is a key link between TCR antigen recognition and the transcriptional paradigm shift of T cell activation (205). Due to its assembly of numerous signaling molecules, LAT has been referred to as the central platform for the "LAT signalosome." (206) LAT is comprised of minimal extracellular and transmembrane domains, and an extensive cytoplasmic region with numerous phosphorylation and protein binding sites (207).

LAT Mutations and Connections to Human Disease

Mutational mapping has allowed identification of tyrosine-phosphorylation residues required for LAT to associate with individual signaling partners. For example, mutation of tyrosine 132 in human T cells results in defective binding to PLCγ-1 (208), tyrosine 171 was essential for PI3K activation (209), tyrosines 110 and 226 are required for ERK activation (208), 171 and 191 required for Gads binding, and 171, 191, and 226 together are required for Grb2 binding (**Figure 5**) (210). Aside from tyrosine residues, study of the human Jurkat T cell line identified 11 serine residues in the cytoplasmic domain that may be key to signal propagation. Cells expressing LAT with S->A mutations at serines 38, 40, 106, 164, and 180 exhibited decreased PLCγ-1 and SLP-76 binding, reduced IL-2 production, but increased ZAP70 phosphorylation (211). The impact of each individual serine residue has yet to be elucidated. A recent study identified as many as 90 putative binding partners for LAT, suggesting the myriad of established roles for residues in this molecule may yet underestimate the importance of LAT in T cell activation (212).

Studies in mice have allowed mechanistic elucidation of the key function of LAT and its structure in T cell development and mature T cell functions (213–215). Completely blocking LAT expression or function in mice led to impairment in T cell development. Mutation of the distal 4 tyrosine residues of LAT to phenylalanine mimicked the phenotype of LAT-/- mice, in which T cell development is completely blocked at the double-negative 3 (DN3) stage (215). Selectively knocking-out LAT expression after the DN3 stage led to impaired development of single positive T cells in the thymus and periphery (216). Conversely, mutations or deletions of LAT in mature post-thymic T cells altered but did not completely impair T cell functions, and even led to autoimmunity and aberrant lymphoproliferation, implicating LAT in not only T cell activation but also regulation (217, 218). In one study, a mutation in tyrosine 136 and impaired PLCγ binding led to MHC-independent constitutive Th2 T cell activation and T cell dependent B cell hyperproliferation and antibody production, ultimately resulting in systemic autoimmune disease (219). Others showed that the same mutation resulted in disruption of thymic positive and negative selection (220). Similarly, a third study showed that mutation of tyrosine 136 blocked PLCγ dependent functions, but allowed for continued ERK signaling, first causing impaired T cell development but later causing a lymphoproliferative disorder (221). The mechanism of negative regulation *via* LAT could be mediated *via* association with Grb2, a known inhibitory signal mediator (222), or through binding and inhibiting the active form of LCK (223). Together these results implicate LAT in both T cell thymic selection as well as subsequent immune regulation, and that complex interactions between LAT and its multiple binding partners maintains a balance between T cell activation and inhibition.

In humans, mutations or polymorphisms in LAT recapitulate the range of mouse phenotypes ranging from autoimmunity to immunodeficiency (224). Higher expression levels of LAT (as assessed by qRT-PCR and flow cytometry) were correlated with severity of aplastic anemia, however it was unclear how this over-expression was regulated or if this was a cause or consequence of disease (225). Loss of the cytoplasmic tail of LAT due to a mutation in exon 5 resulted in immunodeficiency characterized by a decrease in circulating T cells, but simultaneous severe autoimmunity (226). T cells in these patients were still able to induce calcium influx and NF-κB activation, but had aberrant ERK signaling (226). The heterogeneity of disease resulting from LAT-deficiency has led some to distinguish LAT-dependent pathology due to immune hyper-activation from true autoimmunity (227). The mouse studies above do suggest LAT also plays a role in regulating thymic selection and can lead to true autoimmune T cells. A similar role in humans has not been entirely ruled out. These studies underscore the fine balance between pro and anti-inflammatory processes that evolved to maintain homeostasis within the immune system, and that LAT is a key regulator of this balance in T cells.

LAT: Connections to Immunotherapy

As of now, no immunotherapies have modulated LAT to enhance anti-cancer T cell responses. Based on our above understanding, it is possible that changes in LAT would lead to too many off target effects of T cell-based therapies, or persistent non-specific inflammation that would be detrimental to the host. However, careful alterations could also increase T cell activation in response to weak or rare neo-antigens or help adoptively

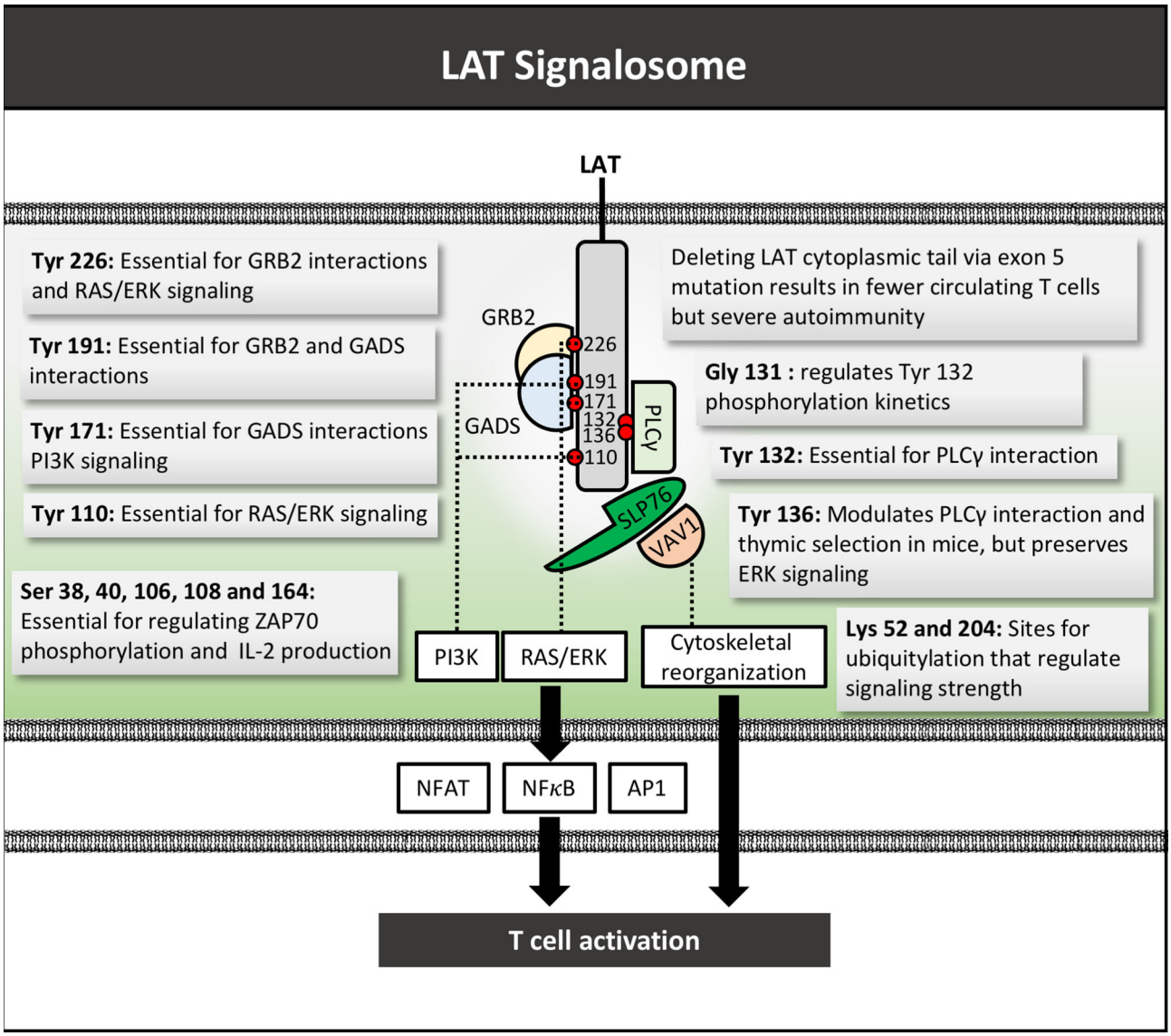

FIGURE 5 | LAT signalosome structure and function. LAT signalosome schematic structure with function of key tyrosine residues depicted by their respective numbers, as well as mutations and respective phenotypes. Several key LAT signaling partners are depicted. Red circles denote phosphate groups. Tyr, Tyrosine; Gly, glycine; Ser, serine; Lys, Lysine.

transferred T cells maintain activation states in solid tumors. These varied effects could be achieved by selectively mutating key tyrosine or serine residues within the cytoplasmic tail. Additional modulations could include replacing lysine with arginine residues, which was shown to decrease ubiquitylation and enhance T cell signaling (228), or mutating residues neighboring key tyrosines to alter phosphorylation kinetics (229). Furthermore, the use of LAT instead of CD3ζ as the intracellular signaling component of CARs could be considered. Extensive pre-clinical testing would be required to ensure any increased T cell activation caused by LAT modulation would not result in intolerable or dangerous side effects and means to kill or turn off such cells should be incorporated as a safety mechanism.

LAT Binding Partners and Downstream Signaling

LAT serves as a major hub after which TCR signaling networks with many other pathways in T cells, such as those downstream of chemokine and cytokine receptors and costimulatory molecules (204). As a consequence, many highly complex, and tightly regulated interactions take place including activation of canonical MAPK, NF-κB, Ca^{++}-mediated signaling pathways that ultimately regulate the nuanced transcriptional profiles characteristic of each T cell phenotype (**Figure 5**) (217, 230). Abnormalities in any one of the many proteins involved in these processes can lead to aberrant T cell phenotypes. Some of the best described interactions between LAT occur with ITK, PLC-

TABLE 1 | Mutations and molecular alterations in the TCR pathways.

Gene	Mutation/Alteration	Structural Outcome	Immunological Outcome	Clinical Outcome	Therapeutic Relevance	Reference
TCRα	G>A at the C-terminus of exon 3 (TRAC domain), chromosomal region 14q11.2	Partial loss of the connecting peptide domain and abolition of the transmembrane and cytoplasmic domains of the TCRα chain - impaired TCR complex assembly	Complete lack of α/β T cells	Patients would be excluded from allo-ACT therapies due to lack of endogenous T cells	Patients lacking T cells could more likely benefit from allo-ACT strategies by demonstrating reduced host v. engrafted T cell responses	(30)
TCR^	p.T48C on α chain and p.S57C on β chain	Creation of second disulfide bond within constant domain	Enhance TCR stability and signaling and T cell mediated tumor killing in cancer models Possibility of expedited T cell exhaustion due to increased TCR strength	Enhanced recognition of lowly expressed or weakly immunogenic tumor antigens	Enhance T cell mediated immunotherapy Engineering this mutation into tumor-reactive TCR to improve antitumor responses	(35)
TCR^	Substitution of leucine/isoleucine residues for 7 hydrophilic residues in TCRα and 10 hydrophilic residues in TCRβ	Increased hydrophobic interactions in the transmembrane domain	Enhanced TCR surface expression and T cell avidity; increased anti-tumor T cell activity *in vitro* Possibility of expedited T cell exhaustion due to increased TCR strength	Enhanced recognition of lowly expressed or weakly immunogenic tumor antigens	Enhance T cell mediated immunotherapy Engineering this mutation into tumor-reactive TCR to improve antitumor responses	(36)
TCR^	Mutating N of the N-glycosylation motif (N-X-S/T) to a glutamine. TCRα: position 84C, 90 and 113 on vα3. TCRβ: position 1.3, 84.5 and 113 on vβ3.	Decreased N-glycosylation of extracellular constant domain	Increased TCR avidity	Enhanced recognition of rare tumor antigens by TCR-pMHC and improved anti-tumor immunity	Enhance T cell mediated immunotherapy Engineering this mutation into tumor-reactive TCR to improve antitumor responses	(37)
TCR^	Exchange human constant region with murine equivalent or 9 amino acids from murine constant region	Prevents native-non-native heterodimerization	Improved pairing of TCR subunits, enhanced CD3/TCR stability, increased anti-tumor activity	Improved outcomes in adoptive transfer approaches using engineered TCRs	Improved outcomes in adoptive transfer approaches using engineered TCRs	(38–39)
TCR^	p.T48C on α chain and p.S57C on β chain	Improved engineered TCR chain pairing, decreased pairing with native TCR chains	Improved engineered TCR T cell tumor antigen recognition and thereby anti-tumor activity	Improved outcomes in adoptive transfer approaches using engineered TCRs	Improved outcomes in adoptive transfer approaches using engineered TCRs	(40)
TCR^	Core peptide targeting of TM domain	Interrupt cohesive interactions between proteins and with TM lipids	Blocks T-cell mediated killing	Prevent autoimmunity	Treatment of autoimmune diseases, could be extrapolated to cancer therapies Use as a strategy to inhibit unwanted activity of TCR-engineered or TIL therapies	(41)
CD3D and CD3E	c.279C>A, c.202C>T leading to p.C93X and p.R68X nonsense codons of CD3D respectively. 2-bp deletion at nucleotide 128 of exon 5 of CD3E leading to frameshift and nonsense codon at residue 56.	Truncation of the extracellular domains of CD3D and CD3E respectively	Total lack of CD3+ thymocytes	SCID	Patients lacking T cells could more likely benefit from allo-ACT strategies by demonstrating reduced host v. engrafted T cell responses	(46)

(Continued)

TABLE 1 | Continued

Gene	Mutation/Alteration	Structural Outcome	Immunological Outcome	Clinical Outcome	Therapeutic Relevance	Reference
CD3Z	38 SNPs in intron 1		Disrupt CD3 expression or ability of CD3 to bind TCR	Association with SLE	Potential use as clinical marker to predict efficacy or development of autoimmunity of ACT T cell therapies	(48)
CD3E	Deletion of 173T in exon 6	Premature stop codon	Lack of T cells	SCID		(50)
CD3G	A>G mutation in initiator codon and G>C mutation at intron 2-exon 3 splice site	Severe truncation or lack of CD3G translation	Low level expression of TCR on T cells	Spectrum from SCID to mild immunodeficiency	Engineering these mutations into T cells or using T cells from individuals harboring these mutations could be an approach to replace the need to knock out endogenous TCRs for allo-ACT strategies	(51)
CD3D	c.202C>T	premature stop codon at residue 68	Selective lack of α/β T cells but preservation of the γ/δ T cell pool		Identify varied necessity of CD3D in α/β vs γ/δ T cell development.	(52, 56–58)
CD3D	G>A at position +5 of intron 2		Selective lack of α/β T cells but preservation of the γ/δ T cell pool			(57, 58)
CD3G	Haploinsufficiency		Effects γ/δ T cell pool			(59)
CD3Z	SNPs and splice variants in 3'UTR, intron 1 (eg rs858554), exon 7			Associated with SLE, RA, and ITP		(48, 60–64)
CD3Z	Hypermethylation			Associated with severe SLE phenotypes; correlates with reduced CD3ζ	Use as clinical marker to predict efficacy of checkpoint, CAR, TIL, or TCR-engineered therapies or potential for developing immunotherapy-mediated autoimmunity	(60)
CD3G	c.205A>T of exon 3	Premature stop codon at residue 69 of CD3γ	Decreased levels of TCR/CD3 expression	Range of disease from asymptomatic immunodeficiency to severe and fatal SCID	Reduced efficacy of TIL and checkpoint-based therapies	(69)
CD3G	Mutation at position -1 of exon 3	Premature stop codon	Disrupted CD3γ expression	Various autoimmune diseases		(70)
CD3G	c.1A>G and c.80G>C	Enrichment of hydrophobic residues at positions 6 and 7 of CDR3 chain	Decreased Treg function; impairment of CD8 T cell development only	Predisposed to autoimmunity	Increased potential for enhanced antitumor efficacy of immunotherapies but also increased risk of developing autoimmunity	(71)
CD3: ITAMS*^	Selective mutagenesis of CD3 ITAMS		Linear relationship between cumulative number of ITAM motifs and T cell proliferation rates	Mouse model with increased propensity for autoimmune disease	Precise regulation of engineered T cell activation	(73)
CD3G	Insertion/deletion in CD3γ promoter (rs66465034)			Increased HCC incidence	Clinical marker for disease, use as marker to predict efficacy of immunotherapies or potential for developing immunotherapy-mediated autoimmunity	(74)
CD3D	rs3181259			Increased recurrence in NSCLC	Clinical marker for disease, use as marker to predict efficacy of immunotherapies or potential for	(75)

(Continued)

TABLE 1 | Continued

Gene	Mutation/Alteration	Structural Outcome	Immunological Outcome	Clinical Outcome	Therapeutic Relevance	Reference
					developing immunotherapy-mediated autoimmunity	
CD3E	rs967591		Correlated with lower CD3ϵ expression	Shorter survival in NSCLC	Clinical marker for disease, use as marker to predict efficacy of immunotherapies or potential for developing immunotherapy-mediated autoimmunity	(76)
CD3Z: ITAMS*^		Selective mutation of one or two ITAMS of CD3ζ in CD19-CD28-CD3ζ CAR		Induction of long-term remission in pre-B acute lymphoblastic leukemia mouse model	Improved CAR or TCR-engineered therapies	(83)
CD3Z^		CAR with FcRγ instead of CD3ζ intracellular domain		Greater anti-tumor efficacy with CDζ	Improved CAR therapies	(85, 86)
CD3Z	rs2949655			Reduced cytotoxicity in response to BiTE treatment	Clinical marker of response to treatment or potential development of autoimmunity	(92)
CD8A	p.G111S		Complete lack of CD8 T cells	Recurrent infections		(103)
CD4/ CD8*^	Missense, non-functional mutations - numerous		Lack of CD4 or CD8 T cell subtypes		Would not respond to checkpoint therapies and would be excluded from autologous ACT strategies	(104, 105)
CD8*^	Decreased sialylation of CD8 stalk region		Increased MHC-I binding affinity		Potential use to increase CD8 T cell activation	(123)
CD4^	p.Q40Y and p.T45W		Increased MHC-II binding affinity		Potential use to increase CD4 T cell activation	(101)
CD8A*^	Fused CD8α to MyD88		Enhanced CD8 T cell function		Improve ACT approaches	(120)
LCK*	Genetic knockout	LCK deficiency	Impaired T cell development, impaired activation induced T cell death		Patients would not benefit from T cell based immunotherapies	(129–132, 149)
LCK	c.1022T>C	Reduced CD4 and CD8 expression	Impaired TCR activation, decreased Treg levels and expansion of an oligoclonal γ/δ T cell population	Severe recurrent infections, autoimmune manifestations and panniculitis;		(138)
LCK	c.188-2A>G	Splice variant	Impaired LCK function and immune dysregulation	Epidermodysplasia verruciformis, recurrent bacterial infections	Patients would not benefit from T cell based immunotherapies	(139)
LCK	Loss of exon 7		Impaired LCK function and immune dysregulation	CVID, SCID	Patients would not benefit from T cell based immunotherapies	(140, 141)
LCK*	Deletion of C-terminal regulatory tyrosine	Truncated Lck lacking Tyr 505	Increase LCK activity, sustained T cell activation, increase IL-2 production	Sustained oncogenesis in thymoma cell line Possibility of expedited T cell exhaustion due to increased TCR and cytokine signaling	Target for therapy in T cell malignancies Engineering this mutation into tumor-reactive CAR- or TCR-engineered T cells could improve antitumor responses.	(142)

(Continued)

TABLE 1 | Continued

Gene	Mutation/Alteration	Structural Outcome	Immunological Outcome	Clinical Outcome	Therapeutic Relevance	Reference
LCK	t(1;7)(p34;q34)	Chromosomal translocation	Increased LCK activity that is correlated with breakpoint location and disease severity	T cell acute lymphoblastic leukemia	Target for therapy in T cell malignancies Engineering this translocation into tumor-reactive TCR-, CAR--TCR-engineered T cells or TILs could improve antitumor responses.	(144)
LCK binding partner^	Mutation of PYAP motif within CD28	Deleted LCK-binding motif in CD28 (ΔCD28) in CARs	Reduced IL-2 production, improved tumor control	Stable disease with pleural mesothelioma	Enhancement of CAR T cell efficacy	(145–147)
LCK	rs10914542		Impairs TCR activation and proliferation	Increase risk of T1D	Predict efficacy of ICI therapy	(151)
LCK	p.G85W of exon 4			Associated with Sjogren syndrome, SLE, RA	Possible increased risk of developing autoimmune complications	(152)
FYN*^	Genetic knockout	Loss of Fyn expression	Promotes differentiation of CD4+ T cells towards Treg (away from Th17)		Activating FYN could promote Th17 phenotype	(163)
ZAP70	Mutations in SH2 and kinase domains: c.169G>A, c.448C>T, c.1602C>T, c.1603G>A, c.1729C>T, c.1763C>A, c.1833G>A, c.1923A>T	Loss or destabilization of protein transcript	Complete lack of functional T cells	Severe immunodeficiency	Patients would not benefit from T cell based immunotherapies	(175–182)
ZAP70	Polymorphisms in coding region (rs17695937, rs13420683) or 3'UTR (rs2278699)			Associated with psoriasis, T1DM, IBD, RA		(183–186)
ZAP70	Intron variant (rs7425883)		Aberrant expression of ZAP70 and enhanced BCR signaling in B cell CLL	Associated with decreased risk of developing non-Hodgkin lymphoma	ZAP70 as prognostic marker of disease Clinical marker for disease, use as marker to predict efficacy of immunotherapies or potential for developing immunotherapy-mediated autoimmunity	(187)
ZAP70*^	Point mutations in second SH2 interdomain, or paired mutations in kinase region		Aberrant thymic selection permissive to higher affinity self-reactive clones		Engineering this mutation into CAR- or TCR-engineered T cells could improve antitumor responses.	(189–191)
ZAP70^	p.Y292F, p.Y315F, p.Y492F	Lack of phosphorylation at key inhibitory residues	De-inhibited ZAP70 signaling	Increased T cell activation	Could be used to enhance T cell activation for immunotherapy	(192)
ZAP70^	p.M414A, p.M414A/p.C405V	Selective inhibition of engineered ZAP70 with PPI-derived inhibitor	Impaired catalytic activity (not observed in Tregs)	Can selectively turn "off" ZAP70 in engineered T cells	Similar approaches could be used to turn off adoptively transferred T cells	(193, 194)
ZAP70^	Tetracycline-inducible ZAP70 promoter	Selectively turn on ZAP70 transcription			Selective over-expression and activation of T cells	(195)
ZAP70^	Fusion of analog-sensitive ZAP70 to the ligand binding domain of the estrogen receptor	Selectively turn on and off ZAP70	Turn on and off ZAP70 in minute-by-minute timeframe		Tight real-time control of engineered T cell activation states	(196)
LAT^	p.Y132F	Defective binding with PLCγ-1			Could be used to fine-tune T cell activation states	(204)
LAT^	p.Y110F and p.Y226F	Required for ERK activation			Could be used to fine-tune T cell activation states	(204)

(Continued)

TABLE 1 | Continued

Gene	Mutation/Alteration	Structural Outcome	Immunological Outcome	Clinical Outcome	Therapeutic Relevance	Reference
LAT^	p.Y171F	Essential for PI3K activation			Could be used to fine-tune T cell activation states	(205)
LAT^	p.Y171F p.Y191F	Required for Gads binding			Could be used to fine-tune T cell activation states	(206)
LAT^	p.Y171F, p.Y191F, p.Y225F	Required for Grb2 binding			Could be used to fine-tune T cell activation states	(206)
LAT^	p.S38A, p.S40A, p.S106A, p.S164A, p.S108A	Decreased PLCγ-1 and SLP-76 binding, increased ZAP70 phosphorylation	Reduced IL-2 production		Could be used to fine-tune T cell activation states	(207)
LAT*^	Mutation of distal 4 Tyr residues to Phe		Mimics LAT-/- in mice; T cell development blocked at DN3 stage		Patients likely to demonstrate weakened responses to T cell based immunotherapies	(209)
LAT*^	p.Y136F	Impaired PLCγ binding	MHC-independent constitutive TH2 activation; T cell dependent B cell hyperproliferation and antibody production	Systemic autoimmune disease	Increase T cell activation	(215)
LAT*^	p.Y136F		Disrupted thymic positive and negative selection			(216)
LAT*^	p.Y136F	Blocked PLCγ dependent functions, ERK signaling maintained	Impaired T cell development	Lymphoproliferative disorder	Increase T cell proliferation	(217)
LAT*^	c.268_269del	Loss of the cytoplasmic tail of LAT and phosphorylation sites	Decreased circulating T cells, aberrant ERK signaling	immunodeficiency with simultaneous autoimmunity	Patients likely to demonstrate weakened responses to T cell based immunotherapies	(222)
LAT^	p.K52R and p.K204R	Decreased ubiquitylation	Enhanced T cell signaling		Enhance T cell activation	(224)
LAT^	p.G131D or p.G131E	Increased phosphorylation kinetics of Y132, increased speed and magnitude of PLCγ activation	Increased sensitivity of T cells to weak antigen stimulation		Enhance T cell activation	(225)
ITK	Gain of function (t(5;9)(q33;q22))			T cell lymphomas	Target for therapy	(227)
ITK	Two patients: 1) p.R29H; 2) p.D500T, p.F501L, and p.M503X	ITK loss of function	Loss of T cell regulation	Lymphoproliferative diseases	Increase expansion of engineered T cells	(228)
ITK	p.Q17X	ITK deficiency		Idiopathic CD4+ T cell lymphopenia		(229)
ITK	c.196C>T in promoter region	Increased ITK transcription		Associated with asthma	Could be used to lower the threshold for T cell activation	(230)
FYB	rs6863066 rs358501		Low levels of LAT-PLCγ-1 interactions; skewing towards aberrant Th2 phenotype	Susceptibility to asthma	Patients likely to demonstrate weakened responses to T cell based immunotherapies	(231)
VAV1^	Deletion of nucleotides 1-67	Creates "oncogenic VAV1"	Decreased calcium mobilization	Linked to oncogenesis	Patients likely to demonstrate suboptimal T cell responses to immunotherapies	(236)
VAV1	p.E59K, p.D517E	Constitutively active or highly stable and overexpressed VAV1	Oncogenesis	Driver mutations in human lung adenocarcinoma	Potential target for therapy	(237)

(Continued)

TABLE 1 | Continued

Gene	Mutation/Alteration	Structural Outcome	Immunological Outcome	Clinical Outcome	Therapeutic Relevance	Reference
ADAP*^		ADAP KO in adoptively transferred T cells	Reduced PD-1 expression, increased anti-tumor efficacy		Improve adoptive T cell therapy	(240)

Detailed tabulation of all major genetic and molecular changes discussed in this review, with the clinical or phenotypic outcomes and potential relevance to immunotherapy as indicated. Of note, this is not an exhaustive list. For additional detailed inquiries, please refer to the references cited in the text.

**studies in mice only.*

^mutational studies or engineered mutations.

AD, Atopic dermatitis; CVID, Common Variable Immune Deficiency; HCC, Hepatocellular Carcinoma; SLE, Systemic Lupus Erythematosus; IBD, Inflammatory Bowel Disease; CD, Crohn's disease; ICB/ICI, Immune Checkpoint Blockade/Inhibitors; T1D, Type 1 Diabetes; X-SCID/SCID, X-Linked Severe Combined Immunodeficiency; PSC, primary sclerosing cholangitis; MSMD, Mendelian Susceptibility to Mycobacterial Disease; MS, Multiple Sclerosis; ITP, Immune Thrombocytopenia; UC, Ulcerative Colitis; RA, Rheumatoid Arthritis; TB, Tubercolosis.

γ1, Grb2, SLP-76, VAV1, and FYB1 (230). Most of these are activating, but the interaction with Grb2 is generally inhibitory to T cell activation, as briefly mentioned above (222). As an example of the spectrum of diseases caused by these downstream proteins, gain of function of ITK is common in T cell lymphomas (231), loss-of-function mutations are associated with lymphoproliferative diseases (232), full ITK deficiency has been linked to idiopathic CD4+ T cell lymphopenia (233), and SNPs in ITK have been associated with asthma (234). Polymorphisms in FYB as well as low levels of LAT-PLC-γ1 interactions have also been implicated in susceptibility to asthma, perhaps due to skewing of T cell polarization towards an aberrant Th2 phenotype (235, 236). VAV1 has strong links to oncogenesis in various tissues (237–239), with mutations causing disruptions in multiple pathways including Ca^{++} signaling (240, 241). It is important to note that many of these downstream mediators are involved in signaling in other immune cell types besides T cells, and receptor driven pathways other than the TCR pathway even within T cells. For example, PLC-γ1 is involved in FcεRI-mediated mast cell degranulation, thus its association with asthma maybe more due to effects on mast cells as opposed to T cells (242). FYB is also expressed in many other immune cell types of both the innate and adaptive system with diverse functions, making cell-type or pathway specific conclusions difficult (243). Any targeting of these molecules to alter T cell function must take into account that not only T cells or the TCR pathway may be affected.

Given the complexity of this stage of T cell activation, no therapies targeting these downstream networks have yet been approved in cancer immunotherapy. However, a few groups have begun to explore distal TCR signaling modulation to benefit immunotherapy. For instance, knocking out ADAP in adoptively transferred T cells reduced PD-1 expression and increased anti-tumor efficacy in mice (244). Other pre-clinical studies have shown that Cis-mediated inhibition of CD8 T cells functions by down-regulating PLC-γ, and targeting this pathway could increase potency of CARs and other adoptive therapies (245). Outside the context of immunotherapy, directly targeting some of these molecules could also have direct anti-neoplastic effects. Much more cell-type specific, and pathway specific studies are needed before targeting these downstream mediators can be used safely and effectively in treatment of human cancers.

CONCLUSIONS

The TCR proximal signaling pathway that regulate T cell function comprise a complex cascade of interactions involving numerous extracellular and intracellular proteins with unique functions. Perturbations such as polymorphisms and mutations in each contribute to a myriad of human diseases ranging from immunodeficiency to autoimmunity, and some even have significant contributions to various malignancies. Understanding the nuanced role of each molecule has allowed for the design of immunotherapies to take advantage of the significant involvement of TCR signaling and cytokine activation of T cells in cancer. The genetic alterations described throughout this review are summarized in **Table 1**.

AUTHOR CONTRIBUTIONS

AK and NL wrote the manuscript and prepared the figures. AC prepared the table and provided editing and input into the manuscript. ED oversaw the entire project providing guidance as well as detailed review and editing of all aspects of the paper. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Advanced Flow Cytometry Assays for Immune Monitoring of CAR-T Cell Applications

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Adoptive immunotherapy using chimeric antigen receptor (CAR)-T cells has achieved successful remissions in refractory B-cell leukemia and B-cell lymphomas. In order to estimate both success and severe side effects of CAR-T cell therapies, longitudinal monitoring of the patient's immune system including CAR-T cells is desirable to accompany clinical staging. To conduct research on the fate and immunological impact of infused CAR-T cells, we established standardized 13-colour/15-parameter flow cytometry assays that are suitable to characterize immune cell subpopulations in the peripheral blood during CAR-T cell treatment. The respective staining technology is based on pre-formulated dry antibody panels in a uniform format. Additionally, further antibodies of choice can be added to address specific clinical or research questions. We designed panels for the anti-CD19 CAR-T therapy and, as a proof of concept, we assessed a healthy individual and three B-cell lymphoma patients treated with anti-CD19 CAR-T cells. We analyzed the presence of anti-CD19 CAR-T cells as well as residual CD19+ B cells, the activation status of the T-cell compartment, the expression of co-stimulatory signaling molecules and cytotoxic agents such as perforin and granzyme B. In summary, this work introduces standardized and modular flow cytometry assays for CAR-T cell clinical research, which could also be adapted in the future as quality controls during the CAR-T cell manufacturing process.

Keywords: CAR-T, CD19, immune profiling, antibody, flow cytometry, staining technology

INTRODUCTION

Adoptive immunotherapy using immune effector cells engineered *ex vivo* to express chimeric antigen receptors (CARs) that mediate the lysis of cancer cells has become an innovative approach in cancer therapy (1). The CD19 antigen expressed on B cells has been the first clinically approved target of CAR-T cell immunotherapy. In comparison to conventional treatment approaches, anti-

CD19 CAR-T cell treatment led to impressive remission rates in patients with precursor B-Cell Acute Lymphoblastic Leukemia (B-ALL), Diffuse Large B-Cell Lymphoma (DLBCL), Primary Mediastinal B-Cell Lymphoma (PMBCL) and Mantle Cell Lymphoma (MCL) (2–5). Spearheaded by this success, the potential of CAR-T cell therapy is currently being investigated in hundreds of clinical trials – the large majority in B cell malignancies (6). However, despite its great promise, CAR-T cell therapy bears several medical and economical challenges. Indeed, CAR-T cell therapy comes with severe, toxic and potentially life-threating adverse effects, which have been observed in a high proportion of patients (2–5, 7). These side effects are caused by cytokine secretion (e.g. IL-1, IL-6) due to immune cell and target interactions, which initiate cytokine release syndrome (CRS), macrophage activation syndrome (MAS) and neurotoxic symptoms (8–12). Beside body-imaging, monitoring of the patient's immune system before and after CAR-T cell treatment and during further course of therapy could help to evaluate the success of the therapy and the status of the applied CAR-T cells. Furthermore, such patient monitoring could help to estimate the systemic immune response and might indicate the risk of immediate adverse effects or long-term complications. Focusing on B cell malignancies, at first the presence of both anti-CD19 CAR-T cells and remaining CD19+ B cells needs to be determined. Secondly, the fitness and activation status of the anti-CD19 CAR-T cells and the interacting patient´s immune cells might help to better understand the therapy course. Furthermore, the expression profiles of immune checkpoint molecules, co-inhibitory and co-stimulatory receptors, and differentiation markers of T lymphocytes are of high interest, because ligand interactions with these molecules can modulate endogenous T cell and CAR-T cell efficacy.

Methods to assess these parameters must be highly standardized, robust, and transferable. Multiparametric immunophenotyping by flow cytometry allows the fast, comprehensive and mostly antibody-based routine analysis of progenitor and immune cells in the peripheral blood, bone marrow or cerebrospinal fluid in healthy people and in patients with leukemia and lymphoma (13–15). However, flow cytometry is prone to variability due to individual operator handling, reagent/antibody issues and data analysis (16). Pre-formulated dry antibody panels that are expert-designed and produced under standardized conditions can contribute to minimizing these sources of human variability and technical issues. DURA Innovations (Beckman Coulter Life Sciences, Brea CA/USA) is a research technology that supports the standardization of flow cytometry through a non-lyophilized layer of pre-formulated, dry antibodies at the bottom of a ready-to-use test tube. In addition, tandem dye conjugates used in the DURA Innovations format ensure consistency without the need for refinement of spillover correction regardless of how experienced an operator might be. These pre-formulated antibody panels can be complemented by the addition of further antibodies in liquid format to match specific requirements related to CAR-T cell therapy, research applications or the quality control (QC) of CAR-T cell production.

In this research study, we apply a comprehensive set of standardized flow cytometry assays to phenotype the cellular immune system. As a proof of concept, we examine the immune cells present in blood samples of three CAR-T cell treated patients suffering from B cell lymphoma in comparison to respective controls. We show that the flow cytometry assays are suitable to track the CAR-T cells, to assess the fitness of the T cell compartment and to measure further accompanying factors in the blood of the patient's immune system. We believe that the novel 13-colour/15-parameter flow cytometry assays could help to monitor the course of CAR-T immunotherapy in the future. Furthermore, the applied assays might also serve as a novel QC tool to monitor the manufacturing of CAR-T cell products.

MATERIALS AND METHODS

Human Blood Samples

EDTA-anticoagulated (Sarstedt, Nümbrecht, Germany) or heparinized whole blood was obtained from a healthy donor and from three patients (DLBCL, PMBCL, transformed Follicular Lymphoma) treated with anti-CD19 CAR-T cells (Axicabtagene ciloleucel or Tisagenlecleucel), which gave informed consent. Ethical approval was given by the local Ethic committee at the University of Leipzig (351/17-ek).

Instrument Settings

In our study, the *Minimum Information about a Flow Cytometry Experiment* (MiFlowCyt) (17) were considered if reasonable. A DxFLEX flow cytometer equipped with 3 Lasers (405/488/638nm), 13 fluorescence detectors and a standard filter configuration (Beckman Coulter Life Sciences, Brea CA/USA) was used in this study. Setup, calibration and quality control procedures (QC) were conducted according to the manufacturer's instructions. In brief, to set up the DxFLEX instrument, Daily QC beads (Beckman Coulter Life Sciences, Brea CA/USA) were used. For compensation, either single color-stained lysed whole blood samples or single color-stained VersaComp antibody capturing beads (Beckman Coulter Life Sciences, Brea CA/USA) were used and an automatic compensation was performed according to the manufacturer's instructions. The filter specifications, the fluorochrome labels used and detectors are shown in **Table 1**.

TABLE 1 | Filter specifications, fluorochrome labels used and detectors applied in this study.

Excitation / Filter	Violet Laser 405 nm	Blue Laser 488 nm	Red Laser 638 nm
450/45 BP	Pacific Blue, SNv428, ViaKrome 405		
525/40 BP	Krome Orange	FITC	
585/42 BP		PE	
610/20 BP	BV605, Superbright 600	ECD	
660/10 BP	BV650		APC
690/50 BP		PC5.5	
712/25 BP			APC-AF700, AF700
780/60 BP	BV785	PC7	APC-AF750, AF750

Leukocyte Counting and Viability

Leukocytes counting and cell viability were performed using the ready-to-use DURAClone IM-count tube containing anti-CD45-FITC, 7-AAD and reference beads (Beckman Coulter Life Sciences, Brea CA/USA). 100 µl of EDTA-anti-coagulated peripheral blood was added to the DURAClone IM count tube (Beckman Coulter Life Sciences), followed by 6-8 sec of mixing and 15 min of incubation at room temperature (RT) in the dark. Next, 2 ml of Versalyse (Beckman Coulter Life Sciences) were added for red blood cell lysis, followed by mixing, 10 min incubation at RT in the dark and data acquisition on the flow cytometer. The obtained CD45+ leukocyte absolute count is used to estimate cellular concentrations for CD45+ subpopulations in all tubes with a CD45 staining.

Surface Marker Immunostaining

The antibody panels TCR, RE ALB, CAR-T1, CAR-T2, NK1, NK2 were used to stain the EDTA blood samples. The CAR-T1, CAR-T2, NK1 and NK2 were custom design panels in dry DURA Innovations format and are not available as off-the-shelf products.

After the addition of further liquid drop-in antibodies to the dry antibody panels, 100 µl of EDTA-anti-coagulated whole blood was added, followed by mixing and 15 min incubation at RT in the dark. Red blood cells were lysed by adding 2 ml of VersaLyse, followed by mixing and incubation for 10 minutes in the dark. Lysed cells were centrifuged at 200 g for 5 min, the supernatant was discarded and cells were washed with 3 ml of phosphate buffered saline (PBS, 10x Gibco, 70011-036). After centrifugation (200 g, 5 min), the supernatant was discarded and cells were resuspended in 0.5 ml of PBS, containing 0.1% formaldehyde prior to acquisition on the flow cytometer.

Intracellular Immunostaining

For the antibody panel CAR-T3 (intracellular proteins) 50 µl of heparinized blood was stimulated using the dry DURActive 1 kit containing phorbol 12-myristate 13-acetate (PMA), Ionomycin and Brefeldin A (Beckman Coulter Life Sciences) for 3 h at 37°C, or control stimulated using PBS. Activated samples were prepared using the PerFix-nc buffer system (please see Beckman Coulter's detailed instructions for the use of PerFix-nc, product ref. B31167). The activated material was fixed with buffer R1 for 15 min. In the meantime, 300 µl of buffer R2 was added to the dry CAR-T3 antibody panel and the additional liquid drop-in antibodies before adding the fixed sample to the CAR-T3 tube. Red blood cell lysis occurs synchronously to permeabilization and staining during incubation for 30 min at RT in the dark. Stained samples were washed with 3 ml of buffer R3, centrifuged (200 g, 5 min) and the cell pellet was resuspended in 0.5 ml buffer R3 prior to acquisition.

Antibodies

The antibodies and dyes used in this study are shown in **Table 2**.

TABLE 2 | Antibodies and dyes used in this study.

Antibody	Clone	Fluorochrome (s)	Volume per test	Product ref.	Vendor
7-AAD	n.a.	n.a.	n.a.	C00162 (DURAClone IM Count)	Beckman Coulter
aCD19 CAR Biotin	n.a.	n.a.	5 µl	130-115-965	Miltenyi Biotec
anti Biotin	REA746	PE	5 µl	130-110-951	
CD3	UCHT-1	APC-AF750, AF750	n.a.	B53340 (DURAClone IM TCR), DURAClone custom	Beckman Coulter
CD4	13B8.2	APC	n.a.		Beckman Coulter
CD8	B9.11	Krome Orange, AF700	n.a,		Beckman Coulter
CD10	ALB1	PC5.5	n.a.	C00163 (DURAClone RE ALB)	Beckman Coulter
CD15	80H5	Pacific Blue	10 µl	B49218	Beckman Coulter
CD16	3G8	FITC	n.a.	DURAClone custom	Beckman Coulter
CD19	J3-119	PC7	n.a.	C00163 (DURAClone RE ALB)	Beckman Coulter
CD20	B9.E9	APC-AF750	n.a.		Beckman Coulter
CD22	SJ10.1H11	APC	10 µl	A60791	Beckman Coulter
CD24	ALB9	APC	10 µl	A87785	Beckman Coulter
CD25	B1.49.9	SNv428	n.a.	DURAClone custom	Beckman Coulter
CD27	1A4CD27	PC5.5	10 µl	B21444	Beckman Coulter
CD28	CD28.2	BV650	5 µl	302946	Biolegend
CD31	WM59	BV605	5 µl	303121	Biolegend
CD34	581	ECD	n.a.	C00163 (DURAClone RE ALB)	Beckman Coulter
CD38	LS198-4-3	APC-AF700	n.a.		Beckman Coulter
CD45	J33	Krome Orange	n.a.	B53340 (DURAClone IM TCR), C00163 (DURAClone RE ALB), DURAClone custom	Beckman Coulter
CD45RA	HI100	BV785	5 µl	304140	Biolegend
CD56	N901	APC-AF750	n.a.	DURAClone custom	Beckman Coulter
CD56	5.1H11	BV785	5 µl	362550	Biolegend
CD57	NC1	FITC	n.a.	DURAClone custom	Beckman Coulter

(Continued)

TABLE 2 | Continued

Antibody	Clone	Fluorochrome (s)	Volume per test	Product ref.	Vendor
CD58	AICD58	FITC	n.a.	C00163 (DURAClone RE ALB)	Beckman Coulter
CD69	TPI.55.3	PC7	n.a.	DURAClone custom	Beckman Coulter
CD95	DX2	BV650	5 ul	305642	Biolegend
CD127	R34.34	FITC	n.a.	DURAClone custom	Beckman Coulter
CD134 (OX40)	Ber-ACT35	BV650	5 µl	563658	BD Biosciences
CD137 (4-1BB)	4B4-1	ECD	n.a.	DURAClone custom	Beckman Coulter
CD155 (PVR)	SKII.4	BV650	5 µl	748275	BD Biosciences
CD197 (CCR7)	GO43H7	BV605	5 µl	353224	Biolegend
CD223 (LAG-3)	11C3C65	ECD	n.a.	DURAClone custom	Beckman Coulter
CD226	11A8	BV785	5 µl	338322	Biolegend
CD274 (PD-L1)	PD-L1	PC7	n.a.	DURAClone custom	Beckman Coulter
CD278 (ICOS)	C398.4A	BV785	5 µl	313534	Biolegend
CD279 (PD-1)	PD1.3	PC5.5	n.a.	DURAClone custom	Beckman Coulter
CD314 (NKG2D)	ON72	APC	n.a.		Beckman Coulter
CD335 (NKp46)	BAB281	PE	n.a.		Beckman Coulter
CD366 (TIM-3)	F38-2E2	SNv428	n.a.		Beckman Coulter
HLA-DR	Immu357	ECD, PC5.5	n.a.		Beckman Coulter
Granzyme B	GB11	ECD	n.a.		Beckman Coulter
INFγ	45.15	FITC	n.a.		Beckman Coulter
IL-2	MQ1-17H12	PC7	n.a.		Beckman Coulter
Perforin	dG9	PC5.5	n.a.		Beckman Coulter
TCRαβ	IP26A	PE	n.a.	B53340 (DURAClone IM TCR)	Beckman Coulter
TCRγδ	IMMU510	FITC	n.a.		Beckman Coulter
TCRVδ1	R9.12	PC7	n.a.		Beckman Coulter
TCRVδ2	IMMU389	Pacific Blue	n.a.		Beckman Coulter
TIGIT	A15153G	BV605	5 µl	372712	Biolegend
TNFα	IPM2	AF700	n.a.	DURAClone custom	Beckman Coulter
ViaKrome 405	n.a.	n.a.	2.5 µl (recons-tituted)	C36614	Beckman Coulter
VISTA	B7H5DS8	Superbright600	5 µl	No longer available	Thermo Fisher

Data Analysis

All dot plots were generated using Kaluza 2.1 analysis software (Beckman Coulter Life Sciences).

RESULTS

Pre-Formulated Dry Antibody Assays for Flow Cytometry

In order to establish a standardized flow cytometry method for CAR-T monitoring, we developed assays that are based on pre-formulated dry antibody panels usefull at all steps of CAR-T cell therapy (**Figure 1**). These flow cytometry assays are produced so that the antibodies are present in dried form at the bottom of the reagent tube and the (blood) sample can be directly added to the pre-formulated antibody mixtures. In our study we established assays consisting of comprehensive immune cell antibody backbone panels, which we complemented with accompanying antibodies to best fit our purpose of phenotyping the cellular immune systems of CAR-treated patients. We have carefully configured all our antibody panels so that antigen density and fluorochrome brightness are reciprocally matched and that spill over situations are minimized or not critical. The exact antibody composition of all established panels together with the fluorochromes used is shown in **Figure 2**.

Leukocyte Viability and Detection of CD19+ B Cells

To test the established assays, we used peripheral blood samples collected from a healthy donor and from a patient suffering from DLBCL treated with CD19-directed CAR-T cells (Axicabtagene ciloleucel). The blood sample from the DLBCL patient was obtained 9 days post-infusion when CAR-T cells typically peak in the peripheral blood (3, 5, 7, 18, 19). The blood samples were added to the test tubes, processed and measured on the flow cytometer as described in the experimental section. First, we determined the number of leukocytes in the blood samples and their viability (IM-count tube) (**Figure 3**). Overall, we found that the anti-CD19 CAR patient had much fewer leukocytes than the healthy control due to prior lymphodepleting therapy (**Figures 3a, A**) and that leukocyte viability was high (>93%) in both processed samples (**Figures 3b, B**). Next, we examined the

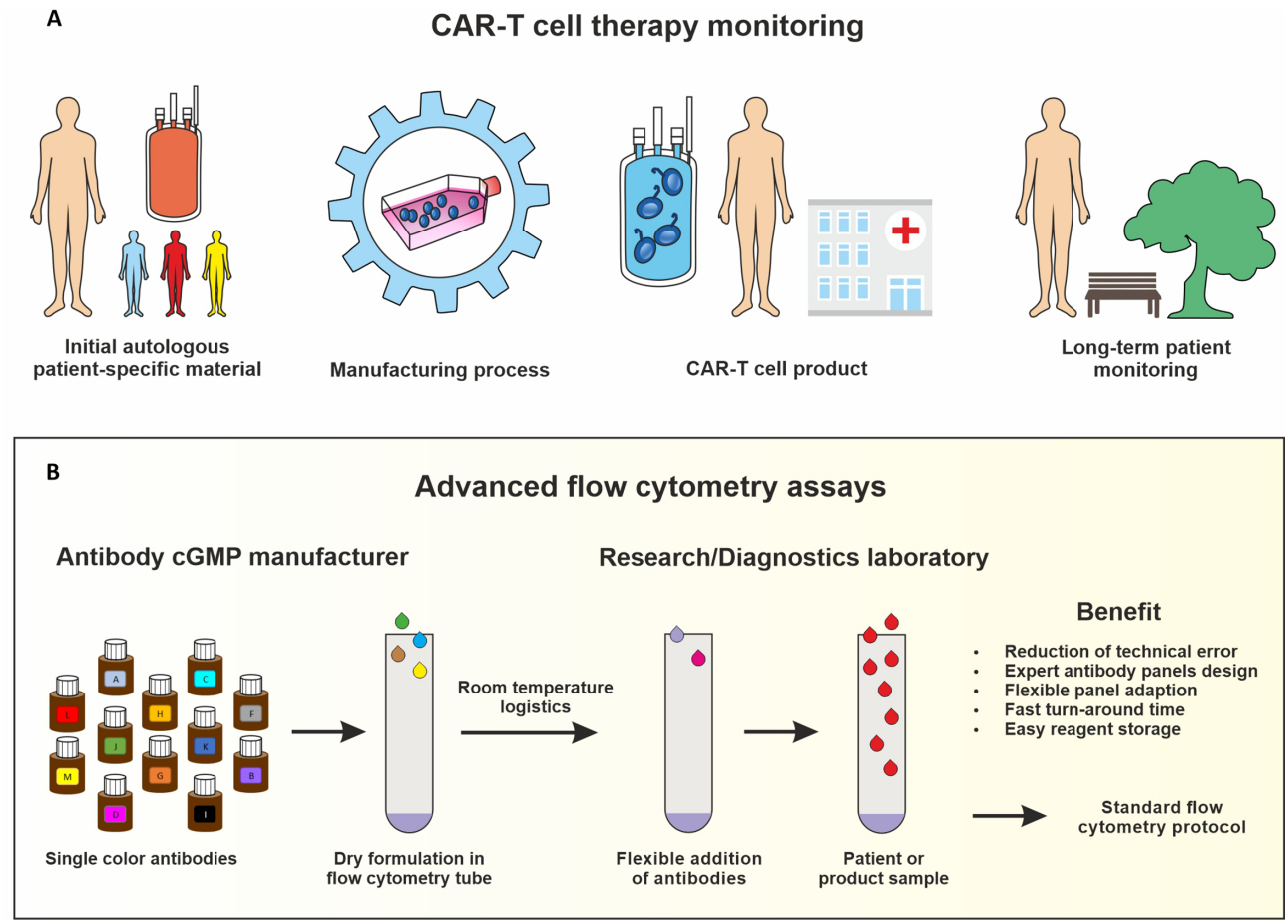

FIGURE 1 | Immune monitoring assays for CAR-T cell production and therapy. Key steps of the CAR-T cell therapy that should be monitored include analyzing the starting leukapheresis patient material, the manufacturing process, the CAR-T cell product release and the long-term patient follow-up **(A)**. Advanced flow cytometry assays based on dry uniform DURAClone antibody panels for standardization which can be complemented with antibodies in liquid formulation for specific purposes **(B)**.

CD19+ B cells in both samples (RE ALB tube) (**Figure 4**). Among the lymphocytes of the healthy individual we detected 3.55% CD19+ B cells (**Figures 4a–d**), which were largely also positive for the B cell maturation markers CD20 and CD22/24 (**Figures 4e–f**). These B cells were mostly negative for CD10, CD34 and CD38 (**Figures 4g–i**). In contrast, we could not find any residual CD19+ B cells in the anti CD19 CAR-T cell treated patient (**Figures 4A–F**). Furthermore, we could also not detect any anti-CD19 CAR+ CD19+ cells in the patient sample (**Figure 4D**).

Assessment of the T Cell Compartment and Anti-CD19 CAR-T Cell Fitness

To investigate the T cell compartment we used a T cell receptor (TCR) antibody panel (**Figure 5**). First, we gated for CD3+ and found that 86.25% and 65.30% of the lymphocytes were T cells in the healthy and CAR treated patient sample, respectively (**Figures 5a–c, 5A–C**). Afterward, we discriminated the CD3+ population in CD4+ and CD8+ T cells and observed a CD4/CD8 ratio of 3.8 (77.09%/20.32%) in the healthy individual (**Figure 5d**). Vice versa, in the CAR treated patient we found an inversed CD4/CD8 ratio of 0.5 (32.02%/64.82%) as can be seen in **Figure 5D**. Furthermore, the T cell activation status was much higher in the CAR treated patient than in the healthy individual as measured by HLA-DR expression (**Figure 5eE**). We found a similar percentage of 38% CD31+CD45RA+ recent thymic emigrants in both samples (**Figures 5f, F**). In the CAR treated patient 20.57% of all T cells were CD27+/CD28+ memory T cells, whereas in the healthy donor 76.41% of all T cells were CD27+/CD28+ (**Figures 5g, G**). Furthermore, we also determined the percentage of alpha/beta T cells and Vd1+ or Vd2+ gamma/delta T cells (**Figures 5h, H, i, I**) and we identified the lack of Vd2 gamma/delta T cells (TCVd2) in the CAR treated patient to be the main difference between both samples (**Figures 5i, I**).

Next, we aimed to characterize the anti-CD19 CAR-T cells in the peripheral patient blood. To this end, we applied the antibody panel CAR-T1 (**Figure 6**). As observed before in **Figure 5**, the healthy donor had much more leukocytes and the cell viability was high in both samples (**Figures 6a–c, A–C**). We also confirmed that the percentage of CD45+/CD3+ T cells

Panel		Violet (405nm)					Blue (488), Yellow-green (561) optional					Red (633-638nm)		
	DURA Clone	Pacific Blue / SNv428	Krome Orange	BV605	BV650	BV785	FITC	PE	ECD	PC5.5	PC7	APC	APC-AF700 /AF700	APC-AF750/ AF750
Cell count /viability	*IM Count*	-	-	-	-	-	CD45	counting beads	-	7-AAD	-	-	-	-
							Leukocytes			Viability				
T cell receptors	*IM TCRs*	TCRVδ2	CD45	CD31	CD28	CD45RA	TCRγδ	TCRαβ	HLA-DR	CD27	TCRVδ1	CD4	CD8	CD3
		TCRγδ subtype	Leukocytes	Rec. thym. emigrants	T Differen-tiation	T Differen-tiation	Pan TCRγδ	Pan TCRαβ	Activation	Differen-tiation	TCRγδ subtype	T Helper	Cytox. T	T
Residual B	*RE ALB*	CD15	CD45				CD58	CAR-CD19	CD34	CD10	CD19	CD22 /CD24	CD38	CD20
		Exclusion of neutrophils	Leukocyte				Abnormal CD58(hi) expression	CAR expression	B matura-tion (early)	B matura-tion	B	Alternative B	B matura-tion	B matura-tion
CAR-T1	*custom*	CD25	CD45	CD197 (CCR7)	CD95	CD45RA	CD127	CAR-CD19	CD137 (4-1BB)	7-AAD	CD69	CD4	CD8	CD3
		Treg(hi), Activation	Leukocyte	Naive and central memory T	Stem cell-like T (naive phenotype)	T Differen-tiation	Treg(lo/-), IL-7R	CAR expression	Stimulatory checkpoint	Viability; DNA stain	Activation	T Helper	Cytox. T	T
CAR-T2	*custom*	CD366 (TIM-3)	CD45	TIGIT	CD155 (PVR)	CD226	CD57	CAR-CD19	CD223 (LAG-3)	7-AAD	CD279 (PD-1)	CD4	CD8	CD3
		Inhibitory checkpoint	Leukocyte	Inhibiting PVR ligand	Immuno-regulatory	Stimulating PVR ligand	Term. Differen-tiation	CAR expression	Inhibitory checkpoint	Viability; DNA stain	Inhibitory checkpoint	T Helper	Cytox. T, NK	T
CAR-T3	*custom*	ViaKrome 405	CD8			CD56	IFNγ	CAR-CD19	Gran-zyme B	Perforin	IL-2	CD4	TNFα	CD3
		viability dye, fixable	Cytox. T, NK			NK, NKTlike	Inflam. cytokine	CAR expression	Cytox. lytic enzyme	Cytox. lytic enzyme	Inflam. cytokine	T Helper	Inflam. cytokine	T
NK1	*custom*	CD25	CD45	TIGIT	CD155 (PVR)	CD226	CD16	CD335 (NKp46)	CD137 (4-1BB)	CD279 (PD-1)	CD274 (PD-L1)	CD314 (NKG2D)	CD56	CD3/14
		Activation	Leukocyte	Inhibiting PVR ligand	Immuno-regulatory	Stimulating PVR ligand	NK	Nat. cytox. receptor, NK	Stimulatory checkpoint	Inhibitory checkpoint	Inhibiting PD-1 ligand	Activating receptor, NK	NK	Exclusion of T and Monocytes
NK2	*custom*	CD366 (TIM-3)	CD45	VISTA	CD134 (OX40)	CD278 (ICOS)	CD16	CD335 (NKp46)	CD223 (LAG-3)	HLA-DR	CD69	CD314 (NKG2D)	CD56	CD3 /CD14
		Inhibitory checkpoint	Leukocyte	Inhibitory checkpoint	Stimulatory checkpoint	Stimulatory checkpoint	NK	Nat. cytox. receptor, NK	Inhibitory checkpoint	Activation	Activation	Activating receptor, NK	NK	Exclusion of T and Monocytes

FIGURE 2 | Antibody panel design. The antibody composition of the designed panels is shown together with the biological implication of each assessed antigen (grey) and the used fluorochromes and lasers. Antibody panels compose of dry antibodies that are off-the-shelf DURAClone products (blue) or custom-made DURAClone products (green). These dry antibody-based panels are complemented with further antibodies in liquid formulation (orange).

in the lymphocyte population was higher in the healthy than in the CAR treated individual (**Figures 6d, D**). When analyzing the CD3+ cells in more detail, we detected no anti-CD19 CAR-T cells in the healthy donor, and therefore no false-positive results of the anti-CD19 CAR-T staining (**Figure 6e**). We further confirmed the high CD4/CD8 ratio of 3.6 (74.50%/20.90%) in the healthy individual (**Figure 6g**). In the CAR treated patient 34.27% of all CD45+/CD3+ T cells were positive for the anti-CD19 CAR receptor (**Figure 6E**). Interestingly, in this anti-CD19 CAR+ T cell population we found a CD4/CD8 ratio of 1.8 (62.70%/35.20%; **Figure 6G2**), whereas in the anti-CD19 CAR- population the CD4/CD8 ratio was only 0.3 (21.78%/71.41%; **Figure 6G1**), which is consistent with our previous data (see **Figure 5**). The CAR-T1 panel is also designed to stain for markers of different effector and memory T cells subpopulations (CCR7, CD45RA, CD127, CD95). Gating for these markers revealed very low percentages of naïve and central memory cells in the patient T cells in comparison to the healthy donor (**Figures 6f, F, h, H**). Next, we identified CD127dimCD25 + T regulatory cells (Tregs) in the CD4+ T cell populations and found Tregs in both samples with Tregs percentages being the highest in the CAR+ patient T cells (**Figures 6i, I1, I2**). Using overlay plots we visualized that expression of the checkpoint molecule CD137 is similar between the CAR patient and healthy donor (**Figure 6kK**), whereas the activation markers CD69 and CD25 were higher in CAR- and CAR+ cells, respectively (**Figures 6lL, mM**).

The purpose of the CAR-T2 panel is to assess the CAR-T cell fitness through staining of T cell exhaustion and immune checkpoint molecules (**Figure 7**). Therefore, the panel is similar in design to the panel CAR-T1 and anti-CD3, -CD4, -CD8, -CD45 and anti-CD19 CAR antibodies are present in the panel CAR-T2 as well (see **Figure 2**). Consequently, the data shown in **Figures 7a–f** and **7A–F** are reproducing the results from **Figure 6**. A key marker of the panel CAR-T2 is CD57, which indicates the terminal differentiation and exhaustion of T cells. For this exhaustion marker we observed a stronger expression in the CAR- T cells of the CAR treated patient in comparison to the

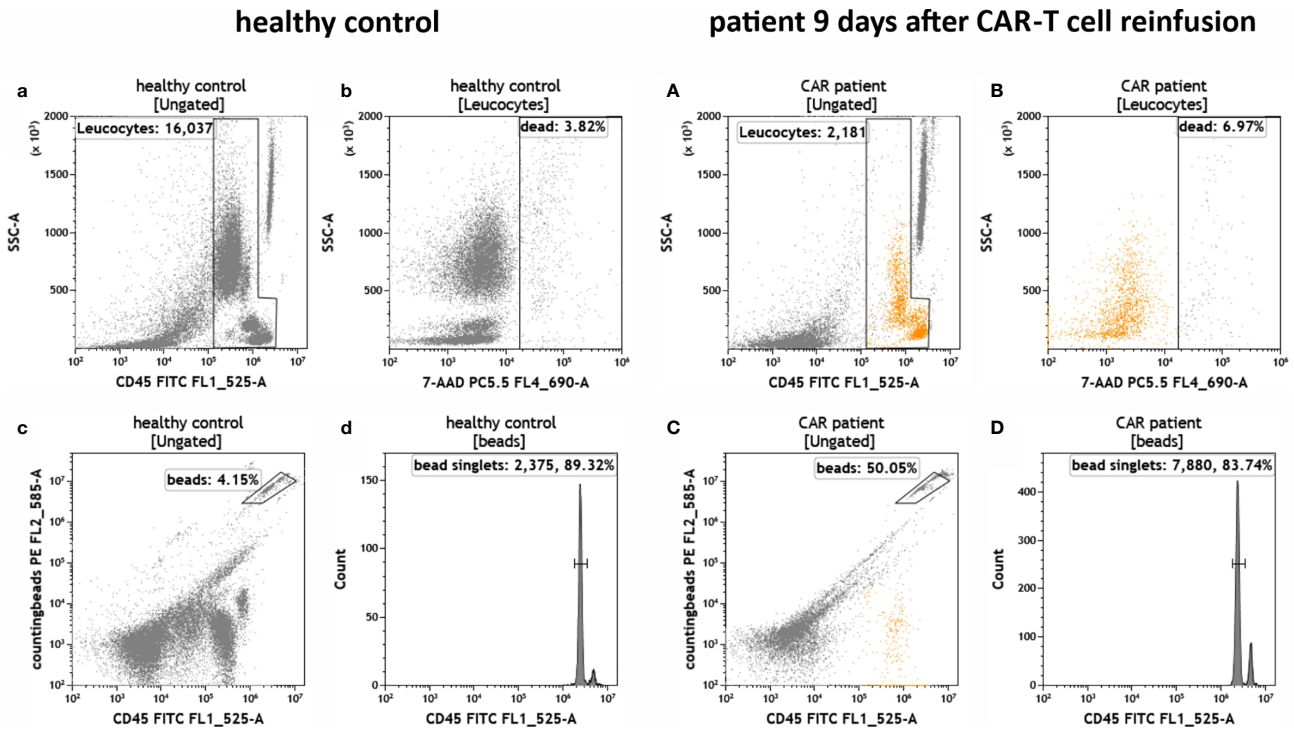

FIGURE 3 | Total number and viability of leukocytes in the peripheral blood of a healthy individual and a patient treated with anti-CD19 CAR-T cells. Flow cytometric identification of leukocytes **(a, A)** and cell counting by bead measurement **(c, C)** in both healthy control **(a–d)** and a patient 9 days after CAR-T cell reinfusion **(A–D)**. Dead cells **(b, B)** and bead doublets **(d, D)** were excluded.

CAR+ T cells, and also for the T cells of the healthy donor (**Figure 7gG**). Furthermore, we measured several inhibitory immune checkpoint molecules (LAG3, PD-1, TIM3) as well as the TIGIT/CD226/CD155 immune checkpoint signaling axis (**Figures 7hH–mM**). For all assessed checkpoint molecules, we found a trend toward higher expression in the T cells of the CAR patient compared to the healthy individual, and a tendency of higher expression in the CAR+ than in the CAR- patient T cells.

To evaluate the effector function of CAR-T cells by measuring intracellular markers we developed the antibody panel CAR-T3 (**Figure 8**). Therefore, blood of the CAR patient 30 days after CAR-T cell re-infusion and blood of the healthy donor was stimulated for 3 h at 37°C in DURActive 1 tubes. The backbone of CAR-T3 was the same as for the other CAR-T tubes. After gating of lymphocytes (**Figures 8a, aa, A, AA**) and single cells (**Figures 8b, bb, B, BB**) T cells were identified (**Figures 8c, cc, C, CC**). As for CAR-T1 (**Figure 6**) and CAR-T2 (**Figure 7**) T cells were lower in the CAR patient compared to the healthy donor (**Figures 8c, cc, C, CC**). Furthermore, the CD4/CD8 ratio was 3.73 (75.17%/20.16%) in the unstimulated healthy control and 3.95 (69.52%/17.60%) in the stimulated one (**Figures 8d, dd**) while for the CAR patient the ratios were 0.45 (27.46%/60.92%) and 0.49 (29.39%/60.94%) (**Figures 8D, DD**), respectively. As expected the CAR-T cell content in the blood of the CAR patient was much lower (**Figures 8E, EE**) than after 9 days of re-infusion (**Figures 6** and **7**). Stimulation of the cells does not alter the amount of intracellular granzyme B (**Figure 8fF**) and perforin (**Figure 8gG**). However, levels of granzyme B (**Figure 8fF**) and perforin (**Figure 8gG**) of the CAR patient sample were higher compared to the healthy donor. For IFN-γ the stimulation leads to an intracellular enhancement in the CAR patient and the healthy donor (**Figure 8hH**) while for IL-2 (**Figure 8iI**) and TNF-α (**Figure 8jJ**) an enhancement was only observed in the healthy control.

Finally, we measured two additional patients suffering from different B-cell lymphomas (PMBCL, transformed Follicular Lymphoma) and who were treated with either Axicabtagene ciloleucel or Tisagenlecleucel after 6 and 7 days, respectively. The key results of the residual B cell, CAR-T1 and TCR panels are presented in **Figure 9**. As the control for these analyses, we used the patient's own cells at day 0 under lymphodepleted conditions before administration of the CAR-T cells. Overall, most data are similar to the above shown results. We could not detect any CD19+ B cells nor anti-CD19 CAR+ CD19+ cells in the samples after treatment (**Figures 9A, B**). Among the CD3+ T cells 29.41% (**Figure 9A**) and 14.76% (**Figure 9B**) of the population were positive for the anti-CD19 CAR receptor after 6 and 7 days, respectively. Furthermore, the T cell activation assessed by HLA-DR expression was much higher after 6 and 7 days compared to the patient cells before CAR-T administration at day 0 (**Figures 9A, B**).

In summary, we have developed antibody panels to deeply characterize patient B, T and CAR-T cells using advanced flow

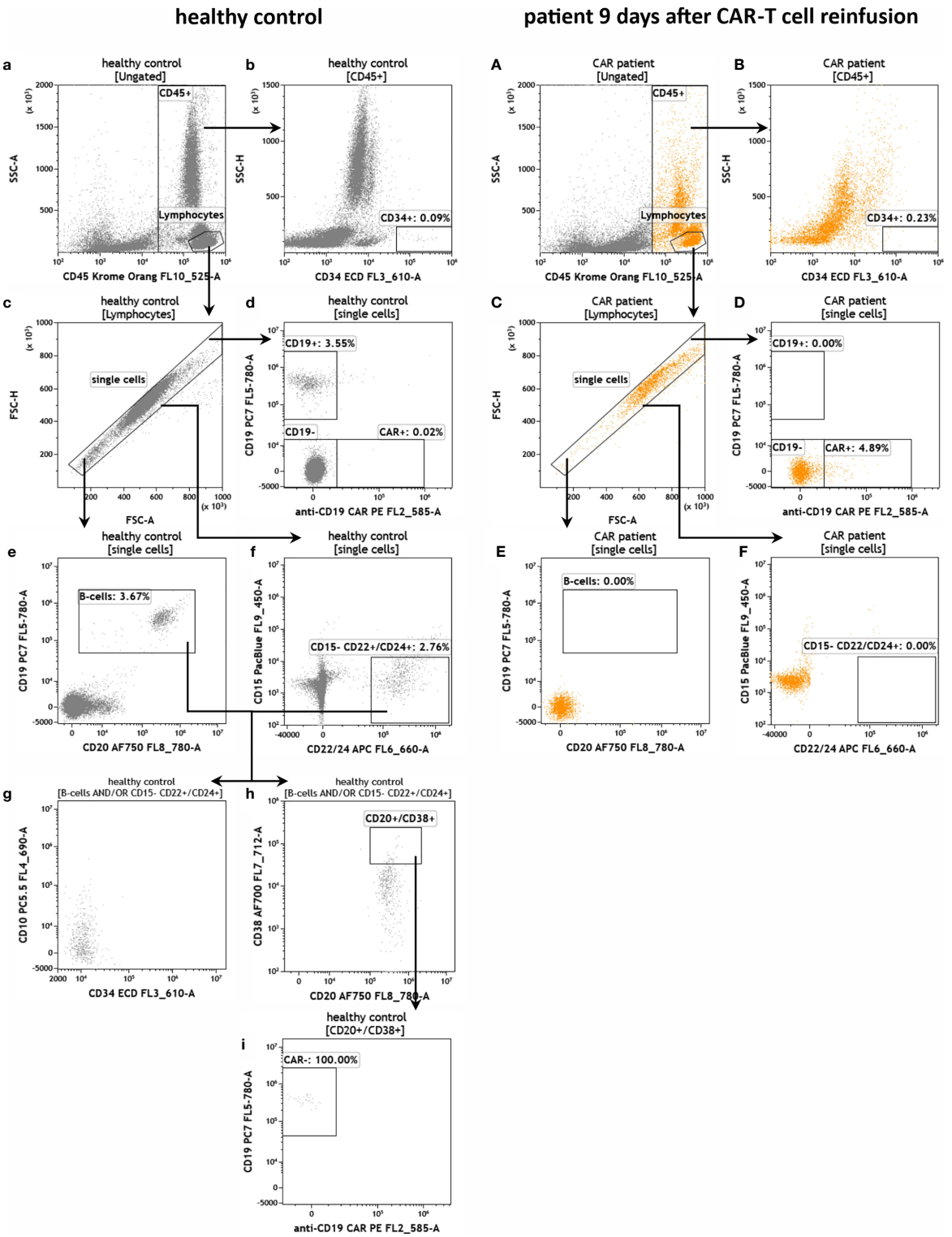

FIGURE 4 | Detection of CD19+ B cells in the peripheral blood of a healthy individual and a patient treated with anti-CD19 CAR-T cells. Flow cytometric gating strategy (illustrated by arrows) and phenotyping of B cells in healthy control **(a–i)** in comparison to residual B cells in a patient 9 days after anti-CD19 CAR-T cell reinfusion **(A–F)**. After lymphocyte identification **(a, A)** CD34+ progenitor cells were detected **(b, B)** and exclusion of doublets was performed **(c, C)**. The presence of potential CAR+ CD19 B cells was evaluated **(d, D)**. B cells and residual B cells were identified by gating CD19, CD20 and CD22/24 **(d, D–f, F)**. If present, B-cells can be further characterized by CD10, CD34, CD38 and anti-CD19 CAR **(g-i)**.

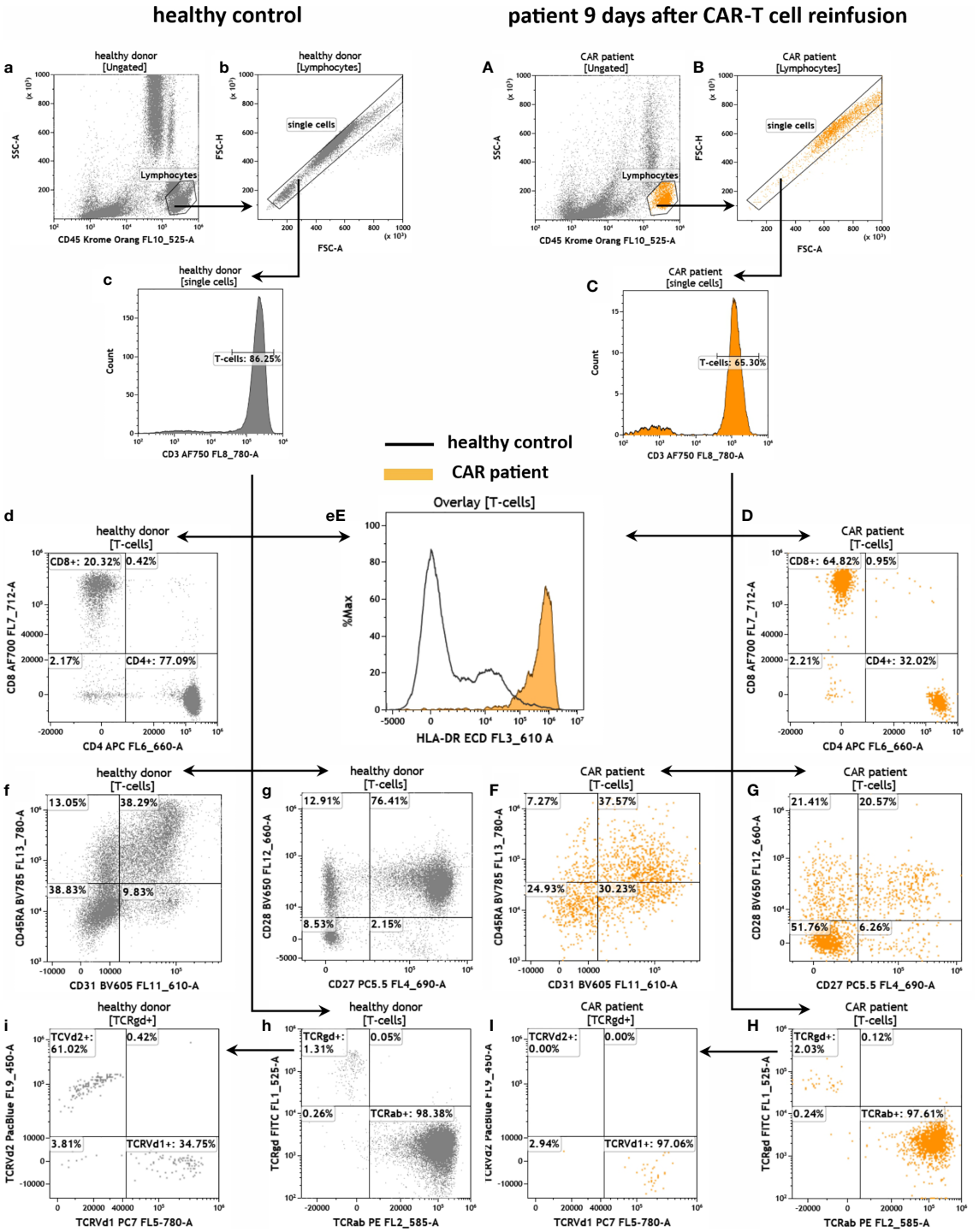

FIGURE 5 | Assessment of T cells in the peripheral blood of a healthy individual and a patient treated with anti-CD19 CAR-T cells by the panel TCR. Flow cytometric gating strategy (illustrated by arrows) and phenotyping of T cells in healthy control **(a–h)** in comparison to a patient 9 days after CAR-T cell reinfusion **(A–H)**. After lymphocyte gating **(a, A)** and exclusion of doublets **(b, B)** CD3+ T cells were identified **(c, C)**. T cells were further discriminated in CD4+ and CD8+ T cells **(d, D)**, CD31+CD45RA+ recent thymic emigrants **(f, F)** and naïve/memory (CD27+/CD28+/-) as well as effector-memory (CD27-/CD28-) cells **(g, G)**. The expression of HLA-DR on T cells **(eE)** was used as a marker for general T cell activation. Furthermore, the numbers of alpha/beta and gamma/delta T cells **(h, H)** as well as Vd1+ and Vd2+ gamma/delta T cells were determined **(i, I)**.

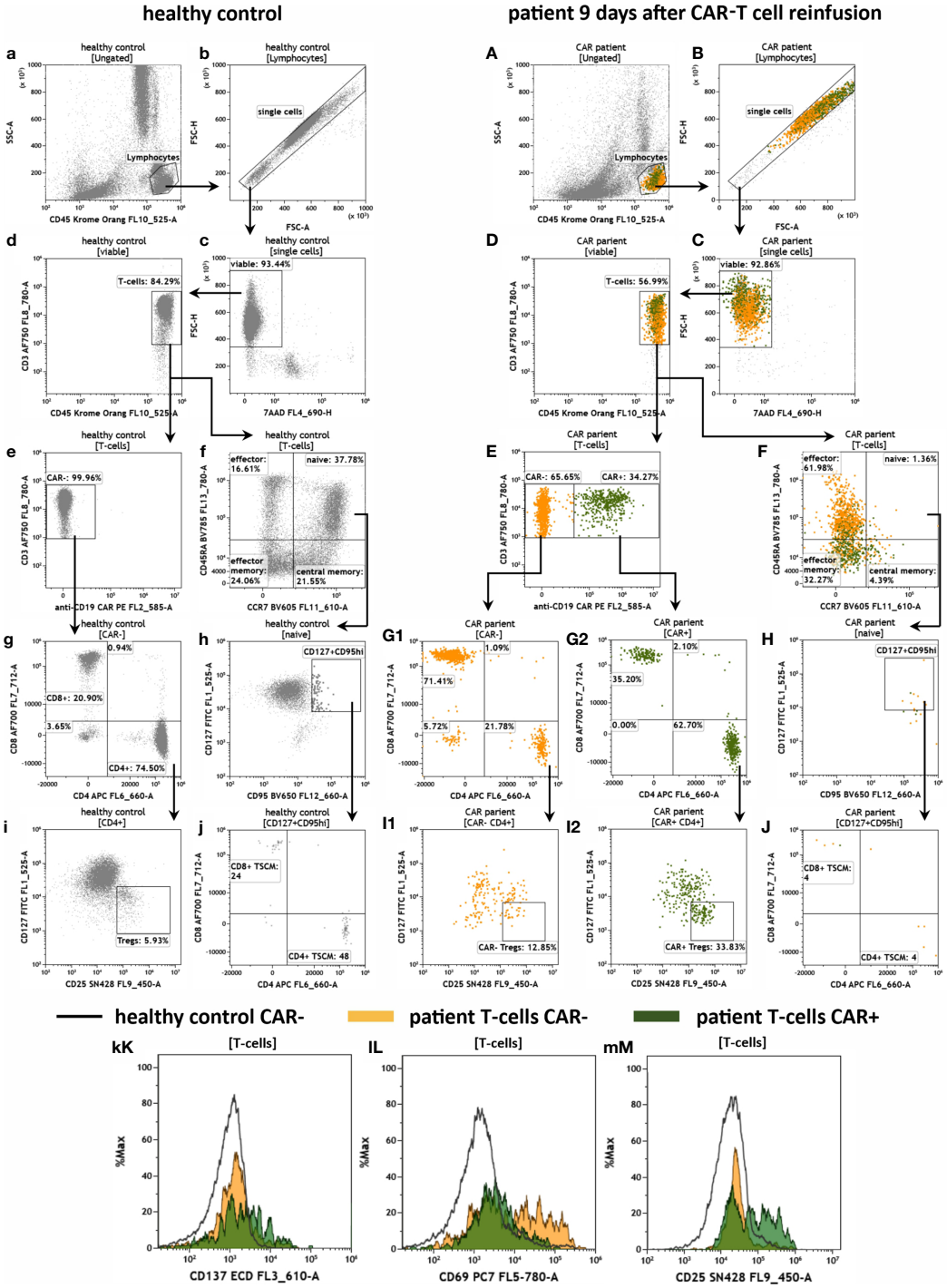

FIGURE 6 | Characterization of anti-CD19 CAR-T cells in the peripheral blood of a patient treated with CAR-T cells by the panel CAR-T1. Flow cytometric gating strategy (illustrated by arrows) and phenotyping of anti-CD19 CAR-T cells in a patient 9 days after CAR-T cell reinfusion **(A–M)** in comparison to a healthy donor **(a–m)**: After lymphocyte gating **(a, A)** and exclusion of doublets **(b, B)** viable **(c, C)** T cells **(d, D)** were identified. The T cells were divided in anti-CD19 CAR- and CAR+ cells **(e, E)**. CAR- and CAR+ T cells were further divided in CD4+ and CD8+ cells **(g, G1** and **G2)**, followed by identification of CD127dimCD25+Tregs in the CD4+ T cell populations **(i, I1** and **I2)**. CAR-T cells and normal T cells maturation stages were assessed by CD45RA and CCR7 expression **(f, F)**. In the naïve compartment, CD127+CD95+ cells are assessed for identification of a stem cell-like T cell memory type **(h, H)** including expression of CD4 and CD8 **(j, J)**. Expression of the checkpoint CD137 and the activation markers CD69 and CD25 is compared by overlay plots between T cells from the healthy donor and patient CAR+ and CAR- T cells. **(kK–mM)**. The healthy control did not have anti-CD19 CAR-T cells. Color code: In the CAR-T cell treated patient, events are assigned to either anti-CD19 CAR- (orange) or CAR+ (green) T cells.

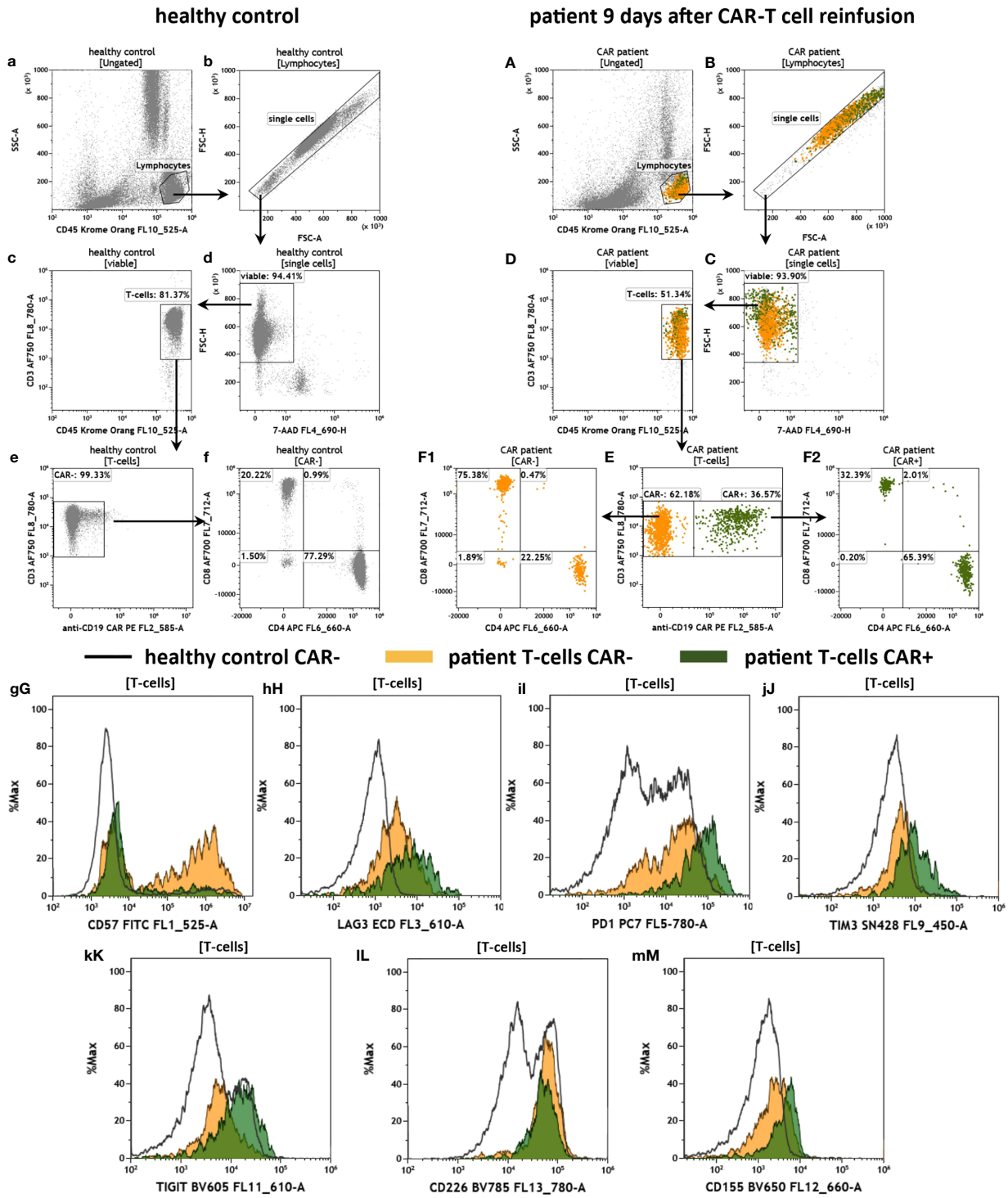

FIGURE 7 | Fitness of anti-CD19 CAR-T cells in the peripheral blood of a patient treated with CAR-T cells by the panel CAR-T2. Flow cytometric gating strategy (illustrated by arrows) and phenotyping of anti CD19 CAR-T cells in a patient 9 days after CAR-T cell reinfusion **(A–M)** in comparison to a healthy control individual **(a–m)**: After lymphocyte gating **(a, A)** and exclusion of doublets **(b, B)** viable **(c, C)** T cells **(d, D)** were identified. The T cells were divided in anti-CD19 CAR- and CAR+ cells **(e, E)**. CAR- and CAR+ T cells were further divided in CD4+ and CD8+ cells **(f, F1, F2)**. T cell expression of the terminal differentiation marker CD57 **(gG)**, checkpoints LAG3 **(hH)**, PD-1 **(iI)**, TIM3 **(jJ)**, and the immune modulating TIGIT **(kK)**, CD226 **(IL)** and CD155 **(mM)** were compared by overlay plots between T cells from the healthy donor and patients CAR- and CAR+ T cells. The healthy control did not have anti CD19 CAR-T cells. Color code: In the CAR-T cell treated patient, events are assigned to either anti-CD19 CAR- (orange) or CAR+ (green) T cells.

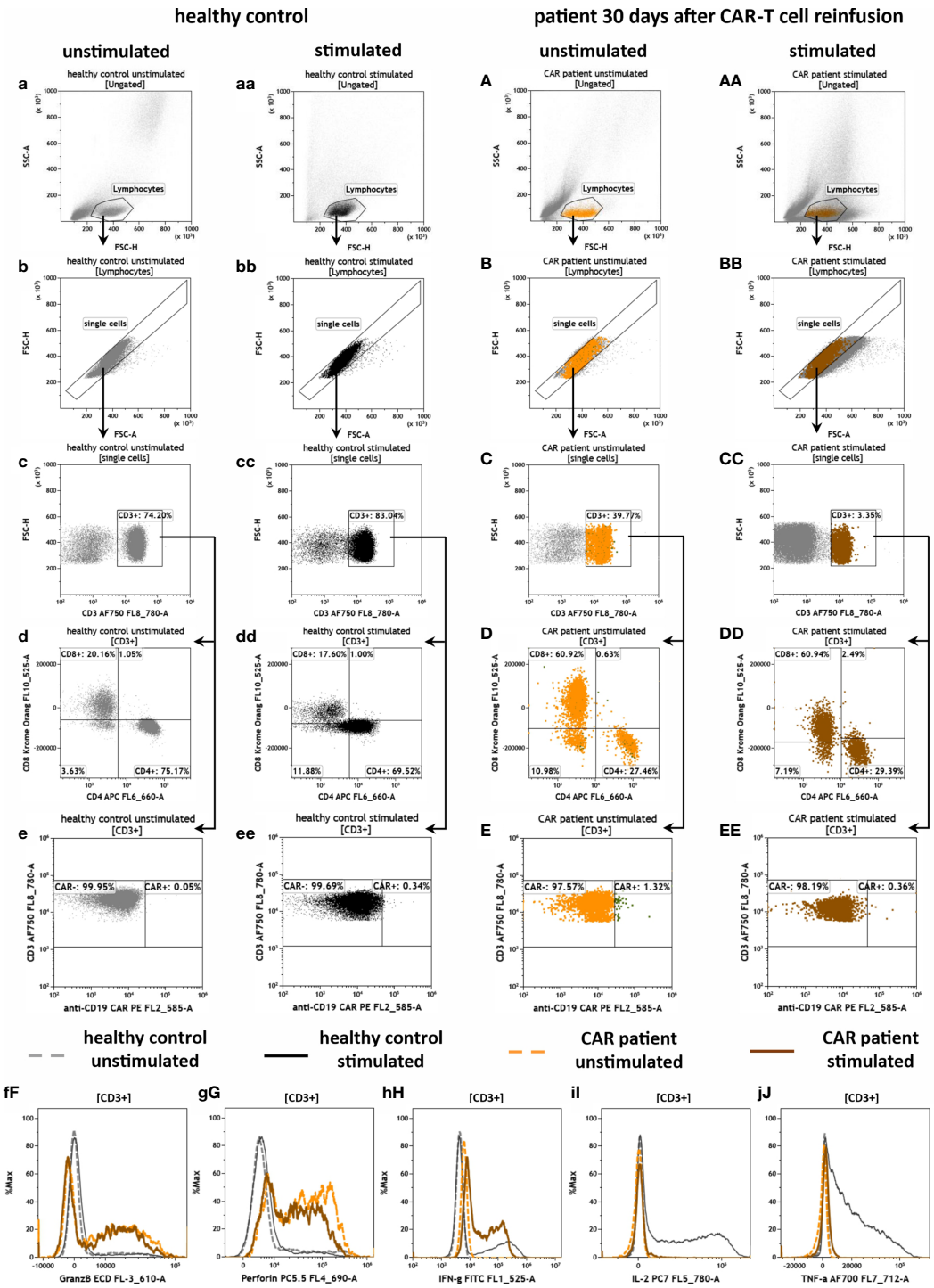

FIGURE 8 | Effector potential of anti-CD19 CAR-T cells in the peripheral blood of a patient treated with CAR-T cells by the panel CAR-T3. Flow cytometric gating strategy (illustrated by arrows) and phenotyping of extra- and intracellular staining of anti-CD19 CAR-T cells in a patient 30 days after CAR-T cell reinfusion **(A–J)** in comparison to a healthy donor **(a–j)**, in both unstimulated cells **(a–j** and **A–J)** or cells that were stimulated with PMA/Ionomycin **(aa–j, AA–J)**: After lymphocyte gating **(a–AA)** and exclusion of doublets **(b–BB)**, T cells **(c–CC)** were identified and further divided in CD4+ and CD8+ T cells **(d–DD)** as well as CAR- and CAR+ T cells **(e–EE)**. The intracellular expression of granzyme B **(fF)**, perforin **(gG)**, IFN-gamma **(hH)**, IL-2 **(il)** and TNF-α **(jJ)** were compared by overlay plots between unstimulated and stimulated T cells from the healthy donor and the anti-CD19 CAR-T cell treated patient. The healthy control did not have CAR-T cells. Color code: dashed gray line = unstimulated healthy donor; black = stimulated healthy donor; dashed orange = unstimulated anti CD19 CAR patient, brown = stimulated anti CD19 CAR patient.

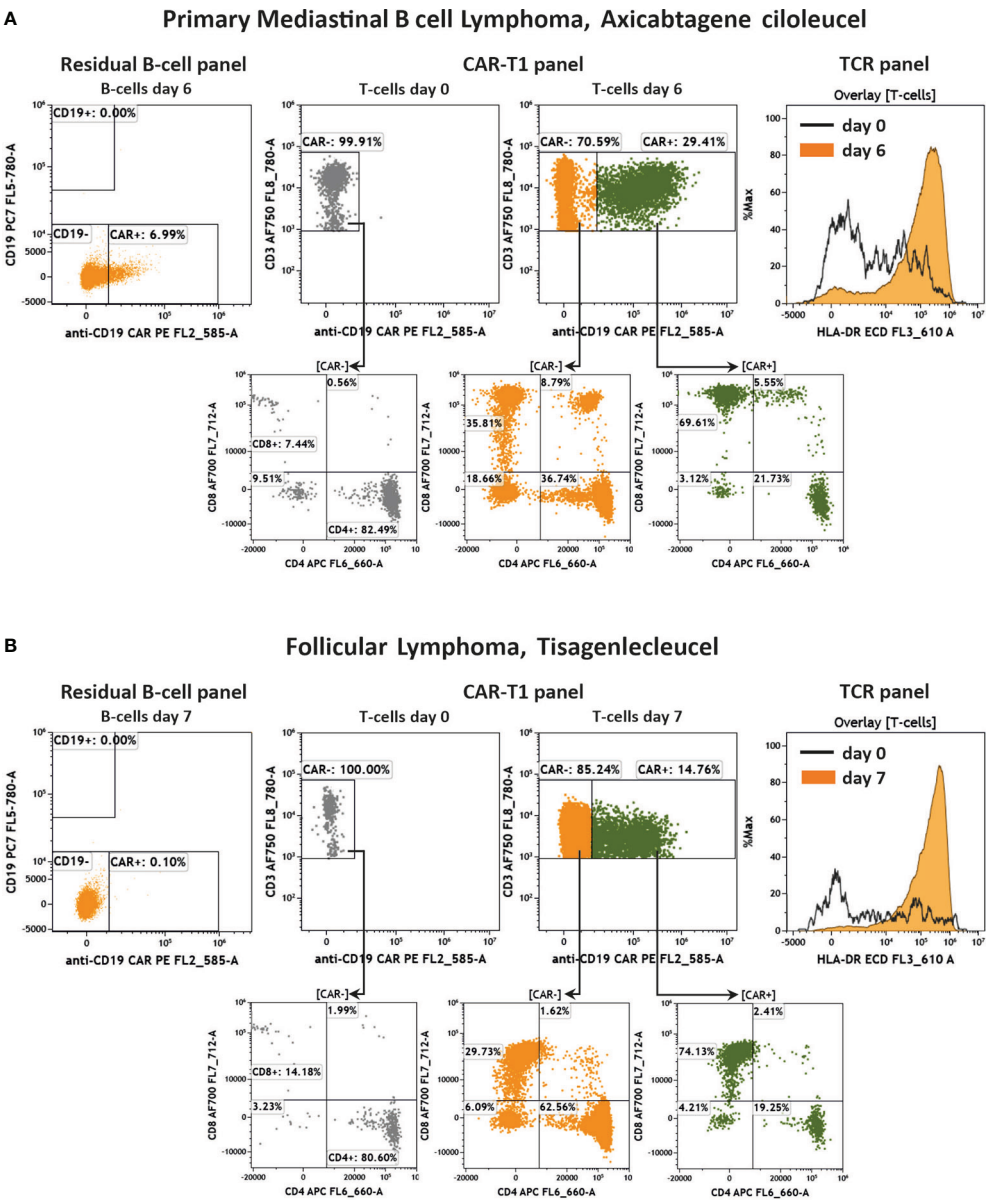

FIGURE 9 | Key results of two additional anti-CD19 CAR-T cell treated patients. Flow cytometric analysis of anti-CD19 CAR+/- CD19+ cells, anti-CD19 CAR-T cells, CD4/CD8 distribution and activation of T cells in two additional patients. Shown are a PMBCL patient treated with Axicabtagene ciloleucel **(A)** and a transformed Follicular Lymphoma patient treated with Tisagenlecleucel **(B)** 6 and 7 days after treatment, respectively, compared to day 0 before administration of CAR-T cells. Gating strategy was performed according to the **Figures 4**–**6**.

cytometry panels and proposed corresponding gating strategies. However, the assays are not limited to these immune cell populations and can be readily expanded to other immune cells important for cancer immunotherapy. As an example, we have also developed antibody panels to phenotype natural killer (NK) cells in the patient blood (see **Supplementary Figures S1** and **S2**). These panels, which assess NK cells through CD56 and CD16 staining, analyze the NK cell status with regards to activating receptors (e.g. NKG2D), cytotoxicity receptors (e.g. NKp46), checkpoint and checkpoint ligand molecules as well as many other the immune modulating molecules. These panels can also be useful for upcoming CAR-NK cell therapies.

DISCUSSION

Immunotherapy using cancer-directed immune cells is one of the biggest innovations in modern cancer therapy. However, there are

still many aspects of CAR-T cell immunotherapy that need to be refined to ensure its lasting success. Indeed, CAR-T cells are genetically modified, living drugs that directly intervene in the patient's immune system. Therefore, monitoring the immune system of a patient after receiving CAR-T cell infusion might provide important additional information for evaluating the efficacy and safety of the therapy. Moreover, because CAR-T therapy is a novel immunotherapeutic approach, monitoring the patient's immune system is as crucial for understanding the long-term effects of the overall concept. To monitor anti-CD19 CAR-T cell content *in vivo*, polymerase chain reaction (PCR) is widely used and PCR-results have been reported to correlate with CAR surface expression measured by flow cytometry (4, 20–26). However, flow cytometry allows the identification and characterization of CAR-T cell subpopulations and of patient's immune cells in a fast way and at a single-cell level. In addition, flow cytometry detects the CAR at the proteomic level and thus can provide information regarding CAR cell functionality (27). Therefore, flow cytometry together with upcoming next-generation sequencing (NGS) approaches (18, 28) enables the comprehensive monitoring of CAR-T cell therapy (29). However, in flow cytometry inter-operator and inter-assay variations can make it difficult to compare data recorded at different times or by different laboratories. Standardization of flow cytometry methods can help to overcome these problems and increase the comparability and reproducibility of acquired data (16). Here we introduced 13 colour/15 parameter flow cytometry assays for standardized research on the fate and immunological impact of CAR-T cells post-infusion.

We characterized CAR-T cells, T cells, B cells and NK cells in the peripheral blood of three patients after the infusion of anti-CD19 CAR-T cells. As expected, the CAR treated patients had only very few leukocytes due to previous chemotherapies and lymphodepletion. We were able to detect the expected amounts of CD19+ B cells in the healthy donor and could not find any residual CD19+ B cells in the anti-CD19 CAR treated patients post-infusion, implicating efficacy of the CAR-T cells. Further, we included the CAR detection in the residual B cell panel to identify potential CAR-positive B cells. In very rare cases it has been described that during the production of the anti-CD19 CAR-T cells residual B cells are also transduced with the anti-CD19 CAR, which could then bind and mask the cell's own CD19 and thereby escape the therapy leading to death of the patient (30). While PCR methods would miss such CAR-transduced B cells, the flow cytometry panel described here could detect them. Furthermore, we observed that the CD4/CD8 T cell ratio in the CAR patients was lower than in the healthy donor, whereas the activation of T cells (as measured by HLA-DR) was clearly higher in the CAR patients. Fourteen to thirty-five percent of the overall patients T cells were anti CD19 CAR-T cells with varying CD4/CD8 ratios between the patients (approx. 0.3 – 1.8). In addition, our flow cytometry assays were designed to measure a large repertoire of T cell fitness parameters, which included exhaustion markers and checkpoint as well as other immunomodulating molecules. We also observed that the DLBCL patient's CAR-negative T cells showed a very high expression of the exhaustion marker CD57, whereas the CAR-positive T cells were low in CD57, which needs to be confirmed in a larger sample cohort. Regarding the expression of checkpoint and immunomodulating markers, we detected a general trend toward higher expression in the patient T cells than in the healthy donor T cells. With the CAR-T3 panel we were able to analyze the cells' cytotoxic potential by measuring intracellular perforin, granzyme B, IL-2, TNF-α and IFN-γ. We found that, in contrast to the healthy donor, the CAR patient's T cells are loaded with perforin and granzyme B, both unstimulated and stimulated, indicating their cytotoxic potential. Furthermore, the T cells of the healthy donor and the CAR treated patient produced IFN-γ after stimulation indicating an activity of the cells.

The goal of this work was to introduce standardized flow cytometry research assays for the study of infused anti-CD19 CAR-T cells including the interacting immune system. Therefore, we designed comprehensive antibody panels and provided detailed gating strategies for all panels using the peripheral blood of a DLBCL patient as an example (**Figures 3–8**). In addition, to show the strength and versatility of the assays, we have measured two more patients with different B cell lymphomas (PMBCL, Follicular Lymphoma), and which were treated with different CAR-T cell products (Axicabtagene ciloleucel or Tisagenlecleucel, **Figure 9**). We acknowledge that our study has several limitations. First of all, the obtained results only represent a snap-shot of one healthy and a very small number of patient donors. For instance, it is well known that the presence and the status of CAR-T cells in the peripheral blood highly vary over time (7). From a technological point of view, it is important to note that the CAR patients had very low leukocytes numbers, which might skew the staining procedure and the comparison to a healthy donor with comparatively higher leukocytes numbers. The low number of leukocytes in the lymphodepleted CAR patients also determines the lower limit of detection (LLOD), which with the achieved number of leukocytes is 1%). Furthermore, here we analyzed B, T and NK cells but other immune cell populations might be of interest to obtain a comprehensive picture of the patient immune status. Taken together, although our data visualize the suitability of the introduced advanced flow cytometry assays, further experiments with more donors and time points are needed to verify the results and to subsequently optimize the antibody panels. Such future work may best be performed in multi-center studies.

In summary, we have introduced flow cytometry research assays for studying anti-CD19 CAR-T cells and the interacting immune system *in vivo*. The underlying principle using pre-formulated, single tube antibody cocktails, makes these assays a promising method for the standardized monitoring of CAR-T immunotherapy and therefore has also been suggested by others (31, 32). The DURAclone technology used here has been successfully applied for the detection of regulatory T cells (Tregs), naïve/memory T cells and other immune cells in the peripheral blood of healthy human donors in previous work (33–35). Furthermore, the feasibility of this technology for immuno-monitoring of cancer patients has been proven by the detection of minimal residual disease (MRD) in myeloma patients and by analyzing blood cells of cancer patients in response to immunotherapeutic nanoparticles (36, 37). In addition, the

introduced technology is not limited to CD19-targeting CAR approaches and can be readily adapted to the evaluation of other CAR immunotherapies. In this regard, the design of the CAR-T 1-3 panels and the RE ALB panel provides a well standardized framework, which allows for drop-in of any specific anti-CAR probes (e.g. soluble CAR-targeted proteins, anti-idiotypic antibodies) labeled with the bright and well-established PE dye. Indeed, it is expected that several new CAR-T cell products will reach market authorization soon. Moreover, the number of upcoming future clinical trials investigating CAR-T cells and other CAR-engineered immune effector cells such as NK cells or macrophages continues to expand (6, 38–40). However, the potential increase in CAR therapies exceeds the current manufacturing capacity and only a fraction of patients who could benefit from CAR-T therapy are currently receiving it (41). Therefore, the *Good Manufacturing Practice (*GMP)-compatible production of CAR-T cells requires continuous process improvement to keep up with the fast-growing medical demand (42). In this regard, first semi-automated cell processing systems have been successfully used for the GMP-compatible, clinical-grade production of CAR-T and CAR-NK cells (7, 43–48). Monitoring CAR-T cell production from the starting leukapheresis material over to the process of production/upscaling to the final product release requires standardized quality control (QC) tools. Thus, we envision that our flow cytometry assays can also serve as novel QC tools during CAR-T cell production, in particular when linked to automated data analysis and machine learning technologies, which is within reach as previously shown (49, 50).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee at the Medical Faculty, Leipzig University Käthe-Kollwitzstraße 82 04109 Leipzig. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

UB, UP, VV, TM, MB, SF, US, and UK designed the research. RW, AB, and AP ran the experiments. UB, RW, AB, MK, ARB, AQ, AP, VV, UP, US, SF, and UK analyzed results. UB, RW, and AB created the figures. UB, RW, AB, US, SF, and UK wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.658314/full#supplementary-material

Supplementary Figure 1 | Characterization of NK cells in the peripheral blood of a healthy individual and a patient treated with anti-CD19 CAR-T cells by the panel NK1. Flow cytometric gating strategy (illustrated by arrows) and phenotyping of NK cells in a patient 9 days after CAR-T cell reinfusion **(A–L)** in comparison to a healthy donor **(a–l)**: After lymphocyte gating **(a, A)** and exclusion of doublets **(b, B)**, as well as T cells and monocytes gating by their expression of CD3 and CD14 **(c, C)**, respectively, NK cells were divided in CD16+/CD56bright and CD16+/CD56dim cells **(d, D)**. CD56bright and dim cells **(d, D)** in both healthy control **(d–l)** and in the CAR patient **(D–L)** were analyzed for the expression of: Activating receptor NKG2D and natural cytotoxicity receptor NKp46 **(e, E)**, checkpoint ligand PD-L1/CD274 **(f, F)**, checkpoints CD137 and PD-1 **(f, F, g, G, j, J)**, the immune modulating molecules TIGIT, CD155, CD226, **(h, H, j–l, J–L)** and the activation marker CD25 **(i, I)**.

Supplementary Figure 2 | Characterization of NK cells in the peripheral blood of a healthy individual and a patient treated with anti-CD19 CAR-T cells by the panel NK2: Flow cytometric gating strategy (illustrated by arrows) and phenotyping of NK cells in a patient 9 days after CAR-T cell reinfusion **(A–L)** in comparison to a healthy donor **(a–l)**: After lymphocyte gating **(a, A)** and exclusion of doublets **(b, B)**, as well as T cells and monocytes gating by their expression of CD3 and CD14 **(c, C)**, respectively, NK cells were divided in CD16+/CD56bright and CD16+/CD56dim cells **(d, D)**. CD56bright and dim cells in both healthy control **(d–l)** and in the anti CD19 CAR patient **(D–L)** were analyzed for the expression of: Activating receptor NKG2D and natural cytotoxicity receptor NKp46 **(e, E)**, the activation markers HLA-DR **(fF)** and CD69 **(g, G)** as well as checkpoints CD278 (ICOS) **(h, H)**, LAG-3 **(i, I)**, CD134 (OX40) **(j, J)**, TIM-3 **(k, K)** and VISTA **(l, L)**.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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Case Report: Prolonged Survival Following EGFRvIII CAR T Cell Treatment for Recurrent Glioblastoma

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Autologous chimeric antigen receptor (CAR) T cells targeted to epidermal growth factor receptor variant III (CAR T-EGFRvIII) have been developed and administered experimentally to treat patients with IDH1 wildtype recurrent glioblastoma (rGBM) (NCT02209376). We report the case of a 59-year-old patient who received a single peripheral infusion of CAR T-EGFRvIII cells and survived 36 months after disease recurrence, exceeding expected survival for recurrent glioblastoma. Post-infusion histopathologic analysis of tissue obtained during a second stage surgical resection revealed immunosuppressive adaptive changes in the tumor tissue as well as reduced EGFRvIII expression. Serial brain imaging demonstrated a significant reduction in relative cerebral blood volume (rCBV), a measure strongly associated with tumor proliferative activity, at early time points following CAR T treatment. Notably, CAR T-EGFRvIII cells persisted in her peripheral circulation during 29 months of follow-up, the longest period of CAR T persistence reported in GBM trials to date. These findings in a long-term survivor show that peripherally administered CAR T-EGFRvIII cells can persist for years in the circulation and suggest that this cell therapy approach could be optimized to achieve broader efficacy in recurrent GBM patients.

Keywords: CAR T cell therapy, glioblastoma, EGFRvIII, recurrent glioblastoma (rGBM), CAR (chimeric antigen receptor), perfusion imaging

INTRODUCTION

Autologous T cells redirected with chimeric antigen receptors (CARs) targeting EGFRvIII represent an investigational treatment paradigm for glioblastoma (GBM), the most common and aggressive adult primary brain malignancy (1). Neither surgery, chemotherapy nor radiation can completely control the disease, and second-line therapies for recurrence remain limited. Though median survival is 9 months after recurrence (2), we report a patient treated with CAR T-EGFRvIII who survived 36 months after disease recurrence.

CARs are synthetic surface receptors, combining artificial extracellular single chain antibody fragments for target cell recognition and intracellular T-cell activation and co-stimulation domains [(3), STM]. CAR T cells have proven highly efficacious against B cell malignancies, leading in 2017 to the FDA approval of two CD19 antigen-specific CAR T cell products in refractory B cell cancers. These results have raised expectations for CAR T in other cancers including GBM (4). At our institution, O'Rourke and colleagues conducted the first-in-human trial (NCT02209376) of autologous T cells redirected to EGFRvIII for rGBM (5). At the time of publication of that trial, three patients remained alive, including one who had remained alive and well without further therapy for more than 18 months. Here we present detailed analysis and an updated clinical course of that patient, with new data on histopathology, survival, duration of CAR T persistence, immunosuppressive responses in the tumor tissue, and perfusion MRI metrics (5). This analysis of a patient who received CAR T for rGBM suggests that a single peripheral infusion of CAR T cells may have on-target anti-tumor activity.

CASE DESCRIPTION

A previously healthy 58-year-old right-handed woman presented to an outside institution for difficulty reading and writing. MRI revealed a 23 cm (4) (4.0 cm max. diameter) contrast-enhancing lesion in the left posterior temporal lobe. She underwent near complete resection without complication, and histopathology confirmed the diagnosis of GBM with positive O (6)-methylguanine-DNA methyltransferase (MGMT) methylation, negative mutant IDH1 (R132H), and 60% EGFRvIII positivity. She completed three months of standard-of-care chemoradiation therapy (Stupp Protocol) and was then referred to the University of Pennsylvania with Karnofsky Performance Status (KPS) of 90, no focal neurologic deficits, and no steroid requirement. Surveillance MRI with dynamic susceptibility contrast (DSC) perfusion imaging revealed elevated rCBV suggesting tumor recurrence 6 months after initial resection (7). She was enrolled in our EGFRvIII-directed CAR T for recurrent GBM trial (NCT02209376).

Twenty-six days after leukapheresis, she underwent intravenous infusion of 9.2 x 10^7 autologous EGFRvIII-directed CAR T cells. Two weeks prior to infusion, she had reported worsening pressure headaches, an increase in word-finding difficulty, and a "whooshing" sound in her left ear. These symptoms improved with a one-week steroid taper. On post-infusion day 7, she reported mild flu-like symptoms, including arthralgia, myalgia, and headache. She was managed conservatively with acetaminophen, which relieved her symptoms. Three months after CAR T cell infusion, the patient experienced increasingly severe headaches, requiring the re-initiation of dexamethasone. MRI demonstrated increased size of the enhancing lesion with increasing FLAIR abnormality, concerning for tumor progression vs. treatment response, the latter supported by decreased tumor relative cerebral blood volume (rCBV) (**Figures 1A, B**). To address her clinical complaints, and to potentially guide further therapy with tissue diagnosis, the patient underwent a second craniotomy on post-CAR T day 104. The pathology was consistent with recurrent GBM. She was discharged home with KPS 80 on post-operative day 2. At her post-operative visit, she reported difficulty with reading, consistent with a partial (quadrant) visual field deficit.

The patient enjoyed good functional status and received no additional chemotherapy for 18 months following her second operation (**Figure 2**). After 15 months, serial MRIs revealed gradual tumor progression (arrows, **Figure 1B**). At 32 months from CAR T infusion, she developed a methicillin sensitive *S. aureus* osteomyelitis in her right lower extremity and required operative debridement. After surgery she had a rapid, unexpected decline, never fully regaining her mental status, and in accordance with her and her family's wishes she was transferred to hospice care. Her overall survival was 36 months from the date of initial tumor recurrence, and 34 months from CAR T infusion.

METHODS

Immunohistochemistry

Five-micron sections of formalin-fixed paraffin-embedded tissue were stained using antibodies against PD1 (Clone NAT105; abcam 52587 1:40 dilution), Foxp3 (clone 206D, Biolegend 320102 1:100 dilution) and EGFR VIII (Cell Signaling 64952, 1:100. Staining was performed on a Leica Bond™ instrument using the Bond Polymer Refine Detection System (Leica Biosystems DS9800). Heat-induced epitope retrieval was done for 20 minutes in ER1 solution (Leica Biosystems AR9961) for PD1 and ER2 solution (Leica Biosystems AR9640) for Foxp3 and EGFR. To quantify IHC results, we used Fiji (ImageJ) to deconvolute hematoxylin from 3,3′-diaminobenzidine staining, followed by thresholding of signal to positive cells to create a binary image. The resulting output was passed through the watershed algorithm followed by automated particle analysis to determine the percent positivity by area.

Radiographic Analysis

The DTI maps (MD, FA), CBV, Cho, Cr maps and FLAIR images were co-registered to contrast-enhanced T1-weighted

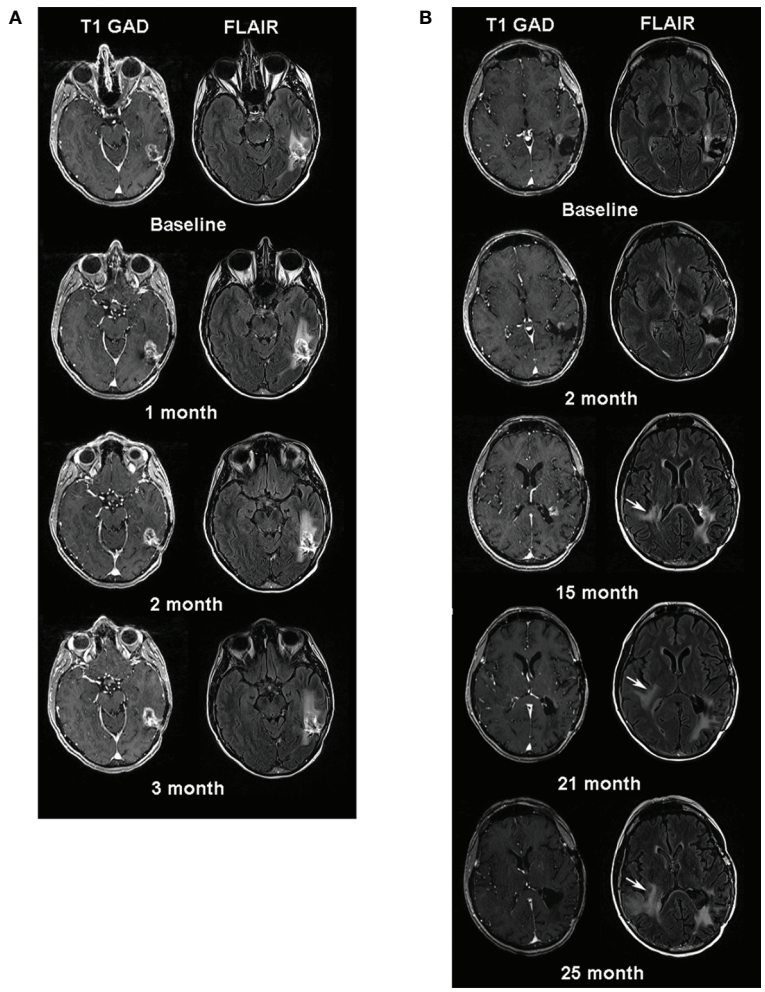


FIGURE 1 | **(A)** Serial MR images one week before (baseline) and three months after CAR T-EGFRvIII infusion. Left sided images are axial post contrast T1 weighted images and right sided images are axial FLAIR images. Note, minimal increase in size of the enhancing lesion in the left temporal lobe at follow-up time-points. **(B)** Serial MRI examinations after the patient's second surgery. The baseline is one month after her second surgery. White arrows at 15, 21- and 25-month follow-up periods indicate the increasing signal abnormality in the right periventricular region on FLAIR images.

images. A semi-automatic segmentation approach was used to generate a mask from the enhancing region of the neoplasm. The CBV values were normalized to the contra-lateral normal white matter to obtain relative CBV (rCBV). The median values of DTI parameters, rCBV and Cho/Cr from the enhancing regions of the neoplasms were estimated at each time point. Additionally, the top 90th percentile rCBV values were computed and reported as rCBVmax. The percent changes for each parameter (MD, FA, rCBV, rCBVmax and Cho/Cr) between the baseline and the subsequent scans (N) were calculated as (N – baseline)/baseline × 100.

Study Approval

All experiments were performed in accordance with the approval by the Abramson Cancer Center Clinical Trials Scientific Review Committee, the Penn Institutional Biosafety Committee, and Institutional Review Board.

RESULTS AND DISCUSSION

Histopathological examination of the tumor tissue obtained 104 days after CAR T-EGFRvIII infusion revealed recurrent and residual malignant glioma on a background of treatment related changes (**Figure 3**). Immunohistochemistry (IHC) showed a reduction in EGFRvIII expression from 78.0% to 3.7% by tumor area (**Figure 3**), suggesting possible on-target activity against antigen-expressing tumor cells. As published in the original clinical trial, we also employed an EGFRvIII-targeted RNA sequencing assay to determine trial eligibility (5). This assay determines the ratio of EGFRvIII transcripts (defined by deletion of exons 2 through 7) to total EGFR transcripts. For this patient, the RNA sequencing assay showed that EGFRvIII transcripts decreased to 13% compared to 60% in the pre-infusion specimen (5, 6). The post-CAR T tumor also had a more prominent T cell infiltrate as measured by CD3 staining (**Figure 3**).

Deep sequencing of the *TCRβ CDR3* gene in the tumor tissue demonstrated an increase in the diversity of the T-cell clones, suggesting that a polyclonal T cell infiltrate had been recruited to the tumor (5). Peripheral blood samples detected CAR T-EGFRvIII cells throughout the 29 months of follow-up (**Figure 2**), indicating that the product engrafted successfully in the patient and recirculated through the peripheral blood (5).

At three years from initial tumor recurrence, this patient's greater than expected survival may have been due to a combination of factors. Positive prognostic factors included a good performance status, a unifocal lesion, and no initial steroid dependence (8). Negative prognostic factors included the lack of IDH1 mutation, unmethylated MGMT on the recurrent tumor, and the large tumor volume (8). Based on the EORTC meta-analysis of clinical trials for rGBM, patients with similar prognostic features have a median overall survival on the order of 8 months from recurrence (8).

In a previously published case report, one patient receiving IL13Rα2-targeted CAR T experienced a complete, though transient, radiographic response for his multifocal rGBM (9). When the patient's tumor did recur after 7.5 months, pathology showed decreased expression of the IL13Rα2 antigen. That case was also notable for evidence of recruitment of endogenous host immunity, with both CAR-expressing T cells and mixed endogenous immune cells present in the cerebral spinal fluid following treatments. In our case, the reduced expression of EGFRvIII antigen and increase in the number and diversity of T cell clones support trafficking of clones in the infusion product and suggest a local specific immune response related to antigen editing (5). Both the presented case and the IL13Rα2 study suggest that recruitment of a non-CAR T host immune infiltrate may constitute part of the therapeutic response to CAR T treatment.

The post-infusion tumor tissue in the current study demonstrated anti-inflammatory adaptations. In particular, the post-CAR T specimen showed a moderate level of programmed cell death protein 1 (PD-1) staining, whereas the baseline tumor had almost no staining (**Figure 3**) (5). Interestingly, this patient's post-CAR T tissue did not demonstrate increases in regulatory T cell (Treg) markers (**Figure 3**), in contrast to other patients in the trial, possibly due to her second stage craniotomy occurring 104 days after CAR T treatment. The four patients with surgery within 13 days of infusion showed an increase in Treg markers, while no patients with later surgeries did so. The timing of and durability of expression of anti-inflammatory adaptive changes following CAR T treatment should be investigated further.

The lack of noninvasive biomarkers for anti-tumoral response remains a major problem in the surveillance of patients undergoing CAR T trials. Conventional MRI may be unreliable for assessing tumor progression post immunotherapy, as the inflammatory changes that accompany immunotherapies may lead to disruption of the blood brain barrier and resultant increasing areas of contrast enhancement and worsening FLAIR signal abnormality (10–12). While still investigational,

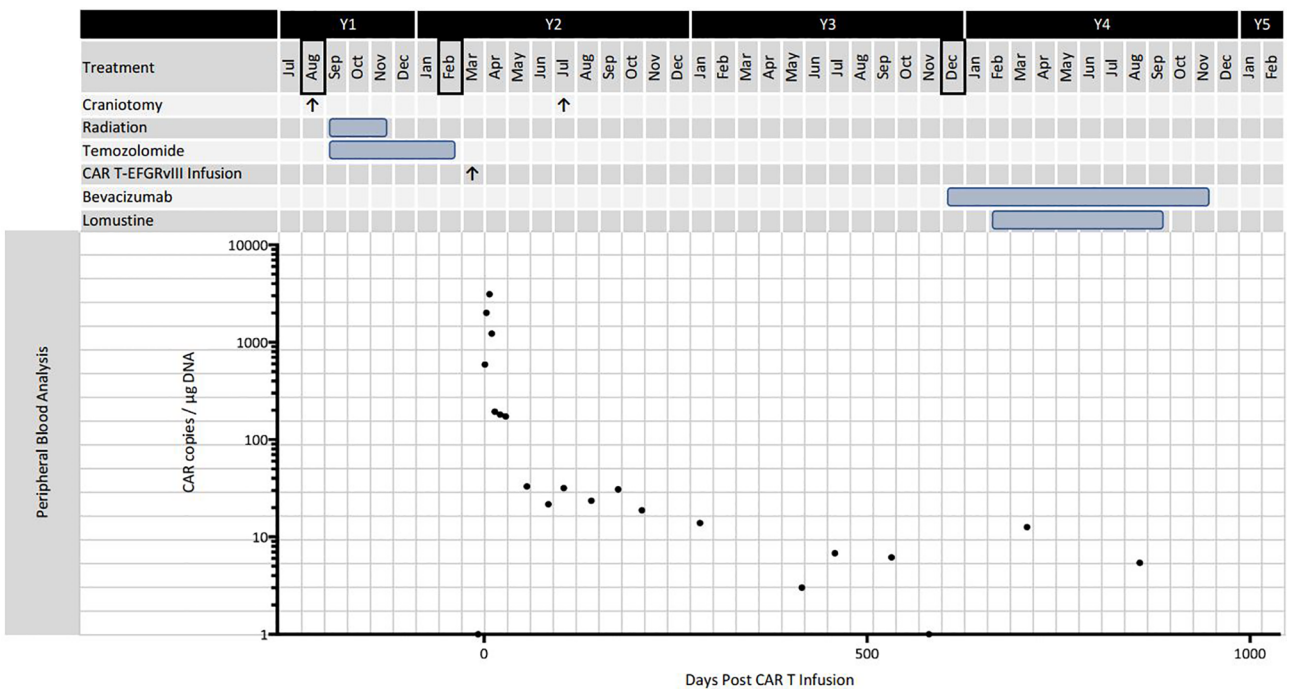
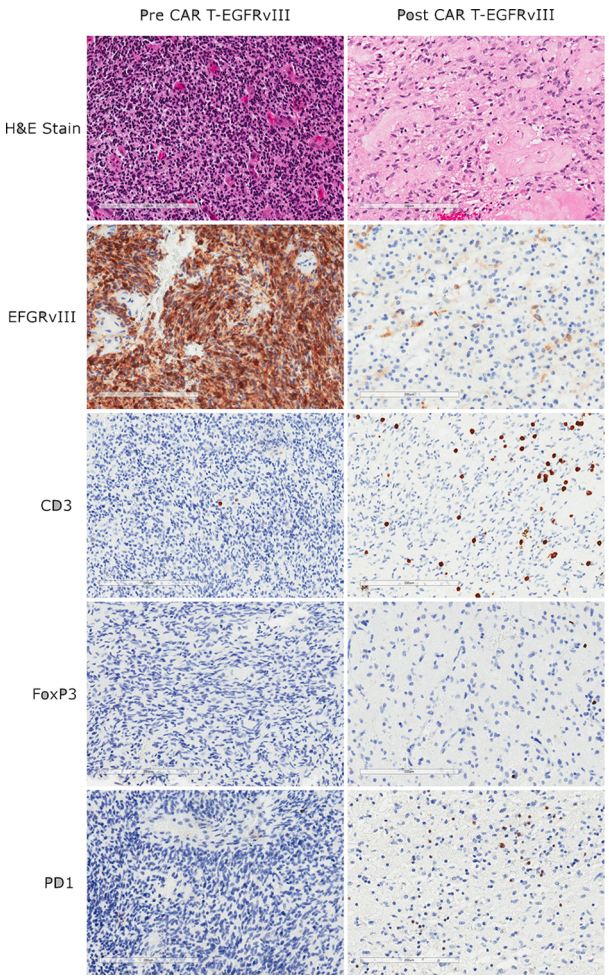


FIGURE 2 | Timeline of disease progression and treatment. Black bordered months indicate diagnosis or definitive progression on surveillance MRI. The graph shows CAR T-EGFRvIII levels in the peripheral blood as measured by qPCR.



IHC Summary				
	% Positive (area)		Intensity	
Stain	Pre	Post	Pre	Post
EGFRvIII	78.00	3.65	2+ to 3+[a]	1+ to 2+[b]
CD3	0.24	3.40	0	3+
FoxP3	0.00	0.32	0	3+
PD1	0.00	0.57	3+	3+

FIGURE 3 | Immunohistochemical (IHC) analysis of EGFRvIII, CD3, FoxP3, and PD1 expression in the patient's tumor before and after CAR T infusion. The left panels show tissue from her original surgery, prior to any treatment. The right panels show tissue from her surgery 104 days after CAR T infusion. Scale bars are 200 μm. Percent positivity by area was calculated by ImageJ as described in the methods, and staining intensity is by pathologist assessment. a: Pre-treatment fields also contain scattered 1+ EGFRvIII positive cells of unknown type. b: The positive cells are of undetermined lineage.

advanced imaging modalities such as diffusion, perfusion and MR spectroscopy may be valuable in assessing CAR T treatment response. For this patient, the reduction in rCBV at early time points following CAR T treatment merits attention (**Figure 4**), as other studies have correlated rCBV to tumor biologic activity (13, 14). The rCBV metric assesses microvascular volume, an indirect measure of tumor angiogenesis (15). A prospective study of 53 glioma patients noted near perfect correspondence of tumor grading by rCBV versus histopathology, with a significant correlation between the mitotic index and rCBV values (13). In a trial of a dendritic cell vaccine in GBM, there were higher rCBV values in patients who progressed compared to those with stable disease (14).

CAR T cells detectable by qPCR persisted at 29 months post infusion, the last sample collected, representing the longest interval of peripheral engraftment that has been reported in CAR T for GBM. This is comparable to the duration of engraftment for anti CD19 CAR T cells as reported in the ELIANA trial (16). A previous study using HER2 directed CAR T cells showed persistence at 12 months (17). The recent clinical trial by Goff et al. of EGFRvIII directed CAR T cells in seventeen patients was notable for one survivor at 59 months but no data on CAR T persistence past 9 months (18). In our patient, the peak level of CAR T cells detected in the peripheral blood occurred at approximately seven days, which is comparable to CD19 CAR T pharmacokinetics (16). This coincided with the patient's mild systemic symptoms, suggesting a cytokine-driven immune response. The CAR T expansion and related inflammatory syndrome are evidence that T cell activation occurred. The authors of the IL13Rα2 trial noted a similar correspondence of flu-like symptoms and cytokine levels with CAR T treatment, although this happened more quickly in their trial, at day one or two post CAR T infusion (9). The more rapid CAR T expansion in the IL13Rα2 study was perhaps due to

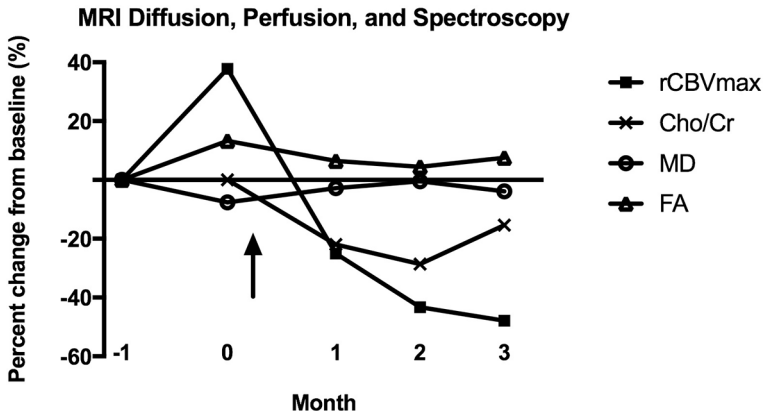


FIGURE 4 | Percent change from baseline for MRI diffusion (MD, FA), perfusion (rCBVmax), and spectroscopy (Cho/Cr) parameters after CAR T-EGFRvIII infusion. No MR spectroscopy was performed at month -1, so the baseline for Cho/Cr is month 0. The arrow indicates the date of CAR T-EGFRvIII infusion. MD, mean diffusivity; FA, fractional anisotropy; rCBVmax, maximum relative cerebral blood volume; Cho/Cr, choline/creatine ratio.

intra-tumoral and intraventricular locoregional delivery, which may have expedited the interaction of CAR T and tumor cells.

While this case suggests evidence for a CAR T therapeutic response in rGBM, increased efficacy may occur with the next generations of CAR T cell therapy. First, strategies targeting multiple antigens may overcome the dual challenges of heterogenous antigen expression and selective deletion. Second, the increase in PD-1 and other immunoregulators following CAR T are suggestive of adaptive changes that may allow tumors to evade the current generation of therapies (5). Approaches that combine CAR T with immune checkpoint blockade agents like PD-1 inhibitors may be one solution for potentiating CART persistence required to result in clinical efficacy. The combination of CAR T-EGFRvIII and PD-1 blockade is currently the focus of a clinical trial at our institution for newly diagnosed GBM (NCT03726515). Tumor-associated macrophages and microglia, accounting for as many as half of the cells in the GBM TME (19–21), also exert a significant immunoinhibitory function, and therapies such as CD40 agonists may re-educate macrophages to destroy tumor stroma and support T cell activation (22). Finally, strategies that deliver T cells or adjuvant treatments directly to the tumor or ventricular system may enhance efficacy (9). Through gene engineering, CAR T cells can be equipped with new features that address the challenges observed in the first generation of clinical trials. The responses seen to date suggest that CAR T therapy can be optimized to make a significant therapeutic impact in GBM.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital of the University of Pennsylvania IRB. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JD, FH, MN, SM, and SH contributed to data acquisition and analysis and the writing of the manuscript. SL, JJM, AD, and JL contributed to data acquisition and the writing of the manuscript. DO'R, ZB, RO'C, MM, and CJ contributed to study design and the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: DO'R and ZB are inventors on patents related to CAR T cells that have been filed by the University of Pennsylvania. JJM consults with or serves on the board of directors of several companies developing CAR T technology. JM and SL are inventors of intellectual property related to CAR T cells that is licensed by the University of Pennsylvania to Novartis.

CHJ reports receiving grants from Tmunity Therapeutics and holds founders stock in Tmunity Therapeutics and DeCART Therapeutics. CHJ also receives personal income from BluesphereBio, Cabaletta, Carisma, Cellares, Celldex Therapeutics, Viracta Therapeutics, Ziopharm and WIRB-Copernicus Group as well as royalties from Novartis.

MCM is an inventor on patent applications related to CAR technology and has received licensing royalties from Novartis corporation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CASE REPORT
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PiggyBac-Generated CAR19-T Cells Plus Lenalidomide Cause Durable Complete Remission of Triple-Hit Refractory/Relapsed DLBCL: A Case Report

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MYC/BCL2/BCL6 triple-hit lymphoma (THL) is an uncommon subset of high-grade B-cell lymphoma with aggressive clinical behavior and poor prognosis. TP53 mutation is an independently poor progonistic indicator in patients with THL, hence novel therapeutic strategies are needed for these patients. CD19-directed chimeric antigen receptor (CAR19)-T cell therapy has shown promising efficacy for relapsed/refractory diffuse large B cell lymphoma (RR DLBCL), but the majority of CAR19-T cell products to date have been manufactured using viral vectors. *PiggyBac* transposon system, with an inclination to memory T cells, offers a more convenient and economical alternative for transgene delivery. We herein report the first case of triple-hit RR DLBCL with TP53 mutation who was treated with *piggyBac*-generated CAR19-T cells and accompanied by grade 2 cytokine release syndrome. The patient obtained a complete remission (CR) in the 2nd month post-infusion and demanded maintenance therapy. Whether maintenance therapy is favorable and how to administrate it after CAR-T cell infusion remain controversial. Preclinical studies demonstrated that lenalidomide could enhance antitumor activity of CAR19-T cells. Therefore, we pioneered oral lenalidomide after CAR19-T therapy in the patient from the 4th month, and he discontinued after one cycle due to side effects. The patient has still kept sustained CR for over 24 months. Our case have firstly demonstrated the feasibility, preliminary safety and efficacy of *piggyBac*-produced CAR19-T cell therapy in triple-hit lymphoma. The innovative combination with lenalidomide warrants further investigation. Our findings shed new light on the possible solutions to improve short-term relapse after CAR19-T cell therapy in RR DLBCL. ChiCTR, number ChiCTR1800018111.

Keywords: triple-hit lymphoma, diffuse large B cell lymphoma, relapsed/refractory, chimeric antigen receptor-T cell therapy, *PiggyBac* transposon system, lenalidomide maintenance

INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of aggressive non-Hodgkin lymphoma, accounting for 30–40% of newly diagnosed cases worldwide (1). Although most patients achieve remission with R-CHOP (rituximab, cyclophosphamide, adriamycin and prednisone) immunochemotherapy, 10–15% exhibit primary refractory disease and 20–35% suffer a relapse (2). For those with relapsed/refractory (RR) DLBCL, the median overall survival (OS) was only 6.3 months with conventional therapy (3). Triple-hit lymphoma (THL) that carries concurrent MYC, BCL2 and BCL6 rearrangements is a relatively rare subset, identified in approximately 1% of DLBCL patients (4–6). These rearrangements result in highly aggressive clinical behavior, resistance to standard chemotherapy and extremely poor outcomes (7). Although high-dose chemotherapy combined with autologous stem cell transplantation (ASCT) remains the standard treatment for RR DLBCL, a series of studies demonstrated no appreciable benefits or even inferior outcomes for patients with THL after ASCT (8–10). TP53 is an important tumor suppressor gene and is proven as an inferior prognostic factor in DLBCL (11). Novel therapeutic strategies are needed to improve survival for these patients with triple-hit RR DLBCL.

CD19-specific chimeric antigen receptor (CAR19)-T cell therapy has offered a new paradigm for the treatment of RR DLBCL. Three second-generation CAR19-T cell products, axicabtagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel) and lisocabtagene maraleucel (liso-cel) have got FDA-approval for DLBCL (12–14). A latest meta-analysis demonstrated that second-generation CAR19-T cell therapy attained a remarkable overall response rate (ORR 55–79%) in RR DLBCL (n = 306), and the median OS was 13.2 months (15). Subgroup analyses demonstrated that CAR19-T cell therapy exhibited consistent efficacy between doublt-hit or triple-hit lymphoma and standard-risk DLBCL, supporting its application in triple-hit RR DLBCL (16, 17). The majority of CAR-T cells used in clinical trials to data are conducted by lentivirus or retrovirus. *PiggyBac* transposon system, as an emerging non-viral methodology for stable genetic modification of human T cells, possesses a large gene-capacity, simple and cost-effective manufacturing, and an inclination to stem-cell memory (SCM) phenotype (18–21). Preclinical studies suggested that *PiggyBac*-generated CAR19-T cells had a potent activity against B-cell malignancies (22–24). However, the efficacy and safety of *piggyBac*-engineered CAR-T cells haven't been reported in human clinical trials. Here, we report the first case with triple-hit RR DLBCL who has received *piggyBac*-generated CAR19-T cell therapy and achieved durable complete remission (CR) for over 24 months.

Whether maintenance treatment is favorable and how to administrate it after CAR19-T cell therapy are hotly debated issues in RR DLBCL. Preclinical studies demonstrated that lenalidomide could enhance antitumor function of CAR19-T cells for lymphoma (25). Lenalidomide has been approved as maintenance therapy after transplantation for multiple myeloma, but not reported as maintenance after CAR-T cell therapy. The patient obtained a CR in the 2nd month and demanded maintenance therapy. Therefore, we have pioneered oral lenalidomide in the patient after CAR19-T cell infusion, which is worth further exploration.

Abbreviations: ABC, activated B-cell; ASCT, autologous stem cell transplantation; CAR19, CD19-directed chimeric antigen receptor; CR, complete remission/response; CT, computed tomography; DLBCL, diffuse large B cell lymphoma; DHL, double-hit lymphoma; R-CHOP, rituximab, cyclophosphamide, adriamycin and prednisone; R-DHAP, rituximab, dexamethasone, cytarabine and cisplatine; R-ICE, rituximab, ifosfamide, carboplatine and etoposide; FISH, Fluorescence *in situ* hybridization; IHC, immunohistochemistry; ORR, overall response rate; OS, overall survival; PET, positron emission tomography; RR, relapsed/refractory; SCM, stem-cell memory; THL, triple-hit lymphoma.

CASE REPORT

Lymphoma Treatment History

A 53-year-old Chinese male patient was diagnosed as follicular lymphoma (grade 3A, stage II, group B) in May 2017 and achieved first CR after two cycles of R-CHOP (**Figure 1**). After given four cycles of R-CHOP, the patient's disease relapsed. The pathologic biopsy and immunohistochemistry (IHC) of celiac lymph nodes revealed DLBCL, activated B-cell (ABC) subtype, and overexpression of MYC, BCL2 and BCL6. Fluorescence *in situ* hybridization (FISH) of the lymphoma tissues also detected the triple rearrangements. Second-generation sequencing of the paraffin-embeded lymphoma tissues indicated 68.70% of TP53 mutation. The patient receieved two cycles of R-ICE (rituximab, ifosfamide, carboplatine and etoposide), and obtained a second CR. Unfortunately, he failed to collect hematopoietic stem cells for ASCT, and his disease progressed again with bone marrow involvement in June 2018. He complained of pain and weakness of both lower limbs, especially the left lower extremity. Subsequently, one cycle of R-DHAP(rituximab, dexamethasone, cytarabine and cisplatine) was administrated. Symptoms of left peripheral facial paralysis and severe headache occurred, and supportive treatment showed no response. The patient then suffered left eyeball pain, but still kept complete self-cognition. Head computed tomography (CT) scans excluded elevated intraocular pressure and intracranial space-occupying lesions. Head magnetic resonance imaging, including plain and enhancement scans, didn't show involvement of the brain parenchyma. The patient was unable to receive lumbar puncture and intrathecal chemotherapy because of pain and weakness of both lower limbs. He was given promptly with high-dose methotrexate intravenously once and single infusion of combined regimens including rituximab, high-dose methotrexate and cytarabine. Positron emission tomography (PET)-CT scans suggested extensive invasion of lymphoma in gastric wall, small intestines and bone marrow, scoring 5 points per Deauville criteria (**Figure 1**). Abdominal CT scans showed an abnormal mass sized as 3.0 × 2.2 cm (**Figure 1**). Repeated IHC of the paraffin-embeded lymphoma tissues was strongly positive for CD19. 1.5% of the cells were of unknown classification in bone marrow cytology, but no abnormal monoclonal B cells were found

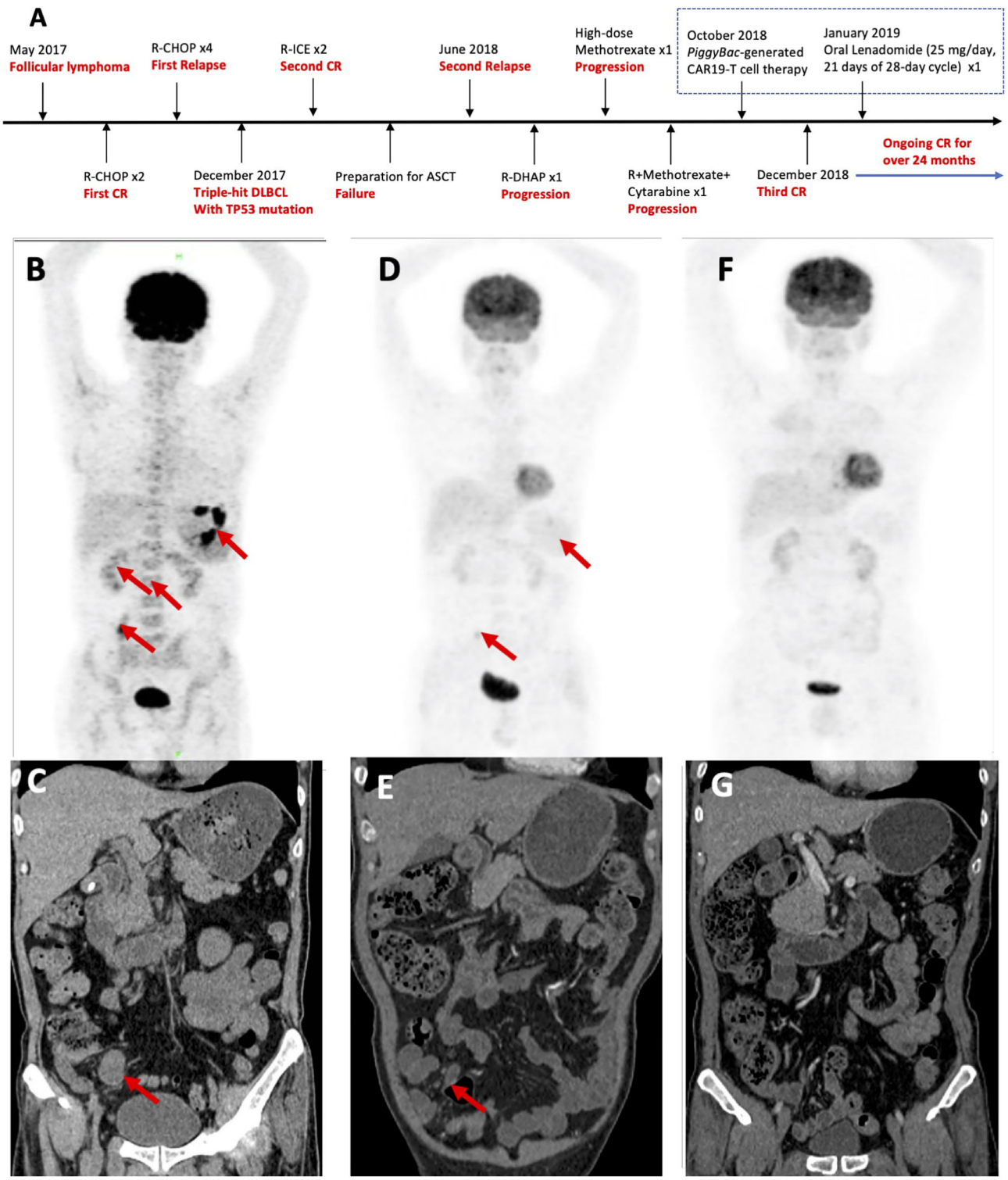

FIGURE 1 | Patient treatment history and response to *piggyBac*-generated CAR19-T cells. **(A)** Patient's disease progression and lymphoma treatment history. **(B)** Pre-treatment PET-CT, showing extensive invasion of tumors in gastric wall (SUVmax 13.2–18.0), small intestines (SUVmax 6.7) and bone marrow (SUVmax 2.6–3.2), scoring 5 points per Deauville criteria. **(C)** Pre-treatment abdominal CT, showing an abnormal mass sized as 3.0 × 2.2 cm (arrow). **(D)** PET-CT in the 1st month post-infusion, showing diminished invasion of tumors in small intestines (SUVmax 4.6) scoring 3 points per Deauville criteria. **(E)** Abdominal CT scans in the 1st month, showing an abnormal mass sized as 1.6 × 1.0 cm (arrow). **(F)** PET-CT scans were consistent with a complete metabolic response in the 2nd month post-infusion. **(G)** Normal Abdominal CT in the 2nd month.

in bone marrow immunophenotyping. FISH showed the triple rearrangements of MYC, BCL2 and BCL6, and 4.51% of TP53 mutation was found in bone marrow. Cerebrospinal fluid examination didn't indicate involvement in central nervous system. Therefore, the patient was enrolled in the phase 1 study (ChiCTR1800018111).

Manufacturing of *piggyBac*-Generated CAR19-T Cells

The CD19CAR incorporated an FMC63 mAb-derived single chain variable fragment, a human CD8α hinge and transmembrane domain, an intracellular 4-1BB (CD137) costimulatory domain, and a cytoplasmic CD3ζ signal (**Figure 2**). The CD19CAR gene was cloned into the *PiggyBac* transposon vector pNB328-EF1α to construct pNB328-CD19CAR, as described (26). Peripheral blood mononuclear cells were collected by leukapheresis from the patient and isolated by Ficoll density gradient centrifugation. T cells were electroporated with pNB328-CD19CAR plasmids and then activated by anti-CD3/CD28 antibodies in KBM581 medium containing 200 U/ml recombinant human interleukin (IL)-2 for 5 days. Thereafter, the activated cells were cultured until meeting the predefined release criteria, including transduction efficiency ≥5%, cell viability ≥70%, negative mycoplasma, negative bacterial and fungal cultures.

CAR19-T Cell Infusion and Patient's Outcomes

The patient was given the lymphodepleting chemotherapy (fludarabine 50mg, days-5 to -3; cyclophosphamide 1,200 mg, day-3). On October 23, 2018 (day 0), the patient received an infusion of *piggyBac*-generated CAR19-T cells at the dose of 1.0×10^6/kg. In the final infused cell products, the CAR transfection efficiency was 42.97%, including 33.28% of $CD4^+CAR^+$ cells and 13.54% of SCM T cells (**Figure 2**).

During days 9 to 11 post-infusion, the patient experienced pyrexia, hypoxia and emesis with a maximum temperature of 39.3 °C, which was rated as grade 2 cytokine release syndrome (CRS) against Lee's criteria (27). As the body temperature rose after infusion, serum IL-6 and C-reactive protein significantly elevated, peaked at 598.5 and 73.8 mg/l, respectively (36-fold and 9-fold over upper limit of normal) (**Figure 3A**). Therefore, intravenous tocilizumab 8 mg/kg and supportive treatment were administrated, and CRS got well controlled. The CAR copies increased dramatically and peaked at 103,000 copies/ug on day 14 (**Figure 3**). The peak of ferritin and lactate

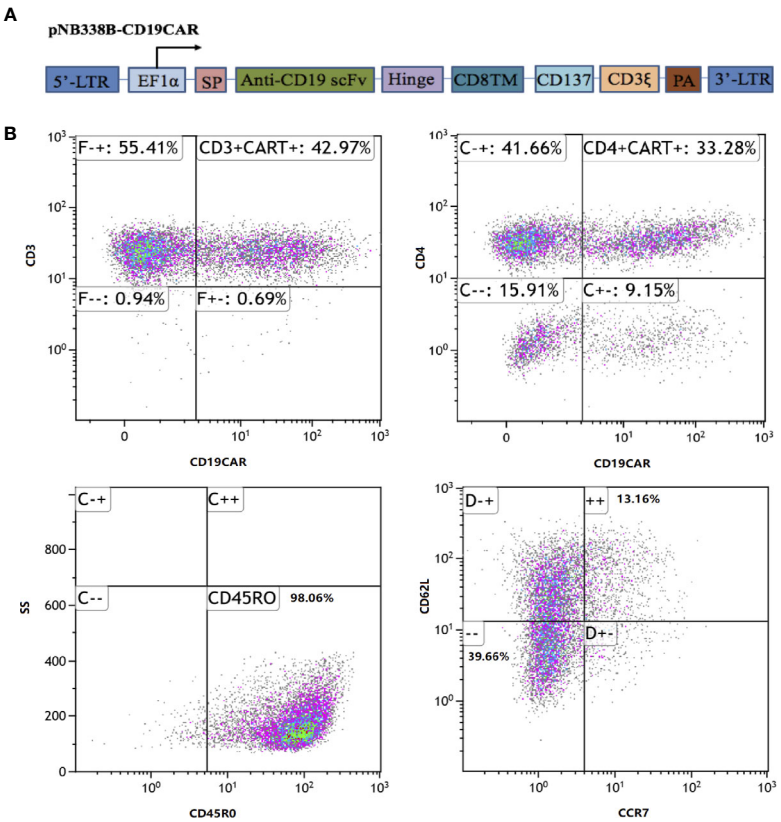

FIGURE 2 | CD19CAR structure and immunophenotype of infused *piggyBac*-generated CAR19-T cell products. **(A)** Schematic diagram of CD19CAR. SP, CDS signal peptide; TM, Transmembrane; PA, SV40 Poly A signal. **(B)** In the final infused products, the CAR transfection efficiency was 42.97%, including 33.28% of $CD4^+CAR^+$ cells and 13.54% of stem-cell memory T cells ($CD45RO^+CCR7^+CD62L^+$).

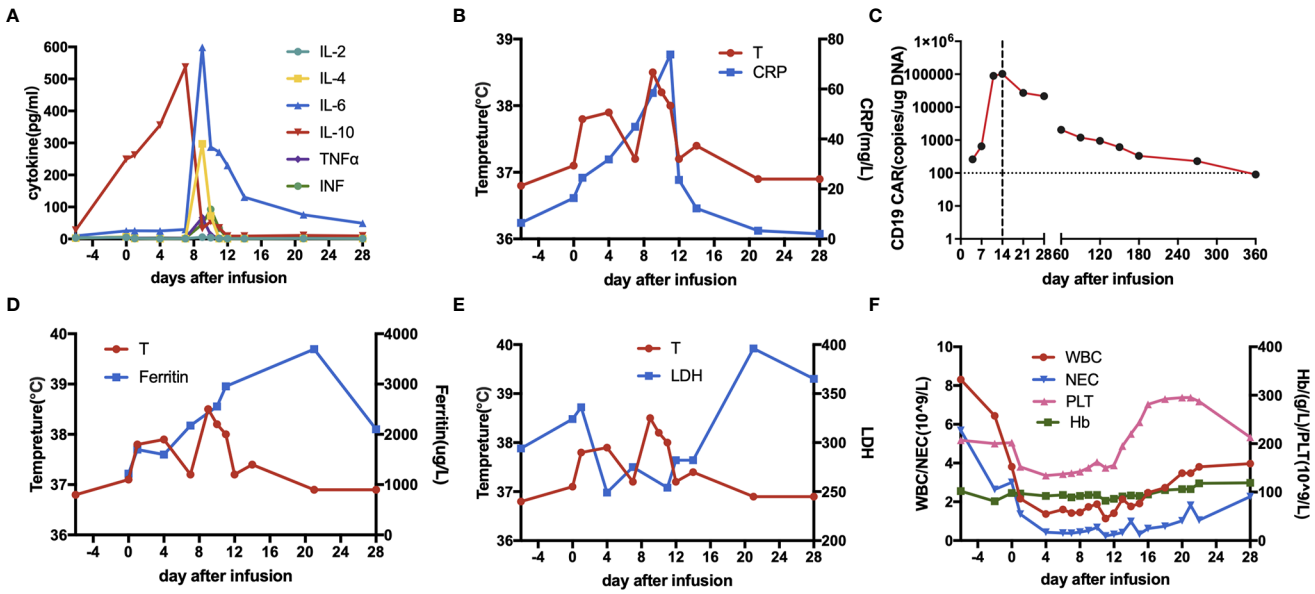

FIGURE 3 | Clinical monitoring after infusion of piggyBac-generated CAR19-T cells. **(A)** Trends of Interleukin(IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor α (TNFα) and interferon (INF) during 28 days after infusion. **(B)** Trend of C-reactive protein (CRP) was associated with temperature (T) rising during 28 days after infusion. **(C)** CAR19 copies in peripheral blood were measured by quantitative polymerase chain reaction. **(D, E)** The peak values of ferritin and lactate dehydrogenase (LHD) after infusion were later than temperature. **(F)** Changes of white blood cells (WBC), neutrophils (NEC), platelet (PLT) and hemoglobin (Hb) during 28 days after infusion.

dehydrogenase were detected on day 24, later than the time when toxicity culminated (**Figure 3D**). Moreover, he developed grade 3–4 hematological toxicities, which were relieved during the 1st month (**Figure 3**).

The patient obtained a partial remission (PR) in the 1st month per Lugano criteria (**Figure 1D**) (28). Bone marrow biopsy and immunophenotyping had no signs of lymphoma involvement, and only 0.5% of the cells of unknown classification were found in bone marrow cytology. In the 2nd month, PET-CT scans were consistent with a complete metabolic response, and abdominal CT scans were normal (**Figure 1F**). Bone marrow examination were normal, including biopsy, cytology, and immunophenotyping. TP53 mutation disappeared and FISH detected no abnormality in bone marrow. In the 3rd month, the patient maintained CR and demanded maintenance therapy. Oral lenadomide(25 mg/day, 21 days of 28-day cycle) was given in the 4th month and the patient discontinued after one-cycle administration due to side effects such as skin rashes, pruritus and painful joints. The CAR copies could be detected in peripheral blood until the 9th month post-infusion by quantitative polymerase chain reaction (**Figure 3**). The patient has still kept durable CR for over 24 months.

DISCUSSION

To the best of our knowledge, this is the first case with triple-hit RR DLBCL who has received *piggyBac*-generated CAR19-T cell therapy and maintained durable CR for over 24 months. It's also the first report about oral lenalidomide maintenance after CAR-T cell infusion.

The 2016 revised World Health Organization guidelines of lymphoid neoplasms classified large B-cell lymphoma with rearrangements of MYC and BCL2 or/and BCL6 in a distinct category to be designated high-grade B-cell lymphoma, also called double-hit lymphoma (DHL) or THL (4). DHL comprises approximately 2–10% of newly diagnosed DLBCL cases, and THL is a rare subset, accounting for almost 1% in DLBCL (5, 6). The largest series of THL to data included 40 patients, suggesting that its clinicopathologic features were similar to DHL and TP53 mutation was an independent predictor of poor prognosis (7). Patients with DHL/THL have significantly suboptimal responses and dismal outcomes with standard first-line R-CHOP chemoimmunotherapy (6, 29, 30). Our case relapsed following initial CR to R-CHOP and transformed to triple-hit DLBCL. High-dose chemotherapy combined with ASCT has historically regarded as the curative chance for patients with chemotherapy-sensitive relapse (31). Therefore, our patient was given R-ICE as salvage regimens prior to ASCT, and the patient achieved second CR. Unfortunately, the patient failed in the bridge to ASCT, and suffered second recurrence, and even became resistant to another three lines of salvage chemotherapy. Novel therapeutic options are urgently needed for these patients with triple-hit RR DLBCL.

CAR19-T cell therapy has emerged as a novel promising immunotherapy exhibiting remarkable efficacy in patients with chemotherapy-refractory DLBCL (**Table 1**). Axi-cel, as the first FDA-approved CAR19-T cell product for RR DLBCL, is generated untilizing retroviral vectors and contains a CD28

TABLE 1 | Representative CAR19-T cell products in B-cell lymphoma.

CAR19-T Cell Products	Axicabtagene Ciloleucel[11]	Tisagenlecleucel[12]	Lisocabtagene Maraleucel[13]
CAR Construct	FMC63(CD19 scFv)/CD28/CD3ζ	FMC63(CD19 scFv)/4-1BB/CD3ζ	FMC63(CD19 scFv)/4-1BB/CD3ζ CD4:CD8=1:1
Vector	Retrovirus	Lentivirus	Lentivirus
FDA-approved indication	RR DLBCL, PMBCL, transformed FL, and HGBCL	RR DLBCL, PMBCL, transformed FL, and HGBCL	RR DLBCL, HGBCL, PMBCL, and FL grade 3B
Clinical Trial	ZUMA-1(NCT02348216)	JULIET(NCT02445248)	TRANSCEND(NCT02631044)
CAR-T Cell Dose	2×10^6 cells/kg	median 3×10^8 cells/kg range (0.1-6.0) $\times10^8$ cells/kg	DL1 5×10^7 total cells[once/twice] DL2 10×10^7 total cells DL3 15×10^7 total cells
Enrolled Patients	119, 108 infused	165, 111 infused	344, 269 infused
Bridging Therapy	not allowed	92%	59%
Lymphodepletion Regimens	Cy 500mg/m^2+Flu 30mg/m^2 on day-5, -4 and -3	Cy 250mg/m^2 +Flu 25mg/m^2 for 3 days or Ben 90mg/m^2 for 2 days	Cy 300 mg/m^2 + Flu 30mg/m2 on day-5, -4 and -3
Efficacy Evaluation	n=101	n=93	n=256
Best ORR	83%	52%	73%
Best CR rate	58%	40%	53%
Median Follow-up	27.1m (IQR 25.7-28.8)	14m (range 0.1-26)	Not reported
Median DOR	11.1m (range 4.2- NR)	NR (95%CI 10-NR)	NR (95%CI 8.6- NR)
Median PFS	5.9m (95%CI 3.3-15.0)	12m-PFS 83%	6.8m (95%CI 3.3-14.1)
Median OS	NR (range 12.8- NR)	12m (95%CI 7.0- NR)	21.1m (range 13.3- NR)
Relapse Rate	53.5% (45/84)	65% responders remain relapse-free at 12m	Not reported
Safety Evaluation	n=108	n=111	n=268
CRS	93%(severe 11%)	64%(severe 22%)	42%(severe 2%)
Neurotoxicity	67%(severe 32%)	23%(severe 12%)	30%(severe 10%)

PMBCL, primary mediastinal B-cell lymphoma; FL, follicular lymphoma; HGBCL, high-grade B-cell lymphoma; Flu, fludarabine; Cy, cyclophosphamide; Ben, bendamustine; DOR, duration of remission;PFS, progression-free survival; m, month(s); IQR, interquartile range; CI, confidence interval; NR, Not Reached.

costimulation domain (12). In the pivotal ZUMA-1 trial, axi-cel showed a striking ORR and CR rate of 83 and 58% (n = 108), respectively. At a median follow-up of 27.1 months, 39% maintained ongoing remission, and the median OS didn't reach. 93% of patients had CRS per Lee criteria (severe 11%), and 67% experienced neurotoxicity (severe 32%), both of which were manageable and largely reversible (32). Tisa-cel is the second FDA-approved CAR19-T cell product for DLBCL, which is manufactured using lentiviral vectors and a 4-1BB costimulation domain (13). In the pivotal JULIET study, 52% of patients achieved an objective response and 40% attained a CR (n = 93) at a median follow-up of 14 months. CRS occurred in 64% of patients (severe 22%) against the Penn grading scale, and neurotoxicity occurred in 23% of patients (severe 12%) (16). The lower incidence of CRS and neurotoxicity compared with axi-cel is possiblely related to 4-1BB costimulation domain ultilized in tisa-cel. Parallel comparison of 4-1BB or CD28 co-stimulated CAR19-T cells for B-cell lymphoma suggests that 4-1BB is more beneficial and tolerated for the clinical performance (33). Therefore, 4-1BB is widely applied in subsequent CAR-T cell products, including lisocabtagene maraleucel(liso-mar) and the *piggyBac*-generated CAR19-T cells in our study. Liso-mar is being actively tested in B-cell lymphoma. In the TRANSCEND trial, liso-mar exhibited a high response rate (73%, n = 268) and low incidences of CRS (42%, severe 2%) and neurotoxicity (30%, severe 10%) (14). Subgroup analyses of the JULIET and TRANSCEND trials demonstrated that patients with DHL/THL responded as similarly well to CAR19-T cell therapy as standard-risk DLBCL (14, 16, 17). These results support the application of CAR19-T cell therapy in doublt-hit or triple-hit RR DLBCL.

The majority of CAR-T cells used in clinical trials to data are conducted by lentivirus or retrovirus. Paralleled comparison of vectors for the generation of CAR-T cells was comprehensively reviewed (34). Viral vectors possess an ideal transduction efficiency and stable transgene expression, but to some extent, they also correlate with a high risk of insertional mutagenesis and potential malignant transformation. The novel non-viral *PiggyBac* transposon system, with decreased integration frequency into proto-oncogenes in human T cells, shows great application potential for stable genetic modification of human T cells (35). *PiggyBac* system consists of two components: a transposon plasmid carrying the target gene and another plasmid encoding the transposase, both of which are introduced into cells by electroporation. Preclinical studies suggested that *PiggyBac*-generated CAR19-T cells had a potent activity against B-cell malignancies (22–24). However, the preliminary efficacy and safety of *piggyBac*-engineered CAR-T cells haven't been reported in human clinical trials. Here, we report the first case with triple-hit RR DLBCL who has received *piggyBac*-generated CAR19-T cell therapy. Limited *in vivo* expansion and persistence of CAR19-T cells is considered as a main possible mechanism of CD19-positive relapse after CAR19-T cell therapy (36). SCM T cells are known to promote superior *in vivo* perliferation and survival of CAR-T cells (37–40). Previous studies indicated that *piggyBac* system had the preference to SCM T cells (21, 23, 41), thus *piggyBac* system could be a feasible optimization for CAR-T manufacturing. In

the case, the final infused cell products contained only 13.54% of SCM T cells and owned *in vivo* lifespan of 9 months, which inadequately explained the patient's exceptional response.

Whether maintenance treatment is favorable and how to administrate it after CAR19-T cell therapy are hotly debated issues in RR DLBCL. The patient obtained a CR in the 2nd month and demanded maintenance therapy. Despite ASCT recommended as the standard care for RR DLBCL, a series of studies demonstrated no appreciable benefits or even inferior outcomes for patients with DHL/THL after ASCT (8–10). Until now there is no evidence to support consolidation with transplantation after CAR-T cell therapy in lymphoma, hence novel strategies for maintenance therapy are warranted to explore. Lenalidomide, an oral immunomodulator, has been approved by FDA as maintenance therapy after ASCT for patients with multiple myeloma based on evidences from two randomized, blinded trials (CALGB100104 and IFM 2005-02) (42–44). Lenalidomide also exhibited activity as maintenance therapy in RR DLBCL, especially in ABC subtype for the reason that lenalidomide strongly inhibited NF-kB signaling, key pathogenesis of ABC subtype (45). Oral lenalidomide maintenance (25 mg/day, 21 days of 28-day cycle) after salvage chemotherapy in patients with RR DLBCL (n = 47) attained 1-year and 5-year PFS of 70 and 53%, respectively (46). Preclinical studies demonstrated that lenalidomide could enhance antitumor function of CAR19-T cells for aggressive B-cell lymphoma, whose mechanisms included augmented cytotoxicity, memory maintenance and persistence, and Th1 cytokine production (25). These results indicated that lenalidomide maintenance after CAR-T cells therapy deserved investigation. Therefore, we pioneered oral lenalidomide in the patient in the 4th month. The patient still maintains CR for over 2 years and his OS is over 3 years.

Several limitations exist in the present study. First, this is a case report, and more cases are needed to observe the overall safety and efficacy of *piggyBac*-generated CAR19-T cell therapy in RR DLBCL. Second, the patient discontinued after one-cycle lenalidomide due to adverse reactions, thus the risks of lenolidomide shouldn't be understated. A multicenter retrospective study indicated that lymphoma patients had similar benefits in ORR, PFS and OS when administrated with 10, 15 or 25 mg/day lenalidomide (47). Oral low-dose lenalidomide maintenance (10 mg) after CAR-T cell therapy deserves further exploration to minimize side reactions. Third, the additive role of lenalidomide maintenance after CAR-T cell therapy warrants controlled trials to verify.

In conclusion, our case, for the first time, has demonstrated the feasibility, preliminary safety and efficacy of *piggyBac*-produced CAR19-T cell therapy in triple-hit lymphoma. Future investigations with large sample sizes are needed to clarify the overall safety and activity of *piggyBac*-genrated CAR19-T cell therapy in RR DLBCL, and the additive effects of lenalidomide maintenance.

Patient Perspective

When I failed to prepare for ASCT, and then suffered the second relapse and didn't respond to the following chemotherapy, I felt so helpless. The outcome of patients with RR DLBCL is dismal, especially for patients with MYC, BCL2 and BCL6 rearrangements and TP53 mutuation. I am glad to participate in the study and has received the *piggyBac*-generated CAR19-T cell therapy. Although I attained CR in the 2nd month, I demanded maintenance treatment in view of my experience of two relapses. My doctors gave me oral lenalidomide in the 4th month, but I discontinued after one course due to some side effects. I still keep CR for over 2 years and I hope that my case will give some inspiration for physicians and patients all over the world.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Union Hospital affiliated to Huazhong University of Science and Technology, Wuhan, China. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YH and HM conceived and designed the study. CL and YS performed data analysis and wrote the paper. JWa contributed imaging interpretation. LT and HJ performed data collection. TG, LL, YW, and LX participated in patient management. LA analyzed the flow results. JWa, ZL, and QQ provided the CAR19-T cell products. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: Authors YS, JW, ZL, and QQ are employed by Shanghai Cell Therapy Group Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PTK7-Targeting CAR T-Cells for the Treatment of Lung Cancer and Other Malignancies

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In spite of impressive success in treating hematologic malignancies, adoptive therapy with chimeric antigen receptor modified T cells (CAR T) has not yet been effective in solid tumors, where identification of suitable tumor-specific antigens remains a major obstacle for CAR T-cell therapy due to the "on target off tumor" toxicity. Protein tyrosine kinase 7 (PTK7) is a member of the Wnt-related pseudokinases and identified as a highly expressed antigen enriched in cancer stem cells (CSCs) from multiple solid tumors, including but not limited to triple-negative breast cancer, non-small-cell lung cancer, and ovarian cancer, suggesting it may serve as a promising tumor-specific target for CAR T-cell therapy. In this study, we constructed three different PTK7-specific CAR (PTK7-CAR1/2/3), each comprising a humanized PTK7-specific single-chain variable fragment (scFv), hinge and transmembrane (TM) regions of the human CD8α molecule, 4-1BB intracellular co-stimulatory domain (BB-ICD), and CD3ζ intracellular domain (CD3ζ-ICD) sequence, and then prepared the CAR T cells by lentivirus-mediated transduction of human activated T cells accordingly, and we sequentially evaluated their antigen-specific recognition and killing activity *in vitro* and *in vivo*. T cells transduced with all three PTK7-CAR candidates exhibited antigen-specific cytokine production and potent cytotoxicity against naturally expressing PTK7-positive tumor cells of multiple cancer types without mediating cytotoxicity of a panel of normal primary human cells; meanwhile, *in vitro* recursive cytotoxicity assays demonstrated that only PTK7-CAR2 modified T cells retained effective through multiple rounds of tumor challenge. Using *in vivo* xenograft models of lung cancers with different expression levels of PTK7, systemic delivery of PTK7-CAR2 modified T cells significantly prevented tumor growth and prolonged overall survival of mice. Altogether, our results support PTK7 as a therapeutic target suitable for CAR T-cell therapy that could be applied for lung cancers and many other solid cancers with PTK7 overexpression.

Keywords: chimeric antigen receptor (CAR), cancer stem cells (CSCs), lung cancer 4, adoptive cell therapy (ACT), tumor-initiating cells (TICs), PTK7

INTRODUCTION

Chimeric antigen receptor (CAR)-modified T-cell (CAR T-cell) therapy is an innovative immunotherapeutic approach that vigorously rejuvenates the long-term pursuit adoptive cell transfer (ACT) for cancer immunotherapy (1, 2). Typical synthetic CAR comprises of single-chain variable fragment (scFv) of a monoclonal antibody (mAb), hinge/spacer and transmembrane (TM), and co-stimulatory and activating signaling domains from one or two co-stimulatory molecules and CD3ζ chain of the T cell receptor (TCR) complex respectively (1, 3). CAR modification confers T cells with "*de novo*" defined antigen specificities independently of both the natural TCR and major histocompatibility complex (MHC) restriction, which not only overcomes the downregulation of Human Leucocyte Antigen (HLA, human MHC) molecules frequently observed in cancer cells, but also widens the repertoire of actionable targets due to scFv-mediated antigen recognition of non-protein epitopes, thus greatly expanding the potentials of ACT for cancer immunotherapy (1, 3, 4). CAR T-cell therapy targeting CD19 antigen has achieved a remarkable therapeutic efficacy in treating relapse or refractory B-cell malignancies, culminating in the regulatory approval of two CAR T-cell products for patients with certain leukemia and lymphoma (1, 2); in addition, CAR T cells targeting other antigens, such as BCMA and CD22, have also exhibited a promising therapeutic potential in treating some type of intractable leukemia and multiple myeloma (5–7). These results have demonstrated that CAR T cells can be artificially generated with desirable characteristics to induce durable and complete responses in cancer patients even with highly refractory disease.

Despite great success in treating hematological malignancy, CAR T-cell therapy in solid tumor is still in its infancy with scant objective response seen (4). Among various factors constraining the efficacy of CAR T-cell therapy in solid tumor, a major obstacle is the lack of appropriate tumor antigens suitable for CAR-T targeting (4, 8). At present, the majority of CAR T-cell targets in solid tumors are overexpressed tumor-associated antigens (TAA) with lower-level expression in normal tissues as compared to tumor tissues, such as HER2, GPC-3, EGFR, mesothelin, PSMA, and IL13Ra2, which greatly limits the maximum safety dosage in order to avoid on-target off-tumor side effect and consequently results in unsatisfactory clinical efficacy (1, 8, 9). In addition, due to the extreme heterogeneous antigen expression and highly genomic instability in solid tumors, tumor cells are prone to produce antigen-loss variants under the immune selection pressure from CAR T-cell therapy, leading to immune escape (8). Therefore, the identification of new target antigens that are not easy to generate immune escape is still a key issue for the successful treatment of solid tumors with CAR T cells.

PTK7, also known as colon carcinoma kinase 4 (CCK-4), is a member of the pseudokinase family of receptor tyrosine kinases (RTKs) that have an intracellular catalytically inactive tyrosine kinase-like domain (10, 11). PTK7 is expressed during embryogenesis but absent from normal vital adult tissues, apart from a subset of immature $CD4^+$ recent thymic emigrants (RTEs) and plasmacytoid dendritic cells (pDCs) and low-level expression on some normal tissues (10–12). Genetic and biochemical studies have demonstrated an involvement of PTK7 in non-canonical Wnt signaling *via* interacting with Wnt ligands such asROR2, Wnt5a, or Wnt3a (13, 14). PTK7 is strongly associated with planar cell polarity (PCP) regulation as PTK7-deficient embryos exhibit severe developmental defects in PCP (15, 16). In addition, evidence is also present for context-dependent roles of PTK7 in the vascular endothelial growth factor (VEGF), semaphorin/plexin, and canonical Wnt signaling pathways (11). Oncogenic functions of PTK7 have been documented in several hematological and solid tumors (10, 11). Recent studies showed that PTK7 is overexpressed in triple-negative breast cancer (TNBC), non-small-cell lung cancer (NSCLC), ovarian cancer (OVCA), cervical cancer, esophageal squamous cell carcinoma (ESCC), and hepatocellular carcinoma (HCC) and enriched in tumor-initiating cells (TICs) from TNBC, OVCA, and NSCLC patient-derived xenografts (PDXs), and its overexpression is associated with poor survival in NSCLC, cervical cancer, ESCC, and HCC (12, 17–22). Bie J et al. found that PTK7 was dramatically upregulated in the ESCC tissues and cancer stem cell (CSC)-like cells and its knockdown reduced sphere formation, promoted apoptosis, and suppressed invasive behavior of tumor cells (17). Chen et al. conducted a large-scale meta-analysis to search the genes specifically overexpressed in lung adenocarcinoma where PTK7 was identified to be the one of overexpressed six genes confirmed by IHC analysis in primary adenocarcinoma samples. Functional investigation revealed that PTK7 knockdown decreased cell viability and increased apoptosis in lung adenocarcinoma cell lines. More importantly, a PTK7-targeting antibody-drug conjugate (ADC) induced sustained tumor regressions in lung and breast tumor xenograft models (12); furthermore, recent studies have documented the success and feasibility of PTK7-based tumor-targeting strategies by using PTK7-specific antibodies or aptamers for *in vivo* imaging or drug delivery (23–25). These pioneering studies strongly support the potential of the PTK7 as an attractive candidate for CAR T-cell therapy that could be broadly applied.

In this study, we developed an alternative approach of exploiting PTK7 as a target for CAR T-cell therapy. The rationale is based in part upon the hypothesis that PTK7

Abbreviations: CAR, chimeric antigen receptor; CSCs, cancer stem cells; scFv, single-chain variable fragment; ACT, adoptive cell therapy; TM, transmembrane; TCR, T cell receptor; MHC, major histocompatibility complex; HLA, Human Leucocyte Antigen; TAA, tumor-associated antigens; CCK-4, colon carcinoma kinase 4; RTKs, receptor tyrosine kinases; PCP, planar cell polarity; VEGF, vascular endothelial growth factor; TNBC, triple-negative breast cancer; NSCLC, non-small-cell lung cancer; OVCA, ovarian cancer; ESCC, cervical cancer, esophageal squamous cell carcinoma; HCC, hepatocellular carcinoma; TICs, enriched in tumor-initiating cells; PDXs, patient-derived xenografts; ADC, antibody-drug conjugate; FBS, fetal bovine serum; HUVECs, human umbilical vein endothelial cells; IHC, immunohistochemistry; tEGFR, truncated EGFR; ROR1, Receptor tyrosine kinase-like orphan receptor 1; EpCAM, epithelial cell adhesion molecule.

expression is enriched on TIC/CSC-like cells, and targeting antigens with enriched expression in TIC/CSC-like cells would achieve a long-term antitumor effect (26). Given the predicted potential and safety of PTK7 as an immunotherapy target, we sought to develop PTK7-specific CAR T-cell therapy for lung cancer and to evaluate its efficacy and safety in *in vitro* and *in vivo* preclinical models.

MATERIALS AND METHODS

Cell Lines

Human NSLCL cell lines H520, H1975, and H1299, SCLC cell lines H446 and H69, pancreatic cancer cell line BxPC3, breast cancer cell line MDA-DB-468, ovarian cancer cell line OVCAR3, CHO, and HEK-293 T cells were purchased from American Type Culture Collection (ATCC) and maintained in DMEM medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin/streptomycin (all from Thermo Fisher Scientific); and all cell lines were cultured at 37°C in a humidified chamber with 5% CO2. Stably transfected PTK7-CHO cell line was constructed by infecting parental CHO cells with lentiviral supernatants containing PTK7 gene (#HG19399-UT, Sino Biological) and sorting for PTK7 expression by using MoFloTM XDP cell sorting system (Beckman Coulter). These cell lines were also infected with the lentiviral supernatants containing Luciferase-IRES-GFP (GL) and were then sorted for GFP expression to obtain GL-expressing cell lines. Human primary normal epithelial cell lines (Mammary, Small Airway, and Renal Epithelial Cells) and human umbilical vein endothelial cells (HUVECs) were obtained from PriCells (Wuhan, China) and cultured according to the supplier's instructions.

PTK7-CAR Construction

Sequences of three humanized mouse antihuman PTK7 antibodies (Hu23, Hu24, and Hu58 with the affinity of 3.9, 1.2, and 2.1 nM, respectively) were obtained from a US patent (US20150315293A1). The variable region sequences of heavy (VH) and light chain (VL) of these antibodies were used to design scFv with the sequence of VH-$(G_4S)3$ Linker-VL. PTK7-CARs containing scFv from Hu23, Hu24, and Hu58 were designated as PTK7-CAR1, PTK7-CAR2, and PTK7-CAR3, respectively. From the 5′-end to 3′-end, each CAR is comprised of the human CD8α signal peptide sequence, PTK7-scFv, hinge and TM regions of the human CD8α molecule, 4-1BB intracellular domain sequence (BB-ICD), and CD3ζ intracellular domain sequence (CD3ζ-ICD) as previously described (27). Following CAR, a truncated tEGFR sequence is included *via* T2A ribosomal skipping sequence in the construct to allow for potential enrichment, tracking, and depletion if needed of transduced T cells (28). DNA encoding the CARs was codon-optimized and synthesized by General Biosystems (Anhui, China) with appropriate restriction sites. The CAR sequences were then cloned into third-generation self-inactivated lentiviral vector pLVEF derived from pRRLSIN.cPPT.PGK-GFP.WPRE vector (Plasmid #12252, Addgene) with replacing its original human PGK promoter with human EF1α promoter from pWPXLd vector (Plasmid #12258, Addgene). As a negative control, lentiviral vector encoding truncated tEGFR was constructed.

Lentivirus Production

High-titer replication-incompetent lentiviruses were produced and concentrated as described previously (29). Briefly, HEK-293 T cells were transfected with pVSV-G (VSV glycoprotein expression plasmid), pRSV-Rev (Rev expression plasmid), pMDLg/p.RRE (Gag/Pol expression plasmid), and pLVEF transfer plasmid using polyethylenimine (PEI, Sigma). The viral supernatant was harvested at 24 and 48 h after transfection and concentrated by using Lenti-X Concentrator (Clontech) in accordance with the manufacturer's instructions.

CAR T-Cell Production

Human PBMCs were obtained from healthy donors under protocols approved by the Institutional Review Board of Harbin Medical University and isolated by density gradient centrifugation over Ficoll-Paque (GE Healthcare). Freshly isolated PBMCs were then activated with antihuman CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a 3:1 ratio for 48 h followed by two sequential transductions with lentiviruses on RetroNectin-coated non-tissue treated plates and maintained in culture in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific) and recombinant human IL-2 (300 U/ml). Fresh media containing cytokine were replenished every other day to maintain T-cell concentration at 0.5×10^6 cells/ml. Five days after transduction, the CD3/CD28 Dynabeads were removed from the culture by magnetic separation, and CAR T cells were propagated for 14 days in total before using for functional assays. To track T cell numbers over time, viable cells were counted using trypan blue.

Flow Cytometry

PTK7 expression on tumor cells was detected by mouse monoclonal anti-PTK7 antibody (clone OTI2E7, Invitrogen) and goat anti-mouse IgG-phycoerythrin (PE)-conjugated antibody (Jackson ImmunoResearch). CAR expression on 293T cells was detected by APC-conjugated rabbit monoclonal anti-EGFR antibody (Clone E01, Sino Biological) and biotin-conjugated goat antihuman IgGF(ab′)2 (Jackson ImmunoResearch) and streptavidin-PE (BioLegend). CAR expression on T cells was detected by BV510-conjugated CD3 (clone UCHT1), APC-Cy7-conjugated CD4 (clone OKT4), and FITC-conjugated rabbit monoclonal anti-EGFR antibody (Clone E01, Sino Biological) and biotin-conjugated goat antihuman IgGF(ab′)2 and streptavidin-APC (BioLegend). The phenotype and effector molecule expression on CAR T cells were detected with a panel of monoclonal antihuman antibodies as follows: BV510-conjugated CD3 (clone UCHT1), BV421-conjugated CD4 (clone OKT4), APC-Cy7-conjugated CD8 (clone SK1), FITC-conjugated rabbit monoclonal anti-EGFR antibody, APC-conjugated CD45RO (clone UCHL1), PE-conjugated CCR7 (clone G043H7), PE-conjugated TIM-3 (clone F38-2E2), APC-conjugated PD-1 (clone EH12.2H7), and PE-conjugated Granzyme B (clone GB11, all from BioLegend). CAR T cells in

peripheral blood from tumor-bearing tumor were detected by BV510-conjugated CD3 and APC-conjugated rabbit anti-EGFR antibody. In most assays, cells were stained with Zombie Aqua™ Fixable Viability Kit (BioLegend) to exclude dead cells from analysis. Flow cytometry data were acquired with a FACSCantoTM system (BD Biosciences) using DIVA software according to the manufacturers' instructions.

Cytokine Release Assays

Control or PTK7-CAR T cells (1×10^5 cells/100 µl media) were co-cultured with an equal number of target cells for 24 h, after which cell-free supernatants were harvested for testing IL-2 and IFN-γ secretion by ELISA kits (R&D Systems) according to the manufacturer's instructions.

Proliferation Assay

Control or CAR T cells were first labeled with 5 µM fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) according to the manufacturer's instructions, and then co-cultured with tumor cells at an effector-to-target ratio of 1:1. CFSE dilution was measured on gated T cells on day 3 using flow cytometry.

In Vitro Killing Assays

For tumor cell killing assays, GL-expressing target cells (1×10^4 cells/100 µl media) were co-cultured with control or PTK7-CAR T cells at the varying effector-to-target ratios in triplicate wells of white 96-well plates. In some assays, it was conducted in the presence of soluble PTK7 protein (OriGene). Target cell viability was monitored 18 h later by using Bright-Glo™ Luciferase Assay System (Promega) according to the manufacturer's instructions. The percent lysis (%) was calculated by using the following equation: 1-[bioluminescence value in sample well (target cells + CAR T cells)/maximum bioluminescence value (target cells alone)].

For human primary normal cell killing assays, target cells were first labeled with 5 µM fluorescent dye CFSE according to the manufacturer's instructions, and then co-cultured with control or PTK7-CAR T cells at the indicated effector-to-target ratios in triplicates. After 18 h incubation at 37°C, mixed cells were harvested and stained with 7-AAD and then subjected to flow cytometric analysis to quantify remaining live (7-AAD negative) target cells. The cytotoxicity was calculated as 100%—the percentage of alive target cells/alive target cells in control wells without effectors.

In Vitro Recursive Cytotoxicity Assays

GL-expressing tumor cells (1×10^5 cells/500 µl CAR-T culture media) were seeded in 24-well tissue culture plates. After overnight plating, 2.5×10^4 (effector-to-target ratio of 1:4) CART cells in 500 µl media were added to the monolayer of tumor cells (round 1). Three days later, when most of the target tumor cells were confirmed to be killed by trypan blue staining, all cells in the well were collected and washed with PBS, resuspended in fresh medium, and added to a new plate seeded with tumor cells for 3 days (round 2). This procedure was repeated one more time, if applicable (round 3). At the end of each round, a duplicate well was harvested for counting of residual tumor cells (GFP^+) and CAR T cells ($CD3^+EGFR^+$) and other phenotypic analysis (granzyme B, PD-1, TIM-3) of CAR T cells by flow cytometry.

In Vivo Tumor Models

All animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Harbin Medical University. Six- to 8-week-old B-NSG mice (NOD-Prkdcscid Il2rgtm1/Bcgen) were obtained from Biocytogen Co., Ltd (Beijing, China) and maintained on a 12 h light-dark cycle in a temperature-controlled high-barrier facility with free access to food and water and treated under specific pathogen-free conditions at the Animal Centre of the Harbin Medical University. The tumor xenograft model was established by subcutaneous (s.c.) inoculation with 3×10^5 H520 or H69 tumor cells suspended in 100 µl PBS. After 7 days, when the tumor was consistently palpable (50–100 mm^3), mice were randomized into three groups (three to five mice per group) and intravenously (i.v.) injected with 5×10^6control or PTK7-CAR T cells suspended in 100 µl PBS and repeated once 1 week later. Mice were weekly monitored for tumor growth by using a caliper for 60 days, and then euthanized by cervical dislocation with blood and tumor harvested for analysis when they seemed moribund or their tumors reached 15 mm in diameter. Tumor volume (V) was calculated according to the following formula: V (mm^3) = 0.5 × length × $width^2$.

Immunohistochemistry (IHC)

Tumor tissues were fixed with formalin and embedded in paraffin. Then, 4 mm thick sections were deparaffinized with xylene and rehydrated in decreasing concentrations of ethanol. After heat-induced antigen retrieval, slides were then blocked by 3% BSA and stained with rabbit monoclonal antihuman CD3ϵ antibody (clone SP162, Abcam) or rabbit polyclonal anti-PTK7 antibody (Invitrogen) in the blocking solution overnight at 4°C. Commercially available normal human tissue microarray (TMA; Shanghai Outdo Biotec) including 20 normal tissues (two to four sections per tissue) were retrieved by EDTA solution (Solarbio) and then stained with the same polyclonal anti-PTK7 antibody (1:200 dilution at 4°C overnight). Slides were then rinsed with Tris-HCl/0.05% Tween-20 buffer and visualized with a horseradish peroxidase (HRP)-conjugated anti-rabbit EnVision+ Kit (Dako). PBS substituted for the primary antibody was used as the negative control.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software (version 7.0). Differences in groups were determined by two-way ANOVA with Tukey's multiple comparison test with $P<0.05$ considered to be a statistically significant. The survival curves were constructed using the Kaplan-Meier method and analyzed by using a log-rank test. All values and error bars represent the mean ± SEM. In the figures, significance of findings was defined as follows: $p > 0.05$; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, or $^{****}p < 0.0001$.

RESULTS

Generation of PTK7-CAR T Cells

To assess the suitability of PTK7 as a target for CAR T cells, we designed three CARs (PTK7-CAR1, PTK7-CAR2, and PTK7-CAR3) each containing ascFv derived from one of three humanized antihuman PTK7 monoclonal antibodies (**Figure 1A**). The PTK7-specific scFv was fused to CD8α hinge and transmembrane domain with intracellular 4-1BB (CD137) co-stimulatory and CD3ζ activating signaling domains in tandem. To facilitate the detection of transduced T cells, a truncated EGFR (tEGFR) tag was included *via* T2A ribosomal skipping sequence. Expression of tEGFR alone served as a negative control. We synthesized full-length DNA encoding each of the CARs and cloned into a self-inactivating lentiviral vector. Then 293T cells were infected with CAR-encoding replication-incompetent lentiviruses where CAR and tEGFR displayed a linear co-expression pattern, indicating that tEGFR is a reliable marker for PTK7-CAR expression (**Figure 1B**). PBMC from healthy donors were then transduced with the lentiviruses following anti-CD3/CD28 bead stimulation, and both CAR and tEGFR expressions were determined by FACS analysis 5–7 days after transduction. We observed a similar linear co-expression pattern of both CAR and tEGFR in each CAR-transduced T cells with CAR transduction efficiency approximately 40–60% and 20–30% in $CD4^+$ and $CD8^+$ T cells, respectively (**Figures 1C** and **S1A**). Although there was a similar CAR expression positivity among three PTK7-CAR

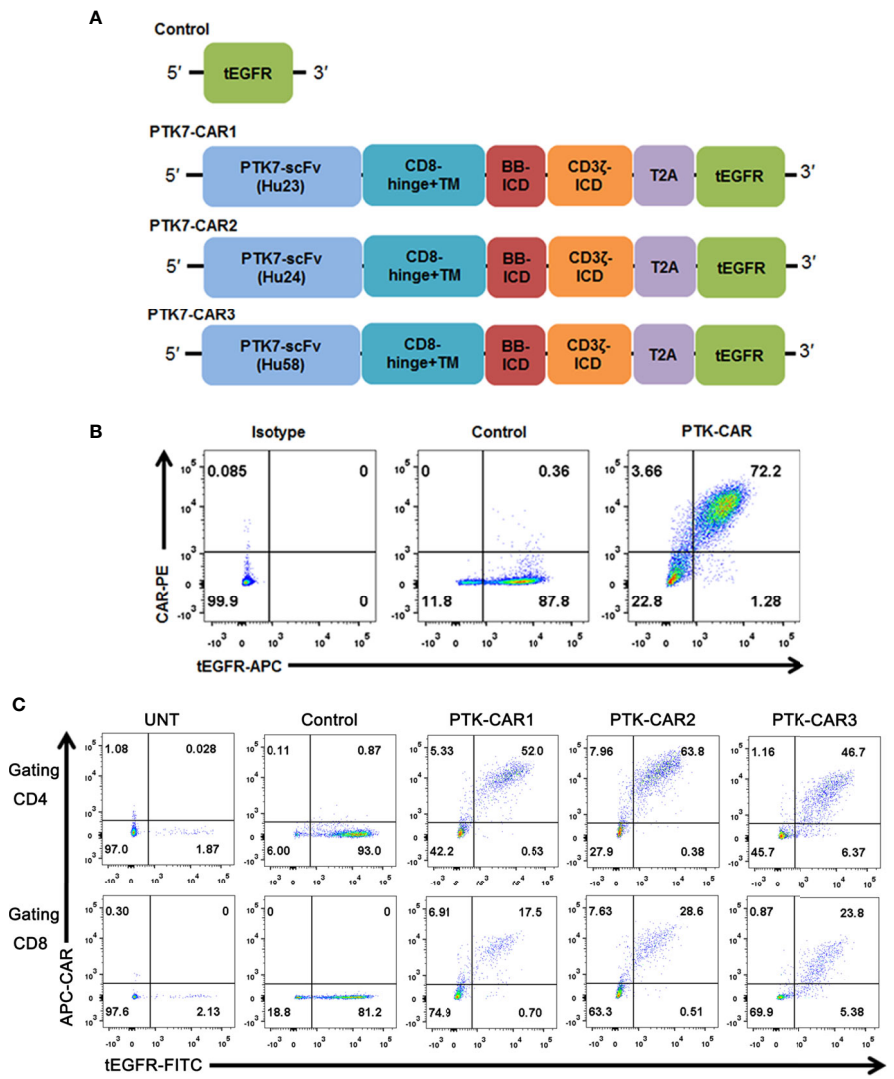

FIGURE 1 | PTK7-CAR generation, cell-surface expression, and transduction of human T cells. **(A)** PTK7-CAR was generated by fusing PTK7-specific scFv to the co-stimulatory signaling domain of the 4-1BB (BB-ICD) and activating signaling domain of CD3ζ (CD3ζ-ICD), a T2A ribosomal skipping sequence, and tEGFR was included for the detection of CAR-modified T cells. **(B)** 293T cells transfected with control or PTK7-CAR constructs express both CAR and the marker gene tEGFR. **(C)** PTK7-CAR expression on transduced human $CD4^+$ and $CD8^+$ T cells was approximately 40–60% and 20–30%, respectively, as determined by tEGFR and CAR co-staining.

candidates, we consistently observed a high CAR expression per cell in the PTK7-CAR2 T cells (**Figure S1B**). Phenotypic analysis showed that PTK7-CAR T cells contained central memory, effector memory, and T stem cell memory, without significant differences among three candidates (**Figure S1C**). In addition, no difference in T-cell expansion without antigen stimulation was seen *in vitro* among control and those candidates (**Figure S1D**).

PTK7-CAR T Cells Secrete Effector Cytokines and Proliferate After Exposure to PTK7-Expressingtumor Cells

To test specific recognition by PTK7-CART cells, we initially exploited PTK7-negative parental CHO cells and stably transfected PTK7-expressing PTK7-CHO cells (**Figure 2**). PTK7-CAR T cells and control T cells of three donors were co-cultured with CHO or PTK7-CHO cells, and effector cytokine IFN-γ and IL-2 release in the supernatants were evaluated after 24 h (**Figure 3A**). PTK7-CAR T cells secreted significant amounts of IFN-γ and IL-2 after exposure to PTK7-CHO cells compared with control T cells; however, parental CHO cells did not stimulate PTK7-CAR T cells to produce effector cytokines, indicating that cytokine production requires both the expression of PTK7 on target cells and PTK7-CAR expression on transduced T cells. We confirmed the above findings using a panel of tumor cell lines naturally expressing the varying levels of PTK7 representative of multiple cancer types, including NSCLC (H520, H1975, H1299), SCLC (H446, H69), pancreatic (BxPC3), breast (MDA-DB-468), and ovarian (OVCAR3) cancer (**Figure 2**). Similarly, PTK7-CAR T cells produced a large amount of IFN-γ and IL-2, which is positively associated with the expression level of PTK7 on respective tumor cells (**Figure 3A**). Notably, PTK7-CAR2 T cells had a trend of producing a higher level of cytokines especially responding to stimulation by tumor cell lines expressing lower level of PTK7 (H1299 and BxPC3 cells), consistent with the higher level of CAR expression per cell in this construct (**Figure S1B**).

We also evaluated the antigen-specific proliferation of PTK7-CAR T cells in response to PTK7-expressing cells. T-cell proliferation was dependent on the expression level of PTK7 on target cells, and tumor cells with a high level of PTK7 expression induced more vigorous T-cell proliferation than that with a lower level of PTK7 expression (**Figure 3B**). Again, PTK7-CAR2 T cells exhibited a trend of more potent proliferation when stimulated with tumor cells expressing a lower level of PTK7.

PTK7-CAR T Cells Specifically Kill PTK7-Expressing Tumor Cells and Retain Effector Function Upon Recursive Target Exposure

We next evaluated the specific killing of PTK7-positive tumor cells by PTK7-CAR T cells in both short-term (18 h) and recursive

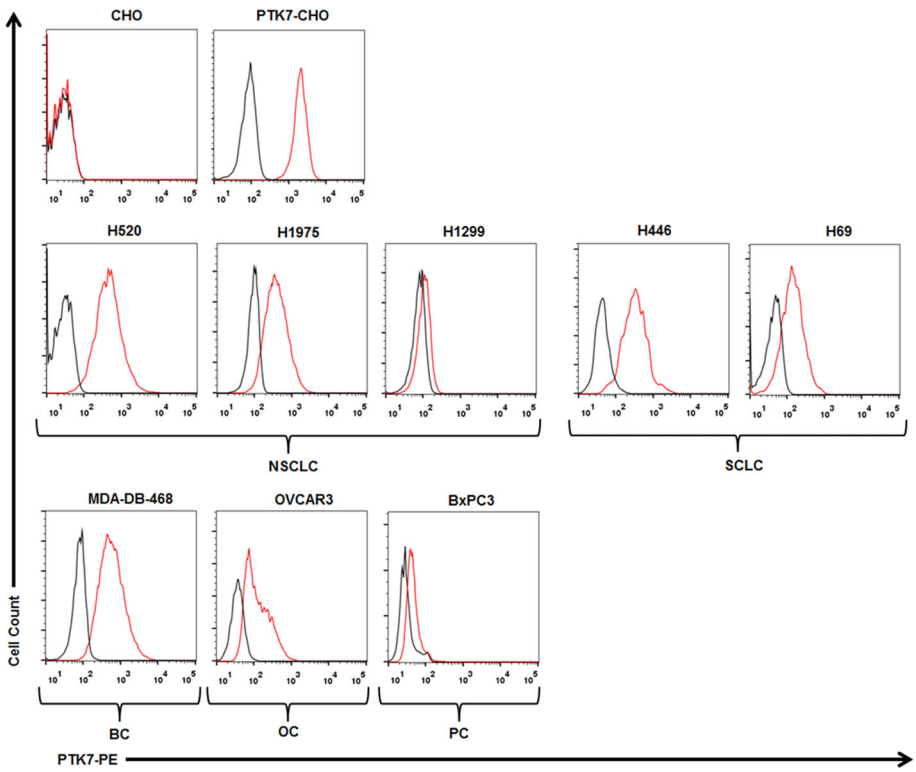

FIGURE 2 | PTK7 is overexpressed on several tumor cell lines. CHO and PTK7-CHO cells served as negative and positive controls, respectively. PTK7 overexpression was observed on NSCLC (H520, H1975, H1299), SCLC (H446, H69), MDA-DB-468 breast cancer (BC), BxPC3 pancreatic cancer (PC), OVCAR3 ovarian cancer (OC) cells. Black and red lines denote the control (secondary antibody alone) and PTK7 staining, respectively.

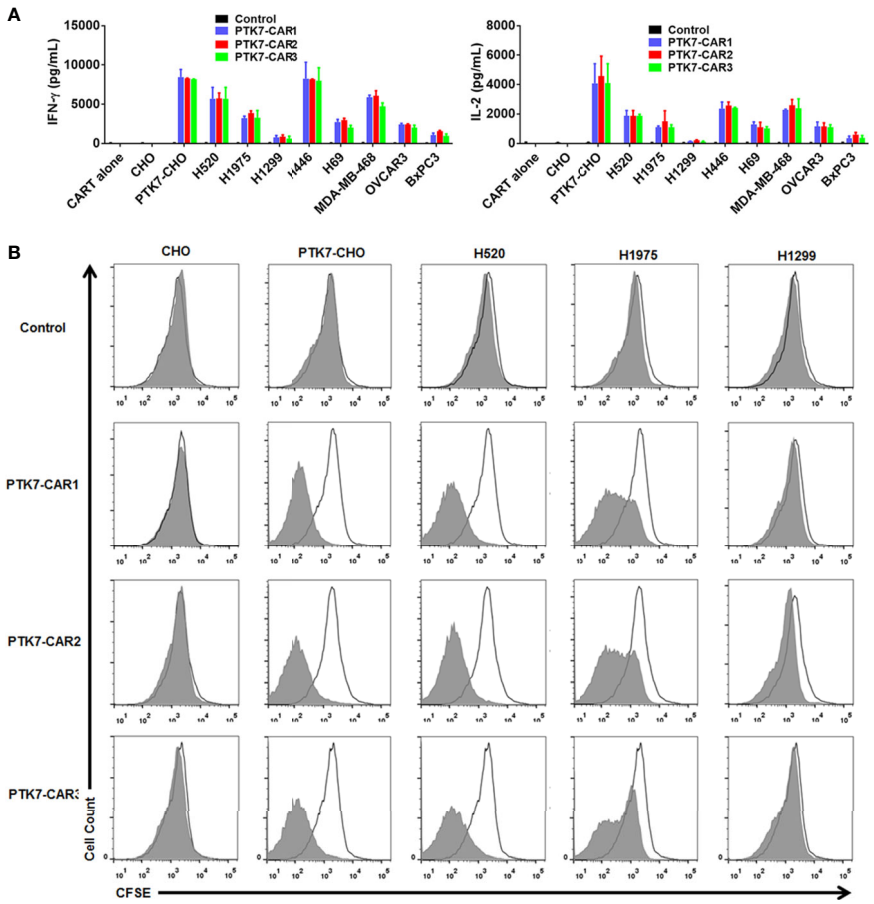

FIGURE 3 | PTK7-CAR T cells release IFN-γ and IL-2 and proliferate in response to PTK7-positive target cells. **(A)** Control or PTK7-CAR T cells from healthy donors (n = 3) were co-cultured with CHO and PTK7-CHO and various PTK7-expressing tumor cell lines for 24 h before performing IFN-γ and IL-2 ELISA. Mean and SEM are shown. **(B)** T cells were labeled with CFSE and co-cultured for 3 days with CHO, PTK7-CHO, H520, H1975, or H1299 cells in the absence of exogenous IL-2, and CFSE dilution was analyzed by flow cytometry. A representative histogram from three independent assays is shown.

long-term (three rounds with each round of 3 days) cytotoxicity assays. In the short-term assays, PTK7-CAR T cells exhibited a robust dose-dependent cytotoxicity against PTK7-expressing PTK7-CHO cells and tumor cells but not parental CHO cells (**Figure 4**). Noticeably, PTK7-CAR2 T cells demonstrated a comparatively higher degree of cytotoxicity against tumor cells expressing the lower level of PTK7 (H69, BxPC3, and H1299 cells). As PTK7 has be reported to be shed from tumor cells in a soluble form (12), we also evaluated the effect of soluble PTK7 on the cytotoxicity of PTK7-CAR T cells, which showed it minimally impacted the tumor killing of these cells (**Figure S2**). Maintenance of specific cytotoxicity and proliferative response exposure to continuous antigen stimulation has been described to be associated with preferential antitumor activity (30, 31). To mimic that context *in vitro*, we performed the recursive long-term cytotoxicity assay where CAR T cells were exposed to recursive target cells at a certain ratio, and tumor cell killing and T cell proliferation served as readouts after each round (**Figure 5A**). We observed that PTK7-CAR2 T cells retained effective through three rounds of tumor challenge, whereas the other two PTK7-CAR T cells failed to control tumor cell growth after the first or second round of challenge (**Figure 5B**). In parallel, PTK7-CAR2 T cells had a better persistence after each round of challenge (**Figure 5C**). PTK7-CAR2 T cells also exhibited superior effector function at the individual cell level as evidenced by higher levels of lytic enzyme granzyme B expression and reduced expression of the exhaustion markers PD-1 and lower percentage of PD-1^{+}TIM-3^{+} cells as compared to the other two PTK7-CAR T cells (**Figures 5D–F** and **S3**).

PTK7-CAR T Cells Mediate Antitumor Activity Against Established Lung Cancer Xenografts

In view of the *in vitro* preferential target-specific recognition and cytotoxicity of PTK7-CAR2 T cells as well as the fact that the antibody from which the scFv used by PTK7-CAR2 is derived has been tested in the clinical trial (12), we evaluated the *in vivo* antitumor activity of these candidate CAR T cells in the xenograft tumor models established from two lung cancer cell lines with distinct antigen expression: H520 and H69 cells with

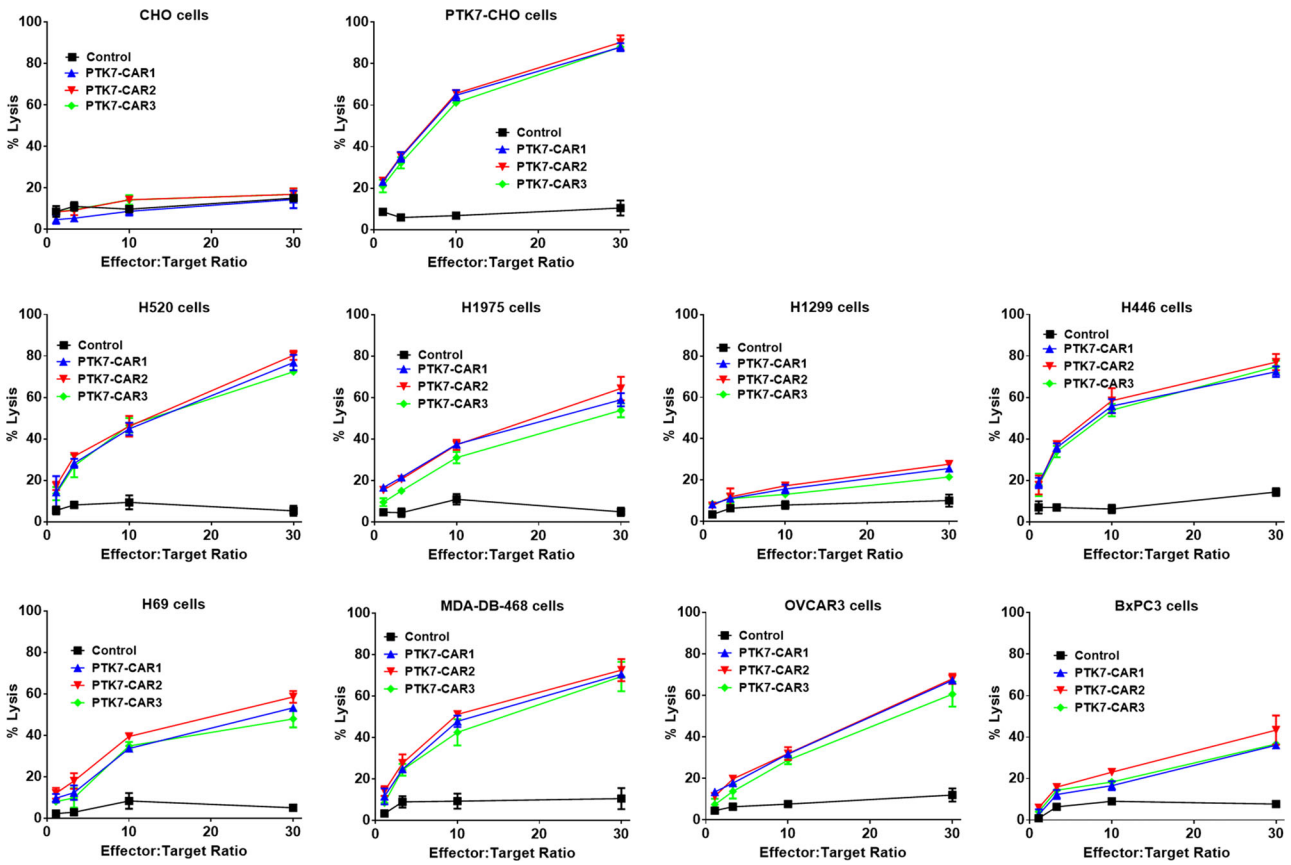

FIGURE 4 | PTK7-CAR T cells kill PTK7-positive tumor cell lines. GL-expressing tumor target cells were co-cultured with control or PTK7-CAR T cells at the varying effector-to-target ratios in triplicate wells of white 96-well plates. Target cell viability was monitored 18 h later by using Bright-Glo™ Luciferase Assay System according to the manufacturer's instructions. The percent lysis (%) was calculated by using the following equation: 1-[bioluminescence value in sample well (target cells + CAR T cells)/maximum bioluminescence value (target cells alone)]. Shown are means ± SEM of % cell killing in triplicate wells.

high or moderate level of PTK7 expression respectively as determined by flow cytometry and IHC staining of cell line-derived xenografts (**Figures 2** and **S4**). NSG mice (n = 3–5/group) were s.c. inoculated with H520 or H69 tumor cells. Seven days later mice started to receive two injection of control or PTK7-CAR2 T cells 1 week apart, and tumor growth was monitored by measuring tumor size. Three independent experiments with T cells from different donors showed that administration of PTK7-CAR2 T cells greatly inhibited tumor growth and significantly prolonged the overall survival of mice bearing H520 ($p<0.0001$) and H69($p<0.001$) tumors (**Figures 6A, B**), culminating in tumor-free survival of more than half of mice at the end of experiment in both tumor models. In contrast, mice treated with control T cells or PBS developed a rapidly progressive tumor, necessitating euthanasia approximately 6 weeks after tumor inoculation, excluding the contribution of allogeneic reactivity to antitumor effect of PTK7-CAR T cells. Accordingly, PTK7-CAR2 T cells exhibited superior initial expansion (day 10 after T-cell infusion) *in vivo* in the peripheral blood and extended persistence when mice were sacrificed (**Figures 6C** and **S5**). In addition, $CD3^+$ T-cell infiltration in tumor xenografts was determined by IHC staining at the endpoint of the experiment, and mice treated with PTK7-CAR2 T cells exhibited a prominent accumulation of T cells within tumor tissues compared to mice treated with control T cells (**Figure 6D**).

Importantly, there was no overt evidence of adverse reaction associated with the infusion of PTK7-CAR2 T cells to mice, as measured by body weight loss and physical signs of toxicity in above animal studies performed (**Figure S6**).

PTK7-CAR2 T Cells Do Not Mediate Detectable On-Target Off-Tumor Toxicity

A previous study shows that a low level of PTK7 expression can be detected in the normal epithelial cells from some tissues, including mammary gland, lung, kidney, esophagus, and urinary bladder. We assessed the expression of PTK7 in the normal human TMA using the rabbit polyclonal anti-PTK7 antibody. Slides were analyzed blindly by an experienced pathologist. Major organs such as heart, brain, lung, liver, and spleen are PTK7 negative, while focal, weak to moderate PTK7-positive staining was observed in the cytoplasm of some normal human tissues (**Figure S7** and **Table S1**). The

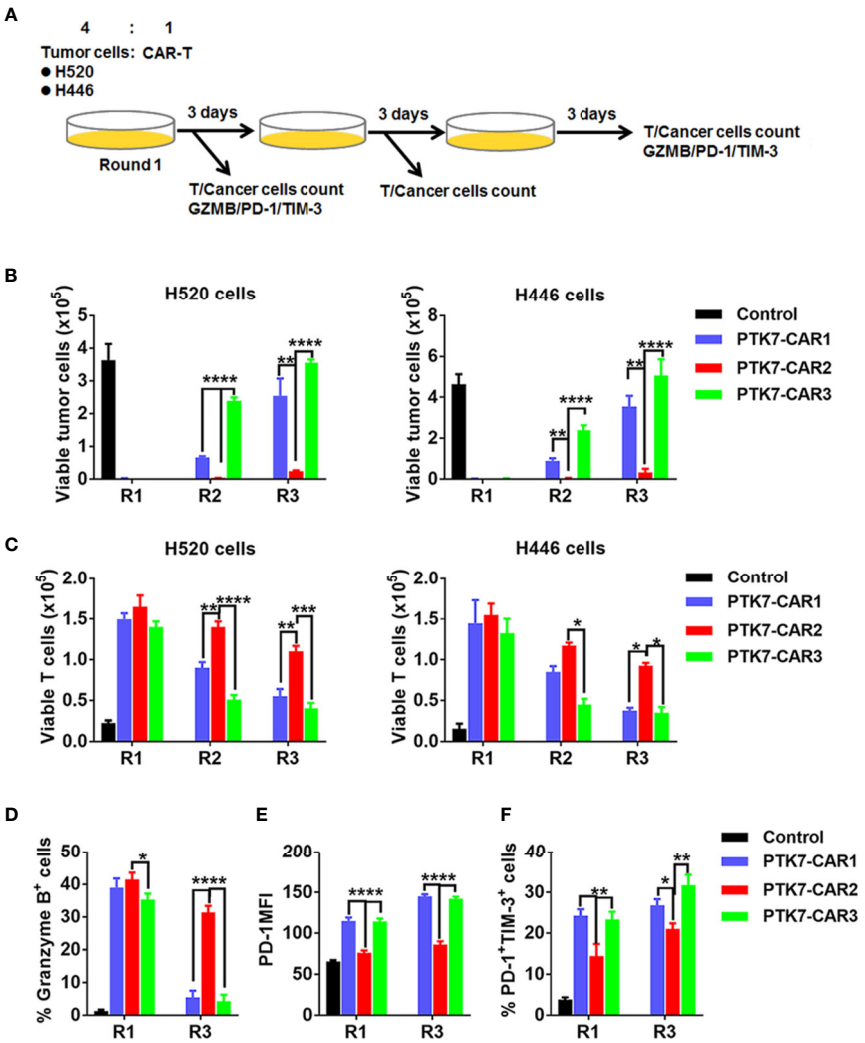

FIGURE 5 | PTK7-CAR2 T cells retain effector function upon recursive target exposure. **(A)** Schematics of the long-term cytotoxicity assay. **(B)** Counts of H520 and H446 target cells after each round of recursive co-culture (rounds 1–3, R1–R3) with control or PTK7-CAR T cells. **(C)** Counts of control or PTK7-CAR T cells after each round of recursive co-culture with target cells. **(D)** Intracellular staining for granzyme B of control or PTK7-CAR T cells at the end of round 1 and 3 co-culture with H520 tumor cells. **(E)** PD-1 expression in control or PTK7-CAR T cells after rounds 1 and 3 of recursive co-culture with H520 tumor cells. **(F)** Percentage of PD-1^{+}TIM-3^{+} cells in control or PTK7-CAR T cells after rounds 1 and 3 of recursive co-culture with H520 tumor cells. Data are shown as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, determined by repeated-measures two-way ANOVA with Tukey's *post hoc* test.

highest expression was observed in the stomach with moderate to strong cytoplasmic staining of gastric epithelium, colon with weak cytoplasmic staining of epithelium, and kidney with weak cytoplasmic staining of tubule epithelial cells. Since on-target off-tumor toxicity is a key limiting factor when developing novel CAR T therapies, we roughly address this concern using a panel of primary human normal cell lines with low-level expression of PTK7 (**Figure S8**). Control or PTK7-CAR2 T cells were co-cultured with the primary human normal epithelial cell lines from the mammary gland (Mammary Epithelial Cells, MECs), lung (Small Airway Epithelial Cells, SAECs), and kidney (Renal Epithelial Cells, RECs) and human umbilical vein endothelial cells (HUVECs), and cytotoxicity assays were performed. Compared to control T cells, PTK7-CAR2 T cells did not exhibit more potent killing against this limited panel of normal human primary cells, except for low-level cytotoxicity of HUVECs that was only observed at the highest effector-to-target ratio tested (**Figure 7**). As not all human tissues with PTK7 expression are represented, these studies are limited but can serve as an initial screen for off-tumor activity.

DISCUSSION

Here, we described the generation and antitumor efficacy of second-generation PTK7-targeting CAR T cells with 4-1BB

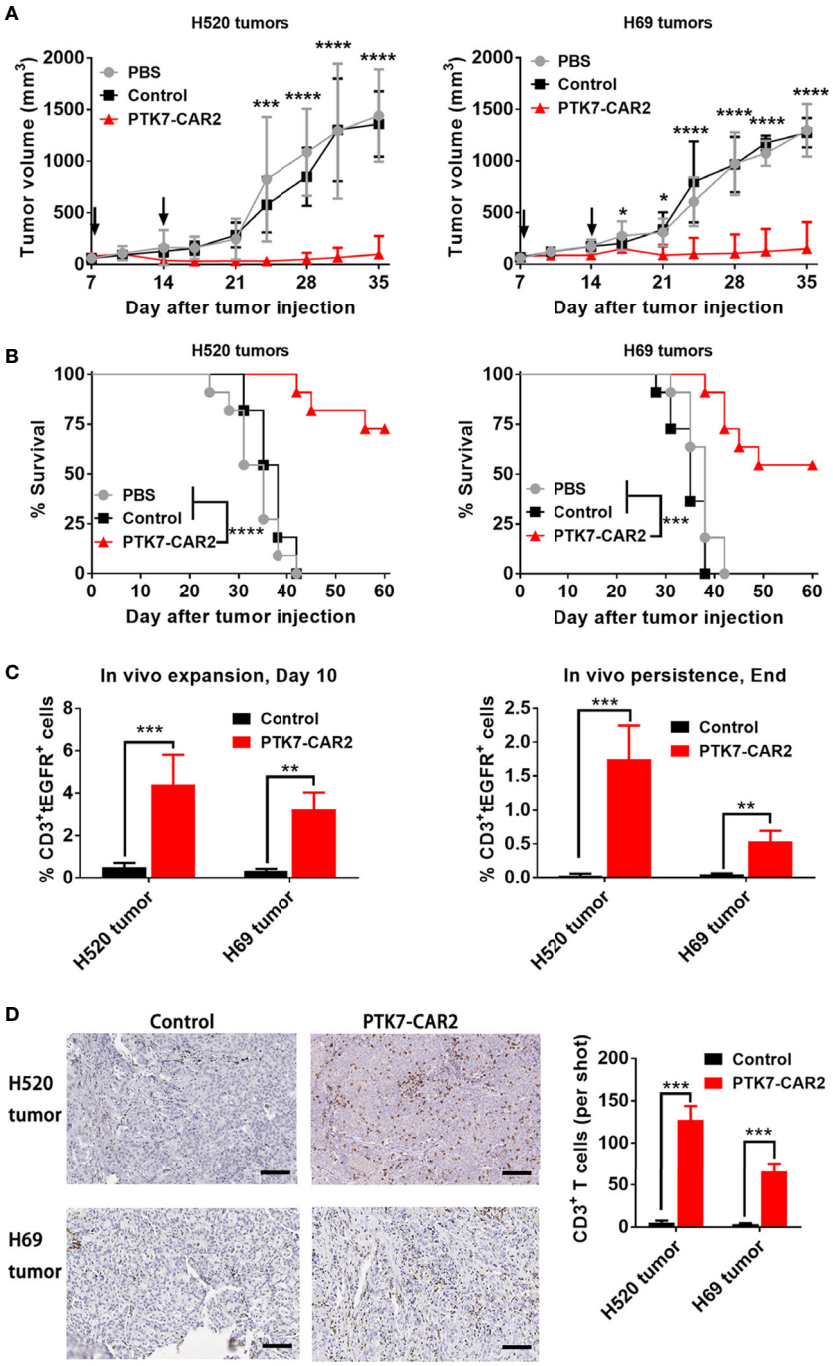

FIGURE 6 | Systemic treatment with PTK7-CAR2 T cells leads to tumor growth control and increased survival of mice in both human tumor xenograft models. **(A)** NSG mice were s.c. implanted with H520 or H69 tumor cells, after 7 days, received two intravenous infusion of control or PTK7-CAR2 T cells (5×10^6 cells in 100 µl PBS) week apart and tumor growth quantified by measuring tumor size. Data are shown as mean ± SEM (n = 5 mice per group). *P < 0.05, ***P < 0.001, and ****P < 0.0001, determined by repeated-measures two-way ANOVA with Tukey's *post hoc* test. **(B)** Kaplan–Meier survival curves summarizing three independent experiments (n = 11 mice per group). ***P < 0.001, and ****P < 0.0001 determined by log-rank test. **(C)** Frequency of human $CD3^+tEGFR^+$ CAR T cells in the peripheral blood collected 10 days after T cell infusion or at the end of experiment. Data are shown as mean ± SEM (n = 4 mice per group). **P < 0.01 and ***P < 0.001, determined by repeated-measures two-way ANOVA with Tukey's *post hoc* test. **(D)** Representative IHC images and quantification of T-cell infiltration in tumor tissues (n = 3) from treated mice harvested at the end of experiment. Scale bars, 100 µm. Data are shown as mean ± SEM (n = 3 mice per group). ***P < 0.001, determined by repeated-measures two-way ANOVA with Tukey's *post hoc* test.

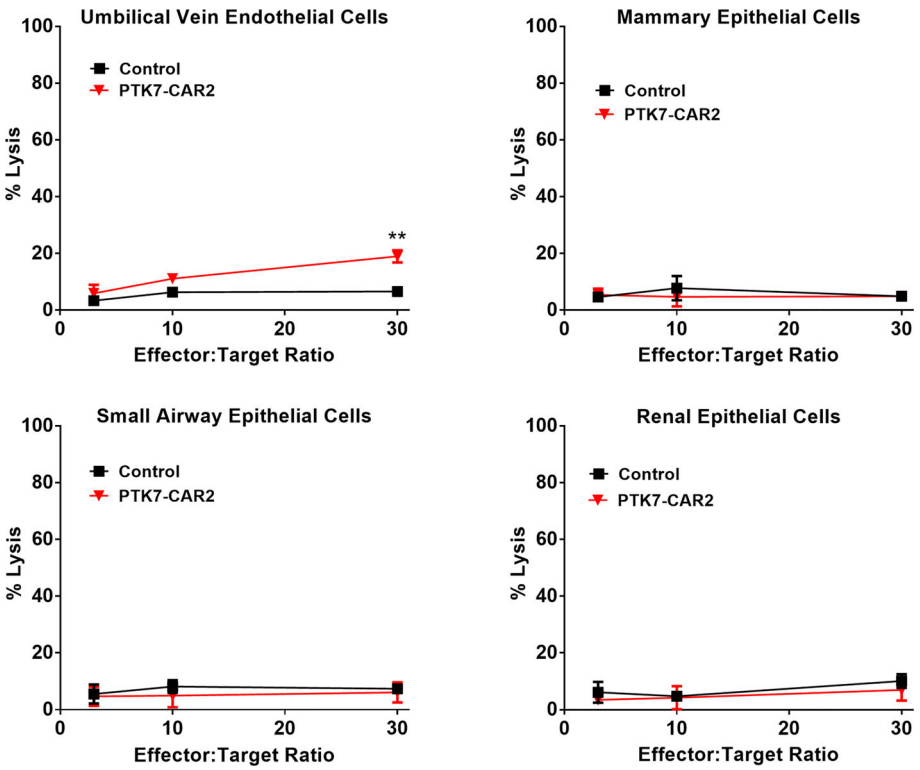

FIGURE 7 | PTK7-CAR2 T cells do not mediate detectable on-target off-tumor toxicity. Control or PTK7-CAR T cells were tested reactivity against a panel of primary human normal epithelial cells or HUVECs in the cytotoxicity assays at the indicated effector-to-target ratios. Shown are mean ± SEM of % cell killing in triplicate wells. **P < 0.01, determined by repeated-measures two-way ANOVA with Tukey's *post hoc* test.

intracellular co-stimulatory signaling domain and demonstrated antigen-specific cytokine production and cytotoxicity against multiple PTK7-positive tool cells and naturally expressing human tumor cells *in vitro*; more importantly, *in vitro* recursive tumor challenge assays pointed to a preferred candidate (PTK7-CAR2) out of three CAR constructs in terms of repetitive target cell killing, CAR T-cell expansion and exhaustion–associated phenotypes, which was previously reported to be associated with *in vivo* antitumor effect of CAR T cells (32). Using *in vivo* lung cancer cell line–derived xenograft models, we showed that PTK7-CAR2 T cells significantly inhibited tumor growth and prolonged overall survival of tumor-bearing mice. The reason why PTK7-CAR2 exhibited a better functionality remains to be explored; however, we consistently observed a high CAR expression per cell in the PTK7-CAR2 T cells (**Figure S1B**), which may underlie the fact that PTK7-CAR2 T cells had a better response to stimulation by tumor cell lines expressing lower levels of PTK7. It is possible that the unique scFv sequence in this construct makes this CAR more easy expression or more stable on the CAR-T cell surface, enabling CAR-T cells a better response in recursive target exposure. We did not evaluate PTK7-CARs integrating CD28 co-stimulation as CD28-containing CAR T cells have undesirable increases in T cell exhaustion markers, limited persistence, and increased possibility of recognizing normal cells with very low levels of antigen as previously reported (30, 33). Although further studies will be needed to evaluate the antitumor efficacy of PTK7-CAR T cells in a more clinically relevant setting such as using PDXs and patient-derived cancer cell lines, our data support PTK7-CAR T cells as a viable therapeutic option for lung cancers and many other solid cancers with PTK7 overexpression given that it is impractical to develop blocking antibodies or small-molecule inhibitors as typically done with receptor tyrosine kinases due to PTK7's lack of catalytic activity.

Several reports have identified PTK7 as a potential antigen target in solid tumors. Previous studies documented that PTK7 is overexpressed in multiple types of solid cancer, and more significantly, its expression is enriched in TICs/CSCs from PDXs or cancer cell lines. As TICs/CSCs with unlimited self-renewal capacity and differentiation potential have been broadly considered to be source to tumor recurrence, metastasis, and therapeutic resistance, it is reasonable to hypothesize that a durable antitumor efficacy would be achieved if specifically targeting TIC/CSCs by immunotherapeutic modalities, including CAR T-cell therapy. In fact, CAR T cells targeting several biomarkers of TICs/CSCs, including CD133, CD24, Receptor tyrosine kinase-like orphan receptor 1 (ROR1), or the epithelial cell adhesion molecule (EpCAM), have been developed and exhibited the excellent antitumor effects in preclinical models (34–40); more importantly, CD133-targeting CAR T cells alone or in combination have demonstrated antitumor

activity in treating patients with CD133-postive metastatic malignancies with controllable toxicities in clinical trials (41, 42). Intriguingly, both PTK7 and ROR1 belong to Wnt ligand binding receptors with important roles in the non-canonical Wnt signaling (10). ROR1 exhibits high and homogeneous cell surface expression in many epithelial tumors with expression profile similar to PTK7, and targeting ROR1 with CAR T-cell therapy improved survival in xenograft models of ROR1$^+$ human tumors with treating lung and breast cancer in an ongoing clinical trial (NCT02706392) (38, 40). Thus, our result documenting a potent antitumor effect of PTK7-CAR T cells adds PTK7 to the kind list of ROR1, which, as a member of Wnt signaling-related pseudokinases, had a characteristic enriched expression in TIC/CSCs and is suitable as a potential therapeutic target for cancer immunotherapy.

On-target off-tumor effect is a major concern when developing CAR T-cell therapy targeting less tolerable TAAs for solid tumors (8). On-target toxicities have been observed in clinical trials with CAR T cells specific for antigens that are shared on some normal tissues (43, 44), and a critical issue to be addressed is whether targeting PTK7 will be safe. Damelin et al. have shown that PTK7 is absent in vital organs, but they detected a low level of expression in esophagus, urinary bladder, kidney, mammary gland, lung, ovary, uterus, and digestive tract with more prominent expression in stromal part (12); in addition, previous studies detected PTK7 expression in human hematopoietic progenitors committed to myeloid and T lymphoid lineages, illustrating the potential for toxicity to normal cells (45–48). Roughly consistent with these previous studies, we also did not detect PTK7 expression in normal human major organs; however, focal, weak to moderate PTK7-positive staining was observed in the cytoplasm of some normal human tissues, including digestive tracts (stomach, esophagus, colon) and kidney. We further evaluated the activity of PTK7-CAR T cells *in vitro* against a normal cell panel that included mammary, lung, kidney epithelial cells, and HUVECs where a low level of lysis against HUVECs was detected only at the highest effector-to-target ratio tested, which is consistent with comparatively higher PTK7 expression on these cells as determined by FACS. As CAR-T cells will first accumulate in the lung through blood vessels, a very high local concentration may be achieved when CAR-T cells are intravenously infused, leading to the blood vessel in the lung attacked by CAR-T cells and consequently on-target off-tumor toxicity. In addition, if applied for lung cancer treatment, a high level of cytokines released from the on-target on-tumor recognition may constitute another important concern as this may induce pulmonary edema, which may be lethal if not diagnosed and treated timely. Although mouse and human PTK7 protein has 90.93% homology in total sequence with 92.98% homology in the extracellular domain, which means that tumor-bearing mouse model should be suitable for the evaluation of on-target toxicity, however, we cannot evaluate the potential toxicity profiles of PTK-CAR T cells in current murine tumor models due to lack of cross-reactivity with murine counterpart of humanized antihuman PTK7 antibodies used to construct scFv part of our PTK-CARs. Positively, Damelin et al. showed that a PTK7-targeting ADC did not exhibit target-dependent toxicity in any of the tissues examined, including those with PTK7 expression (12). As the scFv we used to generate PTK7-CAR2 is derived from the same antibody (Hu24) of that PTK7-ADC, the non-clinical safety profile of PTK7-ADC in that study provides some evidence of safety and potential toxicity estimate of PTK7-CAR2 T cells *in vivo*. Caution still should be taken when translating this PTK7-CAR T cells into clinic considering different mechanisms of action and target recognition sensitivity (potency) between ADC and CAR T cells directing the same targets. In this regard, CAR-T cells targeting epithelial cell adhesion molecule (EpCAM), a tumor-associated antigen overtly presented on the cell surface of various carcinomas, is a typical precedent. Although anti-human EpCAM CAR-T cells unable of recognizing mouse EpCAM eradicated established tumor xenografts without toxicities in the immunodeficient animal models, anti-mouse EpCAM CAR-T cells induced severe pulmonary immunopathology in theimmunocompetent mice due to CAR-T recognition of basal EpCAM expression in normal lung (49). In addition, we may learn from the experience of targeting ROR1 by CAR T cells as these two molecules have similar expression profiles in both normal and tumor tissues (10, 50). Although ROR1-CAR T cells (derived from R12- and 2A-scFv) without cross-reactivity with murine ROR1 exhibited no evident toxicity in NSG mice tumor model, murine ROR1-specific CAR T cells (derived from R11-scFv) induced lethal bone marrow failure due to recognition of ROR1$^+$ stromal cells, which can be rescued by the logic-Gated strategy of CAR construction (38, 40). Thus, the same configuration should be considered when similar results are seen in future investigations of PTK7-CAR T-cell's toxicity profiles; alternatively, tuning scFv affinity and/or concomitantly integrating different co-stimulatory domains may ameliorate the potential concern of on-target off-tumor effect as typical representation for CAR T-cell therapy targeting a range of different antigens including but not limited to HER2, EGFR, CD38 (8, 51, 52). Given the above inherent risks, multiple inducible safety controls that can be built into or applied in conjunction with CAR-T cells should be considered, such as inducible caspase 9 (iCasp9) or tEGFR tag as we integrated in CAR design where tEGFR-expressing CAR-T can be depleted by commercially available antibody cetuximab in case of emergent side effects (53). In sum, further thorough investigations are definitely needed to fully explore the potential toxicities of PTK7-CAR T cells before translating into clinic by using PTK7-CARs with cross-reactivity in mouse and even non-human primate models.

CONCLUSION

Here we describe a PTK7 targeting strategy that is based upon CAR T-cell engineering. This synthetic biology approach overcomes the issues related with PTK7 being pseudokinase unsuited for developing antibody and small-molecule

inhibitors as therapeutic agents, and is supported by the effector functions of modified T-cell in order to deliver PTK7-specific cytotoxicity. In summary, the data presented herein serve as an initial step for future clinical development of PTK7-CAR T-cell therapy safely and efficiently treating PTK7-expressing lung cancer and other malignancies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This study was approved by ethical committees of the Harbin Medical University Cancer Hospital.

AUTHOR CONTRIBUTIONS

Conception and design of studies: YJ, GR, HW, AG. Acquisition, analysis and interpretation: YJ, GL, LF, YL, ME, LW, XL, YYL, YWL, HW, AG. Drafting article: YJ, AG. Critical review and discussion: GR, HW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.665970/full#supplementary-material

Supplementary Figure 1 | (A) The percentage of CAR expression on T cells transduced with control or PTK-CAR lentiviruses. Data are shown as mean ± SEM (n = 5 donors). **(B)** The intensity of CAR expression in both CD4 and CD8 T cells from 3 different CAR constructs. **(C)** CAR T-cell memory phenotypic analysis in both CD4+ and CD8+ cells based on CD45RO and CCR7 expression as follows: Tn/Tscm naive/stem cell memory (CD45RO-/CCR7+), Tem central memory (CD45RO+/CCR7+), Tem effector memory (CD45RO+/CCR7-), Teff effector cells (CD45RO-/CCR7-) (n = 5 donors). **(D)** The in vitro expansion curve of control or PTK-CAR T cells. Data are normalized on the starting input T cell number and shown as mean ± SEM of triplicates from one representative donor. *P < 0.05, ***P < 0.001, determined by two-way ANOVA with Turkey's post hoc test.

Supplementary Figure 2 | Soluble PTK7 antigen does not impact tumor killing of PTK7-CAR T cells in vitro. PTK7-CAR T cell-mediated tumor killing of H520 **(A)** or H1975 **(B)** cells at the effector-to-target ratio of 10 in the presence or absence of 10 μg/mL of soluble purified PTK7 antigen in the short-term cytotoxicity assay.

Supplementary Figure 3 | (A) Representative plots of flow cytometric analysis of intracellular granzyme B (GZMB) expression in control or PTK7-CAR T cells at the end of round 1 and 3 co-culture with H520 tumor cells. **(B)** Representative plots of flow cytometric analysis of PD-1 and TIM-3 expression on control or PTK7-CAR T cells at the end of round 1 and 3 co-culture with H520 tumor cells.

Supplementary Figure 4 | Immunohistochemistry of PTK7 in tumor xenografts. Immunohistochemistry with polyclonal anti-PTK7 antibody was performed on formalin-fixed, processed, and paraffin-embedded (FFPE) tumor tissues from H520 **(A)** or H69 **(B)** xenografts. Scale bar, 100 μm.

Supplementary Figure 5 | Representative plots of flow cytometric analysis of human CD3+tEGFR+ CAR T cells in the peripheral blood collected 10 days (D10) after T cell infusion or at the end of experiment (End) from the H520 **(A)** or H69 **(B)** xenograft tumor models.

Supplementary Figure 6 | Body weights were measured before tumor injection, before CAR T-cell injection, and 7 and 28 days after CAR T-cell injection and compared with PBS or control T-cell treated NSG mice. Lines indicate means ± SEM (n = 5 mice).

Supplementary Figure 7 | Representative micrographs of PTK7 expression in indicated normal human organs assessed by staining with the rabbit polyclonal anti-PTK7 antibody (Invitrogen) at the final concentration of 1 μg/ml. Micrographs are representative of at least 2-3 sections per tissue. Magnification, x 40.

Supplementary Figure 8 | PTK7 expression on the primary human normal epithelial cell lines from mammary gland (Mammary Epithelial Cells, MECs), lung (Small Airway Epithelial Cells, SAECs), and kidney (Renal Epithelial Cells, RECs) and human umbilical vein endothelial cells (HUVECs) determined by FACS analysis. Black and red line denote the control (secondary antibody alone) and PTK7 staining respectively.

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REVIEW
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Optimizing the Clinical Impact of CAR-T Cell Therapy in B-Cell Acute Lymphoblastic Leukemia: Looking Back While Moving Forward

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Chimeric antigen receptor T-cell (CAR-T) therapy has been successful in creating extraordinary clinical outcomes in the treatment of hematologic malignancies including relapsed or refractory (R/R) B-cell acute lymphoblastic leukemia (B-ALL). With several FDA approvals, CAR-T therapy is recognized as an alternative treatment option for particular patients with certain conditions of B-ALL, diffuse large B-cell lymphoma, mantle cell lymphoma, follicular lymphoma, or multiple myeloma. However, CAR-T therapy for B-ALL can be surrounded by challenges such as various adverse events including the life-threatening cytokine release syndrome (CRS) and neurotoxicity, B-cell aplasia-associated hypogammaglobulinemia and agammaglobulinemia, and the alloreactivity of allogeneic CAR-Ts. Furthermore, recent advances such as improvements in media design, the reduction of *ex vivo* culturing duration, and other phenotype-determining factors can still create room for a more effective CAR-T therapy in R/R B-ALL. Herein, we review preclinical and clinical strategies with a focus on novel studies aiming to address the mentioned hurdles and stepping further towards a milestone in CAR-T therapy of B-ALL.

Keywords: chimeric antigen receptor, cytokine release syndrome, neurotoxicity, acute lymphoblastic leukemia, adoptive cell therapy, cancer immunotherapy

1 INTRODUCTION

B-cell acute lymphoblastic leukemia (B-ALL) is characterized by the presence of poorly differentiated abnormal B-cell progenitor cells that have a rapid rate of proliferation in the bone marrow (1). B-ALL is the most prevalent form of acute leukemia in children in the US (2, 3). With around 3000 newly diagnosed cases of children with B-ALL each year, overall survival (OS) surpasses 85% in children (2–4). However, in adult B-ALL patients, OS is not as favorable as it is in children with B-ALL and it ranges from 50% to up to 60% (5, 6). Even though chemotherapy regimens mediate high rates of complete remission (CR), 40% to 50% of adult B-ALL patients ultimately experience disease relapse (7–9). Relapsed or refractory (R/R) cases of B-ALL have unfavorable prognosis with CR rates ranging from around 35% and up to 10% in the first and

second recovery from the disease, respectively (7–9). Therefore, researchers have been investigating other types of treatments capable of mediating better clinical outcomes and mitigated levels of side effects and adverse events.

Cancer immunotherapy first started as an idea to employ the patients' immune system and its components as fighting tools against various types of neoplasms (10, 11). Today, monoclonal antibody (mAb)-based therapies, cancer vaccines, adoptive cell therapy (ACT), and oncolytic viruses are among famous types of cancer immunotherapy that have gained preclinical and clinical success (10). The clinical success of adoptively transferred T cells genetically engineered to express chimeric antigen receptors (CAR-Ts) has created a new era in the treatment of B-cell-based malignancies (12–17). The theory of utilizing T cells as fighting tools for a selective fight against cancer, which started in the early 1990s, has now become a treatment option for certain patients with B-cell-based malignancies (12–20). Over the past few years, the field of CAR-T therapy has progressed remarkably (11, 21). In particular, various CAR generations have been developed and many innovative strategies have been proposed, aimed at improving the safety and efficacy of CAR-T therapy (11, 20–23).

CD19-redirected CAR-T therapy of children and adults with R/R B-ALL results in high remission rates (67% to 93%) (12–17). The capacity of CD19-redirected CAR-Ts for *in vivo* activation, expansion, and robust tumoricidal activity, which results in the mentioned high rates of disease remission even in patients with R/R B-ALL, leads to adverse events and toxicities such as cytokine release syndrome (CRS), neurologic toxicities, and B-cell aplasia (14, 24–27). CRS is a systemic inflammatory condition mediated by various cytokines produced by CAR-Ts and other cells of the immune system (27, 28). C-reactive protein (CRP), interferon-γ (IFN-γ), interleukins (ILs) such as IL-1, IL-2, soluble IL-2 receptor alpha chain (IL-2Rα), IL-4, IL-6, IL-8, and IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), granzyme B, and tumor necrosis factor-α (TNF-α) are among the most important mediators of CRS (27, 28). CRS severity can range from mild levels to high levels necessitating intensive care and urgent medical intervention (27). Moreover, CRS manifests itself as fever, tachycardia, hypotension, hypoxia, etc. (27). Additionally, the pathogenesis of CAR-T-related neurologic toxicities is not very well-known; however, it is evident that this incidence is observed in CRS-developing patients (25, 26). Neurologic toxicities can happen following or before CRS onset and it has been evident that its severity correlates with CRS severity (12, 25). Both of these adverse events are important incidences and they may limit the successful clinical outcomes of B-ALL CAR-T therapy (25, 26). It is also worth mentioning that even though most CAR-T-related adverse events are managed quite efficiently, there might still be room for improvement.

In addition to CRS and neurologic toxicities, B-cell aplasia is another CAR-T-related toxicity occurring during CAR-T therapy of B-ALL (29). B-cell aplasia is the result of on-target off-tumor toxicity of CD19-redirected CAR-Ts against normal B-cells which occurs simultaneously with the targeting of malignant $CD19^+$ blasts (29). B-cell aplasia is considered a tool for measuring the persistence of CAR-Ts after obtaining the desired clinical outcomes (14, 24). Scientific evidence has demonstrated that patients with short durations of B-cell aplasia often suffer from disease relapse (24). Furthermore, B-cell aplasia puts the recipients of CD19-redirected CAR-Ts at risk of various types of infectious diseases (29); therefore, clinical interventions are highly required for mitigating the consequences of this unfavorable event.

Moreover, since the generation of autologous CAR-Ts is not always feasible, allogeneic CAR-Ts may be considered as suitable alternatives (30). However, using allogeneic CAR-Ts is also hindered by two limitations (30, 31). The first limitation is the incidence of graft-versus-host disease (GvHD) which can be life-threatening, and the second limitation is that allogeneic CAR-Ts might be rapidly attacked and eradicated by the immune system of the recipients (30, 31). Both of these hurdles significantly obstruct the tumoricidal activity of allogeneic CAR-Ts; therefore, counterstrategies are highly required for tackling these caveats (30, 31). Herein, we review recent studies that have tried to improve the clinical outcomes of CAR-T therapy in patients with R/R B-ALL by addressing the mentioned limitations. Furthermore, we also discuss preclinical and clinical studies that have investigated other factors such as media design, the duration of *ex vivo* culturing for CAR-T generation, and various phenotype-determining factors that are important for a more efficacious CAR-T therapy in B-ALL and may help achieve better clinical outcomes.

2 CAR-T FUNDAMENTALS

The commercial success story of CAR-Ts started in 2017 when the US Food and Drug Administration (FDA) approved the first CAR-T therapy for medical use (32). To this day, there are five FDA-approved CAR-T products available on the market for five different hematological malignancies (32–38). In particular, *tisagenlecleucel* has been approved for the treatment of certain subjects with B-ALL or diffuse large B-cell lymphoma (DLBCL) (32, 36). *Axicabtagene ciloleucel* is another CAR-T product which has been approved for DLBCL and follicular lymphoma (FL) (33, 38). In addition, *brexucabtagene autoleucel* has been approved for certain patients with mantle cell lymphoma (MCL) or B-ALL whereas *lisocabtagene maraleucel* has been FDA-approved for the treatment of certain individuals with DLBCL (37, 39). Recently, Bristol Myers Squibb's *idecabtagene vicleucel* was FDA-approved for multiple myeloma (MM) (34, 35). *Tisagenlecleucel, axicabtagene ciloleucel, brexucabtagene autoleucel*, and *lisocabtagene maraleucel* use CD19 as their target antigen while *idecabtagene vicleucel* targets B-cell maturation antigen (BCMA) (32–38). It is important to mention that all of these products have been approved for the treatment of certain patients with particular conditions of the mentioned oncological indications (32–38).

CAR-Ts, also known as "*living drugs*", are T cells that have been genetically manipulated to express CARs on their surface

(40). These effector cells combine the precise specificity of mAbs with the cytotoxicity of T lymphocytes, without the need for major histocompatibility complex (MHC); therefore, CAR-Ts are capable of selectively targeting cells proficient in the expression of the desired target antigen while remaining unreactive towards those deficient in target antigen expression (40). CAR-Ts are either redirected towards tumor-associated antigens (TAAs) or tumor-specific antigens (TSAs) of interest expressed on the surface of tumor cells (40). In detail, cell surface-expressed TAAs or TSA are membrane-bound antigens whose expression and surface presentation is independent of MHC molecules (23). Therefore, these antigens can be targeted using mAbs and CAR-Ts (23). On the other hand, intracellular TSAs or TAAs are processed and presented by MHC molecules (41). Targeting such antigens cannot be achieved using CAR-Ts (41). Instead, T cells with engineered T-cell receptors (TCRs) specific for a particular MHC-presented peptide antigen can be beneficial (41).

CARs are synthetic receptors made of an extracellular domain (composed of a targeting domain and a hinge), a transmembrane (TM) domain, and an intracellular domain (composed of a primary T-cell activation domain and one or two co-stimulatory domains) (40). The targeting domain of CARs is mainly composed of a single-chain variable fragment (scFv) of a mAb and is responsible for the selective targeting ability of CARs (40). The hinge is responsible for attaching the targeting domain to the TM domain and is mainly derived from CD8, CD28, IgG1, or IgG4 (40). The TM domain, which acts as the bridging fragment between the extracellular domain and the intracellular domain, can be derived from molecules such as CD8α, CD3ζ, CD28, CD4, and the inducible T-cell co-stimulator (ICOS) (40). Moreover, co-stimulatory domains are considered as the helping hand of the primary T-cell activation domain responsible for CAR-T activation upon target antigen encountering (42–45). In particular, CD28, 4-1BB (CD137), OX40 (CD134), and ICOS have been used as co-stimulatory domains (42–45). It has been demonstrated that co-stimulatory domains possess properties that determine the fate of the engineered effector cells since they can induce memory or effector T-cell phenotype in CAR-Ts (1, 46, 47). Additionally, the CD3ζ part of the TCR CD3 complex, FcεRIγ, CD3ϵ, DAP10, DAP12, the ζ-chain of TCR-associated protein kinase 70 kDa (ZAP70), the lymphocyte-specific protein tyrosine kinase (LCK), and fyn are all among the primary activation domains so far used in the construct of CAR-Ts (48–50).

The structural evolution of CAR-Ts can be described based on their different generations. First-generation CAR-Ts had all of the mentioned domains but they did not have any co-stimulatory domain which resulted in their inadequate antitumor activity and persistence (51). As a solution, second- and third-generation CAR-Ts were designed to have one and two co-stimulatory domains, respectively, resulting in their superior antitumor activity and persistence over their predecessors (52, 53). Fourth-generation CAR-Ts are second-generation CAR-Ts that have an inducer domain for the expression of a cytokine of interest, such as IL-2, in their intracellular domain (instead of a secondary co-stimulatory domain) (54). Fourth-generation CAR-Ts are known as "*T cells redirected for universal cytokine-mediated killing*" (*TRUCKs*) or *armored CARs* since they deliver a transgenic product to the targeted tumor tissue enhancing the tumoricidal activity and efficacy of CAR-Ts (54). Fifth-generation CAR-Ts are structural counterparts of second-generation CAR-Ts but they harbor an intracellular domain of a cytokine receptor (**Figure 1**) (55, 56). With all the good news surrounding this type of therapy for B-ALL, there are remaining challenges such as CAR-T-associated toxicities including CRS and neurotoxicity,

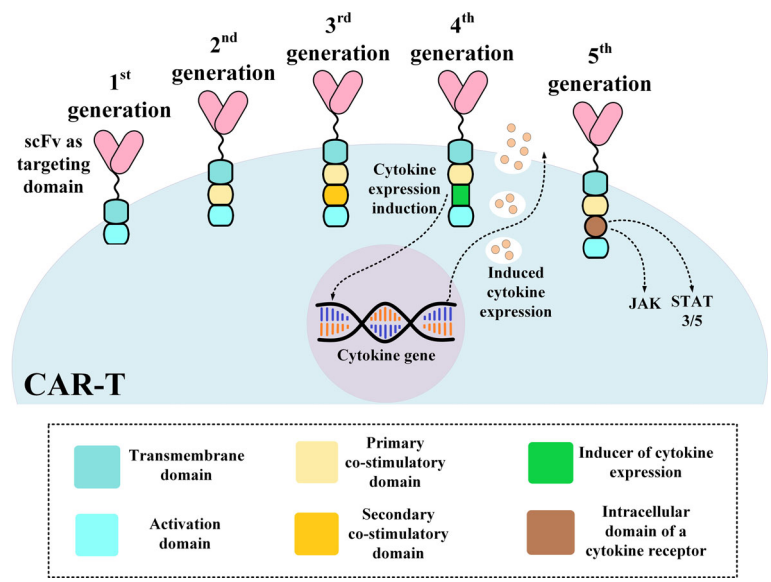

FIGURE 1 | An illustration of different CAR-T generations. CAR, chimeric antigen receptor; scFv, single-chain variable fragment.

which can be mild to life-threatening, the caveats of using allogeneic CAR-Ts, and other factors whose optimization broadens the success zone of B-ALL CAR-T therapy. In the upcoming sections of this article, we will discuss strategies for addressing these hurdles.

3 CLINICAL PROCEDURES FOR IMPROVING THE EFFICACY AND SAFETY OF B-ALL CAR-T THERAPY

3.1 CRS and Neurotoxicity Mitigation

CRS is the most important B-ALL CAR-T therapy-associated toxicity resulting from CAR-T-induced rapid immune activation (57–59). CRS can lead to various serious damages including cardiac toxicity, hyponatremia, and other complications (57–59). Of note, CAR-T therapy-related cardiac toxicity or cardiotoxicity is poorly recognized. However, sinus tachycardia, increase level of blood troponin, left ventricular systolic function (LVSD), profound hypotension, and decompensated heart failure (DHF) are among cardiovascular-related adverse events observed during CAR-T therapies (60). Also, hyponatremia is characterized by serum sodium (Na) levels below 135 mEq/L, and is reported to be common among patients receiving CD19-redirected CAR-T therapy (58). Moreover, neurotoxicity is another CAR-T-associated adverse event that is not yet fully known but the available data indicate that both activated CAR-Ts and endogenous T lymphocytes, and the cytokines secreted by them, may be responsible for post-CAR-T therapy neurotoxicity (61–63). Furthermore, recent single-cell RNA sequencing data have demonstrated that mural cells, that are responsible for the integrity of the brain-blood barrier (BBB), express the CD19 antigen (64). Researchers have added that the expression of CD19 in the brain emerges early in development and continues throughout adulthood (64). Additionally, these data indicate that mouse mural cells exhibit lower levels of CD19 expression which may be responsible for limitations in preclinical neurotoxicity models (64). So far, several clinical strategies, discussed in the upcoming section, have been developed to either prevent CRS and neurotoxicity or to control them and their damaging effects. Furthermore, designing approaches can also be beneficial in the prevention of CAR-T-associated toxicities (50). For instance, instead of the generally used CD3ζ activation domain, researchers have designed and generated CAR-Ts with DAP12 as the activation domain (50). In detail, these second-generation CAR-Ts are made of the natural killer group 2D (NKG2D) ectodomain fused to 4-1BB and the DAP12 cytoplasmic domain (which acts as the activation domain) (50). This design resulted in reduced levels of IFN-γ, TNF-α, and IL-2 release during tumor cell lysis with lower proliferative activity upon repeated antigen stimulation but similar tumoricidal activity in comparison with that of NKG2D CAR-Ts with the CD3ζ activation domain (50). Naturally, NKG2D receptor has the role of a primary activation signal in natural killer (NK) cells whereas it acts as a co-stimulatory signal in T lymphocytes (65). In detail, the NKG2D receptor is a homodimer molecule that interacts with the adaptor molecules DAP10 or DAP12 based on the expressed NKG2D isoform (65). Moreover, mouse and human NK cells, T cells (αβ and γδ), and natural killer T (NKT) cells express a longer isoform of NKG2D that interacts with DAP10 (65). In addition, mouse NK cells also express a shorter splice variant of NKG2D that associate with DAP12 (65). Both DAP10 and DAP12 are responsible for triggering downstream cell signaling cascades in association with NKG2D upon ligand encountering (65).

3.1.1 IL-1 and IL-6 Blockade

Recently, studies have shown that IL-1 and IL-6 released by monocytes and macrophages are the main reasons for CAR-T-associated CRS and *immune effector cell-associated neurotoxicity syndrome* (*ICANS*) (66, 67). In detail, monocytes play a more important role than macrophages in CRS-progressing IL-1 and IL-6 secretion (67). Preclinical data indicate that monocyte ablation or IL-6 receptor blockade using *tocilizumab* can prevent CRS but not lethal neurotoxicity (67). This is because IL-6 receptor blocking antibodies broadly used as the standard procedure for the clinical management of severe CRS cannot penetrate the BBB which results in their inability to induce complete *CAR-T-related encephalopathy syndrome* (*CRES*) remission (67). Furthermore, CRS and CRES are the major safety-related issues of CAR-T therapy which are caused by a rapid increase in the level of multiple cytokines, mainly IL-6, secreted by the activated infused CAR-Ts and mononuclear cells including dendritic cells and macrophages (68). In detail, target cell-induced IL-6 expression and production by CAR-Ts trigger factors that enhance monocyte production and release of IL-6 and other proinflammatory cytokines contributing to CRS and CRES progression (69). Therefore, some researchers have investigated a very novel strategy to address these limitations (69–71). It has been discovered that suppressing the IL-6 gene expression in CAR-Ts significantly decreases the IL-6 release from monocytes and it also reduces the possibility of severe CRS and CRES without reducing the antitumor efficacy of CAR-Ts *in vitro* and *in vivo* (69, 70). Therefore, one research group has generated a CAR construct with IL-6 shRNA (70). The shRNA-IL6-modified CD19-redirected CAR-Ts (ssCAR-T-19) were delivered to a B-ALL patient with skin and testicle extramedullary relapse enrolled in a clinical trial (NCT03919240) (70). The patient was successfully treated by ssCAR-T-19s and this study proposed that these CAR-Ts might be therapeutically useful in efficiently eradicating infiltrating leukemia cells in the skin and testicle with a mild level of toxicity (however, broader patient populations and more careful clinical investigations are required for drawing substantiated conclusions) (70). Furthermore, ssCAR-T-19s have also been investigated in a recent clinical trial (NCT03064269) involving three patients with relapsed central nervous system (CNS) B-ALL (71). The results of this study demonstrated that ssCAR-T-19s can migrate into the CNS and reduce brain leukemic infiltration and significantly eradicate leukemic blasts in the cerebrospinal fluid (CSF) with mildly elevated cytokine levels and only grade 1 CRS (71). Therefore,

this method could be proposed as an approach for reducing the risk of CRES in CAR-T therapy of patients with CNS B-ALL (71). However, in the case of severe CRS occurrence, IL-6 receptor blockade or IL-6 expression inhibition strategies are known to be inadequate approaches (16, 72). Therefore, it is necessary to use high-dose corticosteroid drugs to efficiently control the progression of life-threatening CRS (16, 72). Moreover, recent studies have shown that corticosteroids do not influence the efficacy and kinetics of CAR-Ts in the treatment of B-ALL (73). Additionally, *TO-207* is an investigational drug that can inhibit the abnormal activation of macrophages and monocytes with no negative effects on the activity of T cells (74–77). *In vitro* studies have demonstrated that *TO-207* acts as a multi-cytokine inhibitor by suppressing monocyte-mediated secretion of IL-6 and other inflammatory cytokines (including IL-1β, monocyte chemoattractant protein 1 (MCP-1), IL-8, IL-18, and GM-CSF) without deteriorating CAR-T functionality (74, 78). Furthermore, IL-1 receptor blockade using the IL-1 receptor antagonist immunosuppressive drug *anakinra* has been an effective approach resulting in encouraging preclinical outcomes by preventing both lethal neurotoxicity and CRS (67). The same study has also claimed that *anakinra* and *tocilizumab* exhibit the same level of effectiveness in IL-6 signaling blockade and lethal CRS prevention in preclinical models (67). Moreover, CAR-Ts particularly engineered to secrete IL-1 receptor antagonists have also been investigated in preclinical studies (66). The results have indicated that IL-1 receptor antagonist-secreting CAR-Ts can prevent or mitigate CRS as well as neurotoxicity in a very efficient manner (66). As discussed, IL-1 blockade strategies can be efficiently utilized to prevent life-threatening CAR-T-related toxicities including CRS and neurotoxicity (66). However, it is important to mention that conducting clinical investigations with a high number of patients is a vital step in further validating the preclinical and clinical outcomes discussed in this section.

3.1.2 GM-CSF Blockade

Neurotoxicity and CRS development after CAR-T therapy are directly related to *in vivo* T-cell expansion and subsequent secretion of T-cell effector cytokines such as IL-6, IFN-γ, GM-CSF, and MCP-1 suggesting that even monocytes and macrophages may have contributions in the emergence of such adverse events (15, 79–82). Therefore, the neutralization of such cytokines may be a potential strategy for the management of these CAR-T-related toxicities (79, 83, 84). GM-CSF neutralization reduces the secretion level of CRS-mediating cytokines such as IL-6, IL-8, and MCP-1 (79, 83, 84). Therefore, reductions in the level of these immune cell trafficking mediators can decrease the number of CNS-infiltrating immune cells which results in a reduced level of neuroinflammation (NI) and CRS (79, 83, 84). Sterner et al. have evaluated the effects of GM-CSF neutralization on the functionality of CD19-redirected CAR-Ts using *lenzilumab* and they have demonstrated that *lenzilumab* does not inhibit the function of the CAR-Ts (79). Moreover, they have reported enhanced CD19-redirected CAR-T proliferation and durable control of leukemic disease in patient-derived xenograft (PDX) models after *lenzilumab*-mediated neutralization of GM-CSF (79). Additionally, these researchers expanded their experimental zone by generating CRISPR-Cas9-mediated GM-CSF secretion-deficient (GM-CSF$^{k/o}$) CD19-redirected CAR-Ts (79). These GM-CSF$^{k/o}$ CAR-Ts were capable of maintaining their normal functions, exhibiting enhanced *in vivo* tumoricidal activity, and improving OS as compared with conventional CD19-redirected CAR-Ts (79). Other studies have used other genome-editing strategies such as the *transcription activator-like effector nuclease* (*TALEN*) for the same aim (85). They have demonstrated that their GM-CSF$^{k/o}$ CAR-Ts can mediate a substantially reduced secretion level of GM-CSF leading to a disrupted or decrease macrophage-dependent secretion of CRS-mediating factors such as MCP-1, IL-6, and IL-8 (85). Moreover, CAR-Ts can be engineered to be deficient in GM-CSF expression and produce anti-GM-CSF mAbs. The mentioned findings demonstrate that GM-CSF inhibition might be considered as a potential option for the abrogation of CRS and NI and enhancement of CAR-T function (79). However, it is safe to conclude that in-depth clinical assessments might help researchers understand the suitability of such strategies in clinical settings.

3.1.3 Catecholamine Blockade

Myeloid-derived catecholamines are known as crucial mediators of CRS (86–88). Macrophages secrete and respond to catecholamines *via* their adrenergic receptors upon encountering inflammatory stimuli (86–88). This mechanism results in an increase in the level of cytokine production and secretion (87–89). Therefore, high levels of circulating catecholamines can lead to excessive inflammatory responses (87–89). It has been found that disrupting the key enzymes involved in the synthesis of catecholamines can reduce its circulating level and prevent CRS occurrence (90, 91). For example, myeloid-specific deletion of tyrosine hydroxylase (TH) using *metyrosine* (*MTR*) can reduce the excessive level of this key CRS mediator without impairing CAR-T antitumor activity (91). Additionally, *atrial natriuretic peptide* (*ANP*) can also significantly reduce the circulating level of catecholamines without any interference with CAR-T-mediated therapeutic responses (90).

3.1.4 Application of JAK Inhibitors

As key CRS modulators, inflammatory cytokines including ILs, IFNs, and several growth factors crucially require the JAK tyrosine kinase family for their downstream signaling pathway (92–99). Therefore, the inhibition of the JAK family components can lead to a reduced CRS-related cytokine level (92–99). *Itacitinib* (INCB039110) is a JAK1 inhibitor that is being evaluated in clinical trials for the treatment of GvHD (NCT02614612) (99) and in preclinical investigations for the treatment of inflammatory and autoimmune diseases (97, 98). *Itacitinib* has also been evaluated in combination with other drugs for the treatment of various B-cell malignancies in clinical trials (NCT02018861 and NCT01905813) (95, 96). Recently, studies have evaluated the effects of prophylactic *itacitinib* in the prevention of CAR-T therapy-mediated CRS, and they have

found that *itacitinib* does not impair CAR-T functionality both *in vitro* and *in vivo* and it significantly reduces CRS-associated cytokine levels in a dose-dependent fashion (94). Such data suggest the potential of *itacitinib* as prophylactic therapy for the prevention of CAR-T-related CRS as other studies investigated its various safety aspects (93). Additionally, *ruxolitinib* is also another JAK inhibitor that has recently been used as adjuvant therapy in a case report study involving a patient with B-ALL (92). *Ruxolitinib* has been successful in controlling corticosteroid-resistant CRS without interfering with CAR-T functionality (92). Such studies highlight the role of JAK inhibitors in the prevention and control of CRS and pave the way for more in-depth preclinical and clinical investigations.

3.1.5 Intrathecal (IT) Chemotherapy

IT chemotherapy has been used for controlling refractory CAR-T-associated ICANS that is non-responsive to steroids (100, 101). Shah et al. have administered IT chemotherapy to two CAR-T-receiving patients and demonstrated that it can mediate rapid ICANS resolution without any long-term complications (100). This method can also accelerate the recovery process and reduce the complications of systemic long-term corticosteroid administration (100). Moreover, Yucebay et al. have also reported similar results from a study where IT chemotherapy resulted in the resolution of high-grade neurotoxicity (grade 3-4) of CD19-redirected CAR-T-receiving patients while corticosteroid therapy was inefficient (101). However, both of these studies suggest that this method requires detailed evaluations in future clinical trials (100, 101).

3.1.6 Pretreatment With Antibody-Based Immunotherapy

The occurrence of CRS and neurotoxicity seem to have a direct relationship with the disease burden of patients receiving CAR-T therapy (14, 16, 17). Pretreating patients with antibody-based immunotherapy such as *blinatumomab* or *inotuzumab ozogamycin* (*IO*) before the administration of CAR-Ts has been known to potentially reduce disease bulk, thus minimizing the incidence and severity of the mentioned toxicities (102). *Blinatumomab* is a T-cell-redirecting bispecific antibody (TRBA) that can potentially mediate the elimination of leukemic cells through the engagement of endogenous T cells by simultaneously binding to CD19 on the surface of B-lineage cells and CD3 on cytotoxic T cells (103). Moreover, *IO* is an antibody-drug conjugate consisting of a humanized mAb against CD22 conjugated to the cytotoxic antibiotic agent *ozogamycin* (104). These therapeutic antibodies have shown considerably improved OS rates in comparison with standard therapy (103, 104). Additionally, one study including five adult patients with B-ALL (NCT02772198) has reported that treatment of R/R B-ALL patients with *blinatumomab* and/or *IO* before the beginning of CD19-redirected CAR-T therapy can result in promising response rates (with two out of five patients (40%) achieving minimal residual disease (MRD)-negative CR and two other patients (40%) achieving MRD-positive CR) (102). However, further investigations involving broader patient populations are required for a better understanding of the action mechanisms of antibody-based treatments before CAR-T therapy and their effects on the final clinical outcomes.

3.1.7 Therapeutic Plasma Exchange (TPE)

Recently, it has been shown that TPE (**Figure 2A**) in combination with glucocorticoid therapy can result in the gradual resolution of the CRS-related symptoms of CAR-T therapy recipients (105, 106). These findings suggest that this strategy can be a feasible procedure, at least in patients with severe CRS (grade ≥ 3), even though TPE is not included in CRS management guidelines (105, 106). In a case report, Xiao et al. reported that in a 23-year-old male R/R B-ALL patient, after performing TPE along with the administration of *dexamethasone*, CRS (grade 3) was mitigated and controlled whereas treatment with antiallergic and antipyretic drugs, glucocorticoids, and *tocilizumab* was not effective against the progressing condition of the patient's CRS (105). Moreover, another case report has also reported that TPE has been effective in the treatment of CAR-T-induced CRS of a 4-year-old female patient with R/R B-ALL (107). Additionally, the report of a clinical trial (NCT02349698) involving ten patients has indicated that *tocilizumab*, glucocorticoid, and TPE have been effective in the successful controlling of severe CRS (grade 3-4) developed in four of the patients (108). However, the broader application of this approach for the management of CAR-T-related CRS lies in the outcomes of more comprehensive future clinical studies in this regard.

3.1.8 Hemofiltration

Hemofiltration (**Figure 2B**) has not been generally utilized as a clinical approach for controlling CAR-T therapy-related CRS. However, studies have used hemofiltration to help patients with high-grade CRS (grade ≥4), with developed acute kidney injury, to recover their renal functionality (109). Moreover, a case report has indicated that hemofiltration has been helpful in mitigating severe CRS (grade 4) and preventing multiple organ failure and pulmonary infection in a B-ALL patient after CD19-redirected CAR-T therapy (110). It is important to state that in this case, *tocilizumab* and glucocorticoids were not successful in controlling the mentioned adverse events (110). Furthermore, another study has also proposed that *continuous renal replacement therapy* (*CRRT*) can be an additional approach for controlling CAR-T therapy-related CRS that is resistant to conventional treatment (111). This study has demonstrated that *CRRT* is effective in mitigating sepsis which has a similar pathophysiological mechanism to CRS (111). However, as such studies themselves indicate that there are still various unanswered questions in this field that require addressing (111).

3.1.9 Fractionated CAR-T Infusion

Recently, studies have indicated that there is a relationship between CAR-T infusion dose and the occurrence of CRS (112–114). Therefore, a fractionated dosing scheme can retain high response rates with acceptable tolerability in adult R/R B-ALL patients (113, 114). So far, two clinical trials have evaluated this strategy. The first study (NCT01029366) included R/R B-

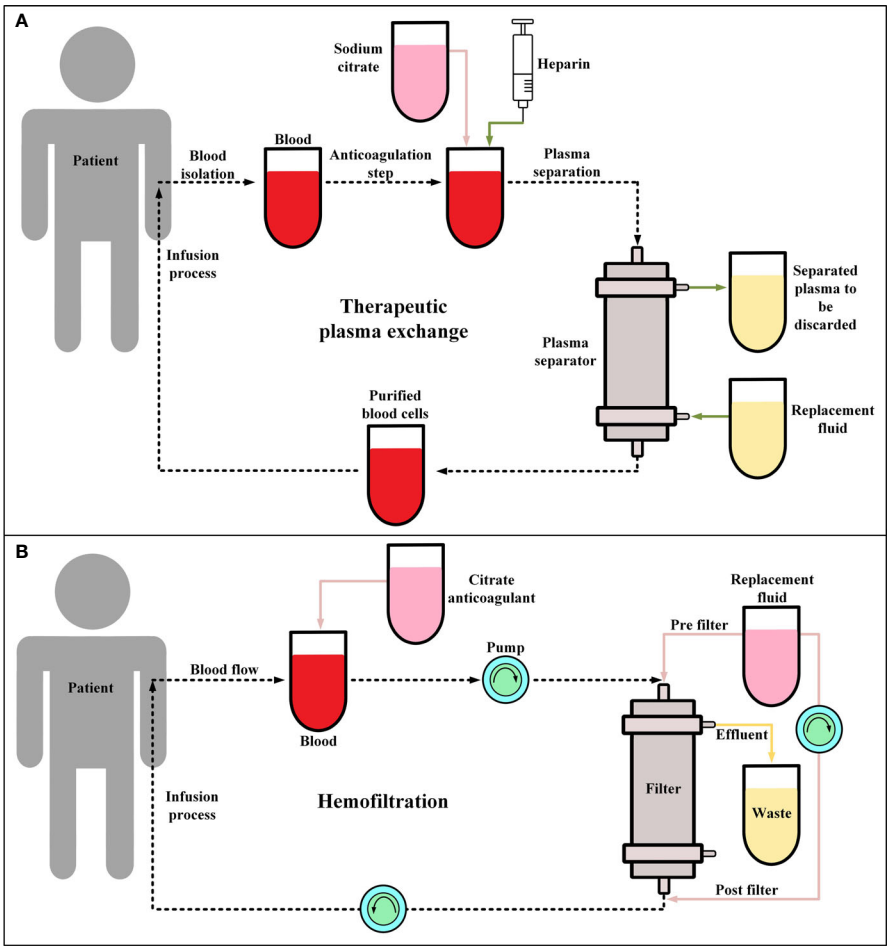

FIGURE 2 | *Therapeutic plasma exchange* (TPE) and *hemofiltration*. **(A)** The general procedure of TPE. Conventionally, in the anticoagulation step, sodium citrate or heparin are utilized. Moreover, the replacement fluid consists of albumin or fresh frozen plasma. **(B)** The procedure of *continuous veno-venous hemofiltration* (CVVH) for hemofiltration. During CVVH, a patient's blood is filtered by the means of highly semipermeable membranes (known as filter) and the ultrafiltrate (wastes) is separated by a process known as convection. Of note, the replacement fluid is added prior to or after hemofiltration.

ALL patients alongside patients with other CD19-positive malignancies and the second one (NCT02030847) only included adults with $CD19^+$ R/R B-ALL (113, 114). In detail, Frey et al. administered *tisagenlecleucel* to adult R/R B-ALL patients after lymphodepletion as either a one-time infusion or fractionated infusions split over 3 days (10% on day 1, 30% on day 2, and 60% on day 3) (113). The total planned CAR-T infusion dose in their study varied with adaptive protocol modifications in response to efficacy and CRS occurrence (113). The second clinical trial included 35 patients in 3 different dosing cohorts which included the low-dose cohort (9 patients), the high-dose single infusion cohort (6 patients), and the high-dose fractionated (HDF) cohort (20 patients) (113). In the low-dose cohort, the patients received single or fractionated dosing, which resulted in manageable toxicity with a 33% CR rate (113). In the high-dose single infusion cohort, half of the patients died due to refractory CRS (grade 4/5) and culture-positive sepsis, and the other half achieved CR (113). Moreover, the HDF cohort had a 90% CR rate and manageable CRS (grade ≤3) (113). Of note, according to Penn Grading System for CRS, grade 4 is characterized by life-threatening complications including hypoxia which necessitates the appliance of mechanical ventilation and hypotension requiring the administration of high-dose antihypotensive agents. Also, grade 5 is defined as CRS-caused mortality (113). The HDF scheme resulted in the highest survival rate with a 2-year OS of 73% and event-free survival of 49.5% (113). The researchers of this study proposed that a fractionated dosing scheme of CTL019, for administration to adult R/R ALL patients, with intrapatient dose modifications can optimize the safety of B-ALL CAR-T therapy without impairing its efficacy (113). Additionally, these clinical trials have demonstrated that a fractionated dosing scheme, where day 2 and day 3 doses are held in response to early CRS, enables individualized dose modifications for achieving a better balance of efficacy and safety in comparison with protocols suggesting a defined single-dose infusion (113).

3.2 Overcoming Graft Rejection

Using autologous CAR-Ts in clinical settings may not always be feasible since the patients may be under different types of treatments and may have malignancies with various levels of severity which might imping on the quality of the final T-cell products and the corresponding clinical outcomes (115). Considering these limitations, allogeneic CAR-Ts, generated from donor-derived T cells, can be used as an alternative even though they tend to have their limitations (115). Such limitations include causing alloreactivity-related adverse events such as host-versus-graft (HvG) and graft-versus-host (GvH) reactions (115). In this section, we will review studies focusing on developing strategies for addressing these limitations.

3.2.1 Conditioning Regimen

Conditioning regimen, also known as lymphodepleting conditioning regimen, is the occasional use of chemotherapeutic agents in cell-based cancer therapies such as CAR-T therapies (regardless of autologous or allogeneic CAR-T therapies) (116). It has been demonstrated that conditioning regimen is an important factor affecting the clinical outcomes of CAR-T therapy (116). Studies have demonstrated that conditioning regimen prior to adoptive cell transfer significantly enhances the efficacy of therapies with *ex vivo*-expanded tumor-infiltrating lymphocytes (TILs) (117). In brief, lymphodepletion chemotherapy provides an appropriate environment for infused T cells to expand by helping create a supportive space in the immune system of the recipients (116–119). Lymphodepleting conditioning regimen acts by several mechanisms including eliminating the recipients' T and B lymphocytes as well as NK cells, the eradication of immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells, inducing co-stimulatory molecules and downregulating indoleamine 2,3-dioxygenase (IDO) in tumor cells, the elimination of homeostatic cytokine sinks such as IL-2, IL-7, and IL-15, and finally promoting the expansion, function, and persistence of the adoptively transferred T cells by eliminating the factors interfering with their activity (117–119).

Fludarabine and *cyclophosphamide* are two chemotherapeutic agents that are occasionally used as the conditioning regimen for B-ALL patients receiving CAR-T therapy (120, 121). Studies have demonstrated that using the combination of these drugs in the lymphodepleting conditioning regimen would result in improved CD19-redirected CAR-T expansion and persistence, better clinical outcomes, and disease-free survival of the patients in comparison with non-*fludarabine* lymphodepleting regimens (120, 121). Others have shown a relationship between elevated in-serum IL-15 levels and clinical response after CD19-redirected CAR-T therapy (122). It is also believed that the probability of achieving a favorable cytokine profile is higher in patients who receive high-intensity lymphodepletion in comparison with those that receive low-intensity lymphodepletion (123). This occurrence in turn is associated with better clinical outcomes (123).

3.2.2 Using Genome-Editing Methods

Genome-editing technologies have been utilized for addressing the unfavorable limitations of using allogeneic T cells (124–127). In this regard, Torikai et al. have utilized the zinc finger nuclease (ZFN) genome-editing technique to eliminate the expression of the endogenous αβ TCR in allogeneic CD19-redirected CAR-Ts (128). They demonstrated this method can prevent allogeneic CAR-T-mediated GvHD without compromising CAR-T functionality or antitumor activity (128). Stepping further, they used the same genome-editing method and disrupted the expression of HLA-A in CD19-redirected CAR-Ts, and demonstrated that this strategy can protect CAR-Ts from lymphodepletion therapy, alongside eliminating the possibility of GvHD (129).

Other researchers have developed practical platforms to demonstrate the applicability of the TALEN genome-editing technique for the production of "off-the-shelf" allogeneic CAR-Ts (130–132). In this regard, Poirot et al. used TALEN for the simultaneous disruption of two genes in allogeneic CAR-Ts (130). First, the researchers disrupted the TCRα constant (TRAC) gene expression which resulted in the elimination of TCRαβ expression; therefore, it abrogated the potential of the allogeneic CAR-Ts for GvHD mediation (130). The second knock-out was aimed at rendering the universal allogeneic CAR-Ts resistant to destruction by a lymphodepleting or immunosuppressive agent (130). CD52, as the second knock-out target, can be specifically targeted using the lymphodepleting mAb *alemtuzumab*, thus enabling the lymphodepletion of recipient(s) and the creation of a receptive environment concurrently or before the administration of the TCR/CD52-knock-out CD19-redirected CAR-Ts (130). Poirot et al. demonstrated that the TCR/CD52-knock-out CD19-redirected CAR-Ts were capable of efficient elimination of $CD19^+$ tumor targets even while accompanied by the chemotherapeutic agent (130). Taken together, this GMP-compatible scalable process can be considered as a general platform for the manufacturing of "off-the-shelf" CAR-Ts from the T cells of healthy third-party donors (130). Similarly, Qasim et al. also generated allogeneic TCR/CD52-knock-out CD19-redirected CAR-Ts from non-HLA matched donors, and demonstrated that these genome-edited universal allogeneic CAR-Ts did not mediate alloreactivity in infant recipients with R/R B-ALL (132). They also reported that these CAR-Ts mediated molecular remission in the recipients with acceptable persistence while being anti-CD52 therapy-resistant (132). Moreover, other researchers have also demonstrated that simultaneous knock-out of TCRαβ expression and deoxycytidine kinase (dCK) can result in the production of allogeneic CD19-redirected CAR-Ts that do not mediate GvHD and are resistant to dCK phosphorylation-dependent lymphodepleting regimens (131).

It is worth mentioning that the results of two Phase I trials using TCR-knockout CD19-redirected CAR-T therapy in both children and adults (NCT02808442 and NCT02746952, respectively) have demonstrated the practicability of using allogeneic genome-edited CAR-Ts for the treatment of patients with R/R B-ALL (133). The results of such trials indicate that allogeneic CAR-Ts can mediate acceptable tumoricidal activity with manageable levels of toxicities and adverse events (133). Such CAR-Ts can be beneficial for the treatment of heavily

pretreated R/R B-ALL patients with rapidly progressing diseases since autologous CAR-Ts may not be accessible in these cases in terms of meeting the adequate cell number or final-product quality (133).

In addition to the abovementioned strategies, CRISPR-Cas9-based genome-editing method can also be utilized for the generation of universal allogeneic CAR-Ts (134–137). One study has used CRISPR-Cas9 to selectively insert the CD19-redirected CAR transgene into the TRAC locus (134). This mechanism results in the deficiency of the endogenous TCR expression and it ameliorates *in vivo* CAR-T functionality while reducing the risk of alloreactivity as compared with retrovirus-mediated random CAR transgene insertion (134). It also postpones the effector T-cell differentiation and exhaustion of CAR-Ts and facilitates the efficient internalization and consequent re-expression of CAR molecules upon multiple target antigen engagement (134). MacLeod et al. made a similar attempt and demonstrated that their TCR-knock-out CD19-redirected CAR-Ts exhibited superior antitumor activity in preclinical mouse models without mediating GvHD (136). Furthermore, researchers incorporated multiple gRNAs into a CAR-encoding lentiviral vector which resulted in the simultaneous knock-out of the endogenous TCR and Beta-2 microglobulin (b2M) of HLA class I, thereby paving the way for a practical strategy for the production of universal allogeneic CAR-Ts (137).

More recently, Georgiadis et al. demonstrated that incorporation of an sgRNA element into the Delta U3 3′ long terminal repeat (LTR) of their CAR-encoding lentiviral vector would result in the production of a self-inactivating lentiviral terminal vector that combines CAR expression with CRISPR-Cas9 effects (135). In detail, after transcription and duplication of the hybrid DU3-sgRNA and electroporation-facilitated delivery of Cas9 mRNA, this platform cleaves the TRAC locus and enables enriching the TCR-knock-out CAR-T population using automated magnetic separation (135). These CAR-Ts would retain their CAR-associated specificity and antileukemic activity, have superior persistence as compared with their conventional counterparts, and would not mediate alloreactivity in the respective recipients (135). However, despite what we have discussed, a recent study by Stenger et al. has indicated that CD19-redirected CAR-Ts harboring endogenous TCR have superior persistence and mediate considerably prolonged leukemia control *in vivo* as compared to their CRISPR-Cas9-mediated TCR-knockout counterparts (138). Conclusively, it can be mentioned that more clinical information is required for drawing more substantiated conclusions.

3.2.3 Virus-Specific T Lymphocytes (VSTs) for CAR Expression

VSTs that harbor their native TCRs can be used as a source of universal allogeneic T cells in CAR-T therapy (139, 140). *Tabelecleucel* (tab-cel®) is a universal allogeneic T-cell immunotherapy using Epstein-Barr virus (EBV)-specific T cells that harbor TCRs specifically targeting EBV antigens (139, 140). It has been demonstrated that CD19-redirected CAR-Ts generated using EVB-specific T cells are well-tolerated with poor mediation of GvHD and CRS suggesting a promising approach for generating universal allogeneic CAR-Ts (140).

VSTs are now being investigated for the prevention of viral infections in recipients of hematopoietic stem cell transplantation (HSCT) since these cells can reconstruct the recipients' impaired immune system and confer immunity against life-threatening viral infections after HSCT without GvHD mediation (141, 142). This feature of VSTs has encouraged researchers to use them as a reliable source for the generation of allogeneic CAR-Ts (142, 143). An ongoing Phase I clinical trial (NCT00840853) has studied VSTs genetically engineered to express CD19-redirected CARs to see if these cells can maintain their antiviral activity alongside exhibiting specific tumoricidal functionality in patients with $CD19^+$ B-ALL after HSCT (142). According to the results of this clinical trial so far, the investigators stated that these CAR-Ts induced no infusion-related toxicities (142). The researchers have also added that vaccinating the recipients of these CAR-Ts with viral antigens may result in enhanced CAR-T expansion and antitumor activity (142). However, this clinical trial is planned to be completed in 2031; therefore, more accurate clinical outcomes can be obtained by then.

3.2.4 Human Induced Pluripotent Stem Cell (hiPSC)-Derived CAR-Ts

Lately, hiPSC-derived CAR-Ts have gained a great deal of attention as universal allogeneic CAR-Ts (144, 145). In detail, peripheral blood-derived T cells are converted to hiPSCs and genetically engineered to express the CD19-redirected CAR with targeted integration into the TRAC locus followed by clonal selection carried out for generating a master hiPSC line, termed TRAC-TiPSC (144, 145). These cells are also genetically manipulated to express a high-affinity form of CD16 with proteolytic resistance for addressing tumor antigen escape (144, 145). The mentioned characteristics of hiPSC-derived CAR-Ts render them incapable of GvHD mediation (144, 145). Taken together, the researchers of these studies indicated that since such CAR-Ts mediated encouraging preclinical results as an effective and safe allogeneic product, it might be feasible to evaluate these CAR-Ts in a Phase I clinical trial in patients with B-cell neoplasms (144, 145).

3.2.5 Placenta Blood-Derived T Cells for CAR Expression

Recently, researchers have demonstrated that T cells isolated from postpartum human placenta/umbilical cord blood can be used for the generation of CD19-redirected CAR-Ts, termed P-CD19-redirected CAR-Ts (146). P-CD19-redirected CAR-Ts are mainly naïve ($CD45RA^+$) T cells and retain their naïve/memory marker expression and harbor a lower expression of effector/exhaustion markers as compared to peripheral blood mononuclear cell-derived CAR-Ts (146). Moreover, these cells also have superior immune tolerance to HLA mismatch and exhibit weakened allogeneic activation resulting in a lower GvHD occurrence possibility (146). Researchers have suggested that these features of P-CD19-redirected CAR-Ts make them a

promising candidate for universal allogeneic CAR-T therapy (146). It is worth mentioning that the endogenous TCR expression of these T cells can be abrogated using the CRISPR genome-editing technique to minimize the risk of endogenous TCR-mediated GvHD (146).

3.2.6 Alloimmune Defense Receptor (ADR)

One strategy for overcoming the issue of cellular immune rejection is the abrogation of immune responses mediated by both activated alloreactive T cells and NK cells that direct the elimination of foreign cells through different mechanisms (147–151). Mo et al. have developed an engineered receptor called ADR that mediates the depletion of activated host T and NK cells through selective recognition of the cell surface receptor 4-1BB since this molecule is temporarily upregulated on the cell surface of activated $CD4^+$ T cells and $CD8^+$ T cells, as well as NK cells (151). The 4-1BB-specific chimeric ADR is composed of a 4-1BB-recognizing fragment derived from 4-1BB ligand (4-1BBL) that is connected to the intracellular CD3ζ chain through a spacer and a TM region (151). *In vitro* and *in vivo* results have indicated that ADR-expressing T cells manage to resist cellular rejection by targeting alloreactive lymphocytes and, on the other hand, they manage to spare resting lymphocytes (151). Furthermore, Mo et al. co-expressed ADR alongside second-generation CD19-redirected CARs, and demonstrated that T cells co-expressing these two chimeric receptors can preserve their independent anti-rejection and antitumor functionality (151). Moreover, T cells expressing only CD19-redirected CARs and T cells co-expressing both CD19-redirected CARs and ADRs have exhibited similar cytotoxic profiles against $CD19^+$ cells, according to Mo and colleagues (151). Conclusively, these researchers have proposed that co-expression of CARs and ADRs can mediate sustained tumor eradication and produce long-term therapeutic benefit in immunocompetent recipients with hematologic malignancies alongside enabling the generation of rejection-resistant off-the-shelf allogeneic CAR-Ts (151).

3.2.7 HLA-Matched or HLA-Haploidentical CAR-Ts

Using HLA-matched or HLA-haploidentical allogeneic CAR-Ts can lead to different clinical outcomes in B-ALL CAR-T therapy (106). Researchers have investigated the use of HLA-matched or HLA-haploidentical allogeneic CD19-redirected CAR-Ts (M-CAR-Ts and H-CAR-Ts, respectively) for the treatment of R/R B-ALL patients to evaluate which one can result in better clinical outcomes (106). Recently, Jin et al. described the first-in-human application of M-CAR-Ts in R/R B-ALL patients before allogeneic hematopoietic stem cell transplantation (allo-HSCT) (106). The results of this investigation demonstrated that using M-CAR-Ts for the treatment of R/R B-ALL patients results in higher CR rates but it also leads to more severe toxicities in comparison with using H-CAR-Ts (106). These researchers also reported that the mild infusion-related toxicities occurring in patients receiving H-CAR-Ts enabled using higher dose infusions in these patients in comparison with the patients receiving M-CAR-Ts (106). It is important to mention that even though these researchers did not observe GvHD or any uncontrolled infusion-related toxicities, there is still a need for paying considerable attention to GvHD and other infusion-related toxicities (106). Other researchers have also investigated the use of H-CAR-Ts (152–154). In particular, Cai et al. and Zhang et al. have reported that using H-CAR-Ts has beneficial therapeutic effects as part of a conditioning regimen for allo-HSCT (152, 153). However, it is suggested that, as compared to M-CAR-Ts, the lower efficacy of H-CAR-Ts may be due to their heterogeneity in mediating graft rejection (106). Additionally, it is yet to be discovered that whether using a stronger immunosuppressive treatment before the infusion of H-CAR-Ts can enhance the efficacy and antitumor activity of these cells (106).

3.3 Overcoming B-Cell Aplasia and Its Consequences

B-cell aplasia, as characterized by the low number or the absence of B cells, is commonly considered as an indicator of a successful CD19-redirected CAR-T therapy which can last for as long as CAR-Ts are potentially functional (29). B-cell aplasia can lead to agammaglobulinemia and hypogammaglobulinemia resulting in an increased risk of sinopulmonary and various other life-threatening infections due to the inability of the patients' immune system to produce antibodies (29). Prevention of serious bacterial infections in patients with hypogammaglobulinemia after CD19-redirected CAR-T therapy and also in patients with primary immunodeficiency (PID) who suffer from impaired immune systems can be achieved through immunoglobulin replacement (14, 15, 155–158). Increasing the level of serum immunoglobulin G (IgG) has been correlated with a considerably lower risk of sinopulmonary infection (29). Serum IgG level of more than 1000 mg/dL (720 mg/dL to 1430 mg/dL IgG level for maintaining an infection-free state) has been known as the optimal concentration needed to provide patients with protection against infections (29). Furthermore, subcutaneous immunoglobulin (SCIg) replacement can be considered as a suitable method for the stabilization of IgG levels, the improvement of health-related quality-of-life scores, and minimizing systemic side effects with increased ease of administration and affordability in comparison with intravenous immunoglobulin (IVIG) replacement (159–163). Moreover, antimicrobial prophylaxis including *levofloxacin* for gram-negative bacteria in patients with neutropenia can be beneficial in the prevention of bacterial infections in patients receiving CD19-redirected CAR-T therapy (25, 164, 165). Similarly, antifungal prophylaxis such as *micafungin* or *fluconazole* for Candida species in patients with neutropenia and *pentamidine* or *sulfamethoxazole* for *Pneumocystis jiroveci* are among common recommendations for fungal infection prevention (25, 164, 165). Also, in the case of viral infections, antiviral prophylaxis such as *acyclovir* is used for controlling herpes simplex virus (HSV)- and varicella-zoster virus (VZV)-related viral infections after CD19-redirected CAR-T therapy (25, 164, 165). Moreover, a recent study has reported prolonged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in a patient who had received BCMA-redirected CAR-T therapy (166). Hensley et al.

stated that even though convalescent plasma therapy and the antiviral agent *remdesivir* was used as treatment options, the patient ultimately died from complications related to this infection (166).

3.4 Overcoming Unintentional Transduction of Leukemic Cells

Unintentional transduction of B-ALL blasts with the CAR transgene during the manufacturing process of CAR-Ts is a rare incidence that leads to the aberrant expression of the CD19-redirected CARs by leukemic cells rendering them resistant to CAR-T-mediated tumor cell eradication (**Figure 3**) (167). Upon this incidence, a mechanism of resistance is conferred through cis binding of the CD19-redirected CAR to the CD19 epitope on the surface of leukemic cells, thus masking the target antigen from being recognized by CD19-redirected CAR-Ts (167). Therefore, the patient in which this incidence was documented for the very first time experienced $CD19^{-}$ disease relapse and underwent salvage chemotherapy, anti-CD22 antibody therapy, and CD22-directed CAR-T therapy (167). However, the patient ultimately died from the complications associated with progressive leukemia (167).

Later in 2020, Ruella et al. developed an anti-CAR19 idiotype CAR (αCAR19) to selectively target and eliminate CD19-redirected CAR^{+} cells (both T cells and unintentionally transduced leukemic cells) (168). This method entails the application of genetically modified T cells (αCAR19) that express CARs that recognize CD19-redirected CAR^{+} cells (168, 169). It has been demonstrated that the αCAR19 T cells can successfully kill both CD19-redirected CAR-expressing leukemic cells and T cells (168). It is worth mentioning that no evidence of reverse killing of αCAR19 T cells by CD19-redirected CAR-expressing T cells has been observed (168). These findings demonstrated that αCAR19 T cells can act as "*cellular antidotes*" and a safe depletion strategy to eliminate common CAR-T engraftment side effects such as prolonged B-cell aplasia and other yet-to-be-known complications as well as CAR-expressing leukemic cells (168, 170). However, broader investigations are still required to safely conclude that this strategy may be efficient in controlling this important unintentional side effect.

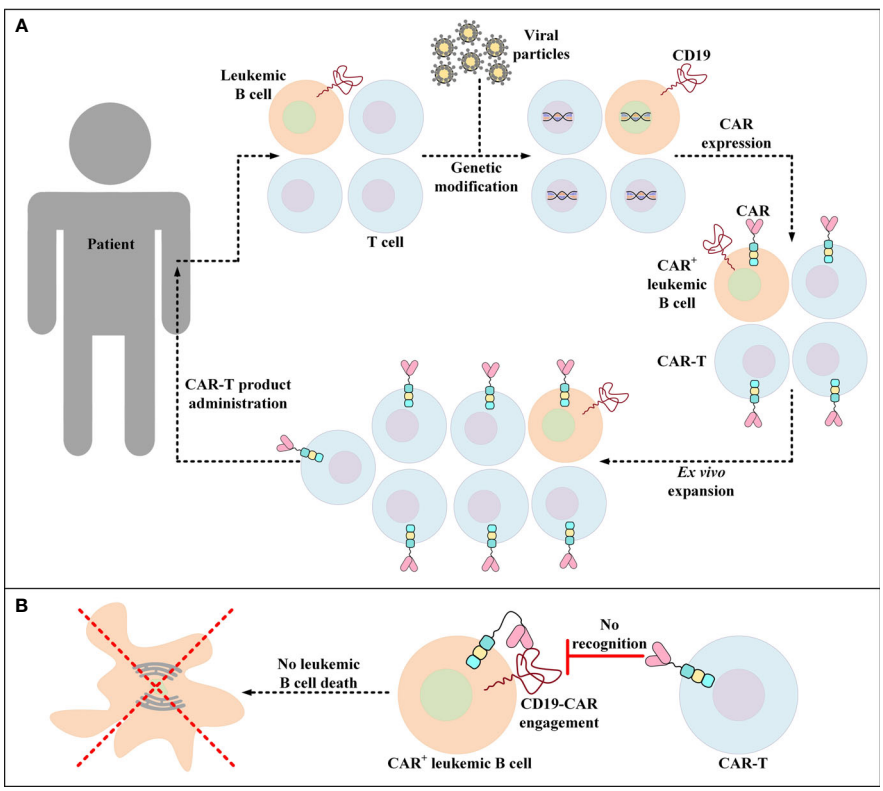

FIGURE 3 | Accidental transduction of leukemic B cells and the emergence of resistance to CAR-T therapy. **(A)** Accidental transduction of leukemic B cells during the process of CAR-T preparation. An error in the isolation of T cells leads to the contamination of the cell pool with leukemic B cells. Moreover, in the process of genetic modification, leukemic B cells are transduced along with the isolated T cells for the expression of certain CD19-specific CARs. **(B)** Resistance of CAR^{+} leukemic cells to a particular CD19-redirected CAR-T product. Following the administration of the developed product into patients, CAR^{+} leukemic cells become resistant to that particular CD19-redirected CAR-T product due to the engagement of their CD19 with their self-expressed CARs (a process known as *epitope masking*). This incident might also occur in the case of CAR-T products that target different target antigens than CD19.

3.5 Phenotype-Changing Factors

The data from various studies propose that there is a strong correlation between the expansion rate of transferred CAR-Ts in the recipient and the subsequent clinical response (14, 24–27). It is also believed that the *in vivo* persistence of functional CAR-Ts is critical for disease relapse prevention (14, 24–27). The phenotypic composition of patient- or donor-isolated T cells and the reinfused product is among multiple factors affecting the *in vivo* expansion and persistence of CAR-Ts and the durability of their antitumor responses (**Figure 4**) (171). In this section, we will discuss various factors affecting the phenotype of CAR-Ts.

3.5.1 $CD4^+$:$CD8^+$ Ratio

Different investigations have demonstrated that human $CD4^+$ T cells and $CD8^+$ T cells consist of functionally and transcriptionally separate subsets with varied *in vivo* proliferation and persistence capacities after *ex vivo* expansion and adoptive transfer (172–177). Sommermeyer et al. have shown that human CD19-redirected CAR-Ts manufactured from purified $CD8^+$ or $CD4^+$ central memory T (T_{CM}) cells or naïve T (T_N) cells are more effective in attacking and killing $CD19^+$ tumors in immunodeficient mice in comparison with CD19-redirected CAR-Ts manufactured from effector memory T (T_{EM}) cells (178). Other studies have also demonstrated that in comparison to T_{EM} cells, $CD8^+$ T_{CM} cells harbor stemness potency alongside showing superior persistence after adoptive transfer (172, 179). This fact proposes the advantages of this subset for providing long-term persistence (172, 179). Moreover, in the first clinical trial assessing the feasibility of selecting and engineering defined T-cell subsets, Turtle et al. conducted a Phase I/II clinical trial to evaluate CD19-redirected CAR-Ts manufactured from separately modified defined $CD4^+$ T-cell and $CD8^+$ T-cell subsets (180). These researchers administered this product in a defined $CD4^+$:$CD8^+$ composition and in a dose-escalation/de-escalation format to adult B-ALL patients after lymphodepletion chemotherapy (180). They found that the infusion of CAR-T comprised of a uniform ratio of $CD4^+$:$CD8^+$ shows a correlation between cell dose and earlier and higher peak expansion of clonally diverse CAR-Ts (180). These findings had not been reported in other investigations in which CAR-Ts were manufactured and infused without the consideration of the $CD4^+$:$CD8^+$ ratio (180). Their results also demonstrated that the infusion of a defined ratio of CD19-redirected CAR-Ts manufactured from $CD8^+$ T_{CM} cells and $CD4^+$ T cells could provide a synergistic improvement in potency (180). Also, the results of preclinical investigations in Raji tumor-bearing immunodeficient mice receiving CAR-Ts manufactured from distinct T-cell subsets have shown that when either $CD8^+$ CAR-Ts or $CD4^+$ CAR-Ts were excluded from the formulated product, the potency of the infused product was significantly reduced (178). It is safe to say that selecting a defined subset of T cells for CAR-T manufacturing with a uniform composition may provide reproducible potency in clinical therapy (180). Moreover, Biasco et al. have recently highlighted the impact of different clonal subtypes of T cells in the final CAR-T product on early antitumor responses and long-term disease-controlling capability of CAR-T therapy (181). These researchers stated that even though T memory stem cells (T_{SCM}) have a low frequency in the final generated CAR-T product, these T-cell clones significantly contribute to the circulating CAR-T pools throughout both early proliferation and prolonged disease-controlling persistence (181). As suggested by Biasco et al., these findings underscore the significant role of T_{SCM} in both early anti-leukemic CAR-T reactions and prolonged disease control (181). However, it is important to mention that CAR-T prolonged persistence, after achieving the desired clinical outcomes and disease control, might lead to cytopenia which should be taken into consideration (182).

3.5.2 *Ex Vivo* Culture Media

The different cell culture media formulations used for the activation and expansion of T cells *ex vivo*, and eventually generating CAR-Ts can be optimized in many ways since they tend to have significant impacts on various aspects of the final T-cell product (183, 184). The cell culture media formulations

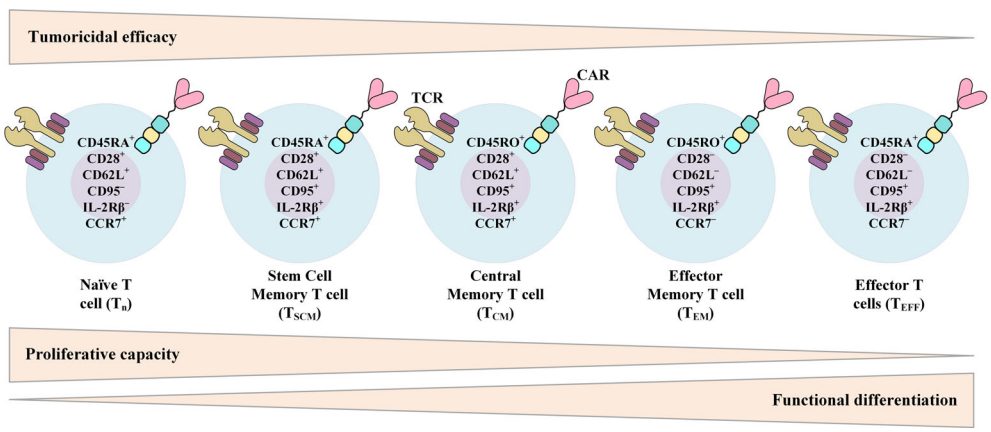

FIGURE 4 | Phenotypic characteristics of different T-cell (or CAR-T) subsets over the course of T-cell differentiation from naïve T cells to effector T cells (T_{EFF}) and their relationship with the proliferative capacity, functional differentiation, and tumoricidal efficacy of T cells. CAR, chimeric antigen receptor; TCR, T-cell receptor.

currently used are dependent on fetal bovine serum (FBS) or human serum (HS) (183, 184). Researchers have investigated the effects of the human transfusion-grade whole blood fraction-derived concentrated growth factor extracts, named Physiologix™ xeno-free (XF) hGFC (Phx), on CAR-T expansion and function (183, 184). This method has been developed to resolve the supply issues of FBS or HS (183, 184). It has been found that Phx enhances T-cell proliferation in both research and clinical-grade media and improves lentiviral-mediated gene transferring and expression (183, 184). Moreover, CAR-Ts expanded *ex vivo* in Phx-conditioned media harbor advanced *in vivo* expansion and antitumor activity as compared to those of CAR-Ts expanded *ex vivo* in HS-conditioned media (183, 184). Phx also enhances the number of naïve T cells ($CD45RO^-/CCR7^+$) and central memory T cells ($CD45RO^+/CCR7^+$) in both $CD4^+$ T-cell and $CD8^+$ T-cell populations which contributes to higher persistence and durability of CAR-Ts resulting in better clinical outcomes (183, 184). Geiger and colleagues have also found that, upon T-cell activation, significant changes occur in the arginine metabolism resulting in a decline in the concentration of the intracellular L-arginine (185). Therefore, these researchers demonstrated that supplementing T-cell culture media with L-arginine can increase the reduced intracellular L-arginine levels in activated T cells and it can induce oxidative phosphorylation instead of glycolysis (185). This effect can result in the central memory phenotype development (185).

The methods currently used for generating FDA-approved CAR-Ts include using anti-CD3 and IL-2 or anti-CD3 and anti-CD28 beads (186). However, these methods result in the generation of CAR-Ts expressing exhaustion markers with an effector phenotype (186). Studies have demonstrated that using IL-7 and IL-15 during the *ex vivo* culturing of T cells would result in the production of CAR-Ts with T memory stem cell phenotype ($CD45RA^+/CCR7^+$) that exhibit superior tumoricidal activity, expansion, and persistence *in vivo* as compared with their counterparts expanded *ex vivo* using IL-2 (187). Others studies have also reported very similar results alongside adding that using IL-7 and IL-15 during the *ex vivo* culturing of T cells also elevates the response rate of the resultant CAR-Ts to anti-PD-1 adjuvant therapies which is mainly due to the anti-PD-1-responsive $CD8^+/CD62L^+/TCF7^+/IRF4^-$ population of the CAR-Ts (188). However, Alizadeh et al. have indicated that T cells expanded with IL-15 preconditioning maintain a less differentiated stem cell memory phenotype ($CD62L^+/CD45RA^+/CCR7^+$) with reduced levels of exhaustion marker expression in comparison with T cells preconditioned with IL-2. They have also stated that using IL-7 or IL-21 alongside IL-15 decreases the favorable influences of IL-15 in the induction of CAR-T phenotype and tumoricidal activity (189).

Additionally, it has been demonstrated that using human platelet lysate (HPL) as a supplement in the culture media of T cells results in a higher number of T cells with central memory phenotype in comparison with human AB serum (ABS)- or FBS-supplemented culturing media (190). It has also been discovered that the presence of various cytokines, such as IL-7, in HPL is responsible for the phenotypic differences observed in the mentioned study (190). Furthermore, Torres Chavez et al. have also obtained similar results indicating that HPL-supplemented *ex vivo* culturing media support T cells to preserve their less differentiated cell phenotype resulting in their longstanding antitumor activity and persistence (191). Above all this, some researchers have demonstrated that treatment of mouse $CD8^+$ T cells with the S enantiomer of the 2-hydroxyglutarate (S-2HG) in *ex vivo* culture significantly enhances their *in vivo* expansion, persistence, and tumoricidal activity (192). Recently, the same group has indicated that S-2HG acts as an immune metabolite and helps clinical-grade allogeneic CAR-Ts maintain their central memory phenotype and exhibit enhanced antitumor activity (193).

3.5.3 *Ex Vivo* Culture Duration

According to the relationship between the state of T-cell differentiation and the potential for *in vivo* engraftment and persistence, some researchers have proposed that reducing the duration of *ex vivo* culturing can limit differentiation and significant loss of stemness and enhance the efficacy of CAR-T therapy (194). Ghassemi et al. have demonstrated that reduced culturing duration helps CAR-Ts have improved *ex vivo* expansion and effector function which are directly correlated with enhanced *in vivo* engraftment and tumoricidal activity (194). These researchers also demonstrated that these CAR-Ts eradicated human B-ALL in a murine xenograft model even at a 6-fold lower dose as compared with their counterparts expanded with the conventional *ex vivo* durations (194). In a nutshell, Ghassemi et al. demonstrated that the antileukemic activity of CAR-Ts is inversely correlated with their *ex vivo* culturing duration (194).

3.5.4 Other Factors

Upon activation, T cells steer their metabolism from fatty acid oxidation to glycolysis which helps them keep their effector function (195). It has been found that T cells with a memory-like phenotype have a low level of glucose uptake in comparison with T cells with an effector-like phenotype (195). Sukumar et al. have demonstrated that activating $CD8^+$ T cells in the presence of 2-deoxyglucose, which is a glycolysis pathway suppressor, can result in the formation of memory T cells (195). Moreover, other researchers have shown that pharmacologic inhibition of the serine/threonine kinase Akt promotes the expansion of TILs with transcriptional, metabolic, and functional features of memory T cells (196). Furthermore, Urak et al. also highlighted the Akt pathway involvement in T-cell differentiation state and memory phenotype formation (197). They demonstrated that the inhibition of Akt using an Akt inhibitor during *ex vivo* expansion of CAR-Ts leads to the production of CAR-Ts expressing higher levels of CD62L and CD28 as compared with CAR-Ts generated without Akt inhibition (197). Moreover, these researchers reported that *ex vivo* Akt-inhibited CAR-Ts exhibit superior antitumor functionality both *in vivo* and *in vitro* as compared with conventional CAR-Ts (197).

Lately, it has been found that the differentiation fate of $CD8^+$ T cells is regulated by the methylcytosine dioxygenase ten-eleven translocation 2 (TET2) (198, 199). TET2 loss results in the formation of memory-like phenotype in $CD8^+$ T cells without any impairment of their expansion or effector function (198, 199). Moreover, TET2-disrupted CAR-Ts have exhibited central memory phenotype at the expansion peak, which highlights the beneficial impact of TET2 suppression in CAR-Ts (198, 199). Furthermore, Kondo et al. have demonstrated that activated effector T cells can be converted into T_{SCM}-like (iT_{SCM}) cells by co-culturing with OP9 stromal cells expressing a Notch ligand such as Delta-like 1 (200–202). It has been found that using IL-7 and IL-15 can significantly be beneficial for the production of iT_{SCM} cells (201). These researchers also indicated that iT_{SCM} cells are tolerance-resistant alongside exhibiting robust engraftment, persistence, and tumoricidal activity *in vivo* (202). More recently, Kondo et al. reported that conventional human CAR-Ts can also be converted into iT_{SCM} CAR-Ts *via* Notch signaling-mediated mitochondrial metabolic reprogramming (203). Additionally, they found that Forkhead box M1 (FOXM1) is involved in the downstream signaling of the Notch pathway (203). FOXM1 is mainly responsible for the metabolic reprogramming of T cells and stem cell memory phenotype induction, and iT_{SCM} CAR-Ts generated using FOXM1 induction are as functional as iT_{SCM} CAR-Ts generated using Notch induction (203). In addition to the mentioned strategies, it is known that the 4-1BB signaling promotes T-cell central memory phenotype formation and preservation in CAR-Ts by promoting oxidative metabolism (204). Therefore, using the 4-1BB co-stimulatory domain in the construct of CAR-Ts helps preserve their central memory phenotype and *in vivo* expansion capacity (46).

4 CONCLUSION

CAR-T therapy will just be another unfulfilled promise without optimized efficacy and efficient toxicity management strategies. This type of therapy offers a new fighting tool with improved targeting capacity capable of fighting R/R B-ALL with durable outcomes (12–20). As outlined in this review, the unfavorable adverse events of B-ALL CAR-T therapy appear as the leading obstacle in the way of its broader therapeutic benefit; therefore, an ideal balance between safety and efficacy is critically required (59). Rapidly evolving research advances in genomic editing methods alongside a better understanding of the mechanisms underlying the discussed toxicities will help us further prevent or manage CRS and neurotoxicity. Moving forward, having a combination of different strategies or an all-in-one unified approach for producing off-the-shelf allogeneic CAR-Ts can decrease the duration of CAR-T production and delivery and eliminate other limitations regarding the generation of autologous CAR-Ts using T cells isolated from heavily treated R/R B-ALL patients (205). A magnificent deal of effort has now been dedicated to these strategies since they can be beneficial for CAR-T therapy in both hematologic malignancies and solid tumors. However, some of these strategies are still in their infancy, as others have been evaluated in preclinical and clinical studies. Furthermore, other aspects of CAR-T therapy can also be improved for having a better CAR-T persistence and antitumor activity by controlling the factors that affect the phenotype of the final T-cell product. Despite all this, the financial limitations and disease relapse mechanisms can still be named as other limitations of B-ALL CAR-T therapy requiring counterstrategies.

AUTHOR CONTRIBUTIONS

PouS: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Validation, Supervision. PooS: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Validation, Supervision. FR: Writing - review & editing, Validation. All authors contributed to the article and approved the submitted version.

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A Bibliometric and Knowledge-Map Analysis of CAR-T Cells From 2009 to 2021

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Objectives: A bibliometric and knowledge-map analysis is used to explore hotspots' evolution and development trends in the CAR-T cell field. By looking for research hotspots and new topics, we can provide new clues and ideas for researchers in this field.

Methods: The articles and reviews regarding CAR-T cells were retrieved and obtained from the Web of Science Core Collection (WOSCC) on October 28th, 2021. CtieSpace [version 5.8.R3 (64-bit)] and VOSviewer (version 1.6.17) were used to conduct the bibliometric and knowledge-map analysis.

Results: 660 authors from 488 institutions in 104 countries/regions published 6,867 papers in 1,212 academic journals. The United States was absolutely in the leading position in this research field. The institution that contributed the most publications was the University of Pennsylvania. Carl H June published the most articles, while Shannon L Maude had the most co-citations. However, there was little cooperation between countries. After 2012, cooperation among various institutions was also small. The journals that published the most CAR-T cell-related papers were *Frontiers in immunology* and *Cancers*. Nevertheless, *Blood* and *The New England Journal of Medicine* were the most commonly co-cited journals. The most influential research hotspots were the research of CAR-T cells in hematological malignancies, the related research of cytokine release syndrome (CRS), CD19, and the anti-tumor activity and efficacy of CAR-T cells. The latest hotspots and topics included the study of CAR-T cells in solid tumors, universal CAR-T cells, CAR-NK cells, CD22, and anakinra (the IL-1 receptor antagonist). The research of CAR-T cells in solid tumors was a rapidly developing hot field. Emerging topics in this field mainly included the study of CAR-T cells in glioblastoma (related targets: IL13Rα2, EGFRvIII, and HER2), neuroblastoma (related target: GD2), sarcoma (related target: HER2), and pancreatic cancer (related target: mesothelin), especially glioblastoma.

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Conclusion: As an anti-tumor therapy with great potential and clinical application prospects, CAR-T cell therapy is still in a stage of rapid development. The related field of CAR-T cells will remain a research hotspot in the future.

Keywords: CAR-T cell, Citespace, VOSviewer, bibliometric, knowledge-map, hotspots, topics

INTRODUCTION

CAR-T cell therapy has developed rapidly in recent years as promising adoptive immunotherapy. It is mainly used to research and treat malignant tumors, especially hematological malignant tumors, and has achieved stimulating clinical effects. In recent 20 years, this therapy has made significant progress in many aspects, mainly in the following aspects: a. CARs have been developed from the first generation to the fifth generation; b. The application of CAR-T cells has been gradually expanded the research and treatment of solid tumors. Compared with hematological malignancies, the biological characteristics of solid tumors are more complex so that CAR-T cells will face more obstacles and challenges in solid tumors (1); c. Improving the efficacy and/or safety of CAR-T cells, some special CARs have been developed based on traditional CARs, such as tandem CARs (2–5), syNotch CARs (6–8), inhibitory CARs (iCARs) (9), AND-NOT CARs (10), and ON/OFF-switch CARs (11–14). d. From single CAR-T cell therapy to combination therapy, for example, combined chemotherapy (15, 16), radiotherapy (17–19), or immune checkpoint inhibitors (20, 21).

As an anti-tumor therapy, the goal of CAR-T cell therapy is clinical transformation and clinical application. In recent years, there has been more and more basic research and clinical research related to CAR-T cells and more papers. Many scholars have reviewed this therapy from many aspects, including how to improve the efficacy and safety of CAR-T cells (22–24), the mechanism and management of related toxic reactions (25–28), the improvement and optimization of CAR structure (29, 30), the selection of targets (31, 32), the influence of TME on CAR-T cells (33, 34), and the research of CAR-T cells in hematological malignancies or solid tumors (35–38).

Bibliometrics is an interdisciplinary science that uses mathematical and statistical methods to analyze knowledge carriers, such as literature quantitatively. Through the comprehensive and objective analysis of most literature on a specific topic by bibliometrics, we can get some vital information. The information includes: a. the contribution of countries/regions, institutions, journals, and authors in this field; b. the collaboration between countries, institutions, or authors; c. the distribution of journals; d. the knowledge base (39–41). Therefore, bibliometrics can help researchers quickly understand a particular field, including research hotspots and evolving trends in this field, and avoid repeated research (42–44).

In this study, CiteSpace [version 5.8.R3 (64-bit)] and VOSviewer (version 1.6.17) were used to analyze the CAR-T cell-related literature and draw the scientific knowledge maps. This study aimed to explore the evolution and development trend of research hotspots in the CAR-T cell field from 2009 to 2021 and seek new hotspots and topics. It was hoped that this study would provide new clues and ideas for the subsequent study of CAR-T cells.

MATERIALS AND METHODS

Data Collection

The data was retrieved and downloaded from WoSCC (Lanzhou University Purchase Edition) on October 28th, 2021. We set the search formula: TS= (CAR-T OR CAR T cell OR CAR-T cell OR CAR T-cell OR CAR-T-cell OR chimeric antigen receptor T cell OR chimeric antigen receptor-T cell OR chimeric antigen receptor T-cell OR chimeric antigen receptor-modified T-cell OR chimeric antigen receptor-transduced T-cell OR chimeric antigen receptor-redirected T cell OR chimeric antigen receptor redirecting T-cell OR chimeric antigen receptor engineered T cell OR chimeric antigen receptor-engineered T-cell). The retrieval time range was from 1980 to October 28th, 2021, and the language was limited to English. The article type was limited to article or review. 7,806 papers (no duplicate) were obtained, including 4,862 articles and 2,944 reviews. Perhaps due to a large amount of data, some functions of CtieSpace [version 5.8.R3 (64-bit)] ran very slowly, so we narrowed the search scope to reduce some literature. We searched in the same way as above, limiting the time from January 1st, 2009 to October 28th, 2021. A total of 6867 papers (no duplicate) were obtained, including 3,980 articles and 2,887 reviews. Except for "The Annual Growth Trend of Publication Outputs", we used the first set of data (years:1980-2021) for analysis; for other studies, we adopted the second set of data (years: 2009-2021) to analyze. It should be mentioned that the second group of data is also significant, including 6867 papers (accounting for 88% of all papers). The retrieved papers were exported in the form of "Full Record and Cited References" and saved in "Plain Text". In addition, these files were named "download_.txt".

Data Analysis

Microsoft Office Excel 2010 was used to manage data and analyze annual publications. Besides, we also used CtieSpace [version 5.8.R3 (64-bit)] and VOSviewer (version 1.6.17) to analyze these data and draw scientific knowledge maps visually.

CtieSpace is a JAVA-based citation visualization software developed by Chaomei Chen, which provides an experimental platform for researching new ideas and comparing existing methods (45). It is one of the most commonly used visual analysis software in bibliometrics. It can analyze the potential literature from multiple angles, observe the research hotspots and trends in a specific field, and visually present them.

The knowledge-map can help researchers intuitively understand the research hotspots and evolution process and forecast the research and development trend of the field of interest (46).

VOSviewer is a free JAVA-based software for bibliometric mapping developed by Nees Jan van Eck and Ludo Waltman in 2009. It focuses more on the visualization of scientific knowledge (47). Moreover, VOSviewer has a powerful ability to handle large maps, which can display large bibliometric maps in an easily interpretable way (47).

RESULTS

The Annual Growth Trend of Publication Outputs

We could know the development trend by counting the CAR-T cells published every year. Setting the retrieval time range from 1980 to October 28th, 2021, we got 7806 papers related to CAR-T cells from WoSCC **(Annexes 1)**. As shown in **Figure 1**, the publications about CAR-T cells are increasing year by year. From 1990 to 2009, the output of publications in this period was meagre, and From 2009 to 2012, the number of relevant papers showed a slow upward trend. From 2012 to 2020, the number of related papers increased rapidly, of which 1,538 papers were published in 2020. By October 28th, 1,496 relevant papers had been published in 2021.

Furthermore, we adopted the second set of data (**Annexes 2**) to analyze. The reasons we had been mentioned in "*Data Collection*" above.

Countries/Regions and Institutions

A total of 488 institutions from 104 countries/regions co-authored 6,867 publications. As can be seen from **Table 1**, the country with the largest output of publications in the United States (n=3554, accounting for 51.8% of the total), followed by China (n=1253, 18.2%), Germany (n=703, 10.2%), England (n=405, 5.9%) and Italy (n=353, 5.14%). The number of publications from the United States far exceeded other countries. Among the top 10 countries, England, Italy, and France had higher centrality, 0.31, 0.54, and 0.4, respectively. It showed that these countries played a strong role as a bridge in the cooperation between countries. 90% of the top 10 countries with the most publications were developed countries. The institution that contributed the most publications was Univ Penn (n=419, 6.10%), followed by Mem Sloan Kettering Canc Ctr (n=299, 4.35%), Univ Texas MD Anderson Canc Ctr (n=263, 3.83%), NCI (n=213, 3.10%), and Univ Washington (n=208, 3.03%). It was worth noting that the top 10 institutions were all from the United States.

As shown in **Figure 2A**, the connection between countries is sparse, indicating that there is little cooperation between countries. From **Figure 2B**, we can see that the purple and gray connections are the most, which indicates that the most intensive years of inter-agency cooperation are 2012 and before 2012, and there is little inter-agency cooperation after 2012.

Journals and Co-Cited Journals

VOSviewer (version 1.6.17) and CtieSpace [version 5.8.R3 (64-bit)] were used to perform the co-citation and co-cited journal

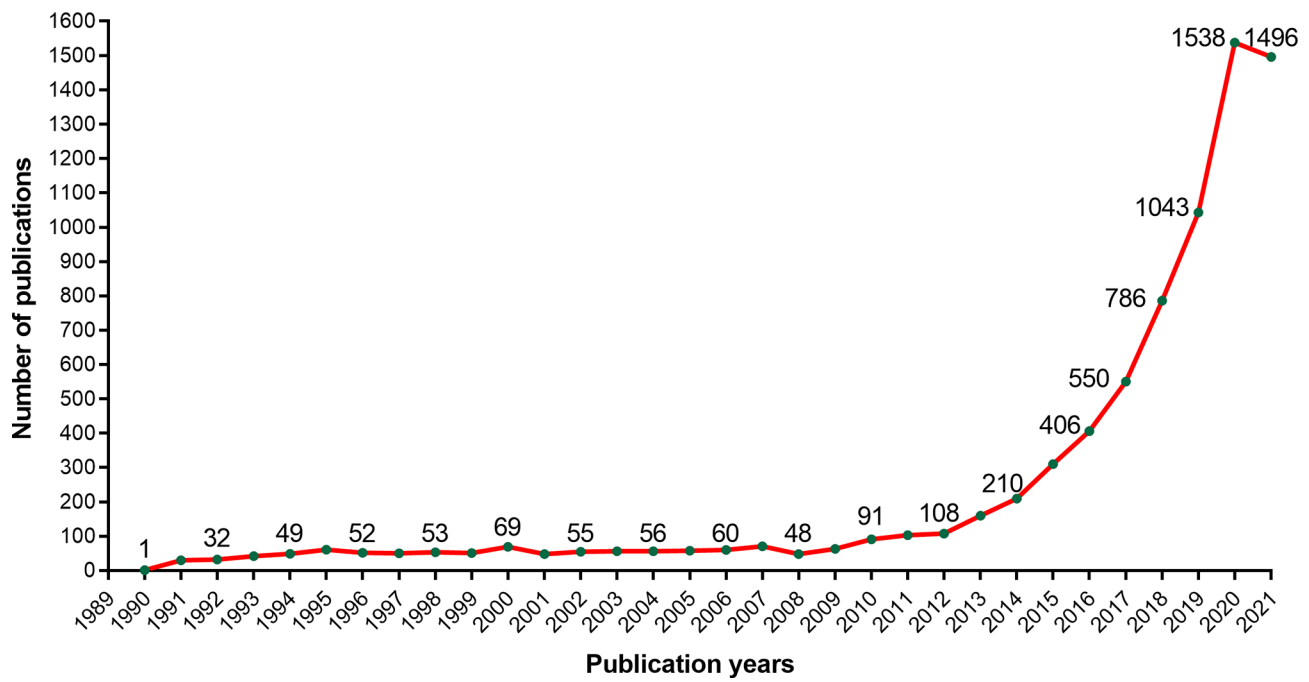

FIGURE 1 | The trend of publication outputs about CAR-T cells.

TABLE 1 | The top 10 countries/regions and institutions involved in CAR-T cells.

Rank	country/region	Count	Centrality	Year	Institution	Count	Centrality	Year
1	USA	3554	0.1	2009	Univ Penn (USA)	419	0.1	2009
2	PEOPLES R CHINA.	1253	0.03	2009	Mem Sloan Kettering Canc Ctr (USA)	299	0.03	2009
3	GERMANY.	703	0.16	2009	Univ Texas MD Anderson Canc Ctr (USA)	263	0.13	2009
4	ENGLAND.	405	0.31	2009	NCI (USA)	213	0.03	2009
5	ITALY.	353	0.54	2009	Univ Washington (USA)	208	0.02	2009
6	FRANCE.	260	0.4	2009	Harvard Med Sch (USA)	194	0	2016
7	JAPAN.	210	0.1	2009	Baylor Coll Med (USA)	168	0.03	2009
8	CANADA.	199	0.1	2010	Fred Hutchinson Canc Res Ctr (USA)	165	0.23	2009
9	AUSTRALIA.	173	0.03	2009	Stanford Univ (USA)	120	0.03	2009
10	SPAIN.	167	0.1	2009	Mayo Clin (USA)	108	0	2010

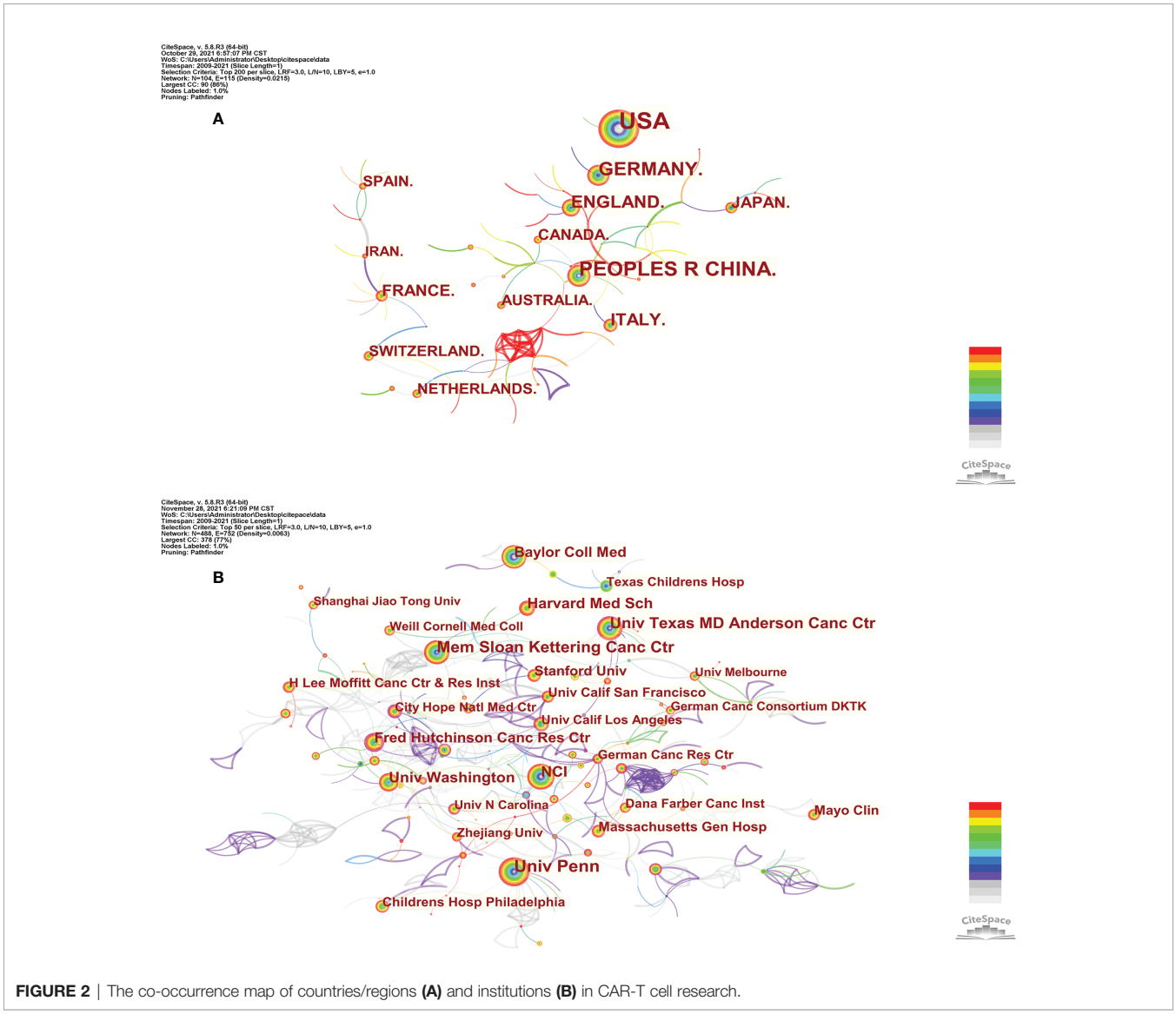

FIGURE 2 | The co-occurrence map of countries/regions **(A)** and institutions **(B)** in CAR-T cell research.

analysis, and finally found the journals with the most published papers and the journals with the most co-citations in this field. The results showed that 6867 papers were published in 1,212 academic journals. **Table 2** shows that the most published papers are *Frontiers in Immunology* (n=296), followed by *Cancers* (n=169), *Molecular Therapy* (n=137), *Blood* (n=134), and *Journal for Immunotherapy of Cancer* (n=118). Among the top 10 journals, eight had published more than 100 papers, and six were located in the Q1 Journal Citation Reports (JCR) region. The density map can well show the most published journals

TABLE 2 | Top 10 journals and co-cited journals related to CAR-T cells.

Rank	Journal	Count	IF(2020)	JCR(2020)	Co-cited journal	Citation	IF(2020)	JCR(2020)
1	Frontiers in immunology	296	7.561	Q2	Blood	46825	23.629	Q1
2	Cancers	169	6.639	Q2	New england journal of medicine	20112	91.253	Q1
3	Molecular therapy	137	11.454	Q1	Journal of clinical oncology	16127	44.544	Q1
4	Blood	134	23.629	Q1	Clinical cancer research	15253	12.531	Q1
5	Journal for immunotherapy of cancer	118	13.751	Q1	Journal of Immunology	13652	5.422	Q1
6	Frontiers in oncology	115	6.244	Q2	Cancer research	12180	12.701	Q1
7	International journal of molecular sciences	107	5.924	Q3	Molecular therapy	11821	11.454	Q1
8	Clinical cancer research	102	12.531	Q1	Proceedings of the national academy of sciences of the united states of america	10602	11.205	Q1
9	Oncoimmunology	97	8.110	Q1	Nature medicine	9392	53.440	Q1
10	Journal of hematology & oncology	87	17.388	Q1	Science	8934	47.728	Q1

IF, Impact Factor; JCR, Journal citation reports.

(**Figure 3A**). In addition, among these journals, the impact factor (IF) of *Blood* (IF=23.629) was highest.

As can be seen from **Table 2**, the most frequently cited journals are *Blood* (n=46825), followed by *New England Journal of Medicine* (n=20112), *Journal of Clinical Oncology* (n=16127), *Clinical Cancer Research* (n=15253), and *Journal of Immunology* (n=13562). Among the top 10 co-cited journals, 8 journals were cited more than 10,000 times, and the cited times of *Blood* far exceeded those of other journals. The density map can well show the most cited journals (**Figure 3B**). Among these journals, 9 journals had an impact factor greater than 11. Among them, the journals with the highest IF were the *New England Journal of Medicine* (IF=91.253), followed by *Nature Medicine* (IF=53.440), *Science* (IF=47.728), *Journal of Clinical Oncology* (IF=44.544), and *Blood* (IF=23.629).

The dual-map overlay of journals can well show the distribution of journals and the relationship between journals and cited journals (the color path represents the cited relationship) (48). **Figure 4** identifies four main reference paths. It indicated that papers published in "Molecular, Biology, Genetics" journals and "Health, Nursing, Medicine" journals were often cited in papers published in "Molecular, Biology, Immunology" journals and "Medicine, medical, Clinical" journals.

Authors and Co-Cited Authors

660 authors co-authored 6,867 publications. As shown in **Table 3**, the most published papers are Car H June (n=133), followed by Gianpietro Dotti (n=97), Stephan A Grupp (n=68), Michel Sadelain (n=62), and Stephen Gottschalk (n=55). The centrality of the top 10 authors was not high. Only Car H June (0.14) and Michel Sadelain (0.14) were more significant than 0.10. From **Figure 5**, we can see a certain degree of cooperation between different authors. Each circle represents one author, and the lines between circles represent cooperation among authors; thicker lines mean closer cooperation, and different colors represent different years.

Co-cited authors are two (or more) authors cited by one or more papers simultaneously. As shown in **Table 3**, the top 10 co-cited authors are cited more than 1000 times. The most frequently co-cited authors are Maude SL (n=2446), followed by Kochenderfer JN (n=1827), Lee DW (n=1729), Porter DL (n=1652) and Brentjens RJ (n=1421). Among the top 10 authors, there were 6 whose centrality exceeded 0.10, of which Brentjens RJ (0.64) was the highest. These co-cited authors with high centrality show annual purple rings in **Figure 6**, indicating that they have played an important role as a bridge.

Keyword Co-Occurrence, Clusters, and Evolution

We can know the research hotspots and directions in this field through the keyword co-occurrence. We extracted 15,542 keywords with VOSviewer. Since chimeric antigen receptor, chimeric antigen receptors, car, cars, chimeric-antigen-receptor, chimeric antigen receptor (car), and chimeric antigen receptors (cars) all meant the same thing, we merged these words. **Table 4** shows that the top 20 keywords appear more than 300 times. The most frequently occurring keywords are chimeric antigen receptor (n=2244), followed by immunotherapy (n=2008), therapy (n=1054), expression (n=958), cancer (n=831), adoptive immunotherapy (n=820) and anti-tumor activity (n=748). These keywords represent the hotspots of CAR-T cell-related research. The density map of keywords can intuitively display these high-frequency keywords (**Figure 7**).

We use VOSviewer to perform network clustering analysis on keywords (minimum number of occurrences of a keywors≥20). **Figure 8** shows a total of 6 clusters with different colors obtained, representing 6 research directions and research scopes. The largest cluster is cluster 1 (red), followed by cluster 2 (green), cluster 3 (blue), cluster 4 (yellow), cluster 5 (purple), and cluster 6 (light blue). There are 157 keywords in cluster 1, including immunotherapy, t-cells, phase-I, solid tumor, glioblastoma, breast cancer, lung cancer, growth-factor receptor, immune checkpoint inhibitors, tumor microenvironment, and dendritic cells. There are 152 keywords in cluster 2, including b-cell, CD19, cytokine release syndrome, neurotoxicity, acute lymphoblastic leukemia, lymphoma, multiple myeloma, management, survival, remissions, chemotherapy, and blinatumomab. There are 147 keywords in cluster 3, including therapy, expression, cancer, activation, lymphocytes, gene therapy, *in-vivo*, responses, differentiation, proliferation, memory, resistance, and cytokines. There are 74 keywords in cluster 4, including chimeric antigen receptor, adoptive immunotherapy, anti-tumor activity, anti-tumor efficacy, persistence, regression, adverse event, CD28

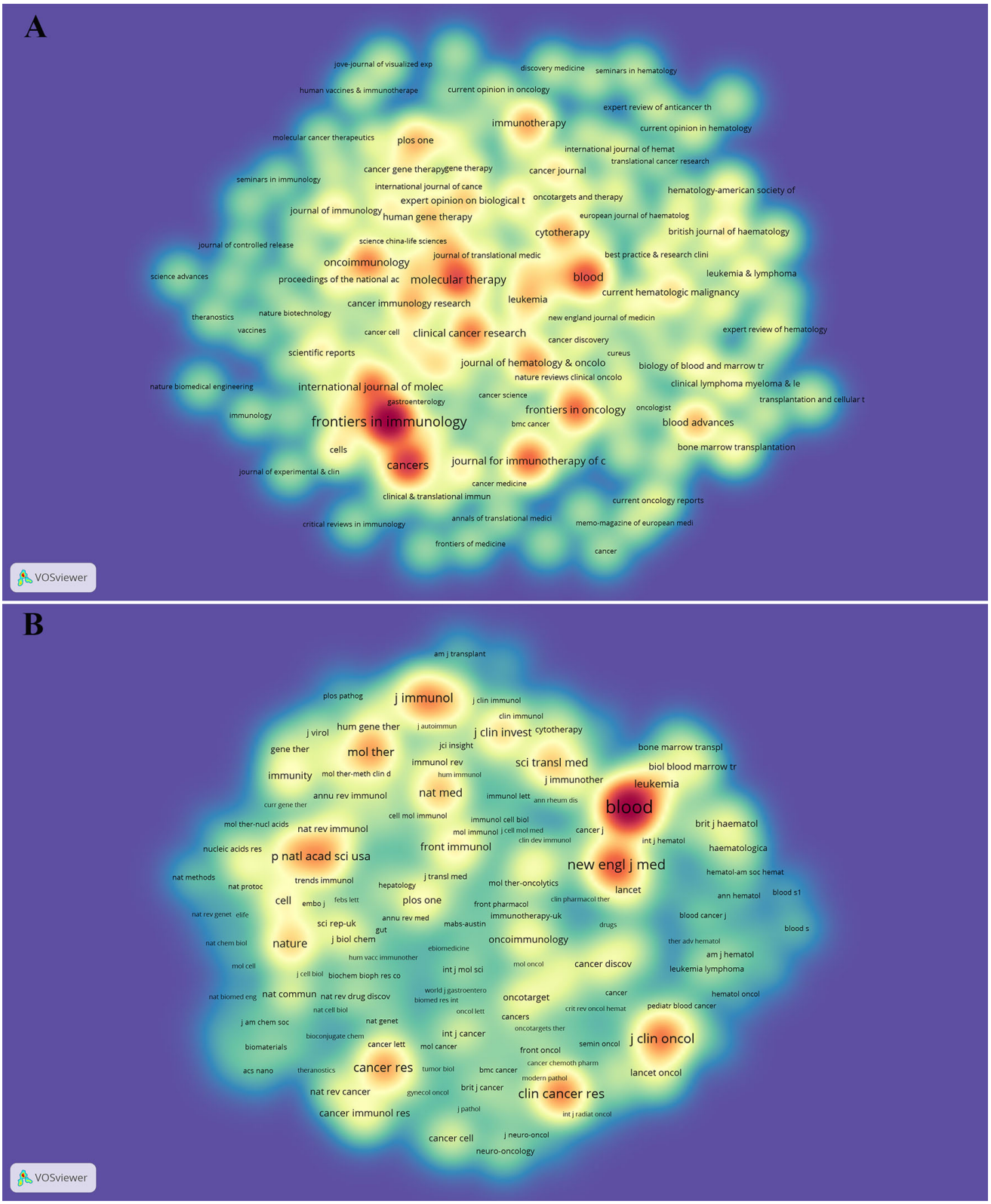

FIGURE 3 | The density map of journals **(A)** and co-cited journals **(B)** in CAR-T cell research. **(A)** shows journals with several publications ≥10; **(B)** shows the journals with citations ≥200.

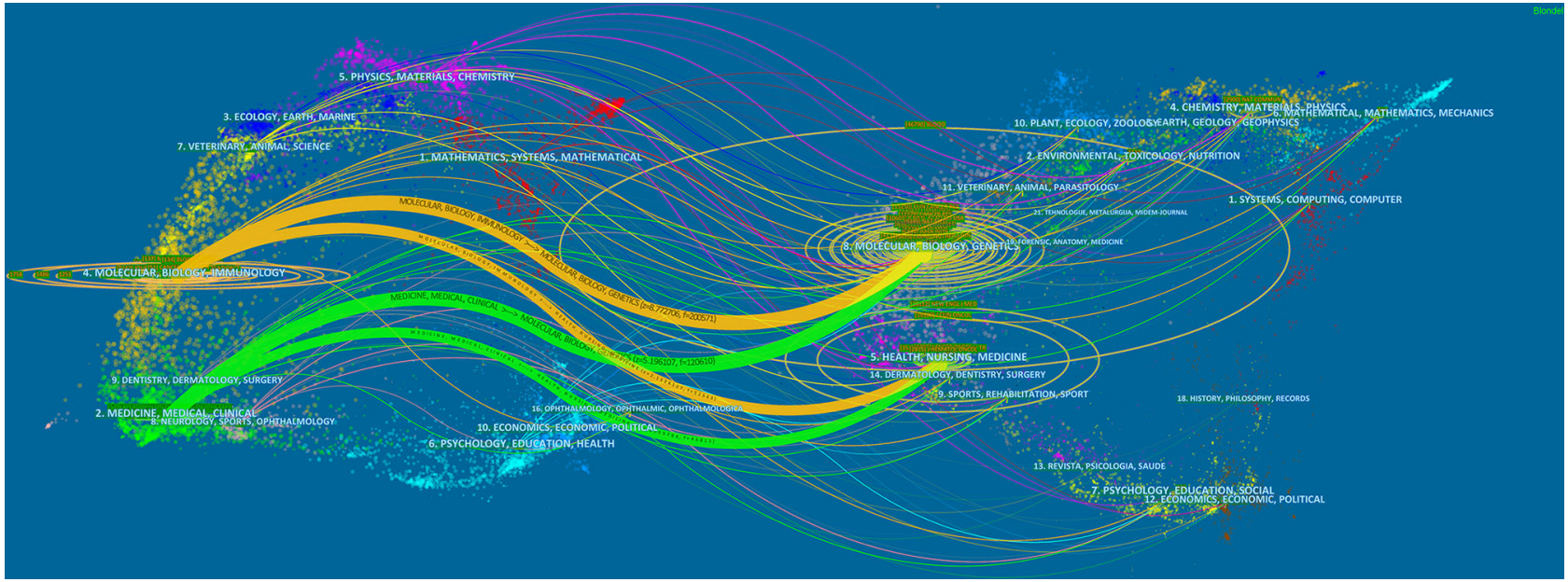

FIGURE 4 | The dual-map overlay of journals on CAR-T cells. Image parameter: a: 2; Source Circle Size: 25; Target Circle Size: 3; Snap to centroids (< Radius): 0. The citing journals are located on the left, and the cited journals are located on the right. The color paths (two orange and two green reference paths) represent the cited relationship.

TABLE 3 | The top 10 authors and co-cited authors of CAR-T cell research.

Rank	Author	Count	Centrality	Co-cited author	Citation	Centrality
1	CARL H JUNE	133	0.14	MAUDE SL	2446	0.55
2	GIANPIETRO DOTTI	97	0.01	KOCHENDERFER JN	1827	0.6
3	STEPHAN A GRUPP	68	0.03	LEE DW	1729	0.28
4	MICHEL SADELAIN	62	0.14	PORTER DL	1652	0
5	STEPHEN GOTTSCHALK	55	0.1	BRENTJENS RJ	1421	0.64
6	BARBARA SAVOLDO	54	0.07	NEELAPU SS	1369	0.1
7	RENIER J BRENTJENS	49	0.01	MORGAN RA	1359	0.3
8	HINRICH ABKEN	48	0.03	GRUPP SA	1312	0.62
9	LAURENCE J N COOPER	48	0	DAVILA ML	1189	0.04
10	MARCELA V MAUS	43	0.03	TURTLE CJ	1119	0.08

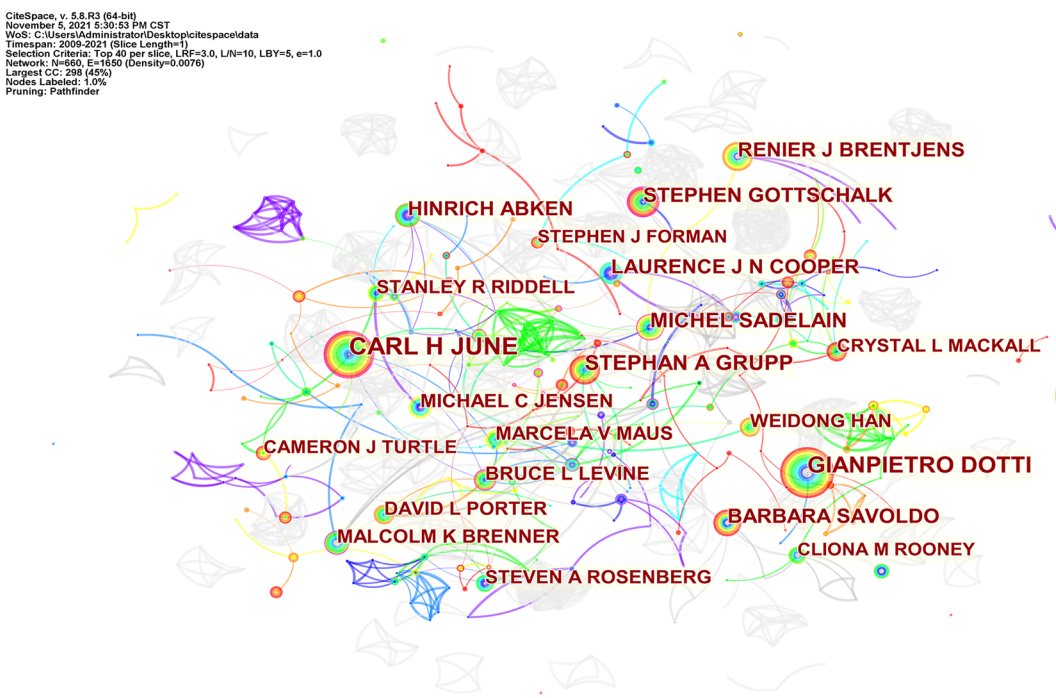

FIGURE 5 | CiteSpace visualization map of authors involved in CAR-T cells.

costimulation, 4-1BB costimulation, suicide gene, and safety switch. There are 42 keywords in cluster 5, including stem-cells, NK cells, *in-vitro*, messenger-RNA, NK-92 cells, modified-cells, and tumor cells. There is only one keyword (toxicity) in cluster 6.

We used CiteSpace to build a timeline viewer for these keywords. The timeline chart can cluster keywords and take time into account. Therefore, it can show the development of keywords in each cluster. Most importantly, it is convenient for us to see the period of a particular topic in a research field and help us explore the evolution track of this field. From **Figure 9**, we can intuitively see the research focus at each stage and evolution track of CAR-T cells.

Co-Cited References and Reference Burst

CiteSpace [version 5.8.R3 (64-bit)] was used to find the top 10 most co-cited references. **Table 5** consisted of three parts representing three fields; top 10 co-cited references related to CAR-T cells, top 10 co-cited references related to CAR-T cells for hematological malignancies, and top 10 co-cited references related to CAR-T cells for solid tumors. These 10 references (The first part of **Table 5**) were cited more than 590 times, among which the top 3 references were cited more than 1,000 times. Moreover, the top 3 references were all from *The New England journal of medicine*, and the first authors of the top 2 references were Shannon L Maude. The titles of these two references were "Chimeric antigen receptor T cells for sustained remissions in leukemia" (49) and "Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia" (50). It was worth noting that 50% of the top 10 co-cited references were from *The New England Journal of Medicine*. The references in the second part were consistent with the first part, mainly about the study of CD19-CAR-T cells

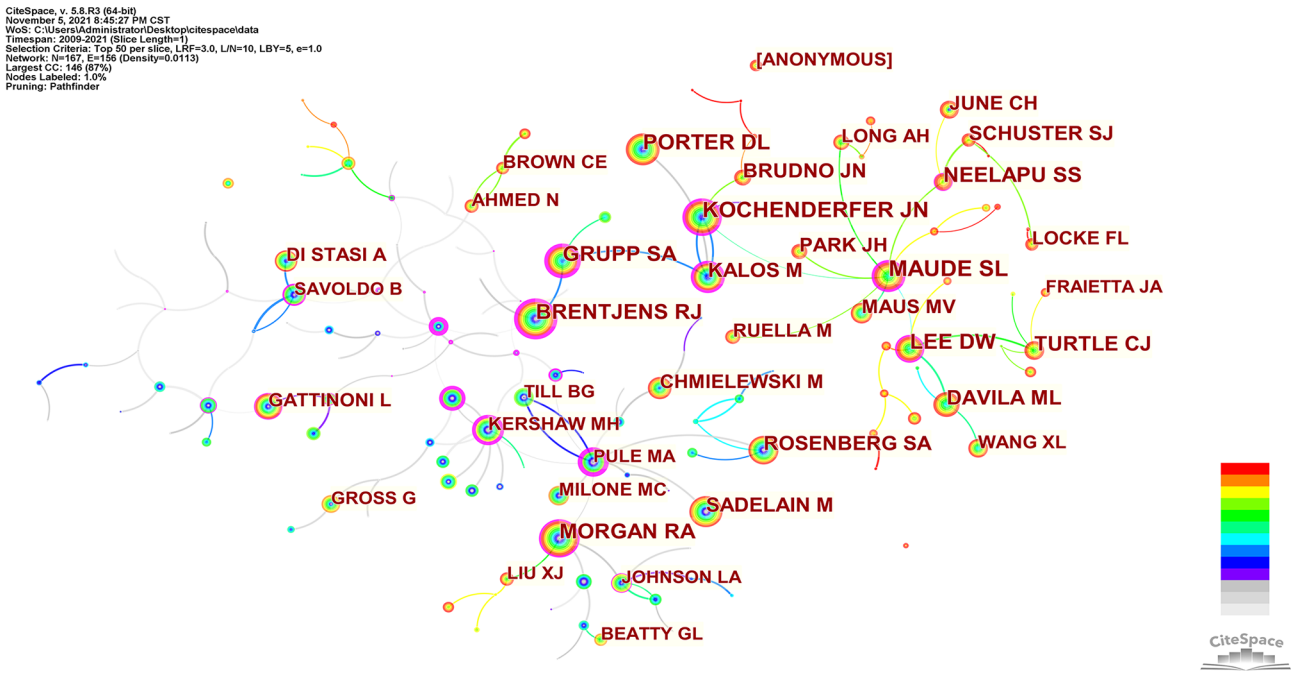

FIGURE 6 | CiteSpace visualization map of co-cited authors involved in CAR-T cells.

TABLE 4 | The top 20 keywords related to CAR-T cells.

Rank	Keyword	Count	Rank	Keyword	Count
1	chimeric antigen receptor	2244	11	b-cell	526
2	immunotherapy	2008	12	cytokine release syndrome	407
3	therapy	1054	13	acute lymphoblastic-leukemia	404
4	expression	958	14	in-vivo	381
5	cancer	831	15	lymphoma	379
6	adoptive immunotherapy	820	16	phase-I	373
7	antitumor-activity	748	17	natural-killer-cells	364
8	t-cells	721	18	CD19	357
9	activation	570	19	dendritic cells	327
10	lymphocytes	563	20	efficacy	326

in hematological malignancies. The third part was the top 10 co-cited references related to CAR-T cells for solid tumors (only including solid tumor-related studies). From this part, we could see that 5 of these 10 papers were about the research of CAR-T cells in brain tumors (glioblastoma and neuroblastoma).

We used CiteSpace (Selection Criteria: Top50; The Number of States: 2; Minimum Duration: 2) to obtain 157 references with the most robust citation bursts for CAR-T cells. **Figure 10** shows the top 50 among them. The titles of top 3 references with the most vigorous citation bursts were "Chimeric Antigen Receptor-Modified T Cells in Chronic Lymphoid Leukemia (70)" (Strength: 222.69; Publication Year: 2011), "Chimeric Antigen Receptor T Cells for Sustained Remissions in Leukemia (49)" (Strength: 219.59; Publication Year: 2014) and "Chimeric Antigen Receptor–Modified T Cells for Acute Lymphoid Leukemia (54)" (Strength: 201.96; Publication Year: 2013). These three references were all from *The New England Journal of Medicine*. Notably, 13 references (26%; Publication Year: 2017 - 2018) in the Top50 were in a citation burst. The citation bursts of 43 references (86%) were from 2011 to 2021. That is, these references were frequently cited within 10 years. All these mean that CAR-T cell-related research fields may continue to receive attention in the future. In addition, we constructed two additional figures, namely "Top 50 References with the Strongest Citation Bursts for CAR-T cells (hematological malignancies)" **(Annexes 3)** and "Top 50 References with the Strongest Citation Bursts for CAR-T cells (solid tumors)" **(Annexes 4)**. Using these two figures, we drew two corresponding tables **(Annexes 5**, **6)** to introduce these references in the state of citation burst. As seen from **Annexes 3, 5**, there are 11 articles in the 14 papers. These articles are mainly about the research of CAR-T cells in hematological malignancies, including efficacy, safety, mechanism and management of related toxicity, and prognosis. The targets involved in these articles are mainly CD19, followed

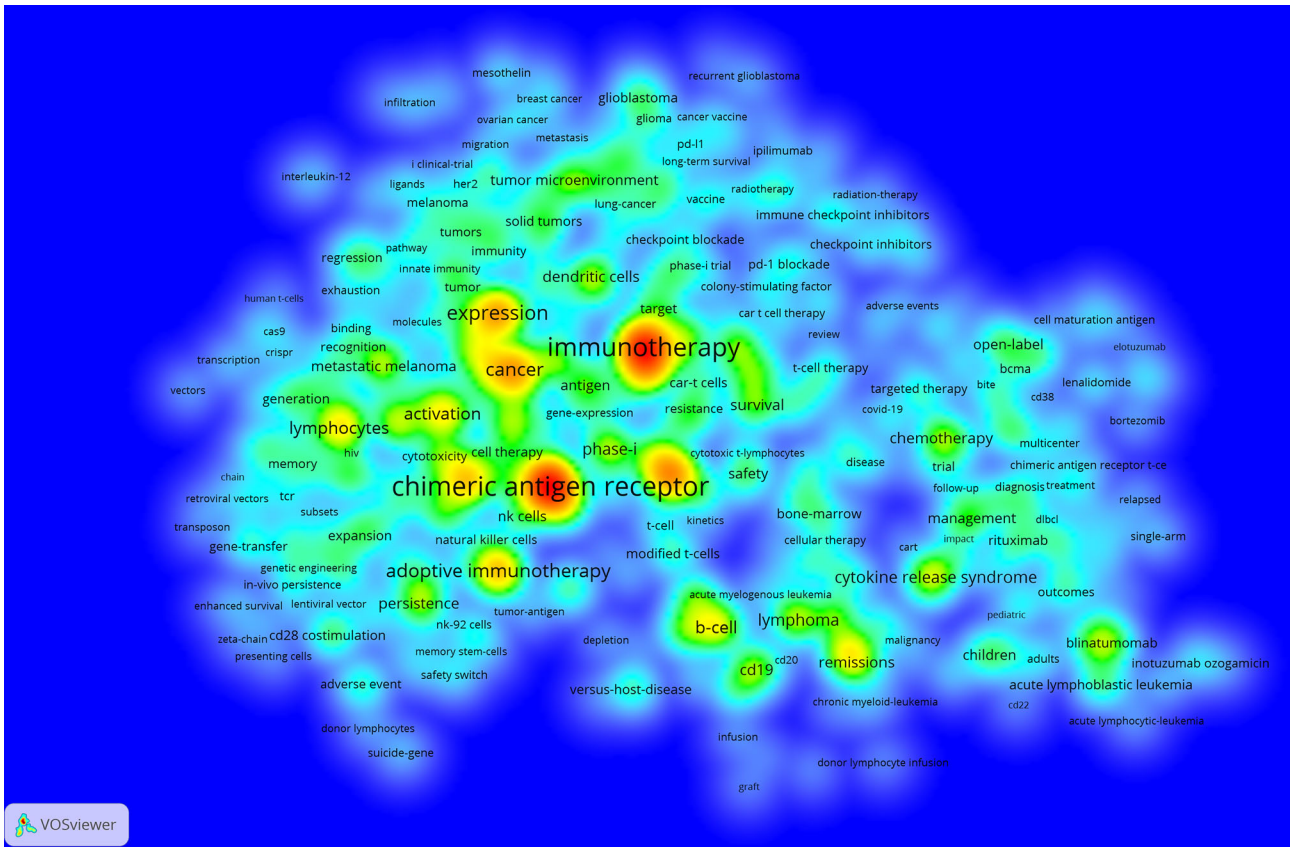

FIGURE 7 | The co-occurrence density map of keywords related to CAR-T cell research. Minimum number of occurrences of keywords ≥20.

by CD22. It can be seen from **Annexes 4, 6** that 10 of the 17 articles are about the research of CAR-T cells in hematological malignancies. It shows that the research of CAR-T cells in hematological malignancies greatly influences the research of CAR-T cells in solid tumors. Moreover, 7 articals are about the research of CAR-T cells in solid tumors, which represent some emerging topics in this field. The solid tumors involved in these articles mainly include glioblastoma (related targets: IL13Rα2, EGFRvIII, and HER2), neuroblastoma (related target: GD2), sarcoma (related target: HER2), and pancreatic cancer (related target: mesothelin), especially glioblastoma.

DISCUSSION

General Information

Through statistics on CAR-T cell papers published every year, we can understand the development trend of this field. CAR-T cells were first proposed and designed by Israeli scientists Eshhar et al. in 1989. At that time, they called the" "chimeric antigen receptor" as "chimeric T-cell receiver (cTCR)" (71, 72). From **Figure 1**, we can see that the annual growth trend of publication outputs related to CAR-T cells is generally on the rise. It can be divided into three stages, including a continuous period (1990-2009), a slow growth period (2009-2012), and a rapid growth period (2012-2020). From 1990 to 2009, the annual number of publications was minimal, indicating that researchers ignored this field, mainly because the technology related to CAR-T cells is still immature. From 2009 to 2012, papers showed slow growth, indicating that this field received attention. After 2012, the number of papers in this field increased every year, which indicates that this field began to receive extensive attention in this period. Since 2012, the CAR-T cell field entered a period of rapid development, which may be mainly due to three events. The first event was that 7-year-old Emily Whitehead, with acute lymphoblastic leukemia (ALL), became the first child patient in the world to receive CAR-T cell therapy. Encouragingly, Emily got complete remission after receiving CD19-CAR-T cell therapy (54). On December 9th, 2013, the second event was that Science selected the top 10 scientific breakthroughs in 2013, and "cancer immunotherapy" ranked first. The third event was a clinical trial in 2014, in which 30 relapsed ALL patients (children and adults) received CD19-CAR-T cells, and the results showed that 90% of these patients achieved complete remission (49). These three major events aroused scientists' enthusiasm for CAR-T cell therapy, which greatly promoted the development of this field. Additionally, as of October 28, 1,496 related papers have been published in 2021. There are still two months left before 2022.

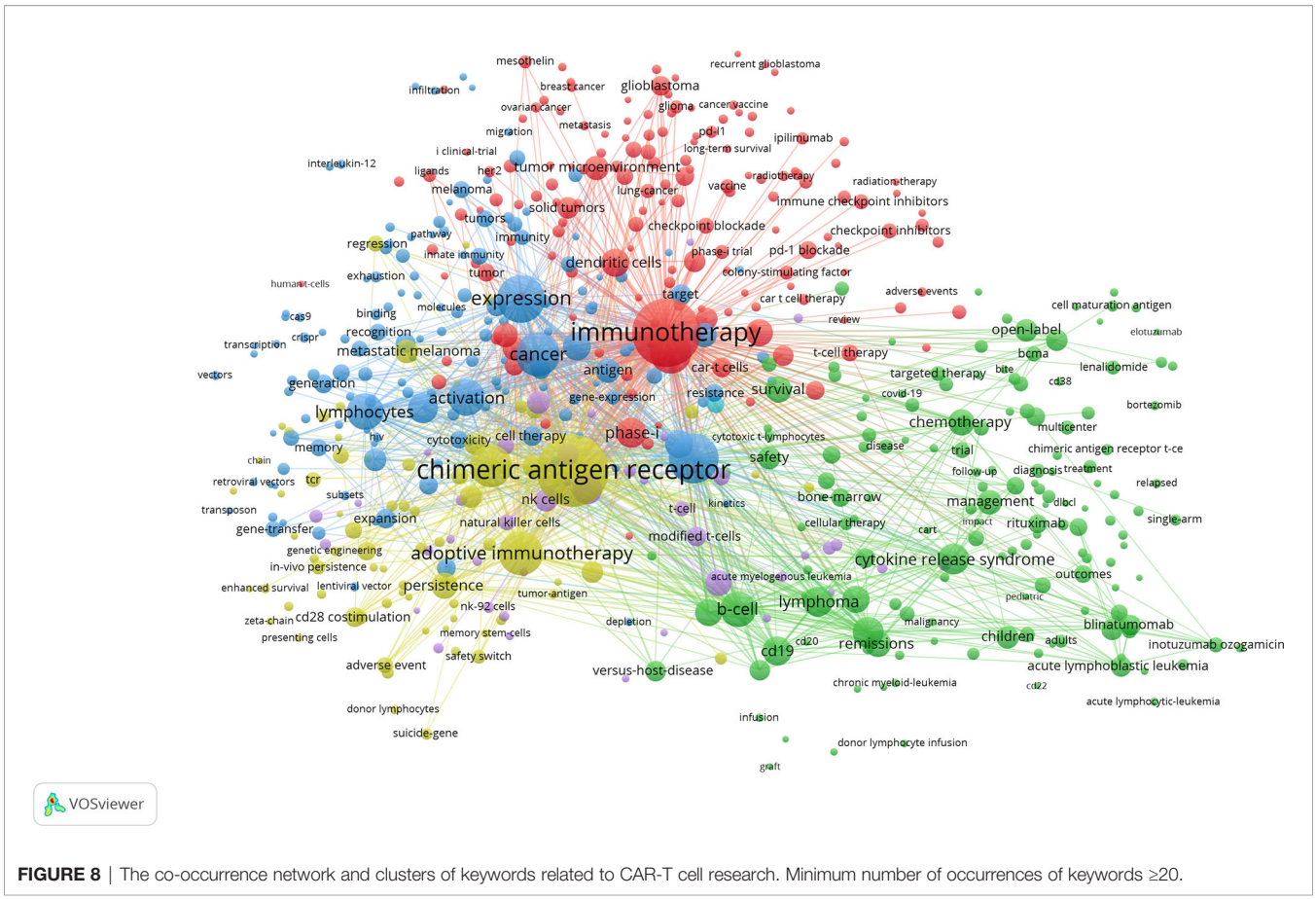

FIGURE 8 | The co-occurrence network and clusters of keywords related to CAR-T cell research. Minimum number of occurrences of keywords ≥20.

According to the current trend, publication output in 2021 will likely exceed that in 2020 and continue to grow positively. It proves that the field of CAR-T cells will continue to receive attention in the future.

This field is also very characteristic in the distribution of countries and institutions. From **Table 1** and **Figure 2A**, the country with the most publications in United States (n=3,554, accounting for 51.8% of the total), followed by China (n=1,253, 18.2%) and Germany (n=703, 10.2%). Moreover, the top 10 institutions were all from the United States, and the institution with the largest output of publications is Univ Penn (n=419, 6.10%). Not only that, so far, five CAR-T cell therapies have been approved by Food And Drug Administration (FDA) for marketing in the United States. All these indicate that the United States is the most influential country in CAR-T cells, and the research in this field is far ahead of other countries. China follows it. In recent years, research in this field in China has also increased significantly (73). From **Table 1**, we can also find an interesting phenomenon that 90% of the top 10 countries with the largest publication outputs are developed countries. The main reason for this phenomenon is that the research and development (R&D) of CAR-T cell-related technology needs a large amount of financial support (74). Besides, CAR-T cell therapy is also an expensive treatment. For example, the cost of treating non-Hodgkin lymphoma (NHL) with Kymriah (tisagenlecleucel, CTL019) or Yescarta (axicabtagene ciloleucel, axi-cel) is about $373,000; The cost of Kymriah in treating ALL is approximately $475,000 (75). High R&D costs and clinical use costs limit the clinical promotion and application of this therapy to some extent. Reducing these costs and making the price of CAR-T cell therapy more affordable is also a difficult question that we are facing (76, 77). As shown in **Figure 2A**, the connection between countries is sparse, indicating little cooperation between countries. From **Figure 2B**, we can see that the purple and gray connections are the most, which indicates that the most intensive years of inter-agency cooperation are 2012 and before 2012, and there is little inter-agency cooperation after 2012. We call for strengthening the exchanges and cooperation between countries and institutions in this field to better promote the development of this field and benefit more cancer patients.

Journals and Co-cited Journals Analysis (**Table 2** and **Figure 3**) showed that the journals that published the most CAR-T cell-related papers were *Frontiers in immunology* (n=296) and *Cancers* (n=169). *Blood* (n=46825) and *The New England Journal of Medicine* (n=20112) were frequently co-cited. Among the top 10 journals with published papers, 6 journals were located in the Q1 JCR region, of which the highest IF was Blood (IF=23.629). The top 10 journals with co-cited times were located in the Q1 JCR region. 9 journals had IF greater than 11,

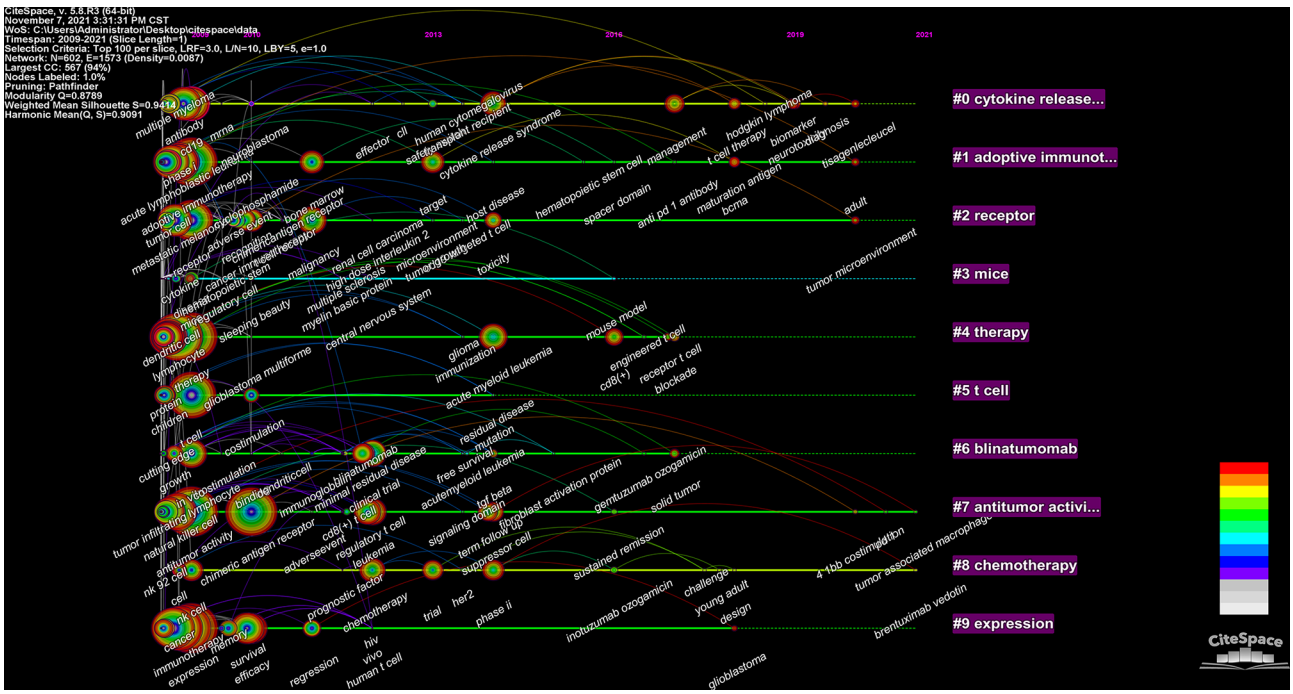

FIGURE 9 | CiteSpace visualization map of timeline viewer related to CAR-T cells.

and 4 had IF greater than 44. The journal with the highest IF was *The New England Journal of Medicine* (IF=91.253). These indicate that many high-quality and high-impact journals are very interested in CAR-T cell-related research. **Figure 4** shows that papers published in " Molecular, Biology, Genetics " journals and " Health, Nursing, Medicine " journals are often cited in papers published in " Molecular, Biology, Immunology " journals and " Medicine, medical, Clinical " journals. It means that the current research related to CAR-T cells mainly focuses on basic research and translational medicine.

In our analysis (**Table 3**, **Figure 5**, and **Figure 6**), Carl H June (n=133) published the most papers, while Shannon L Maude had the most co-citations (n=2446). Among the top 10 co-cited authors, 6 authors played an essential role as a bridge (centrality>0.1). It should be mentioned that the top 10 authors with the most papers had little cooperation with each other; the top 10 authors with the highest number of citations also had little cooperation.

Knowledge Base

Co-citation is a research method to measure the degree of relevance between papers. The knowledge base is a collection of co-cited references (46). In this study, a total of 10 papers related to the field of CAR-T cells were included, which were co-cited most frequently (the first part of **Table 5**), as follows:

Maude et al. published "Chimeric antigen receptor T cells for sustained remissions in leukemia (49)" in *The New England Journal of Medicine* in 2014, which was the most cited paper (1113 citations). This study was a phase I/IIa clinical trial in which 30 relapsed ALL patients (children and adults) received CD19-CAR-T cells (CTL019). The experimental results showed that 27 patients (90%) achieved complete remission, and 19 observed sustained remission for 2 years. In addition, all patients developed cytokine release syndrome (CRS), and CRS could be effectively improved by toxoximab, an IL-6 receptor inhibitor (49). The second co-cited paper, "Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia", was published by Maude et al. (50) in *The New England Journal of Medicine* in 2018. In this phase II clinical trial, 75 patients with CD19+ relapsed or refractory B-cell all received tisagenlecleucel (Kymriah, the first CAR-T cell therapy in the world). The results showed that (50), the total remission rate of these patients was 81%; the 6-month and 12-month overall survival rate were 90% and 76%, respectively; 55 patients (73%) had a grade 3 or 4 tisagenlecleucel-related adverse event. The third co-cited paper, "Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma" was published by Neelapu et al. (51) in *The New England Journal of Medicine* in 2017. In this phase II clinical trial, 101 patients with CD19+ refractory large B-cell lymphoma received axicabtage ciloleucel (Yescarta, the second CAR-T cell therapy in the world). The results showed that (51) the objective response rate (ORR) and complete response rate (CRR) were 82% and 54%, respectively. The 18-month overall survival rate was 52%. The most common adverse events of grade 3 or higher were neutropenia (78%), anemia (43%), and thrombocytopenia (38%). CRS (grade 1~4) occurred in 94 patients (93%) and neurological events (grade 1~4) occurred in 65 patients (64%). The fourth co-cited paper was published by

TABLE 5 | The top 10 co-cited references related to the CAR-T cell field.

Top 10 co-cited references related to CAR-T cells

Rank	Year	Author	Title	Journal	Citation	Centrality
1	2014	Shannon L Maude et al. (49)	Chimeric antigen receptor T cells for sustained remissions in leukemia	N Engl J Med	1113	0.06
2	2018	Shannon L Maude et al. (50)	Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia	N Engl J Med	1065	0.35
3	2017	Sattva S Neelapu et al. (51)	Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma	N Engl J Med	1056	0.25
4	2015	Daniel W Lee et al. (52)	T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial	Lancet	987	0.47
5	2014	Marco L Davila et al. (53)	Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia	Sci Transl Med	743	0.54
6	2013	Stephan A Grupp et al. (54)	Chimeric antigen receptor-modified T cells for acute lymphoid leukemia	N Engl J Med	737	0.57
7	2016	Cameron J Turtle et al. (55)	CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients	J Clin Invest	729	0.03
8	2018	Jae H Park et al. (56)	Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia	N Engl J Med	649	0.35
9	2015	James N Kochenderfer et al. (57)	Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor	J Clin Oncol	626	0.05
10	2013	Renier J Brentjens et al. (58)	CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia	Sci Transl Med	599	0.00

Top 10 co-cited references related to CAR-T cells for hematological malignancies

Rank	Year	Author	Title	Journal	Citation	Centrality
1	2017	Sattva S Neelapu et al. (51)	Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma	N Engl J Med	405	0.07
2	2014	Shannon L Maude et al. (49)	Chimeric antigen receptor T cells for sustained remissions in leukemia	N Engl J Med	389	0
3	2018	Shannon L Maude et al. (50)	Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia	N Engl J Med	386	0.19
4	2015	Daniel W Lee et al. (52)	T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial	Lancet	382	0.23
5	2016	Cameron J Turtle et al. (55)	CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients	J Clin Invest	291	0.01
6	2014	Marco L Davila et al. (53)	Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia	Sci Transl Med	265	0.45
7	2019	Stephen J Schuster et al. (59)	Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma	N Engl J Med	260	0.05
8	2018	Jae H Park et al. (56)	Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia	N Engl J Med	256	0.19
9	2013	Stephan A Grupp et al. (54)	Chimeric antigen receptor-modified T cells for acute lymphoid leukemia	N Engl J Med	251	0.13
10	2015	James N Kochenderfer et al. (57)	Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor	J Clin Oncol	233	0.02

Top 10 co-cited references related to CAR-T cells for solid tumors(only including solid tumor-related studies)

Rank	Year	Author	Title	Journal	Citation	Centrality
1	2016	Christine E Brown et al. (60)	Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy	N Engl J Med	134	0.01
2	2017	Donald M O'Rourke et al. (61)	A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma	Sci Transl Med	120	0.01
3	2015	Nabil Ahmed et al. (62)	Human Epidermal Growth Factor Receptor 2 (HER2) -Specific Chimeric Antigen Receptor-Modified T Cells for the Immunotherapy of HER2-Positive Sarcoma	J Clin Oncol	90	0.03
4	2017	Nabil Ahmed et al. (63)	HER2-Specific Chimeric Antigen Receptor-Modified Virus-Specific T Cells for Progressive Glioblastoma: A Phase 1 Dose-Escalation Trial	JAMA Oncol	71	0.01
5	2016	Leonid Cherkassky et al. (64)	Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition	J Clin Invest	68	0.01
6	2010	Richard A Morgan et al. (65)	Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2	Mol Ther	59	0.09
7	2014	Gregory L Beatty et al. (66)	Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies	Cancer Immunol Res	59	0.02
8	2018	Sarwish Rafiq et al. (67)	Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy *in vivo*	Nat Biotechnol	48	0.01
9	2011	Chrystal U Louis et al. (68)	Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma	Blood	45	0.09
10	2015	Christine E Brown et al. (69)	Bioactivity and Safety of IL13Rα2-Redirected Chimeric Antigen Receptor CD8+ T Cells in Patients with Recurrent Glioblastoma	Clin Cancer Res	43	0

Top 50 References with the Strongest Citation Bursts

References	Year	Strength	Begin	End	2009 - 2021
Pule MA, 2008, NAT MED, V14, P1264, DOI 10.1038/nm.1882, DOI	2008	80.49	2009	2013	
Milone MC, 2009, MOL THER, V17, P1453, DOI 10.1038/mt.2009.83, DOI	2009	74.02	2009	2014	
Till BG, 2008, BLOOD, V112, P2261, DOI 10.1182/blood-2007-12-128843, DOI	2008	70.9	2009	2013	
Carpenito C, 2009, P NATL ACAD SCI USA, V106, P3360, DOI 10.1073/pnas.0813101106, DOI	2009	63	2009	2014	
Sadelain M, 2009, CURR OPIN IMMUNOL, V21, P215, DOI 10.1016/j.coi.2009.02.009, DOI	2009	49.8	2009	2014	
Johnson LA, 2009, BLOOD, V114, P535, DOI 10.1182/blood-2009-03-211714, DOI	2009	55.34	2010	2014	
Brentjens R, 2010, MOL THER, V18, P666, DOI 10.1038/mt.2010.31, DOI	2010	52.53	2010	2014	
Morgan RA, 2010, MOL THER, V18, P843, DOI 10.1038/mt.2010.24, DOI	2010	117.03	2011	2015	
Kochenderfer JN, 2010, BLOOD, V116, P4099, DOI 10.1182/blood-2010-04-281931, DOI	2010	96.38	2011	2015	
Jensen MC, 2010, BIOL BLOOD MARROW TR, V16, P1245, DOI 10.1016/j.bbmt.2010.03.014, DOI	2010	50.57	2011	2015	
Porter DL, 2011, NEW ENGL J MED, V365, P725, DOI 10.1056/NEJMoa1103849, DOI	2011	222.69	2012	2016	
Kalos M, 2011, SCI TRANSL MED, V3, P0, DOI 10.1126/scitranslmed.3002842, DOI	2011	176.13	2012	2016	
Kochenderfer JN, 2012, BLOOD, V119, P2709, DOI 10.1182/blood-2011-10-384388, DOI	2012	146.4	2012	2017	
Brentjens RJ, 2011, BLOOD, V118, P4817, DOI 10.1182/blood-2011-04-348540, DOI	2011	124.56	2012	2016	
Savoldo B, 2011, J CLIN INVEST, V121, P1822, DOI 10.1172/JCI46110, DOI	2011	109.78	2012	2016	
Di Stasi A, 2011, NEW ENGL J MED, V365, P1673, DOI 10.1056/NEJMoa1106152, DOI	2011	87.65	2012	2016	
Louis CU, 2011, BLOOD, V118, P6050, DOI 10.1182/blood-2011-05-354449, DOI	2011	69.29	2012	2016	
Robbins PF, 2011, J CLIN ONCOL, V29, P917, DOI 10.1200/JCO.2010.32.2537, DOI	2011	62.59	2012	2016	
Till BG, 2012, BLOOD, V119, P3940, DOI 10.1182/blood-2011-10-387969, DOI	2012	59.4	2013	2017	
Topalian SL, 2012, NEW ENGL J MED, V366, P2443, DOI 10.1056/NEJMoa1200690, DOI	2012	51.56	2013	2017	
Pegram HJ, 2012, BLOOD, V119, P4133, DOI 10.1182/blood-2011-12-400044, DOI	2012	48.58	2013	2017	
Grupp SA, 2013, NEW ENGL J MED, V368, P1509, DOI 10.1056/NEJMoa1215134, DOI	2013	201.96	2014	2018	
Brentjens RJ, 2013, SCI TRANSL MED, V5, P0, DOI 10.1126/scitranslmed.3005930, DOI	2013	159.4	2014	2018	
Sadelain M, 2013, CANCER DISCOV, V3, P388, DOI 10.1158/2159-8290.CD-12-0548, DOI	2013	72.31	2014	2018	
Kochenderfer JN, 2013, BLOOD, V122, P4129, DOI 10.1182/blood-2013-08-519413, DOI	2013	63.8	2014	2017	
Kloss CC, 2013, NAT BIOTECHNOL, V31, P71, DOI 10.1038/nbt.2459, DOI	2013	61.36	2014	2018	
Cruz CRY, 2013, BLOOD, V122, P2965, DOI 10.1182/blood-2013-06-506741, DOI	2013	60.94	2014	2017	
Lamers CHJ, 2013, MOL THER, V21, P904, DOI 10.1038/mt.2013.17, DOI	2013	53.59	2014	2017	
Haso W, 2013, BLOOD, V121, P1165, DOI 10.1182/blood-2012-06-438002, DOI	2013	51.14	2014	2017	
Morgan RA, 2013, J IMMUNOTHER, V36, P133, DOI 10.1097/CJI.0b013e3182829903, DOI	2013	50.73	2014	2017	
Davila ML, 2014, SCI TRANSL MED, V6, P0, DOI 10.1126/scitranslmed.3008226, DOI	2014	126.55	2015	2019	
Maus MV, 2014, BLOOD, V123, P2625, DOI 10.1182/blood-2013-11-492231, DOI	2014	57.18	2015	2017	
Fedorov VD, 2013, SCI TRANSL MED, V5, P0, DOI 10.1126/scitranslmed.3006597, DOI	2013	49.89	2015	2018	
Maude SL, 2014, NEW ENGL J MED, V371, P1507, DOI 10.1056/NEJMoa1407222, DOI	2014	219.59	2016	2019	
Lee DW, 2014, BLOOD, V124, P188, DOI 10.1182/blood-2014-05-552729, DOI	2014	68.93	2016	2019	
Lee DW, 2015, LANCET, V385, P517, DOI 10.1016/S0140-6736(14)61403-3, DOI	2015	68.65	2016	2018	
Kochenderfer JN, 2015, J CLIN ONCOL, V33, P540, DOI 10.1200/JCO.2014.56.2025, DOI	2015	63.96	2016	2018	
Qasim W, 2017, SCI TRANSL MED, V9, P0, DOI 10.1126/scitranslmed.aaj2013, DOI	2017	49.75	2018	2021	
Neelapu SS, 2017, NEW ENGL J MED, V377, P2531, DOI 10.1056/NEJMoa1707447, DOI	2017	157.38	2019	2021	
Maude SL, 2018, NEW ENGL J MED, V378, P439, DOI 10.1056/NEJMoa1709866, DOI	2018	157.17	2019	2021	
Park JH, 2018, NEW ENGL J MED, V378, P449, DOI 10.1056/NEJMoa1709919, DOI	2018	86.37	2019	2021	
June CH, 2018, NEW ENGL J MED, V379, P64, DOI 10.1056/NEJMra1706169, DOI	2018	73.72	2019	2021	
June CH, 2018, SCIENCE, V359, P1361, DOI 10.1126/science.aar6711, DOI	2018	69.11	2019	2021	
Fraietta JA, 2018, NAT MED, V24, P563, DOI 10.1038/s41591-018-0010-1, DOI	2018	63.48	2019	2021	
Neelapu SS, 2018, NAT REV CLIN ONCOL, V15, P47, DOI 10.1038/nrclinonc.2017.148, DOI	2018	63.28	2019	2021	
Norelli M, 2018, NAT MED, V24, P739, DOI 10.1038/s41591-018-0036-4, DOI	2018	59.1	2019	2021	
Hay KA, 2017, BLOOD, V130, P2295, DOI 10.1182/blood-2017-06-793141, DOI	2017	53.28	2019	2021	
Fry TJ, 2018, NAT MED, V24, P20, DOI 10.1038/nm.4441, DOI	2018	50.69	2019	2021	
Schuster SJ, 2017, NEW ENGL J MED, V377, P2545, DOI 10.1056/NEJMoa1708566, DOI	2017	50.62	2019	2021	
Giavridis T, 2018, NAT MED, V24, P731, DOI 10.1038/s41591-018-0041-7, DOI	2018	49.54	2019	2021	

FIGURE 10 | The top 50 references with the strongest citation bursts involved in CAR-T cells. The Blue bars mean the reference had been published; the red bars mean citation burstness.

Lee et al. (52) in *Lancet* in 2015. In this phase I clinical trial, 21 patients with CD19+ B-ALL or non-Hodgkin lymphoma (NHL) received CD19-CAR-T cells. The results showed that (52) CD19-CAR-T cells had effective anti-tumor activity and were feasible and safe. The fifth co-cited paper was published by Davila et al. (53) in *Science Translational Medicine* in 2014. This phase I clinical trial mainly evaluated the efficacy of CD19-CAR-T cells in B-All patients (the overall complete remission rate was 88%) and put forward the diagnostic criteria and management scheme of severe cytokine release syndrome (sCRS) (53). The sixth co-cited paper was published by Grupp et al. (54) in *The New England Journal of Medicine* in 2013. This study was a case report. Two children with relapsed and refractory pre-B-cell ALL received CD19-CAR-T cell therapy, and both patients got complete remission. Nevertheless, one of them relapsed two months after receiving treatment (54). The seventh co-cited paper was published by Turtle et al. (55) in *The Journal of Clinical Investigation* in 2016. This phase I clinical trial proved for the first time that it was feasible to select different T cell subsets (CD4+T cells and CD8+T cells) to construct CD19-CAR-T cells (55). The eighth co-cited paper was published by Park et al. (56) in *The New England Journal of Medicine* in 2018. The phase I clinical trial mainly showed the long-term follow-up outcomes of relapsed B-ALL patients who received CD19-CAR-T cell therapy and evaluated the safety of this therapy (56). The ninth co-cited paper was published by Kochenderfer et al. (57) in the *Journal of clinical oncology* in 2015. The phase I clinical trial mainly evaluated the safety and effectiveness of CD19-CAR-T cells in the treatment of advanced CD19+B cell malignancies (57). The tenth co-cited paper was published by Brentjens et al. (58) in *Science Translational Medicine* in 2013. This phase I clinical trial proved that CD19-CAR-T cells had a significant anti-tumor effect in relapsed B-ALL patients (58).

In general, the top 10 most co-cited papers (the first part of **Table 5**) are all clinical experimental studies of CD19-CAR-T cells in treating hematological malignancies. The emphases of these studies mainly include evaluating the efficacy of CD 19-CAR-T cells in patients with hematological malignancies, evaluating the adverse reactions in the treatment process, and how to deal with these adverse reactions. Furthermore, it can also be seen from **Table 5** that the references in the second part are basically consistent with the first part. The third part is the top 10 co-cited references related to CAR-T cells for solid tumors (only including solid tumor-related studies). From this part, we can see that 5 of these 10 papers are about the research of CAR-T cells in brain tumors (glioblastoma and neuroblastoma).

The Analysis of Hotspots and Emerging Topics

Keywords can reflect the research hotspots and directions in a specific field. From **Table 4**, the top 20 keywords appear more than 300 times. These keywords represent the research hotspots in the field of CAR-T cells. The more representative keywords include chimeric antigen receptor, immunotherapy, cancer, expression, activation, CRS, ALL, lymphoma, phase-I, CD19, anti-tumor activity, efficiency, and NK cells. From these keywords, we can summarize the general situation of CAR-T cell-related fields, including a. CAR-T cell therapy, which is anti-tumor immunotherapy; b. The activation of CAR-T cells and the expression of CARs are important factors for the function of CAR-T cells; c. CRS is a common and most studied adverse reaction of CAR-T cell therapy (78, 79); d. CAR-T cell therapy is widely used in the research and treatment of hematological malignancies (80, 81); e. Presently, most clinical trials related to CAR-T cells are in phase-I (can be verified in clinicaltrials.gov); f. Anti-tumor activity and efficacy are the research emphases in this field (82, 83); g. CD19 is the most commonly used target in treating hematological malignancies (80, 84); h. CAR-NK cell therapy is currently a research hotspot (85, 86).

The density map of these keywords can show the high-frequency keywords in this field more intuitively (**Figure 7**). The network clustering analysis of keywords (totally divided into 6 clusters) can intuitively show this field's research direction and scope. As shown in **Figure 8**, we get 6 clusters. The keywords of cluster 1 (red) are mainly about the research of CAR-T cells in solid tumors. The keywords of cluster 2 (green) are mainly about the research of CAR-T cells in hematological malignancies. The keywords of cluster 3 (blue) are mainly CAR-T cells' primary research and mechanism research in tumors. The keywords of cluster 4 (yellow) are mainly related to improving the efficacy and safety of CAR-T cells. The keywords of cluster 5 (purple) may be the study of some immune cells related to CAR-T cells. There is only one keyword, " toxicity ", in cluster 6 (light blue). These six clusters represent CAR-T cells' research focus and scope to some extent. In addition, from this ranking, we can see that CAR-T research in solid tumors has become one of the critical research focuses. Because compared with hematological malignancies, there are more solid tumors and more patients with solid tumors. The timeline viewer (**Figure 9**) of keywords can help us see the time of a topic in this field and help us explore this field's evolutionary trajectory.

References with intense citation bursts refer to the sudden increase of citations of certain documents in a certain period, which can help us find emerging topics and research topics that have attracted much attention in a certain field (87). This study obtained 157 references with the most powerful citation bursts and selected the top 50 among them (**Figure 10**). The paper (Strength: 222.69) with the strongest citation burstness was a clinical experimental study published by Porter et al. (70) in *The New England Journal of Medicine* in 2011. They developed a second-generation CAR, with CD19 as the target and 4-1BB as the co-stimulatory molecule. This study proved that (70) CD19-CAR-T cells containing the 4-1BB signal domain had strong and durable anti-leukemia activity. More importantly, this study provided an important basis for the ongoing study of CD19-CAR-T cells in the treatment of B-cell tumors. Up to now, 13 papers (26%) in the top 50 are still in a state of citation burst, and the citation burstness of 12 papers has lasted for 3 years (years: 2019-2021). These 13 papers represent the latest research topics related to CAR-T cells. According to a Ranking by burstness strength (from high to low), the No.1 paper (strength: 157.38)

was published by Neelapu et al. (51) in *The New England Journal of Medicine* in 2017. The results showed that axicabtagene ciloleucel (Yescarta) had an exciting therapeutic effect on CD19+ refractory large B-cell lymphoma. Furthermore, this study also evaluated the security of Yescarta in detail. The second-ranked paper (strength: 157.17) was published by Maude et al. (50) in *The New England Journal of Medicine* in 2018. The study proved that tisagenlecleucel (Kymriah) produced a high remission rate and lasting remission for CD19+ relapsed or refractory B-cell ALL patients and evaluated the safety of Kymriah in detail. The third-ranked paper (strength: 86.37) was published by Park et al. (56) in *The New England Journal of Medicine* in 2018. This study mainly showed the long-term follow-up outcomes of patients with relapsed B-cell ALL who received CD19-CAR-T cell therapy and evaluated the safety of this therapy. The fourth-ranked paper (strength: 73.72) was published by June et al. (88) in *The New England Journal of Medicine* in 2018. This review introduced chimeric antigen receptor therapy from aspects of immuno-oncology, CAR-T cell-related toxic reactions, cell engineering, and synthetic biology. The fifth-ranked paper (strength: 69.11) was also published by June et al. (89) in *Science* in 2018. This review introduced the opportunities and challenges of CAR-T cells in human cancer treatment. The sixth-ranked paper (strength: 63.48) was published by Fraietta et al. (90) in *Nature Medicine* in 2018. This study explored the clinical response of CD19-CAR-T cells in chronic lymphocytic leukemia (CLL) patients and the determinants and mechanisms related to drug resistance. The seventh-ranked paper (strength: 63.28) was published by Neelapu et al. (91) in *Nature Reviews Clinical Oncology* in 2018. This review mainly introduced CAR-T cell-related toxic reactions' evaluation and coping strategies. The eighth-ranked (strength: 59.1) was published by Norelli et al. (92) in *Nature Medicine* in 2018. This study mainly explored the mechanism and treatment of CD19-CAR-T cell-related CRS and neurotoxicity. The ninth-ranked paper (strength: 53.28) was published by Hay et al. (93) in *Blood* in 2017. It mainly explored the dynamics, biomarkers, risk factors, and mechanism of severe CRS(sCRS) in CD19-CAR-T cell therapy, and the tenth-ranked paper (strength: 50.69) was published by Fry et al. (94) in *Nature Medicine* in 2018. This study confirmed the clinical activity of CD22-CAR-T cells in CD19-/CD19+ B-ALL for the first time. The eleventh-ranked paper (Strength: 50.62) was published by Schuster et al. (95) in *The New England Journal of Medicine* in 2017. The study mainly explored the efficacy of CD19-CAR-T cells in adult lymphoma. The twelfth-ranked paper (strength: 49.75) was published by Qasim et al. (96) in *Science Translational Medicine* in 2017. Qasim et al. used gene-editing technology to construct CD19-CAR-T cells and at the same time destroyed the TCR and CD52 of these T cells. These special CD19-CAR-T cells (universal CAR19 T cells) could escape the host immunity and reduce the risk of graft-versa-host disease (GVHD). The thirteenth-ranked paper (strength: 49.54) was published by Giavridis et al. (97) in *Nature Medicine* in 2018. This study explored CRS's mechanism and intervention measures (especially related therapeutic drug "anakinra") related to CAR-T cells.

According to the above analysis and the related analysis of **Annexes 5, 6**, the following important information can be obtained: a. The high-quality and high-impact research related to CAR-T cells mainly focuses on the clinical experimental studies of CD19-CAR-T cells in hematological malignancies. Related research hotspots include efficacy, safety, drug resistance, and the mechanism and management of related toxic reactions (50, 51, 56, 70, 90, 91); b. CRS is a common and most studied CAR-T cell-related toxic reaction (78, 79). Related studies mainly focus on the mechanism, diagnosis (biomarkers), and intervention (especially related therapeutic drug "anakinra") of CRS (92, 93, 97); c. CD19 is the most commonly used target of CAR-T cells in treating hematological malignancies (80, 84). Furthermore, CD22 is also a significant target (94); d. Universal CAR-T cells (96) and CAR-NK cells (85, 86) are also the research hotspots; e. The research of CAR-T cells in solid tumors is also a hot field that has developed rapidly. Emerging topics in this field mainly include the study of CAR-T cells in glioblastoma (related targets: IL13Rα2, EGFRvIII, and HER2), neuroblastoma (related target: GD2), sarcoma (related target: HER2), and pancreatic cance (related target: mesothelin), especially glioblastoma. Furthermore, from the cluster analysis of keywords (**Figure 8**), it can be seen that the research enthusiasm of CAR-T cells in solid tumors can even be compared with that in hematological malignancies.

Limitation

First of all, this study is a bibliometric study, and CiteSpace and VOSviewer cannot wholly replace system retrieval. Secondly, all data was retrieved and downloaded from the WoSCC database, which would miss some papers not included in this database. However, WoSCC is the most commonly used database in scientific econometric analysis, and the data from WoSCC can represent most information to some extent (98). Finally, because of the large amount of data, CtieSpace ran very slowly in some functions, so we reduced some data (only 12%). All of these may reduce the credibility of this study. Nevertheless, the visual analysis based on reference data can help researchers intuitively understand the CAR-T cell field's research hotspots, evolution process, and development trend.

CONCLUSION

In a word, CAR-T cell therapy is an anti-tumor therapy with great potential and clinical application prospects, which is still in a highly developed stage at present. The related research of CAR-T cells will still be a hot field in the future. The following is a summary of knowledge points and research hotspots in the field of CAR-T cells:

a. CAR-T cell-related research is an important research field that many developed countries are interested in, especially the United States is in an absolute leading position. The institution that contributed the most publications is the

University of Pennsylvania. However, there is little cooperation between countries. After 2012, cooperation among various institutions is also small;

b. The journals that published the most CAR-T cell-related papers are *Frontiers in immunology* and *Cancers*. Nevertheless, *Blood* and *The New England Journal of Medicine* are the most commonly co-cited journals;
c. CAR-T cell therapy is a kind of clinical application research. Many high-quality and high-impact journals are very interested in CAR-T cell-related research; especially high-quality clinical trial papers are the most popular;
d. Currently, the research related to CAR-T cells mainly focuses on basic research and translational medicine;
e. The high-quality and high-impact research related to CAR-T cells mainly focuses on the clinical experimental studies of CD19-CAR-T cells in hematological malignancies. Related research hotspots include efficacy, safety, drug resistance, and the mechanism and management of related toxic reactions;
f. CRS is a common and most studied CAR-T cell-related toxic reaction (67, 68). Related studies mainly focus on the mechanism, diagnosis (biomarkers), and intervention (especially related therapeutic drug "anakinra") of CRS;
g. CD19 is the most commonly used target of CAR-T cells in the treatment of hematological malignancies. Furthermore, CD22 is also an important target;
h. Universal CAR-T cells (85) and CAR-NK cells (74, 75) are also the research hotspots;
i. The research of CAR-T cells in solid tumors is also a hot field that has developed rapidly in recent years. Emerging topics in this field mainly include the study of CAR-T cells in glioblastoma (related targets: IL13Rα2, EGFRvIII, and HER2), neuroblastoma (related target: GD2), sarcoma (related target: HER2), and pancreatic cancer (related target: mesothelin), especially glioblastoma.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LM: Writing-Original draft preparation, manuscript, investigation, and figure preparation. JZ: manuscript, investigation, and figure preparation. ZZ: Investigation and figure preparation. SW: Investigation. FT: Investigation. MT: Investigation, Methodology, Supervision. YL: Conceptualization, Methodology, Supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.840956/full#supplementary-material

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Bibliometric Analysis of Chimeric Antigen Receptor-Based Immunotherapy in Cancers From 2001 to 2021

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Background: Chimeric antigen receptor (CAR)-based immunotherapy has shown great potential for the treatment of both hematopoietic malignancies and solid tumors. Nevertheless, multiple obstacles still block the development of CAR-based immunotherapy in the clinical setting. In this study, we aimed to summarize the research landscape and highlight the front lines and trends of this field.

Methods: Literature published from 2001 to 2021 was searched in the Web of Science Core Collection database. Full records and cited references of all the documents were extracted and screened. Bibliometric analysis and visualization were conducted using CiteSpace, Microsoft Excel 2019, VOSviewer and R software.

Results: A total of 5981 articles and reviews were included. The publication and citation results exhibited increasing trends in the last 20 years. *Frontiers in Immunology* and *Blood* were the most productive and most co-cited journals, respectively. The United States was the country with the most productive organizations and publications in the comprehensive worldwide cooperation network, followed by China and Germany. June, C.H. published the most papers with the most citations, while Maude, S.L. ranked first among the co-cited authors. The hotspots in CAR-based therapy research were multiple myeloma, safety and toxicity, solid tumors, CAR-engineered immune cells beyond T cells, and gene editing.

Conclusion: CAR-based immunotherapy is a promising treatment for cancer patients, and there is an emerging movement toward using advanced gene modification technologies to overcome therapeutic challenges, especially in solid tumors, and to generate safer and more effective universal CAR-engineered cell products.

Keywords: chimeric antigen receptors (CARs), CAR-based immunotherapy, bibliometric analysis, solid tumors, natural killer cells, safety and toxicity, gene editing

INTRODUCTION

Among different cancer therapy strategies, immunotherapy has attracted great attention from clinical researchers worldwide. Adoptive cell transfer (ACT) therapies represent one of the most promising treatments, among which chimeric antigen receptor (CAR)-based therapy has shown great potential. The concept of CAR means that an engineered receptor with predefined specificity is grafted onto immune cells through gene editing to endow the cells with the ability to recognize target molecules without MHC restriction (1). The CAR structure consists of 3 major parts, specifically, the extracellular region with the single-chain fragment variant (scFv) that recognizes the antigen, the fundamental hydrophobic alpha helix transmembrane domain, and the core intracellular domain that recruits and phosphorylates downstream protein molecules by conformational changes (2). CAR was first proposed by the Israeli scientist Eshhar in 1989 (3) and was soon widely studied but went through a long period before being used in clinical practice. The first generation contained only the CD3ζ intracellular domain, which led to hypoproliferation and hypocytotoxicity (4); this was improved in the 2nd and 3rd generations by adding one or both costimulation signals, including CD28, CD134 and CD137 (5–7). The 4th generation, also known as T cells redirected for universal cytokine-mediated killing (TRUCKs), includes the expression of a desired cytokine upon CAR activation (8), while in the fifth generation, the addition of the intracellular domains of cytokine receptors such as interleukin (IL)-2 receptor β-chain (IL-2Rβ) fragments has greatly strengthened the activation and proliferation ability of CAR-T cells (9). Of note, in 2017, the FDA approved the use of two CD19-targeting CAR-T products, CTL-019 (Kymriah) from Novartis and Yescarta from Gilead, in pediatric relapsed or refractory acute lymphoblastic leukemia (AML) and adult relapsed or refractory large B cell lymphoma, respectively (10, 11), which indicated that the CAR-T era had finally arrived.

Beyond CAR-T cells, other immune effector cells with CAR modification were under study simultaneously, including natural killer (NK) cells, invariant NK T (iNKT) cells, γδ T cells, macrophages and dendritic cells (DCs). These cell types display different characteristics that outperform T cells in particular aspects. For example, the risk of graft versus host disease (GVHD) mediated by the T-cell receptor can be completely avoided when NK-cells, iNKT or γδ T cells are applied (12). Moreover, multiple killing mechanisms beyond perforin and granzyme, the modulation of antitumor immunity through secretion of cytokines or direct penetration of solid tumors in response to tumor-derived chemokines make it possible to obtain better curative effects in these cell platforms for CAR-based immunotherapies (13). In fact, dozens of clinical trials of different CAR-based immune effector cells are under evaluation (12, 14).

Although tremendous efforts have been invested in CAR-modified cell therapy in cancers, the most promising clinical results have come mostly from hematopoietic malignancies and seldom from solid tumors (15). Research teams from all over the world have been exploring the reasons and potential solutions, but the most feasible ways out of this dilemma remain unclear. To better answer this question, it is necessary to understand the majority of the research foundation and build up a multidimensional research network through longitudinal and global perspectives to illustrate future directions. By bibliometric analysis, a comprehensive and effective scientific information analysis method, we can quantitatively evaluate the contributions of authors, institutions and countries and the links among them (16). More importantly, such analysis could provide a valuable basis for defining the frontiers of and trends in the research field (17).

To date, only a few scientometric studies that focused on CAR-T cells in CAR-based therapy have been presented. Here, we performed a more comprehensive bibliometric analysis of CAR-based immune cell therapy based on sufficient literature and updated data through different analysis methods, aiming to draw a global research network map and determine the next pivotal frontiers.

MATERIALS AND METHODS

Data Source and Search Strategies

A literature search was performed using the Web of Science Core Collection (WoSCC) database on October 1st, 2021. To conduct a thorough search, we used the following strategy: TS = (tumo$r* OR cancer* OR carcinoma* OR sarcoma* OR neoplas* OR malignanc*) AND TS=((chimeric antigen receptor*) OR (chimeric T cell receptor*) OR "CAR-T" OR "CAR-T cell" OR "CAR therapy" OR (chimeric NK cell receptor*) OR "CAR-NK" OR "CAR-iNKT" OR "CAR-γδT" OR "CAR-macrophage" OR "CAR-M" OR Kymriah OR Yescarta). Only articles and reviews written in English and published from 2001 to 2021 were eligible to be included. Two researchers (ZO and LQ) manually screened the titles, abstracts and full texts to exclude irrelevant literature and discussed any potential disagreements. Finally, 5981 documents were included. The screening strategy is shown in **Supplementary Figure 1**.

Data Extraction and Bibliometric Analysis

Full records and cited references of all the documents in WoSCC were collected and downloaded in txt or BibTeX format and then imported to CiteSpace 5.8R1, 64 bits (Drexel University, Philadelphia, PA, USA), Microsoft Excel 2019, VOSviewer 1.6.17 (Leiden University, The Netherlands) or R (Version 4.0.2), according to the software required for data analysis and visual analysis.

Microsoft Office Excel was used to analyze the trends of annual publications and citations of the included literature. The Bibliometrix and Biblioshiny packages in R were used to conduct collaboration network analysis among countries. VOSviewer software was used to analyze country or organization distributions, author contributions, core journals, keyword co-occurrences with coauthorship, co-citation, and co-occurrence analyses in default settings and displayed visualizations of cooperative networks of these items. We also performed the co-citation analysis in co-cited references and the combination of co-cited references and keywords by CiteSpace software, with the following settings: The time slices were from January 2001 to December 2020, with 1 year per slice. In each slice, a modified g-index ($g2 \leq k\,Si \leq_g^c{}_i$, $k \in Z+$, $k = 25$) was set.

The maintenance threshold for burst detection in authors and references was set to 4 years. Other parameters were set to the default settings.

Research Ethics

We conducted the study using scientometric data with no *in vivo* data from animal or human subjects. Therefore, permission from the ethics committee was not necessary in this study.

RESULTS

Overview and Analysis of the Publication and Citation Trends

A total of 7255 studies were identified after a thorough search of the WoSCC database. Document types other than articles and reviews, non-English papers and irrelevant studies were excluded (**Supplementary Figure 1**). No duplicated studies were found. Finally, 5981 studies were included in the bibliometric analysis. The total number of citations for the retrieved articles was 224,968, and the mean citations per article was 37.61. The H-index of all the selected publications was 197.

As shown in **Figure 1**, the annual number of publications concerning CAR-based immunotherapy slowly increased from 45 in 2001 to 95 in 2011. From 2012 to 2020, the number of publications per year grew rapidly, reaching 1111 in 2020. The number of publications in 2021 was slightly lower than that in 2020, perhaps because the search included only three quarters of 2021. However, since the correlation between number of publications and publication year was significant (correlation coefficient $R^2 = 0.9516$), it is convincing that publication this year will establish a new maximum. Correspondingly, the number of annual citations exhibited a similar upward trend, steadily increasing from 40 in 2001 to 2999 in 2011, exploding from 3987 in 2012 to 50991 in 2020, and exhibiting a small decline to 46976 in 2021, with an even stronger correlation (correlation coefficient $R^2 = 0.9666$).

Overall, the publication and citation number results indicated that research on CAR-based immunotherapy in cancer is still in a rapid growth phase, provoking great attention for future research.

Analysis of Productive Journals

We found that a total of 5981 papers concerning CAR-based immunotherapy in cancer were produced by 1030 journals. Among these publication sources, the journal *Frontiers in Immunology* ranked first in production (252, 4.21%), followed by *Cancers* (155, 2.59%), *Molecular Therapy* (130, 2.17%) and *Blood* (127, 2.12%). Of these, the highest impact factor (IF, 2020) among the top 10 most productive journals was earned by *Blood* (22.113), followed by *Journal for Immunotherapy of Cancer* (13.751), *Cancer Research* (12.701) and *Clinical Cancer Research* (12.531, **Table 1**). Half of the top 10 citing journals belonged to Q1 in journal citations reports (JCR), and 70% were from the US (**Table 1**). Furthermore, as listed in **Table 1**, all of top 10 co-cited journals were cited over 8000 times, accounting for more than 30% of the total citations, with *Blood* (37,798, 8.15%) again being the highest, and *New England Journal of Medicine* (16,573, 3.57%) and *Clinical Cancer Research* (15,154,

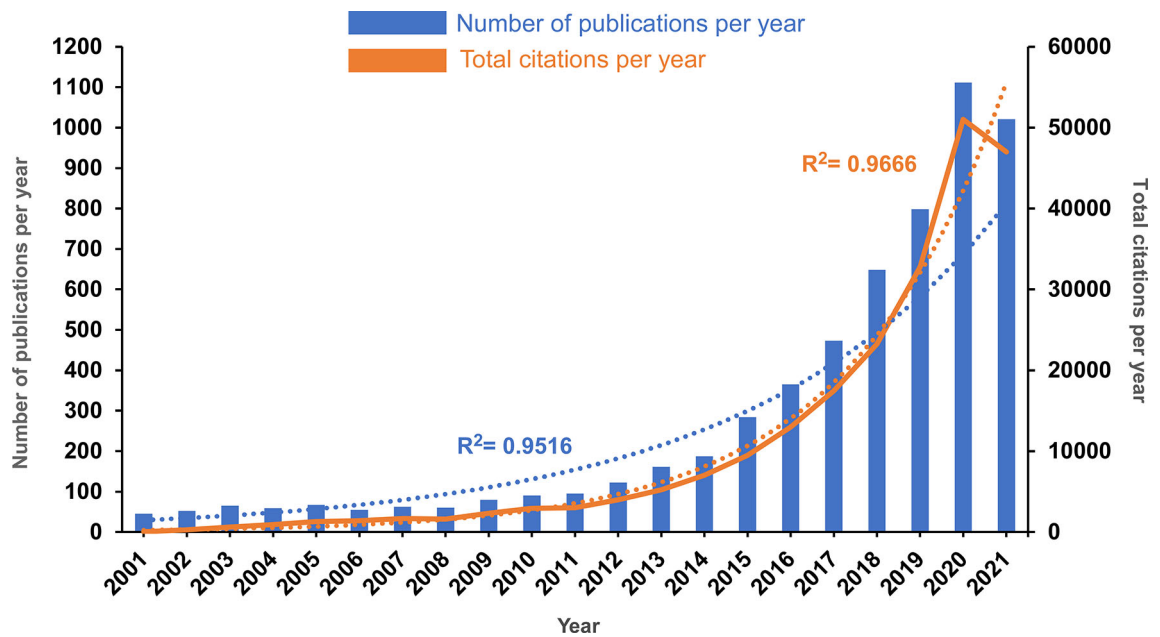

FIGURE 1 | The annual publication and annual citation trends in the past 20 years. The blue bars represent the number of publications per year, and the orange solid curve represents the total number of citations per year. The blue and orange dotted lines represent the trend-fitted curves using exponential functions. The correlation coefficients (R^2) are displayed in the figure.

TABLE 1 | Top 10 journals and co-cited journals related to CAR-based immunotherapy.

Rank	Journal	Counts (%)	IF (2021)	JCR	Country	Rank	Co-cited Journal	Counts (%)	IF (2021)	JCR	Country
1	Frontiers in Immunology	252(4.21)	7.561	Q2	Switzerland	1	Blood	37798 (8.15)	22.113	Q1	US
2	Cancers	155(2.59)	6.639	Q2	Switzerland	2	New England Journal of Medicine	16573 (3.57)	91.245	Q1	US
3	Molecular Therapy	130(2.17)	11.454	Q1	US	3	Clinical Cancer Research	15154 (3.26)	12.531	Q1	US
4	Blood	127(2.12)	22.113	Q1	US	4	Journal of Immunology	13870 (2.99)	5.422	Q2	US
5	Clinical Cancer Research	111(1.85)	12.531	Q1	US	5	Cancer Research	13614 (2.93)	12.701	Q1	US
6	Journal for Immunotherapy of Cancer	111(1.85)	13.751	Q3	UK	6	Journal of Clinical Oncology	13454 (2.90)	44.544	Q1	US
7	International Journal of Molecular Sciences	99(1.65)	5.923	Q2	US	7	Molecular Therapy	10809 (2.33)	11.454	Q1	US
8	Oncoimmunology	98(1.63)	8.110	Q1	US	8	Proceedings of the National Academy of Sciences of the United States of America	10597 (2.28)	11.205	Q1	US
9	Cancer Research	96(1.60)	12.701	Q1	US	9	Science	8492 (1.83)	47.728	Q1	US
10	Frontiers In Oncology	93(1.55)	6.244	Q2	US	10	Nature Medicine	8465 (1.82)	53.440	Q1	US

IF, impact factor; JCR, journal citation reports; US, The United States; UK, The United Kingdom.

3.26%) ranking second and third. Ninety percent of these co-cited journals had IF values over 10 and belonged to Q1 in JCR. We also visualized the top 50 citing and co-cited journals with a spectral density map (**Figure 2**) and found that some of the journals co-occurred in both maps, for example, *Blood*, *Frontiers in Immunology*, *Clinical Cancer Research* and *Cancer Research*, indicating that these journals have maintained close contact with the CAR-based immunotherapy research field.

Analysis of Active Countries and Organizations

The literature included for analysis was produced by 83 countries over the last two decades (articles coauthored by individuals from more than one country or organization were counted multiple times by VOSviewer). Among these countries, the United States published the most papers (n=3088), accounting for 38.82% of the included studies and the most citations (163148), which outnumbered the total citations from the 2nd to 10th countries (**Table 2**). The next most productive countries included China (n=1099, 13.82%), Germany (n=661, 8.31%) and the United Kingdom (n=366, 4.6%), and all top 10 countries exceeded the average number of publications (n=95.83). Interestingly, China ranked 2nd in publications but the last in average document citations (n=17.68), while Australia and Canada displayed the opposite pattern, ranking 2nd and 3rd in citation per paper (n=49.41 and 48.30, respectively) with fewer publications (n=177, 2.23% and 160, 2.01%, respectively). These may be related to access to the literature and the restriction of language selected.

To explore the collaborations between countries, we used Bibliometrix and Biblioshiny packages and VOSviewer to analyze the data and generate visual outputs. As shown in **Figure 3A**, the collaboration network among countries was complicated and extensive. The cooperation centers, displayed by the junctions of red lines, are mainly located in North America (US), Asia (China), Europe (Germany) and Oceania (Australia). The country coauthorship network of the top 30 countries was automictically clustered into 5 categories, as indicated by 5 colors (**Figure 3B**). The time-overlay visualization map showed that the production of these countries has been concentrated within the last 5 years (**Figure 3C**). Considering these observations together with the clustering results, it was supposed that the green cluster (mostly Asian countries) was more newly active than other clusters, while in each cluster, some of the members have become more active in the last 3 years, such as China in the blue cluster (**Figures 3B, C**).

A total of 4557 organizations produced at least 1 paper related to CAR-based immunotherapy in cancer. The top 10 most productive institutions are shown in **Table 2**. All of these institutions are located in the US and have published over 100 papers in the past 20 years, with the number of publications ranging from 100 (Stanford University) to 344 (University of Pennsylvania), total citations ranging from 3861 (Harvard University) to 35010 (University of Pennsylvania), and citations per paper ranging from 25.40 (Harvard University) to 118.93 (National Cancer Institute).

The cooperation of the top 100 research institutions was likewise analyzed by a coauthorship network. Six clusters were displayed (**Figure 3D**), mainly indicating regional collaboration. The largest cluster (red) consists mainly of institutions in the US, the blue cluster in Germany, the green cluster in China, the purple cluster in Australia, the light blue cluster in the UK and the yellow cluster for institutions in Houston, Texas (US). Among these clusters, the red, yellow and purple clusters were most active from approximately 2015 to 2017, while the green and blue clusters were more active in the last 3 years, indicating that institutions in the US, UK and Australia began CAR-T research earlier, but those in China and Germany, such as Zhejiang University, Shanghai Jiao Tong University, Sun Yat-Sen University, and the German Cancer Consortium (DKTK), were close behind and have produced more newer studies (**Figure 3E**).

Analysis of Authors and Coauthorship

A total of 26367 authors participated in publishing the literature involved in this analysis, and 22.2% of these authors have published more than one paper. The top 100 most productive

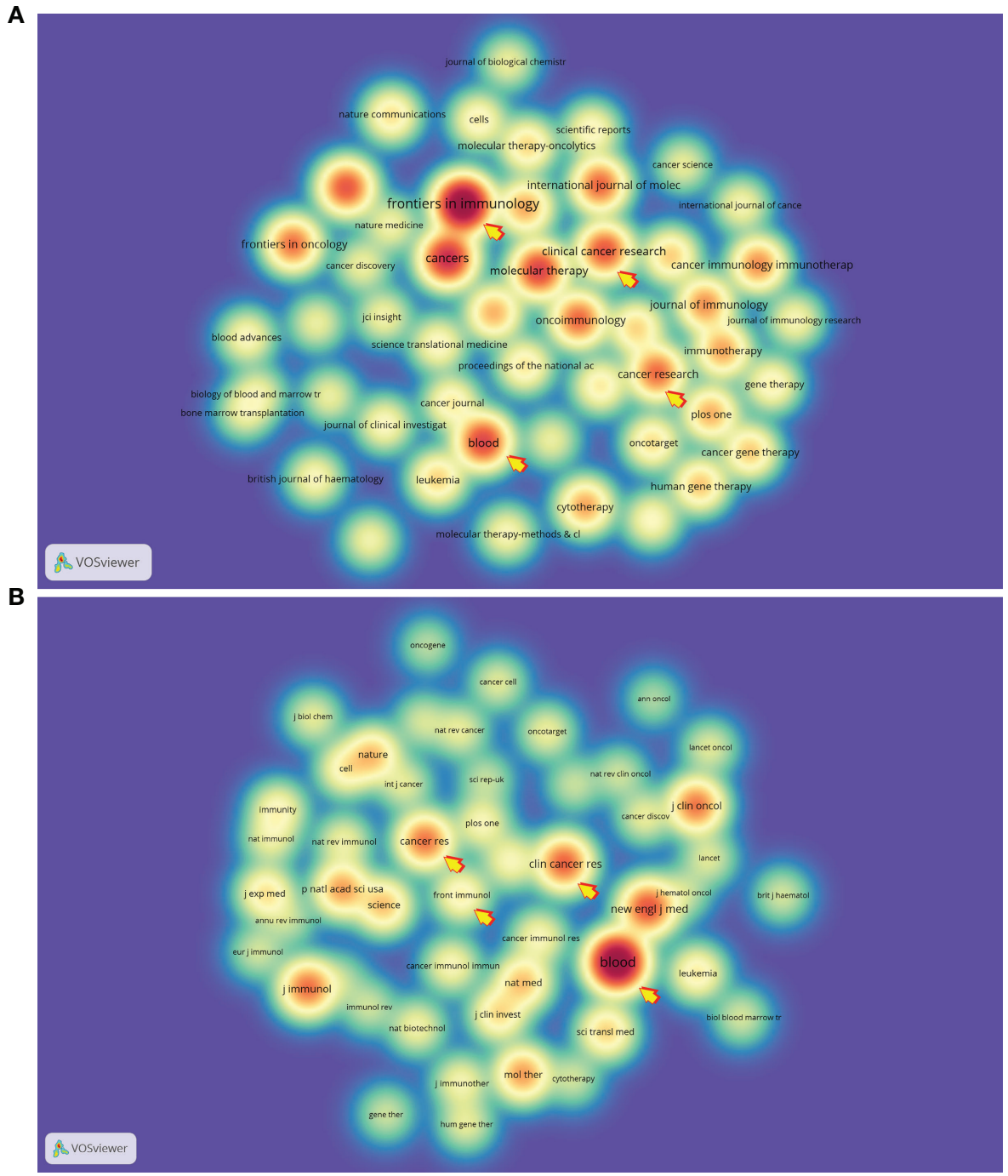

FIGURE 2 | The spectral density map of **(A)** citing and **(B)** co-cited journals. The arrows point to the journals that are present in both panels.

authors and co-cited authors are displayed in spectral density maps in **Figures 4A, D**, while the details of the top 20 authors in both rankings are listed in **Table 3**. Among the top 20 most productive authors, 36 to 104 papers per person were authored, receiving 1589 to 22985 citations, while the total citations of the co-cited authors ranged from 899 to 2868. Notably, June, C.H., one of the pioneers of 4-1BB-CAR-T cells (18), had the most published papers (104) with the most citations (22985) and ranked the 17th among co-cited authors. In addition, 4 other authors, including Brentjens, R.J., Sadelain, M., Grupp, S.A. and Rosenberg, S.A., were also within the top 20 in both rankings, demonstrating their academic authority in this research field.

TABLE 2 | Top 10 countries and organizations related to CAR-based immunotherapy.

Rank	Country	Counts (%)	Total citations	Citations per article	Rank	Organizations	Counts (%)	Total citations	Citations per article
1	US	3088(38.82)	163148	52.83	1	University of Pennsylvania (US)	344 (2.12)	35010	101.77
2	China	1099(13.82)	19425	17.68	2	Memorial Sloan Kettering Cancer Center (US)	243 (1.50)	21389	88.02
3	Germany	661(8.31)	26204	39.64	3	National Cancer Institute (US)	204 (1.26)	24261	118.93
4	UK	366(4.60)	12985	35.48	4	The University of Texas MD Anderson Cancer Center (US)	197 (1.21)	13305	67.54
5	Italy	308(3.87)	10609	34.44	5	Baylor College of Medicine (US)	160 (0.99)	13651	85.32
6	Japan	228(2.87)	7516	32.96	6	University of Washington (US)	160 (0.99)	10614	66.34
7	France	211(2.65)	6626	31.40	7	Harvard University (US)	152 (0.94)	3861	25.40
8	Australia	177(2.23)	8746	49.41	8	Fred Hutchinson Cancer Research Center (US)	131 (0.81)	10604	80.95
9	Canada	160(2.01)	7728	48.30	9	Texas Children's Hospital (US)	111 (0.68)	10173	91.65
10	Netherlands	145(1.82)	5689	39.23	10	Stanford University (US)	100 (0.62)	4349	43.49

US, The United States; UK, The United Kingdom.

The cooperation networks among authors and among co-cited authors were grouped into 7 and 3 clusters according to the closeness of their connections (**Figures 4B, E**), respectively. When combined with the overlay visualization maps of year (**Figure 4C**), it reflected that the brown and light blue clusters were most active in approximately 2014, and the blue, red, yellow and green clusters expanded over time, while the orange and purple clusters mainly published literature in the past 3 years but lack strong connections with other clusters. Among the co-cited authors, Maude, S.L. ranked first, but the centralities of all co-cited authors were below 0.1 (0-0.04), indicating that multiple researchers began to publish within a short period; thus, no single author dominates the researching field. This was also verified by the burst of co-cited authors because these researchers shared the same burst period or overlapped with each other (**Figure 4F**). The burstiness strength ranged from 52.08 (Rossig, C.) to 131.97 (Kalos, M.) among the top 25 co-cited authors with the strongest bursts (**Figure 4F**) but was inconsistent with the top 20 co-cited authors in **Table 3** because several authors did not reach the threshold of 4 years of burst maintenance.

Analysis of the Research Field From Co-Cited References and Keywords

To explore where researchers have been and where they are going in CAR-based immunotherapy, we analyzed the co-citation network of references and keywords by CiteSpace and VOSviewer. First, among 5981 original documents, 1873 cited references from 2665 citing papers were selected automatically by CiteSpace to form the co-citation network (**Figure 5A** and **Supplementary Figure 2**). The top 20 co-cited references were all published in internationally renowned journals (IF>14.808, Q1), with over 344 citations (**Table 4**). Most of these papers were clinical studies, with only one preclinical study and one review. The top 10 co-cited references with updated citation data related to hematopoietic malignances and solid tumors were also displayed in **Tables S1**, **S2**, respectively. In addition, the top 25 references with the strongest citation bursts showed that the majority were frequently cited in the last decade, suggesting that the research field is still progressing (**Supplementary Figure 3**). The included references were clustered into 13 groups based on their major research topic (**Figure 5A** and **Supplementary Figure 2**). Cluster #4, predefined specificity (mean year = 2000), #12, CC chemokine receptor 4 (mean year = 2002) and #3, genetic modification (mean year = 2006) appeared initially, followed by #1, receptor-modified T cell (mean year = 2011) and #10, novel agent (mean year = 2012) and #5 clinical trial (mean year = 2014), while #6, natural killer cell (mean year = 2015), #7, gene editing (mean year = 2015), #11, acute myeloid leukemia (mean year = 2015), #0, solid tumor (mean year = 2016), #2, cytokine release syndrome (mean year = 2017), #8, multiple myeloma (mean year = 2017) and #9, T cell exhaustion (mean year = 2017) were new focuses of research. The top 3 most cited references of each cluster are also displayed by the 3 largest circles in each group (**Figure 5A**).

Keywords are also essential in hotspot analysis. The top 100 keywords with the highest co-occurrence were calculated by VOSviewer and are displayed in **Figure 5B**, showing a comprehensive but clear network. The detailed data of the top 25 co-occurrence keywords are listed in **Table 5**. Among them, the basic concepts "chimeric antigen receptor", "immunotherapy" and "cancer" ranked in the top 3 (**Table 5**), while "cytokine release syndrome", "tumor microenvironment", "multiple myeloma" and "glioblastoma" seemed to represent the forefront of the research field (**Figure 5B**). Moreover, the top 25 keywords with the strongest citation bursts are listed in **Table 5**, showing that most of the keywords were derived 10 years ago, and some of them still have high occurrences, such as "*in vivo*" (2002-2017), "antitumor activity" (2010-2017), and "adoptive immunotherapy" (2011-2016). The target antigens used in the published CAR-based immunotherapies in both liquid cancers and solid tumors were summarized in **Table S3**. Co-occurrence analysis was applied to reveal the popularity of these targets.

Unfortunately, these keywords are general and only partially relate to the frontiers of CAR-based immunotherapy. Therefore, to sufficiently unveil the hotspots, we performed mixed scientometric analysis with co-cited references and keywords and displayed the result with an overlay network map (**Figure 6**). Different from the former 13 clusters, 14 out of 16 clusters are shown in this section (2 irrelevant clusters were hidden), together with the most correlative keywords. The keywords were mainly concentrated in earlier times, especially for cluster #0 labelled "CAR-T cells" and #2 labelled "multiple myeloma", but the new keywords with fewer occurrences should not be neglected. Generally, the clusters could be mainly divided into the

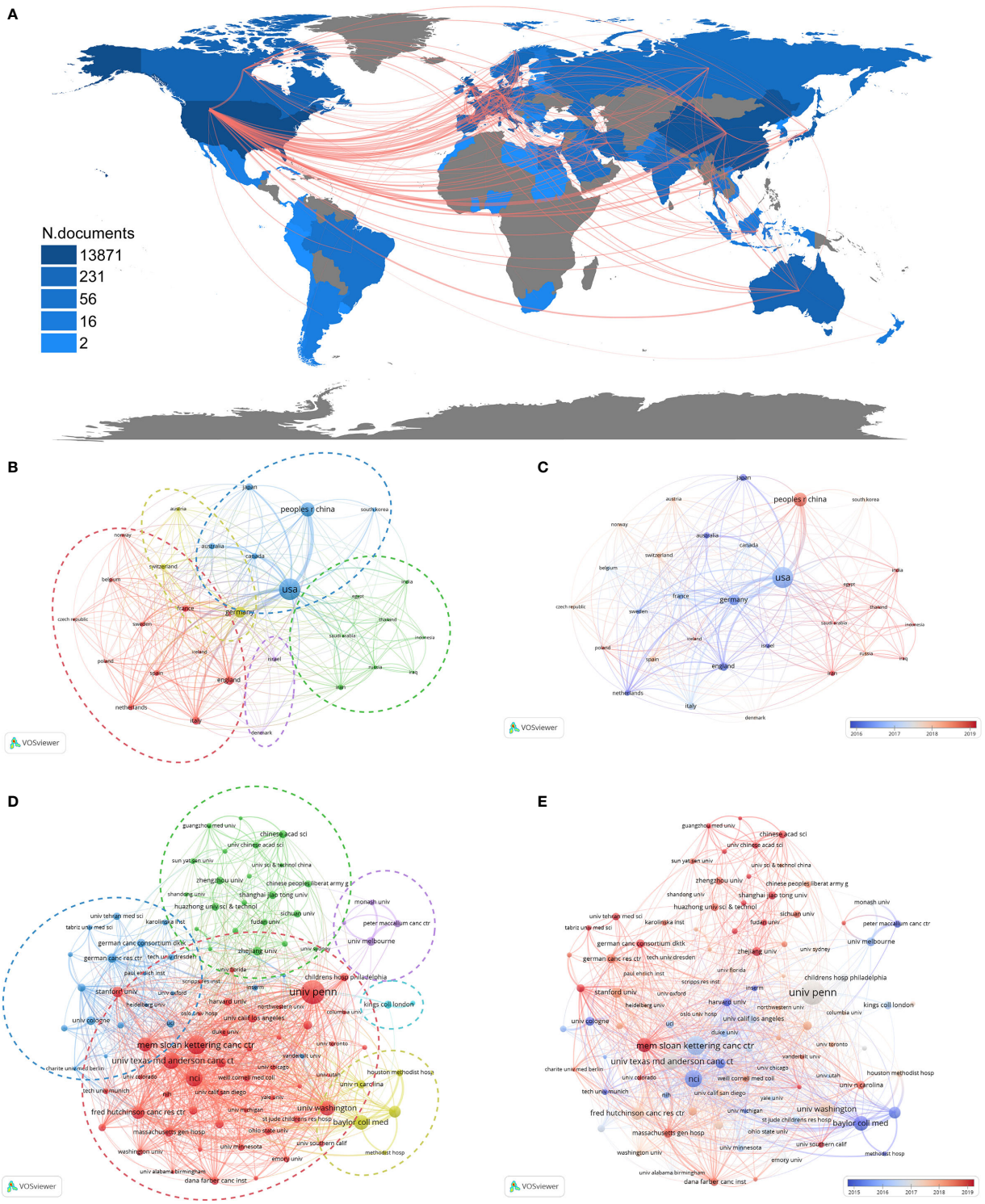

FIGURE 3 | Collaboration networks among countries and among institutions. **(A)** The collaboration map among countries. Color shades represent the number of publications in each country, and red lines indicate cooperation between two countries. Each node in **(B–E)** represents a country or an institution, and each line represents a link between two countries or institutions. The dotted circles in different colors in **(B, D)** indicate the corresponding clusters of countries and institutions. **(B)** The cooperation network among the top 30 most productive countries. **(C)** The time-overlay map of the cooperation network among the top 30 most productive countries. **(D)** The cooperation network among the top 100 most productive institutions. **(E)** The time-overlay map of the cooperation network among the top 100 most productive institutions.

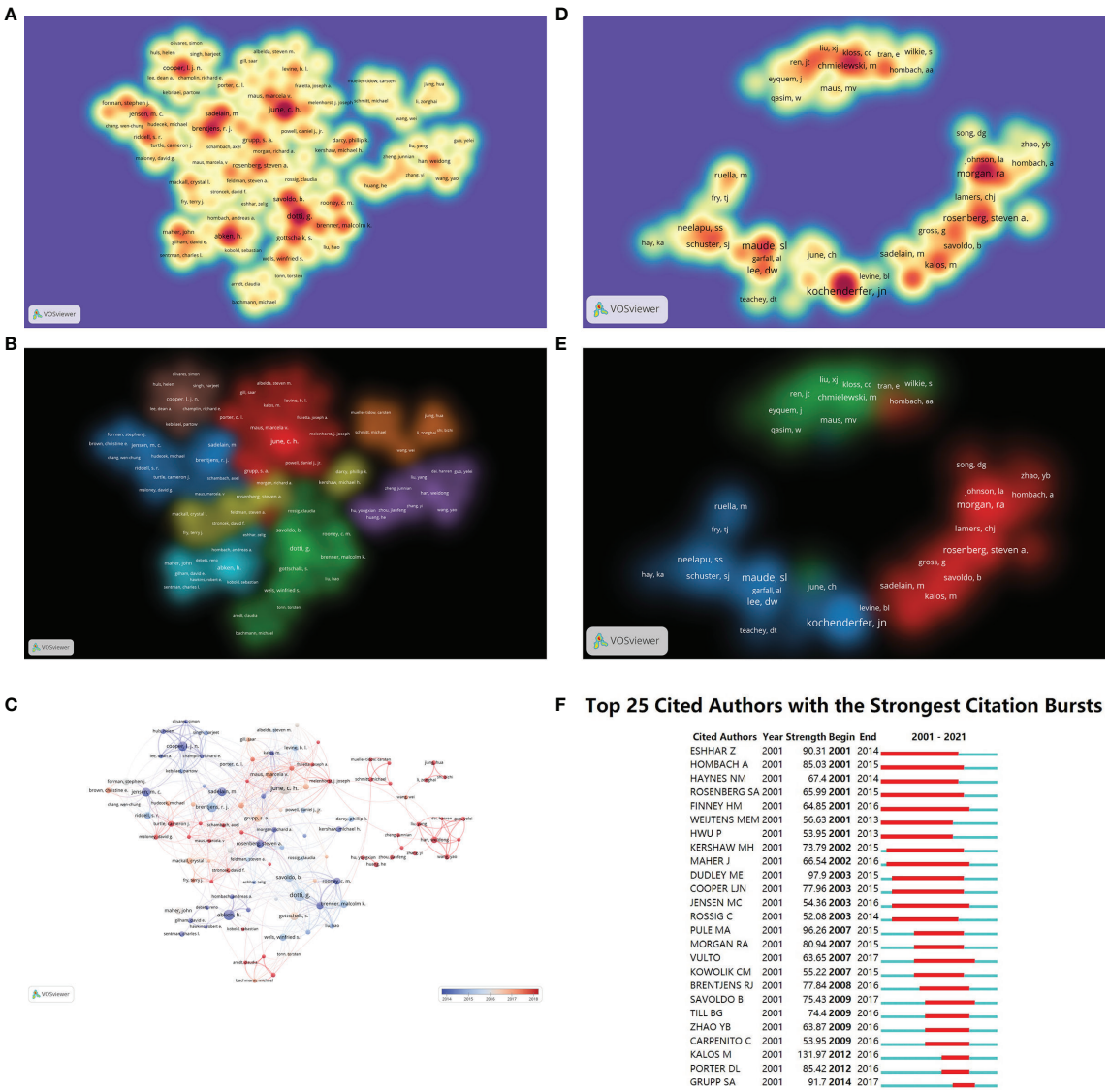

FIGURE 4 | Scholar cooperation maps. **(A)** The spectral density map of the top 100 most productive authors. **(B)** The cluster-overlay map of the top 100 most productive authors. **(C)** Time-overlay maps of the top 100 most productive authors. Each node represents an author, and each line represents the link between two authors. **(D)** The spectral density maps of the top 100 most co-cited authors. **(E)** The cluster-overlay map of the top 100 most co-cited authors. **(F)** The top 25 cited authors with the strongest citation bursts.

following parts: the basic concept of CAR (including cluster #0 and #16, and the related keywords "chimeric antigen receptor", "adoptive immunotherapy", "antitumor activity", etc.), solid tumor (including #1, #6, #10, #11, #13 and #15, and keywords "solid tumor", "glioblastoma", "tumor microenvironment", "fibroblast activation protein", etc.), hematopoietic malignancies (including #2, #7, #8 and #12, and keywords "acute myeloid leukemia", "multiple myeloma", "bcma", etc.), safety (#3 and keywords "cytokine release syndrome", "neurotoxicity", "management", etc.), clinical trials (including #4 and keywords "phase ii trial", "open label", etc.), CAR-based innate cell therapy (including #5 and keywords "natural killer cells", "colony stimulating factor", etc.) and genetic modification (#6 and keywords "CRISPR", "Cas9", "suicide gene", etc.). Furthermore, the most recent clusters, including #1 (mean year = 2016), #6 (mean year = 2017), #10 (mean year = 2017) and #15 (mean year = 2018), mainly focus on the treatment of solid tumors. We then took a closer look at cluster #15, which represented the leading edge of CAR-based immunotherapy in solid tumors. There were 5 cited references in #15 with between 11 and 57 citations, mainly related to B7-H3-targeted CAR-T cells in brain tumors (**Table 6**). The top 5 citing references that cited the 5 cited references mentioned above are also listed in **Table 7** and were equally important because they cover the same

TABLE 3 | Top 20 most relevant authors and co-cited authors related to CAR-based immunotherapy.

Rank	Author	Counts	Total citations	Citations per article	Rank	Co-cited author	Total citations	Centrality	Burstiness strength
1	June, Carl H	104	22985	221.01	1	Maude, Shannon L	2868	0.04	–
2	Dotti, Gianpietro	98	9797	99.97	2	Kochenderfer, James N	2848	0.02	50.56
3	Abken, Hinrich	80	4126	51.58	3	Brentjens, Renier J	2118	0.04	77.84
4	Brentjens, Renier J	68	9711	142.81	4	Lee, Daniel W	1886	0.02	–
5	Cooper, Laurence JN	64	4216	65.88	5	Porter, David L	1855	0.02	85.42
6	Savoldo, Barbara	60	7139	118.98	6	Rosenberg, Steven A	1807	0.03	65.99
7	Sadelain, Michel	59	12791	216.80	7	Morgan, Richard A	1787	0.01	80.94
8	Jensen, Michael C	58	7525	129.74	8	Neelapu, Sattva S	1441	0.01	–
9	Gottschalk, Stephen	53	3164	59.70	9	Turtle, Cameron J	1349	0.02	–
10	Brenner, Malcolm K	49	6685	136.43	10	Sadelain, Michel	1226	0.01	32.14
11	Grupp, Stephan A	49	9528	194.45	11	Grupp, Stephan A	1213	0.01	91.7
12	Rosenberg, Steven A	48	13476	280.75	12	Davila, Marco L	1127	0.01	43.95
13	Riddell, Stanley R	46	7788	169.30	13	Schuster, Stephen J	1064	0.03	–
14	Rooney, Cliona M	46	6835	148.59	14	Kalos, Michael	999	0.00	131.97
15	Maus, Marcela V	43	4328	100.65	15	Brudno, Jennifer N	996	0.01	–
16	Heslop, Helen E	42	6617	157.55	16	Chmielewski, Markus	960	0.01	–
17	Han, Weidong	42	2055	48.93	17	June, Carl H	945	0.01	–
18	Maher, John	41	1589	38.76	18	Kershaw, Michael H	907	0.01	73.79
19	Wels, Winfried S	41	3061	74.66	19	Gattinoni, Luca	905	0.01	42.02
20	Forman, Stephen J	40	3850	96.25	20	Brown, Christine E	899	0.02	–

-, the burstiness strength was missing because the threshold of maintenance of co-cited authors was set to 3 years.

topics, indicating that these studies were closely related to the frontier of this research.

Taken together, these findings provide a better view of the development and front lines of research on CAR-based immunotherapy in cancers.

DISCUSSION

The past 20 years have witnessed the initiation and major expansion of the research field of CAR-based immunotherapy. In the first decade, major emphasis was placed on transferring concepts and theories into clinical practice, including developing the 2nd and 3rd generation of CAR-T cells (19), establishing preclinical studies in different kinds of cancers (20) and receiving FDA approval for the first time for CAR-T cells targeting CD19 (10, 11); thus, relatively few studies were published in this interval. After the success of CD19 CAR-T in B-cell non-Hodgkin lymphoma (B-NHL) in 2008 (21), in refractory chronic lymphocytic leukemia (CLL) in 2011 (22) and in refractory and relapsed ALL in 2013 (23), studies of CAR-based cell therapy entered the fast lane. In 2017, the FDA approved two CD19 CAR-T products, CTL-019 (Kymriah) and Yescarta, indicating the beginning of the commercialization of CAR-based cell therapy. In addition, preclinical studies and clinical trials of CAR-modified immune effect cells have emerged rapidly (12, 24). Faced with the extreme proliferation of research information, we conducted a bibliometric analysis to define the complex collaboration networks and predict the next research hotspots.

The results in this study were based on bibliographic data from published articles and reviews. Therefore, where these studies were published are of great importance, because the core sources provide basic and important evidence for the research field and affect future directions (25). According to the results, *Frontiers in Immunology* has published the most literature related to CAR-based immunotherapy and has been active in recent years, indicating that this journal has focused substantially on this field, providing an effective publishing platform for academic communications. On the other hand, the most cited journals played key roles in linking to and informing the following research (26). Our results showed that both the top 10 most cited journals and the journals with most cited references were world-famous and of high quality, confirming the importance of CAR-based immunotherapy in cancers in the future. Of note, some journals ranked first on both lists, indicating that they might act as leaders and advocates to advance the research on this topic. *Blood*, the most popular co-cited journal and the journal with the highest IF among the top 10 most productive sources, was the pioneer publishing original research on CAR-T cell therapy in hematopoietic malignancy science at the very beginning of this field (27) and continued to publish novel research on CAR-based immunotherapy (28).

Based on our analysis of cooperation among countries, institutions and authors, we may identify some trends in CAR-based immunotherapy in cancers. First, the number of countries involved in this field of research on a global scale has been growing. The collaboration networks of countries and organizations illustrated that CAR-based cell therapy has attracted attention from researchers worldwide. However, most of the published works have originated from a few countries and organizations. In the past 2 decades, production from the top 10 countries accounted for 81% of all publications, and those from the top 10 most productive institutions, which were all from the US, accounted for over 10% of all publications, implying the leadership of these countries and organizations and an imbalance of academic resources. It is noteworthy that China, the only

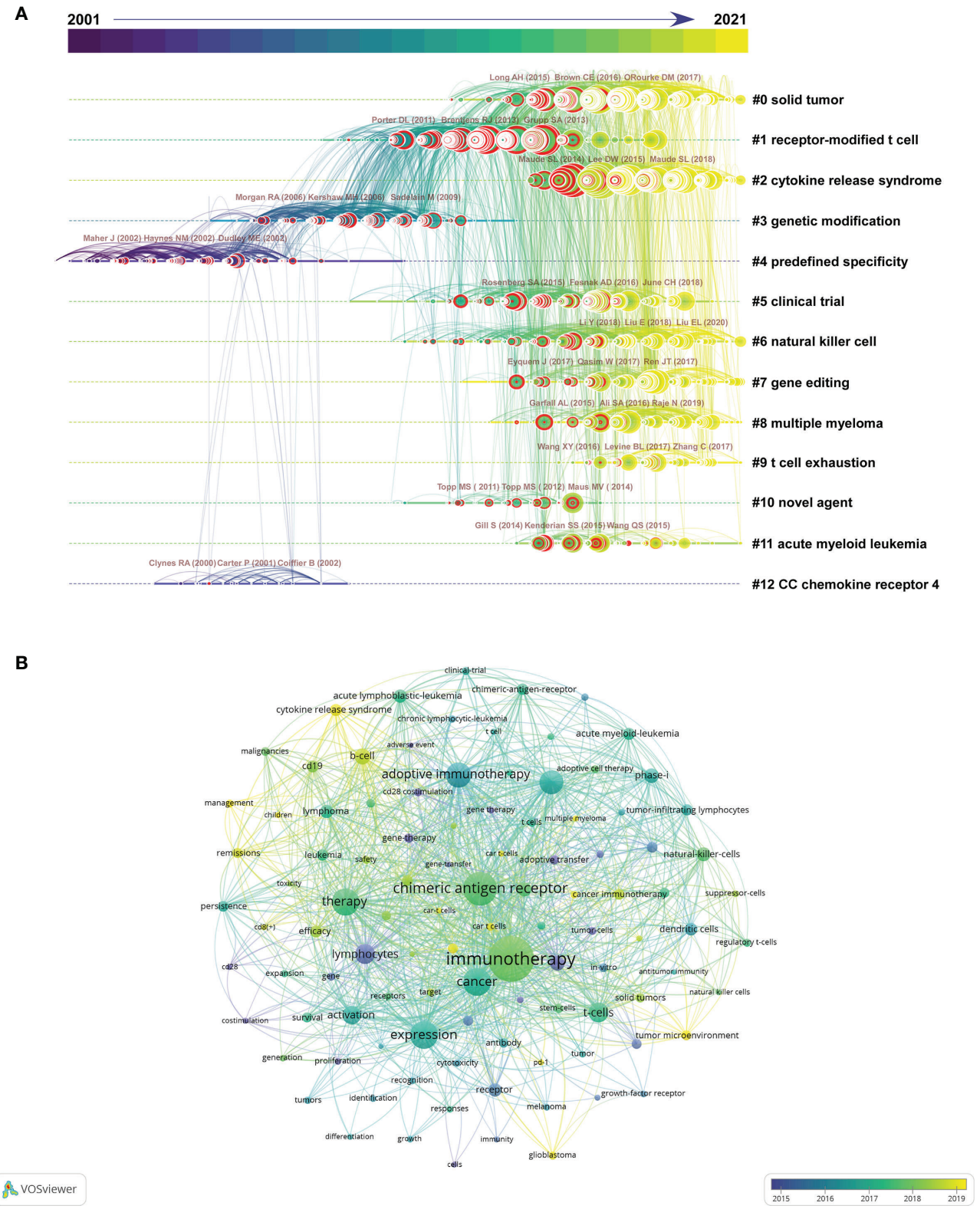

FIGURE 5 | Knowledge map of co-cited references. **(A)** Timeline view of 13 clusters of co-cited references and the corresponding tags. Each node represents an article or review, and the red circle around the node represents a reference burst. Each line represents a link between two studies. The top 3 co-cited references of each cluster are also displayed above the nodes. **(B)** The time-overlay network map of the top 100 co-occurring keywords.

TABLE 4 | Top 20 co-cited references related to CAR-based immunotherapy.

Rank	Reference	Citations	Author	Year	Type	Journal	IF	JCR
1	Chimeric antigen receptor t cells for sustained remissions in leukemia	958	Maude Shannon L	2014	Article(CT)	The New England Journal of Medicine	91.245	Q1
2	T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial	813	Lee Daniel W	2015	Article(CT)	LANCET	79.321	Q1
3	Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia	799	Maude Shannon L	2018	Article(CT)	The New England Journal of Medicine	91.245	Q1
4	Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma	766	Neelapu Sattva S	2017	Article(CT)	The New England Journal of Medicine	91.245	Q1
5	Chimeric Antigen Receptor–Modified T Cellsfor Acute Lymphoid Leukemia	662	Grupp Stephan A	2013	Article(CT)	The New England Journal of Medicine	91.245	Q1
6	Efficacy and Toxicity Management of 19-28z CAR T Cell Therapy in B Cell Acute Lymphoblastic Leukemia	637	Davila Marco L	2014	Article(CT)	Science Translational Medicine	17.956	Q1
7	CD19 CAR–T cells of defined CD4+:CD8+ composition in adult B cell ALL patients	579	Turtle Cameron J	2016	Article(CT)	Journal of Clinical Investigation	14.808	Q1
8	Chemotherapy-Refractory Diffuse Large B-Cell Lymphoma and Indolent B-Cell Malignancies Can Be Effectively Treated with Autologous T Cells Expressing an Anti-CD19 Chimeric Antigen Receptor	559	Kochenderfer James N	2015	Article(CT)	Journal of Clinical Oncology	44.544	Q1
9	CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia	539	Brentjens Renier J	2013	Article(CT)	Science Translational Medicine	17.956	Q1
10	Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia	497	Porter David L	2015	Article(CT)	Science Translational Medicine	17.956	Q1
11	Chimeric Antigen Receptor–Modified T Cells in Chronic Lymphoid Leukemia	475	Porter David L	2011	Article(CR)	The New England Journal of Medicine	91.245	Q1
12	Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia	469	Park Jae H	2018	Article(CT)	The New England Journal of Medicine	91.245	Q1
13	Regression of Glioblastoma after Chimeric Antigen Receptor T-Cell Therapy	398	Brown Christine E	2016	Article(CR)	The New England Journal of Medicine	91.245	Q1
14	Chimeric antigen receptor T–cell therapy — assessment and management of toxicities	398	Neelapu Sattva S	2017	Review	Nature Reviews Clinical Oncology	66.675	Q1
15	Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma	393	Schuster Stephen J	2019	Article(CT)	The New England Journal of Medicine	91.245	Q1
16	B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor–transduced T cells	390	Kochenderfer James N	2012	Article(CT)	Blood	22.113	Q1
17	T Cells with Chimeric Antigen Receptors Have Potent Antitumor Effects and Can Establish Memory in Patients with Advanced Leukemia	387	Kalos Michael	2011	Article(CT)	Science Translational Medicine	17.956	Q1
18	Chimeric Antigen Receptor T Cells in Refractory B-Cell Lymphomas	371	Schuster Stephen J	2017	Article(CT)	The New England Journal of Medicine	91.245	Q1
19	4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors	362	Long Adrienne H	2015	Article	Nature Medicine	53.440	Q1
20	A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma	344	O'Rourke Donald M	2017	Article(CT)	Science Translational Medicine	17.956	Q1

CT, clinical trial; CR, case report; IF, impact factors; JCR, journal citation reports.

TABLE 5 | Top 25 co-occurrence keywords and top 25 keywords with strongest citations burst related to CAR-based immunotherapy.

Rank	Keyword	Occurrences	Total link strength	Rank	Keyword with strongest citations burst	Strength	Begin year	End year
1	chimeric antigen receptor	2005	24140	1	chimeric receptor	41.93	2001	2013
2	immunotherapy	1894	22855	2	monoclonal antibody	40.14	2001	2012
3	cancer	949	11358	3	lymphocyte	38.51	2001	2013
4	expression	946	10455	4	single chain	17.7	2001	2013
5	therapy	919	10055	5	tumor cell	17.56	2001	2011
6	adoptive immunotherapy	820	9905	6	signal transduction	16.04	2001	2009
7	antitumor-activity	765	9259	7	cell	15.89	2001	2008
8	CAR-T cells	719	8938	8	receptor	15.41	2001	2010
9	natural killer cells	607	7642	9	adoptive transfer	15.39	2001	2015
10	t-cells	599	7256	10	*in vivo*	30.17	2002	2017
11	lymphocytes	578	6375	11	tumor necrosis factor	19.25	2002	2010
12	activation	537	6020	12	cd28 costimulation	20.96	2003	2015
13	phase-i trial	490	6308	13	proliferation	17.7	2003	2013
14	adoptive cell therapy	467	5897	14	cancer regression	18.72	2007	2015
15	b cell	430	5047	15	*in vivo* persistence	16	2007	2016
16	acute lymphoblastic leukemia	418	5093	16	gene therapy	35.2	2009	2015
17	*in-vivo*	379	4434	17	metastatic melanoma	18.49	2009	2015
18	cytokine release syndrome	338	3907	18	cd28	18.25	2010	2015
19	receptor	337	3779	19	antitumor activity	15.92	2010	2017
20	tumor infiltrating lymphocytes	334	4325	20	adoptive immunotherapy	38.6	2011	2016
21	lymphoma	334	3889	21	adverse event	18.28	2011	2014
22	survival	333	3877	22	persistence	20.26	2012	2016
23	dendritic cells	322	4243	23	clinical trial	15.8	2013	2016
24	tumor microenvironment	318	4081	24	modified t cell	19.27	2015	2018
25	gene therapy	318	4042	25	CAR-T	22.13	2019	2021

developing country in the top 10 most productive countries, has actively taken part in this research field, especially in recent years. However, the lack of high-quality studies, as indicated by the low number of citations per study, evidently weaker connections with other countries in the collaboration maps and few top researchers with highly co-cited productions, suggested that these latecomers in such research areas should pay more attention to innovations and cooperation to find the way out.

The 2021 Dan David Prize award was presented to 3 pioneers, Rosenberg, S.A., Eshhar, Z. and June, C.H., for their great contributions to the development of CAR-T therapy. While Rosenberg established the foundation of adaptive cell transfer therapy 50 years ago (29), Eshhar empowered cytotoxic T cells (CTLs) to recognize antigens in an MHC-independent manner by gene transfer, thus becoming one of the researchers who invented the first generation of CAR-T cells (4, 30). Subsequently, Sadelain, M. and Campana, D. designed 2nd-generation CARs, and June was devoted to the clinical translation of CAR-T cells in cancer therapy (31). In our study, these authors were identified as the most productive or co-cited authors or authors with the strongest citation burst, which further validated the accuracy of our analytical results.

The most pressing question in this study is what the front lines of CAR-based immunotherapy in cancer are. Co-cited references (i.e., papers cited by the same study) provided the knowledge base and informed the following research (32). The top 20 co-cited references and top 25 references with the highest citation bursts were both mainly clinical case series or case reports focusing on the clinical application of CAR-T cells in ALL (33), CLL (34), lymphoma (35), and safety or toxicity management (36), indicating that the major successes of CAR-based immunotherapy have been in hematopoietic malignancies. According to the clustering results, the directions of the research field have switched from predefined specificity, CC chemokine receptor 4, genetic modification, receptor-modified T cells and clinical trials to natural killer cells, gene editing, acute myeloid leukemia, solid tumors, cytokine release syndrome, multiple myeloma and T cell exhaustion. Keyword co-occurrence can also help to identify popular research subtopics (37), but in this study, we found it insufficient to provide meaningful information. Interestingly, when we combined the keywords with co-cited references and conducted a mixed analysis, we were surprised to find 14 novel clusters, which were not exactly the same as the former clusters, that better illustrated the frontiers of the specific research field with correlated keywords. These appealing subtopics included multiple myeloma, safety and toxicity, solid tumors, CAR-engineered immune cells beyond T cells, and gene editing.

Among hematopoietic cancers, multiple myeloma (MM) has always been considered an almost incurable malignancy of plasma cells because most patients will eventually relapse or become refractory to multiple treatments (38). The emergence of CAR-T cells brought hope for patients with relapsed refractory MM (R/R MM), but conventional targets, such as CD19, may be invalid because of their infrequent expression on these cancer cells. The use of B cell maturation antigen (BCMA), a member of the tumor necrosis factor superfamily that is widely expressed on the surface of multiple myeloma cells but has limited expression on normal human cells and no expression on hematopoietic cells, might be the key breakthrough, as suggested by multiple clinical trials using BCMA-targeting CAR-T therapy (39–41). Allogeneic hematopoietic stem cell transplantation (allo-HSCT)

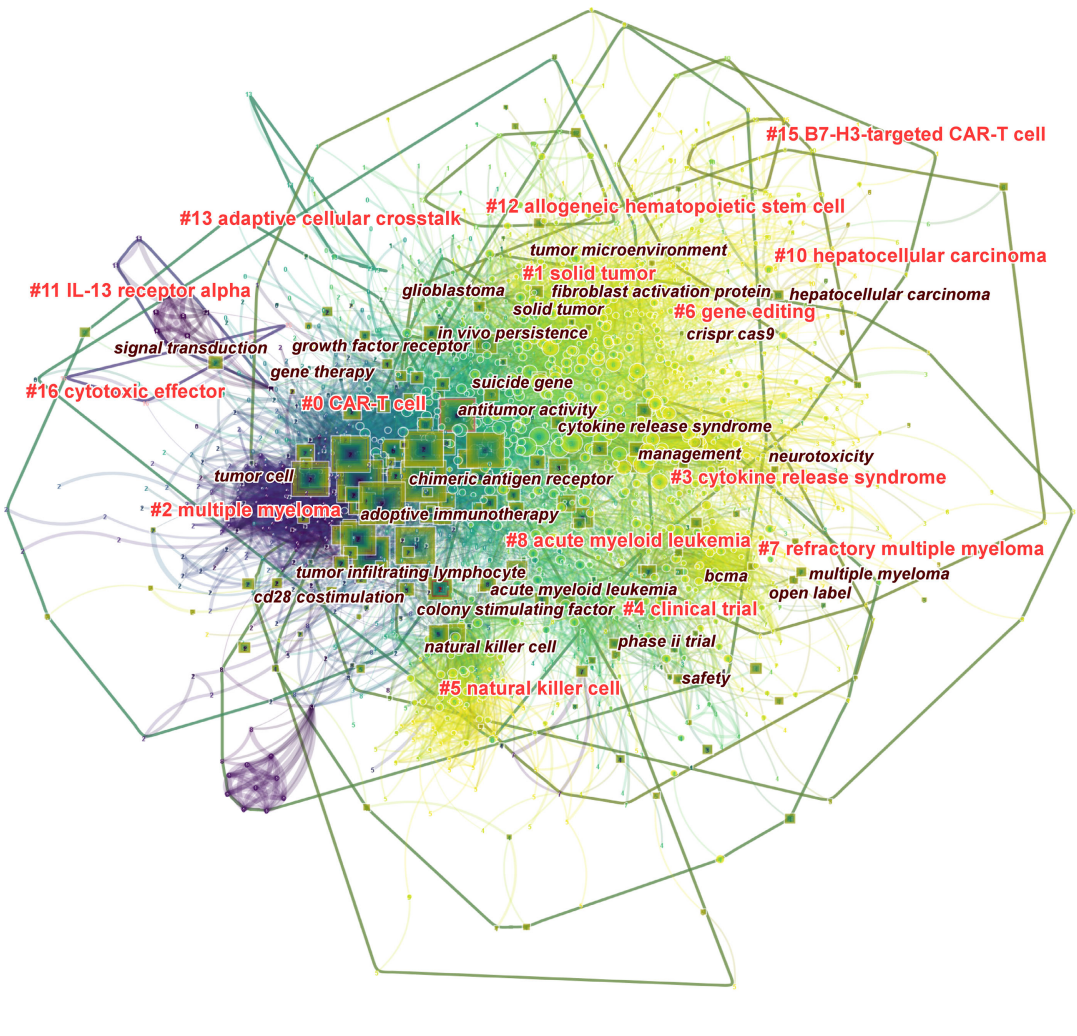

FIGURE 6 | The mixed visualization map of both co-cited references and keywords. Each node represents an article or review, each square represents a keyword, and each frame represents a cluster. The size of each node and square represent the numbers of co-citations and co-occurrences, respectively. The labels of the clusters and the most relevant keywords were also displayed adjacent to the frames. The color indicates the publication year of the articles.

TABLE 6 | Cited References in cluster 15 of the overlay analysis of co-cited references and keywords.

Rank	Reference	Citations	Author	Year	Type	Journal	IF	JCR
1	CAR T cells targeting B7-H3, a Pan-Cancer Antigen, Demonstrate Potent Preclinical Activity Against Pediatric Solid Tumors and Brain Tumors	57	Majzner Robbie G.	2019	Article	Clinical Cancer Research	12.531	Q1
2	Antitumor Responses in the Absence of Toxicity in Solid Tumors by Targeting B7-H3 *via* Chimeric Antigen Receptor T Cells	46	Du Hongwei	2019	Article	Cancer Cell	31.743	Q1
3	B7-H3 as a Novel CAR-T Therapeutic Target for Glioblastoma	17	Tang Xin	2019	Article	Molecular Therapy: Oncolytics	7.200	Q4
4	B7-H3-redirected chimeric antigen receptor T cells target glioblastoma and neurospheres	12	Nehama Dean	2019	Article	EBioMedicine	8.143	Q2
5	Eradication of Tumors through Simultaneous Ablation of CD276/B7-H3-Positive Tumor Cells and Tumor Vasculature	11	Seaman Steven	2017	Article	Cancer Cell	31.743	Q1

IF, impact factors; JCR, journal citation reports.

TABLE 7 | Top 5 citing References in cluster #15 of the overlay analysis of co-cited references and keywords.

Coverage	Reference	Citations	Author	Year	Type	Journal	IF	JCR
4	B7-H3-targeted CAR-T cells exhibit potent antitumor effects on hematologic and solid tumors	18	Zhang Zongliang	2020	Article	Molecular Therapy: Oncolytics	7.200	Q4
4	Targeting B7-H3 immune checkpoint with chimeric antigen receptor-engineered natural killer cells exhibits potent cytotoxicity against non-small cell lung cancer	10	Yang Shuo	2020	Article	Frontiers in Pharmacology	5.810	Q1
4	Route of 41BB/41BBL costimulation determines effector function of B7-H3-CAR.CD28z T cells	8	Nguyen Phuong	2020	Article	Molecular Therapy: Oncolytics	7.200	Q4
4	MEK inhibitor augments antitumor activity of B7-H3-redirected bispecific antibody	5	Li Hongjian	2020	Article	Frontiers in Oncology	6.244	Q2
4	Chimeric antigen receptor T-cell therapy in glioblastoma: current and future	3	Li Long	2020	Review	Frontiers in Immunology	7.561	Q2

IF, impact factors; JCR, journal citation reports.

is another hotly debated topic. The combination of allo-HSCT and CAR-T therapy seem to provide benefit for patients with advanced diseases, particularly high-risk B-cell acute lymphoblastic leukemia (B-ALL) (42). However, the ideal application sequence of the two landmark therapies, the optimal therapeutic window for post allo-HSCT CAR-T infusion, the value of CAR-T in treating peri-transplantation minimal residual disease (MRD), and the utility of CAR-base technology in treating graft-versus-host disease (GVHD), the most frequent complication after allo-HSCT, remain unclear (43).

The safety and toxicity of CAR-based therapy are ongoing concerns for researchers. The most frequently observed side effects in clinical trials are cytokine-release syndrome (CRS) and CRS-related encephalopathy syndrome (CRES), also named immune effector cell-associated neurotoxicity syndrome (ICANS). The supraphysiologic stimulation caused by CAR molecules with high affinity for antigens may lead to the overproduction of cytokines, such as IFN-g, IL-2, TNF-a, MIP-1, and GM-CSF (44). Moreover, it may elevate other proinflammatory cytokines, including IL-6, IL-8 and IL-10, generated by other bystander immune cells, resulting in even more severe hyperactive immune disorders, hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS) (45). To cope with these life-threatening side effects, the current strategy includes pharmacological interventions, such as anti-IL-6R mAb (tocilizumab), anti-IL-6 mAb (siltuximab) or corticosteroids, and supportive care depending on the CRS grade (46). Nevertheless, as it would be unwise to wait for the appearance of CRS, priority was given to preventative technologies, for example, establishing a better predictive system based on valid biomarkers (47), improving the "safer CAR" construct with "suicide switch" or "remote-controlled switch" (48, 49), and preventing side effects prophylactic drug administration (50), all of which require further confirmation in clinical trials.

The clinical breakthroughs of CAR-based immunotherapy in hematopoietic cancers have not been duplicated in solid tumors. Several core problems remain unsolved, including tumor-associated antigen (TAA) heterogeneity, restriction of immune cell trafficking and infiltration and an immune-suppressive microenvironment (51). New strategies have been explored to cope with these challenges. Next-generation sequencing technologies, including immunoproteomics, DNA/RNA sequencing and whole-exome screening to identify somatic mutations in tumor cells, have helped researchers discover the neoantigens or neoepitopes used as TAAs (52, 53). However, TAAs are often shared with normal tissue. The high affinity of CARs that rely on scFv could be dangerous when the vital healthy tissues become the attacked targets even with low antigen expression (54). Moreover, stronger affinity of the CARs demonstrated increased anti-tumor efficacy, but may well result in on-target off-tumor toxicity (55). The balance between the anti-tumor functions of CAR-engineering cells and safety is under active study (56). Of note, our results have distinguished that B7-H3, also known as CD276, might be a promising therapeutic target for CAR-based therapy, and there is evidence that it carries no risk of on-target off-tumor toxicity (57, 58). Recently, our research team also found that SAHA, a pan histone deacetylase inhibitor, could enhance B7-H3.CAR-T cells in solid tumors (59). Elsewhere, novel approaches have been investigated to overcome biological barriers in solid tumors, for example, using local delivery systems; applying anti-vasculature agents, chemokines or oncolytic viruses; and equipping effector immune cells with the ability to generate chemokines or heparinase to degrade the extracellular matrix (60). The most difficult obstacle lies in the tumor microenvironment (TME). The harsh physical conditions (hypoxic, poorly vascularized and with excessive interstitial pressure), immune-suppressive cell components [myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), regulatory T cells (Tregs), etc.] and abnormal metabolism caused by nutrient deprivation create a hostile environment for effector cells, leading to their impaired persistence and terminal exhaustion (61). Four major lines of the researches related to this issue are under examination: combination therapies with exogenous antagonists or cytokines, removal of specific immunosuppressive factors in effector cells, modification of CAR structure to avoid immune suppression, and the recently discovered CAR exosomes derived from CAR-T cells which displayed satisfying cytotoxic capability without PD-1 expression (51, 60–63).

In addition to T lymphocytes, recently, researchers have focused on CAR-engineered innate or innate-like immune cells, including NK cells, macrophages, dendritic cells, NKT cells and γδT cells (12, 13). Of note, CAR-NK therapy has

made progress in both liquid cancers and solid tumors (12, 64, 65). First, the superior safety of CAR-NK therapy was evidenced by the low incidences of CRS, neurotoxicity and GVHD and relatively low on-target off tumor toxicity (66). Second, CAR NK cells can kill tumor cells through CAR-dependent and NK cell receptor-dependent mechanisms, such as inducing apoptosis of target cells by releasing TNF-α, inducing ADCC mediated by CD16, and activating other immune cells by producing IFN-γ, which may kill off-target tumor cells (67). The most appealing advantage is the potential to provide "off-the-shelf" CAR-engineered products generated from the NK-92 cell line, peripheral blood mononuclear cells (PBMCs), umbilical cord blood (UCB), and induced pluripotent stem cells (iPSCs) (14, 68). Further studies should focus on enhancing the tumor infiltrating ability, extending the memory of short-lived NK cells, and optimizing the manufacturing procedure of these products (12, 24, 68).

The improvements discussed above are greatly supported by the rapid improvement of CAR modification technologies. Thanks to the development of gene editing techniques, from retroviral vector-mediated or lentiviral vector-mediated gene transfer to nonviral methods for gene engineering, many of the problems mentioned above may be solved in the near future (69). The NOT gate (70) or AND gate (71) design of dual CAR constructs protects normal tissues from CAR-T cells, while OR gate designs, including bicistronic CAR (72) and tandem CAR (73), enhance the detection capability of tumor-specific T cells to prevent tumor escape. Using transcription activator–like effector nucleases (TALENs) to disrupt the expression of the panlymphocyte molecule CD52 and the αβ T cell receptor (TCRαβ) in CAR-T cells, Qasim et al. generated the universal CAR (74), which represented a step forward to the off-the-shelf, allogeneic CAR-modified products. Moreover, the Nobel prize awarding CRISPR–Cas9 technology also plays an important role in CAR editing, such as knocking out the PD-1 gene to avoid suppressive signaling in the TME (75). Unlike other randomly integrating vectors, the CRISPR–Cas9 system could conduct efficient sequence-specific interventions, such as directing a CD19-specific CAR to the T-cell receptor α constant (TRAC) locus to generate universal CAR-T cells (76). This system could also be adopted in modifying other immune cells, such as NK cells or macrophages, to knock out immune checkpoints or enhance the expression of stimulatory cytokines, activating signals or homing receptors (68, 75, 77).

The major limitations of the CAR-based immunotherapy and the corresponding potential strategies discussed above are summarized in **Table S4**. Although we conducted a relatively thorough analysis in this study, its limitations should not be neglected. First, the included studies were limited to articles and reviews written in English and recorded in the WoSCC database, which may exclude some valuable studies, but we believe that this would not affect the general trend substantially, as systematic reviews have reported similar findings. Second, patent information was not included in this study and may need further comprehensive analysis. Finally, even with three different analytical tools, it is difficult to completely avoid bias introduced by machine algorithms.

CONCLUSION

In summary, a bibliometric analysis of CAR-based immunotherapy in cancer from 2001 to 2021 was performed through automatic analysis software. We found an increasing interest in this field worldwide, with the US being the leading country with the most publications and China being one of the most active major participants. The collaboration networks among institutions and among authors were close and comprehensive. The cutting-edge directions and hotspots in the field are multiple myeloma, safety and toxicity, solid tumors, CAR-engineered immune cells beyond T cells, and gene editing. We expect safer and more effective CAR-engineered products to be introduced to clinical application in the near future, bringing hope for patients with advanced cancers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

ZO, LQ, SF, and JL designed the study. ZO and LQ conducted the literatures searching, data extraction and re-examination. ZO, HR, TL, HL, QL, FW, TC, and YY performed the bibiometric analysis. ZO, BL, SR, SK, and LY performed the visualization. ZO wrote the manuscript. LQ, SF, and JL reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.822004/full#supplementary-material

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REVIEW
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Harnessing the Anti-Tumor Mediators in Mast Cells as a New Strategy for Adoptive Cell Transfer for Cancer

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The emergence of cancer immunotherapies utilizing adoptive cell transfer (ACT) continues to be one of the most promising strategies for cancer treatment. Mast cells (MCs) which occur throughout vascularized tissues, are most commonly associated with Type I hypersensitivity, bind immunoglobin E (IgE) with high affinity, produce anti-cancer mediators such as tumor necrosis factor alpha (TNF-α) and granulocyte macrophage colony-stimulating factor (GM-CSF), and generally populate the tumor microenvironments. Yet, the role of MCs in cancer pathologies remains controversial with evidence for both anti-tumor and pro-tumor effects. Here, we review the studies examining the role of MCs in multiple forms of cancer, provide an alternative, MC-based hypothesis underlying the mechanism of therapeutic tumor IgE efficacy in clinical trials, and propose a novel strategy for using tumor-targeted, IgE-sensitized MCs as a platform for developing new cellular cancer immunotherapies. This autologous MC cancer immunotherapy could have several advantages over current cell-based cancer immunotherapies and provide new mechanistic strategies for cancer therapeutics alone or in combination with current approaches.

Keywords: mast cells, adoptive cell transfer, cancer immunotherapy, FcεRI, IgE

ADOPTIVE CELL TRANSFER FOR CANCER IMMUNOTHERAPY

The use of autologous cells that can be targeted to tumors and induce apoptosis is an emerging therapeutic option to treat malignancies (1). From 2017 to 2018, there was a > 112% increase in the number of cell-based active agents in the global cancer immunotherapy pipeline. Most cells being investigated for autologous cancer immunotherapy have both pro- and anti-tumor mediators,

Abbreviations: MC, Mast Cell; MCs, Mast Cells; ADMC, Adipose-Derived Mast Cells; IgE, Immunoglobin E; TNF-α, Tumor Necrosis Factor Alpha; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; AMCIT, Autologous MC Cancer Immunotherapy; ACT, Adoptive Cell Transfer; CAR, Chimeric Antigen Receptor; CAR T, Chimeric Antigen Receptor T Cells; TIL, Tumor-Infiltrating Lymphocyte; CRS, Cytokine Release Storm; EFS, Event-Free Survival; ORR, Overall Response Rate; PFS, Progression-Free Survival; OS, Overall Survival; ADCC, Antibody-Dependent Cellular Cytotoxicity; FDA, US Food and Drug Administration; NK, Natural Killer Cells; DC, Dendritic Cells.

their elevated numbers correlated with positive or negative patient outcomes, and strategies investigated to either inhibit their presence in tumors or utilize them for their anti-tumor properties. This strategy of adoptive cellular transfer (ACT) is typified by the use of autologous, peripheral T cells engineered *ex vivo* to express a transmembrane chimeric antigen receptor (CAR) composed of an extracellular, antigen-specific single-chain antibody and an intracellular T cell signaling domain (CAR T) (2). The use of CAR T-cell therapies has been approved by the Food and Drug Administration for children with acute lymphoblastic leukemia and adults with advanced lymphomas (3). Other T-cell based strategies, such as tumor-infiltrating lymphocyte (TIL) and engineered T cell receptor therapies are also being investigated (4). Several non-T immune cells also have potential anti-tumor activity. For example, dendritic cells (DC) modified *in vitro* with specific tumor-associated antigens to generate an immune response for cancer-cell elimination has led to clinical trials testing their safety and efficacy (5). Natural killer cells (NK) can eliminate cancer cells with surface markers associated with oncogenic transformation and have been investigated in clinical trials in patients with hematological malignancies or solid tumors (6). Peripheral blood eosinophils and neutrophils, containing potent mediators utilized by the immune system for pathogen destruction, have recently been demonstrated to have antitumorigenic activity (7, 8). As mentioned above, strategies to control tumor macrophages have resulted in numerous clinical trials in cancer patients to eliminate them alone or in combination with other therapies (9–11). Strategies to deplete macrophages are typified through inhibition of the CSF-1/CSF-1R signaling pathway. In general, depleting strategies have had limited success as unwanted removal of beneficial macrophages in non-tumor sights is a challenge (12). Conversely, other studies have hypothesized the anti-tumor capabilities of macrophages could be exploited and thus examined employing them as a form of ACT (13). While cytotoxic macrophages proved effective in animal models, this observation did not translate to humans (14). Recent strategies using CAR are intended to polarize pro-tumor and immunosuppressive M2 phenotype to a M1 phenotype with phagocytic functions, target cancer specific biomarkers, and induce an adaptive immune response (15, 16). In short, most cells being investigated as new platforms for cancer immunotherapy exert both pro- and anti-tumor effects. Therefore, the challenges moving forward in utilizing these cells is to remove the pro-tumor activity and/or enhance their anti-tumor functions. A summary table on the history of cell types being explored or used as cancer immunotherapy is shown in **Table 1**.

CHALLENGES WITH CELL-BASED CANCER IMMUNOTHERAPIES

While the numbers of autologous cells to target and inhibit cancer cell growth *in vivo* continues, so do the unanticipated roadblocks and challenges emerge. One challenge associated with CAR T cell therapies is the potentially life-threatening side-effect loosely defined as cytokine release syndrome (CRS). The CRS is induced by a systemic release of inflammatory cytokines associated with the T cell infusion and proliferation (and other T cell stimulants) (29). Also, the overwhelming majority of unique tumor antigens reside inside tumors, out of the reach of cells targeting them. This has led to efforts to identify and optimize delivery methods such as "*in situ* vaccination" at the tumor site hypothesized to release the inner tumor-associated antigens (30–33). Relatedly, most tumor antigens are promiscuous being found in and on cancerous and non-cancerous cells. This off-target phenomenon can result in serious or even fatal outcomes. An example of this is relates to an early trial in which T-cells were targeted to melanoma-associated antigen 3 (MAGE-A3) on metastatic cancers. Nervous system cells also express a similar MAGE-A12. As a result, T cells also invaded patients brain tissue resulting in the death of 2 out of 9 patients (34). The CAR T cell target CD19 is found on normal and malignant B cells. This can lead to lower immune cell numbers and side effects, such as a higher risk of infection when healthy cells are destroyed (35). Cancer cells are readily accessible to immune cells in blood as they circulate as individual cells or small clumps of cells compared to much larger solid tumors. Thus, another consideration in ACT development is the ability of the targeted cells to enter inside the tumor and release their anti-tumor mediators and killing at multiple locations.

ALLERGY, CANCER RISK, AND THE EMERGENCE OF TUMOR TARGETING IGE'S FOR CANCER IMMUNOTHERAPY

Epidemiological studies investigating a correlation between atopic disease (e.g. serum IgE levels) and several types of cancer have demonstrated either a protective role or as a risk factor depending on the location (36–38). The retrospective, epidemiological observations that dominate the literature in general evaluated self-reported allergy histories, total IgE measurements, and/or skin prick tests and risks of cancer. An emerging area of research that suggests that patients with "ultralow" IgE serum levels have an associated with high rates of new malignancies not observed in mice (39–41). Specifically, patients with IgE deficiency and negative skin prick tests had a higher rate of malignancy than patients who had IgE deficiency with positive skin tests (41). This is important as a hallmark of IgE mediated functional responses of tissue mast cells (MCs) is the skin prick test which would support the possibility that IgE bound to MCs may have a role in tumor surveillance. As the epidemiological evidence linking atopic status to cancer risk continues to evolve (increased, decreased, or no association) so have the proposed hypotheses attempting to relate the possible mechanism linking allergy to cancer (37).

The development of atopy is initiated by the generation of IgE which binds FcεRI on MCs and basophils to induce allergic

TABLE 1 | Chronological history of cell-mediated cancer immunotherapy strategies.

Cell type	Year	Clinical trials	Strategy for targeting	Mechanism of action	Targeted cancer	Refs
Bacteria cells	1891	n/a	Injection of heat-killed cultures of bacteria into tumors to stimulate immune response.	Coley's toxins released through the stimulation of TLRs on immune cells	Sarcoma, lymphoma, testicular carcinoma, etc.	(17)
T cells	1974	n/a (First cell-mediated cancer immunotherapy)	T cells exposed to histocompatible, virus-infected target cells lysed lymphocytic choriomeningitis-infected cells *in vitro* and *in vivo*.	T-cell activation and release of perforin and granzymes	Lymphocytic choriomeningitis	(18)
NK cells	1975 to present	n/a	Endogenous type-C viruses in tumor led to immune cells reactivity in mice.	Tumor cell lysis with NK cells by secretion of IFN-γ, TNF-α, GM-CSF, and chemokines	YAC-1 lymphoma cell line	(19)
Mycobacteria	1990 and 1998	FDA approved ORR*=50% PFS**=30m	Attenuated live culture of bacteria injected in tumors to stimulate the innate immune response.	Macrophages phagocytosis	Non-muscle invasive bladder cancer	(20)
Cytolytic T lymphocytes (CTLs)	1991	n/a	Melanoma cells transduced with MZ2-E were recognized and killed by CTLs.	CTL activation and release of perforin and granzymes	Human melanoma	(21)
T cell targeted immunomodulators	1996-present	>60 FDA approved antibodies ORR=12%-70%	Anti-PD-1/L1, anti-CTLA-4, Bispecific T-cell Engager (BiTE) antibodies, etc.	T-cell activation and release of perforin, granzymes, etc.	Colon carcinoma, fibrosarcoma, melanoma, bladder cancer	(22)
Antigen presenting cells (APC)	2010	FDA approved ORR= 32% OS***	GM-CSF/PAP fusion proteins induce APC activation and mobilized anti-PAP T cells.	Stimulation of T-cell immune response against PAP and release of perforin and granzymes	Prostate Cancer	(23)
Dendritic cell (DC) vaccine	1989-present	FDA approved	Immunization of mice with DC pulsed with unfractionated tumor proteins induced protective immunity against subsequent *in vivo* tumor cell challenge.	Antigen presentation by MHC I and CD8+ T cell secretion of perforin, granzymes, etc.	Malignant lymphomas stages III and IV, Breast cancers, etc.	(24)
Dendritic cells	2010-2020	Phase II completed	DC pulsed with melanoma specific peptides or tumor cell lysate stimulate response to melanoma cells.	Antigen presentation by MHC I and CD8+ T cell secretion of perforin, granzymes, etc.	Brain tumors	(25)
CAR T cells	2010-present	FDA approval 2017 and 2018. ORR= 72% PFS=9.2 m	T cells with chimeric antigen receptor to B cell CD19.	T-cell activation and release of perforin, granzymes, etc.	CD19+ B cell acute lymphoblastic leukemia	(26)
Neutrophils	2010-present	n/a	The anti-tumor activity of alemtuzumab was shown to be primarily dependent on the ADCC mediated by neutrophils *in vivo*.	G-CSF GM-CSF	B-cell lymphocytic leukemia	(8)
Macrophages	2011-present	Used in several clinical trials as a combinatorial immunotherapy	Macrophages manipulated with antibodies or reprogrammed with metabolic/epigenetic substances to repolarize towards an anti-tumor phenotype	Downregulation of pro-tumor cytokines; Upregulation of anti-tumor cytokines	Pancreatic, melanoma, ovarian cancer, etc.	(27)
Oncolytic viral particles	2015	FDA approved ORR=16%	Viral particles modified to express GM-CSF for patients with melanoma	GM-CSF	Metastatic melanoma	(28)
Eosinophils	2019	n/a	Adoptive transfer and cytokine neutralizations.	IL-5 INFγ	Colorectal cancer	(7)
CAR Macrophages	2020	n/a	Macrophages with chimeric antigen receptor to HER2/*neu* induced anti-tumor activity.	Phagocytosis, MHC II, TNF, INFγ	HER2+ ovarian cancer, CD19+ leukemia	(15)

**ORR: overall response rate.*
***PFS: progression-free survival.*
****OS: overall survival.*
n/a, not applicable.

mediator release which induces allergy inflammation when encountering allergen. Tumor targeting IgE's are being developed in an attempt to harness the diverse acquired responses mediated through IgE (e.g. parasite expulsion), the success of targeting cancer tumor markers with humanized IgG as a therapeutic strategy (42), and the epidemiological evidence suggesting a protective role for atopy against some malignancies (43). The IgE isotype has several potential advantages over IgG antibodies approved by the FDA on the market to treat various cancers such as the low serum levels of IgE (generally 100,000 fold lower than IgG) that result in less competition for FcεR occupancy, lack of inhibitory FcεR, and induces a different anti-tumor immune response compared to IgG (44, 45). Currently, there are over 10 IgE antibodies derived from patients or

produced to target tumor-specific that have been assessed using *in vitro* and *in vivo* cancer models (**Tables 2** and **3**) For example, Fu et al. investigated the serum levels of IgE in patients with pancreatic cancer and revealed the cytotoxic effect of the purified IgE against this type of cancer cells (49). The synthesized human tumor-specific IgE's such as MOv18 IgE for ovarian carcinoma (47), Trastuzumab and C6MH3-B1 IgE's for breast (50), colon (58), and ovarian (47) cancers, Cetuximab IgE for breast and epidermoid carcinoma (52), anti-hCD20 for human B-cell lymphoma (53), anti-PSA for human prostate cancer (55), have been investigated by many research groups (**Tables 2** and **3**). Of note, the MOv18 IgE specific for the folate receptor alpha (FRα) was demonstrated to have anti-tumor effects *in vitro* and *in vivo* and is in phase 1 clinical trials testing with early data demonstrating demonstrated safety and efficacy in ovarian cancer patients (64). The survival of FRα-positive xenograft-bearing mice was increased in the presence of monocytes (48). Systemic treatment with MOv18 IgE induced TNF-α and IL-10 upregulation in tumors and significantly upregulated TNF-α, MCP-1 and IL-10 levels in bronchoalveolar lavage fluid using an *in vivo* xenograft model (65). Further *in vitro* studies examined the anti-tumor mechanism of IgE and demonstrated pro-inflammatory signals and tumor cell killing by human monocytes (66). An IgE targeting the tumor-associated antigen SLC3A2 induced FcεRI-mediated degranulation using a rodent cell line transfected with human receptor and triggered with SLC3A2-positive cell lines (58). The antibody did not trigger human basophil activation using unfractionated peripheral blood from cancer patients. In each of these studies, the mechanistic emphasis was on IgE-monocyte-mediated anti-tumor effects *via* IgE Fc-mediated ADCC.

MC IN CANCER; EVIDENCE FOR BOTH ANTI- AND PRO-TUMOR ROLES

As mentioned above, MCs are the final tissue effector cell in FcεRI-IgE allergic responses through the release of histamine and other noxious mediators. Their ability to release these mediators is also controlled by non-IgE and non-receptor mechanisms that are less common and include hypoxia, adenosine, and certain chemokines within the tumor milieu (67). MCs possess both pro-tumor and anti-tumor mediators, are found in large numbers in and around many types of tumors, and studies have variously suggested MCs should be targets for inhibition/depletion or exploited as an anti-tumorigenic strategy (67). There are various studies that showed MCs have an anti-tumorigenic role in ovarian cancer (68), clear-cell renal cell carcinoma (ccRCC) (69), B cell lymphoma (70), skin cancer (71, 72), renal cancer (73), oral squamous cell carcinoma (OSCC) (74, 75), non-small-cell lung cancer (NSCLC) (76, 77), intestine cancer (71, 78), lung cancer (79), melanoma (80–82), prostate cancer (83–85), colorectal cancer (86), and breast cancer (57, 87–92) (**Figure 1A**). Patients with elevated MC counts had a significantly better event-free survival (EFS) compared to those with fewer MCs in several tumor types. Several unique phenotypic characteristics of MCs could contribute mechanistically to anti-tumor effects. Human MCs are unique in that they have prestored, releasable (through FcεRI) tumor necrosis factor alpha (TNF-α), histamine, and tryptase within their granules. The biggest impediment to using TNF-α as an anti-cancer agent is its systemic toxicity and strategies that limit its systemic distribution through local administration in patients have been investigated (93). Histamine induces the

TABLE 2 | *In-vitro* studies of IgE dependent cancer immunotherapy.

Year	Recombinant IgE	Name	Effector cells against cancer cells	Target cancer	Ref.
1991	Anti-HIV gp120	n/a	Human blood basophils and using IgE pathway for cancer immunotherapy	H2712 mouse mammary carcinoma	(46)
1999	Anti-FRα	MOv18 IgE	Human basophils and platelets against IGROV1 cell line	Ovarian carcinoma	(47)
2003	Anti-FRα	MOv18 IgE	Monocytes, eosinophils against human ovarian carcinoma cell line IGROV1	Human ovarian cancer	(48)
2008	IgE from patient	n/a	Peripheral blood mononuclear cells against HPAC cell line	Human pancreatic cancer	(49)
2009	Anti-HER2/*neu*	Trastuzumab IgE	Monocytic cell line U937 against SKBR3; Rat basophilic leukemia MC (RBL-SX38) expressing human FcεRI, against murine colon adenocarcinoma cell line CT26-HER2/*neu*	Human HER2/*neu* positive breast and colon cancers	(50)
2011	anti-FRα	MOv18 IgE	RBL SX-38 against ovarian carcinoma IGROV-1 cell line	Ovarian carcinoma	(51)
2012	Anti-EGFR	Cetuximab IgE	Purified human monocytes and MC, U937 and RBL-SX38 cell lines against EGFR epidermoid and breast cancer cell lines	Human breast cancer and epidermoid carcinoma	(52)
2012	Anti-hCD20	n/a	Primary human MC and eosinophils derived from umbilical cord blood against VU-3C6 hybridoma and OCI-Ly8 lymphoma cancer cell lines	Human B-cell non-Hodgkin lymphoma	(53)
2012	Anti-HER2/*neu*	C6MH3-B1	MC of transgenic mice strains that express human FcεRI against murine mammary carcinoma cells that express human HER2/*neu* (D2F2/E2)	Breast and ovarian cancer	(54)
2013	Anti-PSA	AR47.47 IgE	RBL-SX-38 cells sensitized with anti-PSA IgE and challenged with PSA or artificial molecules containing multiple epitopes of PSA	Human prostate cancer	(55)
2017	Anti-FRα	rMOv18 IgE/IgG2b	RBL-2H3 targeting WAG adenocarcinoma and ovarian tumor	FRα+ cancers	(56)
2019	Anti-HER2/*neu*	Trastuzumab IgE/C6MH3-B1 IgE	Human primary skin/adipose derived MC against breast cancer cell lines	Breast cancer	(57)
2021	SF-25	SLC3A2	RBL-SX-38 cell, basophils, cancer cell lines and *in vivo* xenograft models	Colon cancer (others)	(58)

n/a, not applicable.

TABLE 3 | *In-vivo* studies of MC/IgE dependent cancer immunotherapy.

Year	IgE	Name	Animal	Anti-tumor mechanism/details	Target cancer	Ref.
1999	Anti-hFRα	MOv18 IgE	Mouse	Human peripheral blood mononuclear cells (PBMC) against IGROV1	Human ovarian carcinoma	(47)
2012	Anti-hHER2/*neu*	C6MH3-B1	Mouse	Mast cells of transgenic mice that express functional human FcεRI against D2F2/E2	Human breast and ovarian cancer	(54)
2012	Chimeric mouse-human anti-hMUCI	n/a	Chimeric mouse-human	Administration of anti-hMUC1 IgE significantly reduced growth of MUC1+ tumors in hFcεRI transgenic mice	Human breast carcinoma	(53)
2013	Anti-hPSA	AR47.47 IgE	Mouse	Mice immunized with PSA alone or in combination with anti-PSA IgE demonstrated effector cells' activation but not systemic anaphylaxis	Human prostate cancer	(55)
2014	Anti-hFRα	MOv18 IgE	Cynomolgus monkey	Human and monkey PBMC against human U937 and IGROV1 cell line	Human ovarian carcinoma	(59)
2015	Anti-hFRα	MOv18 IgE	Human	In clinical trials phase I since 2015	Human ovarian cancer	(60)
2016	Anti-hHER2/*neu*	Trastuzumab/ cetuximab IgG	Dog	HER-2 mimotope vaccines used in canine to assess safety and efficacy	Human HER2 positive breast cancer	(61)
2017	n/a	n/a	Mouse	Mice lacking multiple MC proteases (e.g. tryptase) exhibited higher extent of melanoma colonization compared to wild type animals	Mouse melanoma	(62)
2017	Anti-hFRα	hMOv18 IgE/ IgG2b	Immunocompetent rat	Anti-folate receptor-α IgE, but not IgG recruits macrophages to attack tumors *via* TNF-α/MCP-1 signaling	Human FRα+ cancers such as ovarian	(56)
2019	Rat anti-hCSPG4 IgE	n/a	Rat	Immunocompetent mice bearing CSPG4+ tumor received systemic doses of IgE	Human melanoma, glioblastoma, and breast carcinoma	(63)
2021	SF-25	SLC3A2	Mouse	SLC3A2-specific IgE demonstrated cytotoxicity against tumor cells and longer overall survival	Colon cancer	(58)

n/a, not applicable.

differentiation of immature myeloid cells and suppresses their ability to support the growth of tumor allografts (71). Increased histidine decarboxylase (which produces histamine) gene expression is associated with better relapse-free and overall survival in breast cancer patients and histamine treatment reduces tumor growth and increased apoptosis in xenograft breast cancer models (94). Mast cell tryptase alters the morphology and reduces the proliferation of human melanoma cells (82). We and others have demonstrated human MC release copious amounts (2,500-4,000 pg/ml from 10^5 cells) of granulocyte-macrophage colony-stimulating factor (GM-CSF); also, an anti-tumor mediator investigated in over 50 clinical trials (95). Mast cells showed direct antitumor effects *in vitro* and decreased angiogenesis and recruitment of NK and T cells *in vivo* (80).

In contrast, other studies have suggested a pro-tumorigenic role of MCs in different cancers (**Figure 1A**) with increased MC populations in certain tumor microenvironments associated with poor patient prognosis (96–106). These studies investigated the expression of MC markers (e.g. chymase/tryptase expression, FcεRI, c-KIT, etc.) in tumor tissues using immunohistochemistry, flow cytometry, immunoblotting, or RT-PCR techniques (67, 107, 108). In general, most published studies that attribute a pro-tumorigenic role for MC rely on correlations with increased MC numbers at a single time point, dependent on the tumor type, stage, and cancer microenvironment-and patient outcomes (**Figure 1B**). A "snapshot" analysis demonstrating an increase or decrease in MC numbers based on immunohistochemistry and subsequent association with a specific prognosis cannot be relied on to predict if these cells have a beneficial or deleterious effect. Observing an increase in MC numbers paralleled by a poor prognosis (or vice versa) demonstrates a correlation, not a causation between numbers and prognosis. Studies are needed to assess the effects selectively knocking down (i.e. CRISPR) the pro-tumorigenic and/or upregulation of anti-tumorigenic mediators from human MCs. Nonetheless, MCs are one of the first cells to infilitrate the tumor microenvironment and possess such a wide range of receptors and molecules with diverse functions that mediate tumor responses that adds to the controversial role they play in the disease (109).

Another issue surrounding the analysis of the MCs role in cancers relates to conclusions drawn from MC knockout studies, with constraints in results observed depending on the model (110–112). In some cases, a pro- and anti-tumor effect was observed in the same tumors (67, 113, 114). In addition, differences in MC phenotypic and functional responses between mice and humans have been well documented (111, 115–123). For example, Fcγ receptor expression and functional responses mediated by them on mouse and human MCs and monocytes are vastly different (124–126). Further, mouse MCs have a diverse range of various proteases (127) while human MCs principally express three proteases (tryptase, chymase, and carboxypeptidase-A) (128). Histamine is released from human MCs, while both serotonin and histamine are liberated in reasonable amounts from MCs in mice, and both contribute to the physiological effects in anaphylactic reactions, respectively in these species. Interleukin-3 has a profound effect on murine MC differentiation and function not observed with human MCs. Of course, cancer therapeutic strategies require animal models to determine efficacy of drug targets, safety, biodistribution, etc.

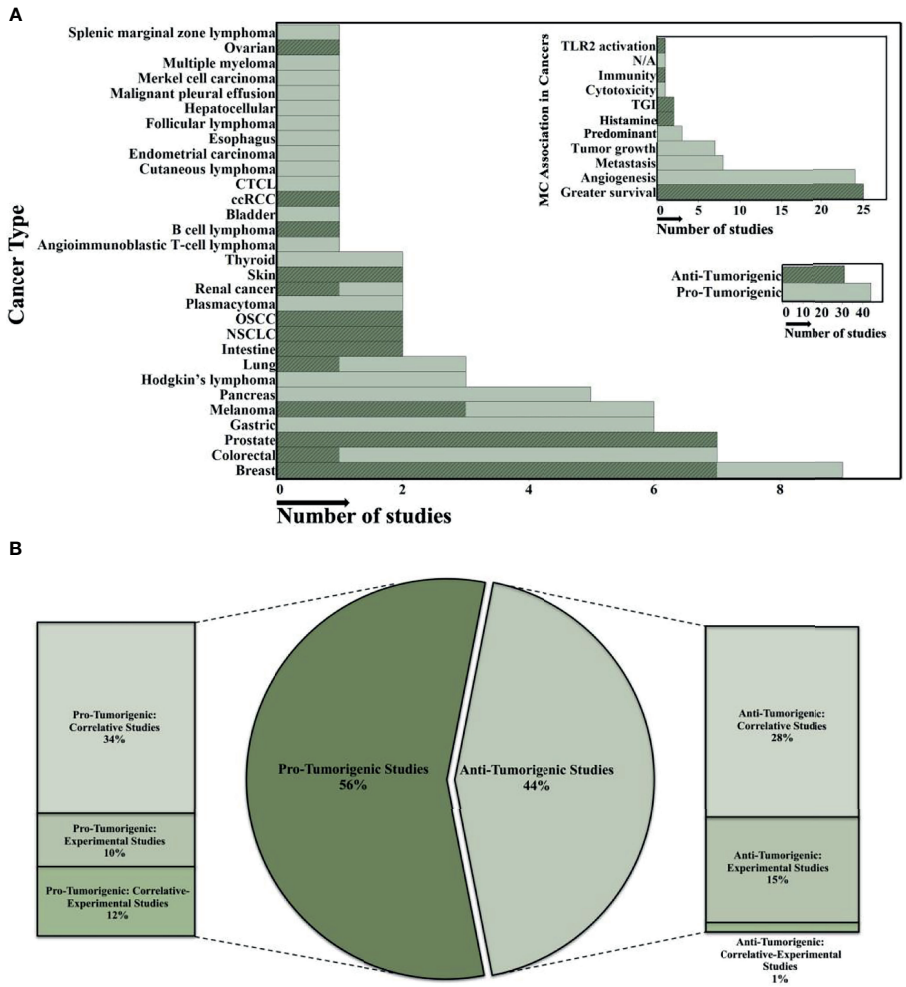

FIGURE 1 | Overview of the role of human MC in different cancerous microenvironments. **(A)** The histograms summarize the data analysis from 75 published studies on MC's anti- or protumorigenic role in the various human cancer microenvironments. The y-axis shows cancer types and MC association in different tumor environments in the large and small histogram-top, respectively. The x-axis indicates the number of studies (all histograms). Highlighted regions demonstrate the number of anti-tumorigenic studies. JMP software was used to show the distribution of number of studies and finding across the categorical variables such as cancer type and MC association in tumor microenvironments in the 75 published studies. **(B)** The Bar-Pie chart illustrates the percentage of the 75 published studies which focused on either anti- or protumorigenic effects of MCs in various cancer microenvironments. In all studies, descriptive analysis is the primary evaluation strategy for MCs role in different cancer microenvironments. In the second step, most of the studies investigated either the Correlative, Experimental, or combination (Correlative-Experimental) approaches. Cutaneous T Cell Lymphomas (CTCL); clear-cell Renal Cell Carcinoma (ccRCC); Oral Squamous Cell Carcinoma (OSCC); Non-SmallCell Lung Cancer (NSCLC); Toll-Like Receptor 2 (TLR2); Tumor Growth Inhibitor (TGI). Predominant is predominancy of the numbers of infiltrated MCs that was investigated in some studies showing the pro-tumorigenic effect on some cancers at certain stages.

But caution must be taken when extrapolating data from mouse models of cancer, especially when focused on MC numbers and MC Fc-specific mechanisms.

COULD MCS MEDIATE THE EFFICACY OF ANTI-TUMOR IGE'S AND IN IGE TUMOR SURVEILLANCE?

The mechanisms underlying the anti-tumor effects of therapeutic IgE's are mostly attributed to monocyte and macrophage infiltration and subsequent IgE-mediated activation of these cells around tumors (56, 65, 129, 130). This hypothesized mechanism seems counter-intuitive to current evidence that demonstrates tumor-infiltrating myeloid cells promote, rather than inhibit-cancer progression (10). FcεRIα-positive macrophages have been identified as critical infiltrating cells that induce tumor progression in squamous cell carcinoma (131) [although evidence is presented that the anti-FcεRI antibody used in this study was not specific for FcεRI on macrophages (132)]. As is the case with MC, macrophages may initiate, promote, or suppress the development of cancer, possess both pro (e.g. VEGF, EGF, and TGF-β) and inhibitory (e.g. nitric

oxide), and have been implicated to mediate angiogenesis, invasiveness, metastasis, and acquired resistance to therapeutic strategies largely based on correlations between cell numbers and patient outcomes (133–135).

The hypothesis that monocytes/macrophages mediate anti-tumor efficacy to tumor IgE's is also premised on the surface expression of FcεRI on monocytes/macrophages that controls their effector functions. However, the expression of FcεRI on primary human monocytes has been reported to be low (<10% in non-atopic patients), or not at all, compared to primary human MC and the expression level on monocytes is 10 to 100-fold less than observed for peripheral blood basophils from the same subjects (136, 137). While human monocytes can be manipulated to increase FcεRI expression *in vitro (*66) it is unknown if primary, tissue macrophages express FcεRI to any degree in humans. It also cannot be assumed the expression of IgE receptors will stay the same after entry and maturation in the tissues as monocytes undergo phenotypic changes upon tissue entry as they mature into macrophages (138). Others have shown human tissue macrophages do not express FcεRI (139–141). Here, another difference between species relates to reports in rodent studies that support the conclusion that macrophages can mediate anaphylaxis in mice; a phenomenon not described in humans (142, 143). One study showed that the responses of human alveolar macrophages involving IgE *in vitro (*144, 145) was most probably mediated by FcεRII (CD23) which has lower affinity for IgE, is distinct functionally from FcεRI (146, 147), and would help explain the RBC-rosetting most of these older studies used to determine IgE binding (148, 149). Lastly, other tissue cells besides MC have been reported to express FcεRI (e.g. Langerhan cells) and the low affinity receptor for IgE (150). Human basophils (and in some cases eosinophils) express FcεRI they are not normally found in tissue but are recruited following certain pathological mechanisms (151). Human eosinophils have demonstrated FcεRI expression (and have anti-tumor properties (7)) but only from donors with eosinophilia and lymphomas (152). Thus, the likelihood of tumor specific IgE binding to human monocyte-derived, tissue macrophages with unknown FcεRI expression to mediate effects seems less likely given many other IgE binding cells are present. MCs [with almost 100% FcεRI expression (57)] are as abundant or more abundant in the tumor microenvironment than macrophages depending on the tumor type. For example, the rodent form of IgE MOv18 reduced lung metastases in a syngeneic rat tumor model expressing human FRα which was attributed to TNFα, IL-10, and MCP-1 released by MOv18-triggered monocytes (56). However, the cytokine profile induced in BAL by MOv18 (TNFα, MCP-1, and IL-10) could very likely include a contribution from lung MCs which we and many others have shown produce such cytokines upon FcεRI stimulation (57, 153–156). We thus propose the binding of tumor targeted IgE Fc to human MC FcεRI and subsequent triggering of this receptor upon tumor engagement mediate the anti-tumor effects of therapeutic IgE's given the demonstrated high amounts of FcεRI on primary human MCs in the tumor milieu (157), the high numbers of FcεRI (>1 x 10^5/cell) that require only ≈100 receptors for full activation (67, 158), the affinity of this interaction (159), the juxtaposition of MCs with cancers cells (67), and the anti-tumor mediators released from MCs (160). Infusion of IgE into patients is hypothesized to increase surface expression of MC FcεRI as this receptor is dependent on serum IgE levels (158, 161).

STUDIES USING TUMOR TARGETING IGE'S AND MCS

Attempts to utilize anti-tumor mediators from MCs for cancer cell targeting was first examined using a mouse–human chimeric IgE specific for CD20 and the epithelial antigen MUC1. Cord blood-derived MCs sensitized with anti-hCD20 IgE are cytotoxic to CD20 tumor cells *in vitro (*53). We used adipose-derived mast cells (ADMC) sensitized with human anti-HER2/*neu* IgE which bound to and released MC mediators when incubated with HER2/*neu*-positive human breast cancer cells (SK-BR-3 and BT-474) resulting in TNF-α mediated, tumor cell apoptosis (57). Importantly, monomeric (shed) HER2/*neu* and serums from HER2+ breast cancer patients did not induce ADMC degranulation, suggesting that such an interaction will not trigger systemic anaphylaxis.

WILL MC BE ADDED TO GROWING LIST OF TUMOR TARGETING CELLULAR IMMUNOTHERAPY?

As discussed above the variety of cell types being investigated as new strategies for cancer immunotherapy continues to increase. MCs are similar to tumor associated macrophages as discussed above in that both have both pro- and anti-tumor capabilities and correlative studies led to assumptions regarding their role in various cancers (16). Because of this, initial efforts were aimed at depleting or repolarizing these cells as a therapeutic, anti-tumor strategy. MCs are presently at the apparently contradictory position in which rationale arguments could be made for inhibiting their numbers in the tumor milieu or increasing their numbers and harnessing their natural associated anti-tumor mediators within them. Yet from our perspective informed decisions as to deplete, increase, or repolarize MCs cannot be made until more studies assess their functional role in cancer models. As with human macrophages, human MC may need to be "repolarized" from a Type I hypersensitivity-associated cell type to an anti-cancer cell through up or down regulation of certain mediators. To this end, transfection/transduction of primary MCs has only recently been achieved using human peripheral blood derived MCs (162). The conditions that will now allow us to manipulate MC so that maximal anti-tumor activity is conferred and/or potential deleterious mediators can be deleted are being explored in our laboratory.

We propose human MCs as another cell type to be used in ACT for cancers in which tumor specific IgE's are available or

could be made. To do this, autologous MCs could be obtained from adipose tissue or cultured from peripheral blood and expanded *ex vivo*. Anti-tumor capabilities could be increased or deleterious mediators downregulated during expansion. FcεRI-positive MCs are then sensitized with IgE targeting antigens found on tumors. The tumor targeting MCs would then injected into the patient and become active upon FcεRI-IgE crosslinking. This autologous MC cancer immunotherapy (**Figure 2**) would result in the release of anti-tumor mediators within the tumor milieu (see graphical abstract). Recently we have demonstrated up to 6 x 10^6 human ADMC can be injected i.v. into mice with no toxicological effects. The ADMC, sensitized with human IgE recognizing the breast cancer antigen HER2/*neu*, shrink HER2/*neu*-positive tumors *in vivo* using a xenograft mouse model (manuscript submitted). Since human GM-CSF is not active in mice (163) the anti-tumor effects we have observed are expected to be stronger in humans in which GM-CSF would be fully active (164). This approach may enhance anti-tumor immunity through epitope spreading of cancer antigens. Importantly, this strategy may spurn new areas of research through transformation or manufacturing of tumor-targeted IgE's. Harvesting adipose tissue from patients is not difficult, commonly performed, and increasingly being used for a wide variety of clinical applications (165, 166). Recently, we have demonstrated peripheral blood, CD34-positive stem cell derived MC also have anti-tumor activity providing a second source of autologous MCs (data not shown).

ADVANTAGES OF AMCIT AS A NEW CANCER IMMUNOTHERAPY APPROACH

There is a growing list of human IgE antibodies targeting cancer antigens that have been fully characterized which provide the targeting needed to transport the MCs to the tumor sites (43). It should be noted with caution when examining anti-tumor effects experimentally that the use of certain tumor targeted IgE's is limited as human IgE does not bind mouse FcεRI receptors (167). Second, the *in vitro* incubation of MCs with IgE for targeting is extremely stable (168) and allows for the saturation of FcεRI binding, thus maximizing the effect while preventing patient IgE binding. IgE also stays bound to MCs for several months *in vivo* (169–173). Third, the adipose stem cells may be cryopreserved, reconstituted, and differentiated into ADMC while retaining expression of introduced genetic modules (data not shown). This is an important characteristic, as it greatly enhances the "logistics" of the potential therapy in that patient cells could be transduced, cryopreserved for shipping, and reconstituted when needed for therapy. Fourth, MC activation is hypothesized to induce acute inflammation and destruction of cancer cells in the tumor microenvironment due to the release of multiple mediators. The presence of dead tumor cells would allow uptake and presentation of tumor antigens by antigen presenting cells as with dendritic cells that elicit an adaptive, long lasting immune response not only to the targeted antigen but also to other tumor antigens due to epitope spreading. This would increase due to the local release of GM-CSF from MCs (174, 175) and the release of regulatory T-cell function suppressors (176). Of course, the use of tumor IgE's alone or using tumor IgE-sensitized MCs as proposed here has the obvious potential to induce a systemic allergic response. Strategies to delete select mediators in human MCs are underway but with caution as it is simply not known if those with potential "toxic" side effects also have potent anti-tumor effects. Lastly, this strategy has the potential to circumvent challenges associated with current ACT strategies in which hyperactive T cells create a cytokine storm (177), reduced chances of off-target binding (not expressed on normal cells; e.g. CD19) (178), and avoid the lack of expansion and/or persistence of autologous cells (as with NK cells) (179).

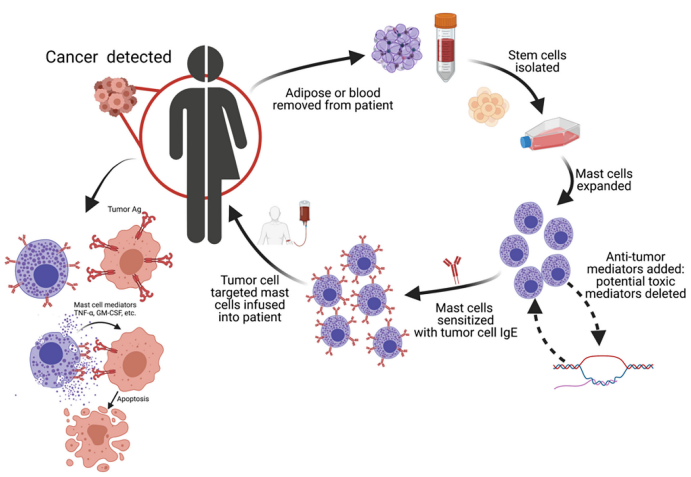

FIGURE 2 | Autologous MC cancer immunotherapy; a potential new platform for cancer therapy. We propose using MCs as a new cell type for adoptive cell transfer for cancers in which tumor-specific IgE's are available. MC progenitors are obtained from patient, MCs expanded and polarized to enhance cytotoxicity and/or minimize systemic toxicity, and re-polarized MCs reinfused into patient.

CONCLUSIONS AND FUTURE DIRECTIONS

While the role of MCs in all cancer pathogenesis is still unclear, future studies are needed to examine if *ex vivo*-derived MCs possess anti-tumor capabilities. Questions remain regarding the possibility of systemic MC activation, although this issue can be overcome as discussed above with prophylactic anti-histamines, as is common practice (180–183). An alternative clinical strategy could be to first add IgE, followed by the MCs. Antigen levels on target cells may vary in patients, which could minimize cell targeting and activation. It is not anticipated that high levels of antigen will be needed, as human MCs require ≈100 FcεRI receptors to aggregate for a full activation response (158) and all FcεRI will be saturated so that patient IgE binding will not occur. Shed antigen in serum may also "mask" the MC-bound IgE without inducing degranulation, however blocking future binding. That said, this remains unlikely given the *in vivo* studies using IgE antibodies to tumor antigens do not suggest masking (50, 58, 184, 185).

There are myriad reasons to speculate on the many potential roadblocks that could arise during the development of the AMCIT as a new cancer immunotherapy strategy. But it is important to highlight similar misgivings, inaccurate predictions regarding toxicity, and major setbacks in the early years of CAR T-cell therapy (177, 186, 187). The emergence of CAR-T immunotherapy was met with skepticism and progressed only gradually based on incremental insights over many years. Even though unexpected toxic effects in Phase 1 studies can quell any new therapy, the unfortunate reality is that it can take time to distinguish toxic effects as was the case in the first CART-19 trials (186, 188, 189). The point is that it is impossible to predict what, if any, side effects might occur *in vivo* with ADMC until studies to assess their role are performed. We believe the need for novel therapies that bring new mechanisms to combat cancer pathologies are important to investigate given the continued morbidity and mortality associated with this disease.

AUTHOR CONTRIBUTIONS

Conceptualization: CK. Methodology: MF, MM, EA, KD, TK, and CK. Investigation: MF, MM, EA, KD, TK, DM, and CK. Writing-original draft: MF and CK. Writing-review and editing: MM, MF, EA, KD, TK, DM, and CK. Funding acquisition: CK and TK. Resources: DM, TK, and CK. Supervision: DM, TK, and CK. All authors contributed to the article and approved the submitted version.

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Human Tumor Targeted Cytotoxic Mast Cells for Cancer Immunotherapy

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The diversity of autologous cells being used and investigated for cancer therapy continues to increase. Mast cells (MCs) are tissue cells that contain a unique set of anti-cancer mediators and are found in and around tumors. We sought to exploit the anti-tumor mediators in MC granules to selectively target them to tumor cells using tumor specific immunoglobin E (IgE) and controllably trigger release of anti-tumor mediators upon tumor cell engagement. We used a human HER2/*neu*-specific IgE to arm human MCs through the high affinity IgE receptor (FcεRI). The ability of MCs to bind to and induce apoptosis of HER2/*neu*-positive cancer cells *in vitro* and *in vivo* was assessed. The interactions between MCs and cancer cells were investigated in real time using confocal microscopy. The mechanism of action using cytotoxic MCs was examined using gene array profiling. Genetically manipulating autologous MC to assess the effects of MC-specific mediators have on apoptosis of tumor cells was developed using siRNA. We found that HER2/*neu* tumor-specific IgE-sensitized MCs bound, penetrated, and killed HER2/*neu*-positive tumor masses *in vitro*. Tunneling nanotubes formed between MCs and tumor cells are described that parallel tumor cell apoptosis. In solid tumor, human breast cancer (BC) xenograft mouse models, infusion of HER2/*neu* IgE-sensitized human MCs co-localized to BC cells, decreased tumor burden, and prolonged overall survival without indications of toxicity. Gene microarray of tumor cells suggests a dependence on TNF and TGFβ signaling pathways leading to apoptosis. Knocking down MC-released tryptase did not affect apoptosis of cancer cells. These studies suggest MCs can be polarized from Type I hypersensitivity-mediating cells to cytotoxic cells that selectively target tumor cells and specifically triggered to release anti-tumor mediators. A strategy to investigate which MC mediators are responsible for the observed tumor killing is

described so that rational decisions can be made in the future when selecting which mediators to target for deletion or those that could further polarize them to cytotoxic MC by adding other known anti-tumor agents. Using autologous human MC may provide further options for cancer therapeutics that offers a unique anti-cancer mechanism of action using tumor targeted IgE's.

Keywords: breast cancer, mast cell, IgE, FcεRI, TNF-α, AllergoOncology, adoptive cellular transfer, cell-based cancer immunotherapy

INTRODUCTION

The tumor microenvironment is a complex mixture of resident stromal cells, infiltrating hematopoietic cells, a heterogeneous population of cancer cells, and tissue mast cells (MCs) arising from hematopoetic progenitors. Mast cells are unique immune cells that secrete a diverse array of biologically active compounds that can stimulate, modulate, or suppress an immune response. The role of MCs in most forms of cancer has been investigated and depending on the model and experimental design, they appear to exhibit either a pro- or anti-tumorigenic role (1–4). This is due in part to observational/correlative studies performed in humans and contradictory roles concluded from rodent MC *in vivo* models given the known differences in human vs. murine MCs (5, 6). Increased histamine decarboxylase gene expression, responsible for MC histamine expression, is associated with better relapse-free and overall survival in breast cancer (BC) patients (7). Histamine thus reduces tumor growth and increases apoptosis of cancer cells *in vivo (*8). Further, human MC granules contain stored, releasable (through FcεRI) tumor necrosis factor alpha (TNF-α) (9). In addition, we and others have demonstrated that human MC release significant amounts (2,500-4,000 pg/ml from 10^5 cells) of granulocyte-macrophage colony-stimulating factor (GM-CSF) upon FcεRI exposure (10). This is relevant as both TNF-α and GM-CSF suppress tumor cell proliferation, induce tumor regression, enhance anti-tumor co-therapies, and have been investigated in over 50 clinical trials (11, 12).

Biologics are being investigated for cancer immunotherapy and the number and variety of FDA-approved, humanized Abs to treat various cancers continues to grow (13). While all are of the human IgG class, IgE has several potential advantages over IgG which has led to the development of several tumor targeted, humanized or chimeric IgE's with distinct anti-tumor immune responses compared to IgG's (14, 15). While the contribution of MCs in studies examining the anti-tumor effects of IgE have been largely overlooked, it is highly plausible that the IgE Fc binding to MC-FcεRI mediates the anti-tumor effects given the affinity of this interaction (16), the juxtaposition of MCs to tumor cells (17), and the anti-tumor mediators within MCs that are released by the interaction of IgE with tumor antigens (10, 18).

These observations form the rationale for these studies: that MCs play a beneficial and exploitable role in cancer microenvironments. This potential anti-tumor effect from the addition of *ex vivo* derived, tumor-targeted, IgE-sensitized MCs into tumor proximity has not been investigated. We thus hypothesized that MCs could be polarized to direct their anti-tumor mediators within the local tumor habitat with a targeted release dependent on a controllable triggering system activated only when encountering tumor-specific antigens. To explore this concept, we used both *in vitro* and *in vivo* models targeting human epidermal growth factor receptor 2 (HER2/*neu*) which is already a target for several IgG-based immunologics for cancer therapy (19). We demonstrate that autologous human MCs have potent anti-tumor properties that can be controllably released upon tumor cells engagement. The MC-induced apoptosis was dependent on tumor IgE, occurred *in vitro* and *in vivo*, and revealed a unique array of tumor genes affected by MCs incubation. MCs could also be genetically manipulated to potentially decrease pro-tumor or toxic mediators and conversely to increase anti-tumor mediators. We present evidence that autologous MCs can be polarized to direct their hyper-inflammatory, anti-tumor function as a novel strategy for cancer cell immunotherapy.

MATERIALS AND METHODS

Kinetics of Tumor Antigen-Induced MC Mediator Release

Human MCs cultured from peripheral blood (BDMC) obtained from healthy adult volunteers as described after informed consent was obtained under a protocol approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases (2009-I-0049) (20). When cultures reached at least six weeks of age they were sensitized with or without 0.1 μg/ml human anti-HER2/*neu* IgE (from Absolute Antibody, Boston, MA) as described previously with adipose-derived MCs (ADMC) and skin derived MCs (10). After 1 h, cells were washed and filtered (40 μm) HER2/*neu*-positive BT474 (ATCC, Manassas, VA) or SK-BR-3 (SL032; Genecopoeia, Inc, Rockville, MD) cells were added at the indicated number and mediator release and apoptosis measured as described (21). All experiments were performed in duplicate from three separate donors and significant differences ($p<0.05$) were determined using the Student *t*-test.

Abbreviations: MCs, Mast cells; BC, Breast cancer; ADMC, Adipose-Derived mast cells; BDMC, Peripheral blood derived mast cells; HER2/*neu*, Human epidermal growth factor receptor 2; FcεRI, High affinity receptor for IgE; IgE, Immunoglobin E; TGF, Transforming growth factor; TNF-α, Tumor necrosis factor alpha; GM-CSF, Granulocyte-macrophage colony-stimulating factor; ACT, Adoptive cell transfer; CAR T, Chimeric antigen receptor t cells.

Production of Luciferase-Transduced Cancer Cell Lines

BT474 cells were transduced with the lentiviral vector pTK1261 (CMV-Luc-GFP/BSD) containing Firefly luciferase, and the fusion GFP/Blastcidine selection marker gene. Cells were washed with media containing Blasticidin S (20 μg/ml) four times to remove non-infected cells. The incorporation of the Firefly gene was confirmed by a luciferase assay that resulted the light intensity of 3,545 relative light units (RLU)/μg, compared to 13 RLU/μg of control, non-infected cells revealing approximately 100% of BT474-1261 cells expressed luciferase protein (**Supplementary Figure 3**). Luciferase/GFP dual-labeled SK-BR-3 cancer cell line was purchased from GeneCopoeia, Inc. Rockville, MD.

Time Lapse Confocal Microscopy

To assess the ability of anti-HER2/*neu* IgE sensitized MCs to induce cell death of HER2/*neu* expressing tumor cells, MCs (1.5×10^5) were sensitized with 0.1 μg/ml of anti-HER2/*neu* IgE or psIgE for 2 h. Filtered (40 μm) BT474-1261 or SK-BR-3 breast cancer cells (5×10^4) on coverslips were labeled with MitoTracker™ Red (1 μM, ThermoFisher Scientific) for one hour. The washed MCs were labeled with CellTracker™ Deep Red (1 μM) for one hour, washed, and added to the cancer cells in medium containing 7 μM of CellEvent™ Caspase 3/7 Green (to detect activated caspase-3/7 in apoptotic cells; Invitrogen) for 1 h according to the manufacturers protocol. Time-lapse photography was recorded over a period of up to four days in a live cell chamber as described (10).

Scanning Electron Microscopy

Cocultures of ADMC and filtered BT474-1261 were treated as above, washed, and fixed with 2.5% glutaraldehyde and 4% formaldehyde in PBS for 2 h. Following three rinses with distilled water, the samples were dehydrated through a gradient series of ethanol (50%-100%) and further dehydrated using a critical point dryer and hexamethyldisilazane (HMDS) to preserve the cell surface protrusions. Specimens were set on stubs by conductive double-sided carbon tape and covered by 10 nm thick gold-palladium by a sputter coater (Leica Microsystems, IL, USA). Cells were examined using a field emission scanning electron microscope (Zeiss Auriga FIBFESEM, Zeiss, NY, USA) at 4 kV.

Maximum Tolerated Dose, *In Vivo* Imaging and Efficacy of ADMC

No studies have examined the maximum tolerated dose (MTD) of human MCs *in vivo*. To assess this MTD, female Nu/Nu mice weighing 20–28 g between 8 and 16 weeks of age were bred and maintained in a pathogen-free animal facility. Mice (6/group) were injected i.v. with or without the indicated number of cells in PBS with an insulin syringe with a 28 Ga needle and following isoflurane anesthesia. Serums were collected before and after injection for analysis and weights taken daily for comparison between control and ADMC injected mice.

For *in vivo* imaging female Nu/Nu mice were implanted with 60 day 17-β-estradiol pellets (Innovative Research of America) subcutaneously 3 days before implantation of luciferase-transduced BT474-1261 tumors. Tumors were implanted as single cell suspensions (2×10^6 cells) into the subcutaneous sacral region or into the inguinal mammary fat pads. When tumors reached ≥200 mm^3 mice were injected intratumorally or intravenously with IgE-sensitized (0.1 μg/ml for 2 h), CellBrite 680-labelled ADMC using the indicated number of cells in PBS. For *in vivo* optical imaging, mice were anesthetized with inhalation of isoflurane mixed with oxygen, and maintained under anesthesia during imaging. The CIVIS-Spectrum optical imaging system with excitation filter and emission filter at 675nm, 720 nm, respectively, was used to conduct fluorescence imaging before and after the injection of ADMC and *in vivo* optical imaging was taken at different time points. After each imaging session, animals were recovered from anesthesia and placed in the normal housing cage between imaging time points. At the end of the last imaging time point, animals were euthanized, and tumor tissue was collected for histology and flow cytometry analysis. For 2D optical image analysis, regions of interest were drawn on the tumor region of each animal, and the total radiant efficiency (excitation normalized fluorescence signal) was measured using Living Imaging software (PerkinElmer, Inc.), and plotted against imaging time points. In addition, to colocalize the fluorescence signal in tumor, 3D fluorescence imaging was also conducted using the IVIS-Spectrum imaging system, followed immediately by a CT imaging using an *in vivo* microCT imaging system (Quantum-GX, PerkinElmer, Inc.) with animals kept under anesthesia in a multi-modality imaging shuttle. The 3D optical images and CT images were co-registered to localize the fluorescence signal in tumor regions.

To assess the anti-tumor effects of ADMC in tumor bearing mice, tumors were generated as above. When tumors reached ∽200 mm^3, mice (4/group) mice were injected i.t. with PBS or HER2/*neu* sensitized ADMC ($1x10^6$) at day 0 and tumor volume and mean survival were assessed. Primary tumor volumes (TV) were calculated according to the National Cancer Institute (NCI; Bethesda, MD) protocol [TV= (length x $width^2$)/2], where "length" and "width" are the long and short diameters of the tumor mass in millimeters. The significance of the differences in tumor volume was determined using the two-tailed Student's *t*-test and survival by the non-parametric Peto-Peto-Wilcoxon Log-Rank test. Samples of blood were taken for MC mediator analysis and toxicological indicators (22). Tumors were removed to assess the presence of MCs by H&E and immunohistochemistry with anti-tryptase Abs (clone G3; a gift from Lawrence Schwartz, VCU Health Systems) that do not cross react with mouse MC tryptases (23).

Gene Expression Profiling of mRNA

RNA sequencing was performed on BT474-1261 cells *in vitro* and BT474-1261 tumors from *in vivo* experiments. Anti-HER2/*neu* IgE, PBS, or psIgE sensitized ADMC (2×10^6) were incubated with filtered BT474-1261 cells (1×10^6) or tumors (>200 mm^3) at days one and four. Preparations of the ADMC used for these experiments were from male donors and the BT474-1261 cells are female so that the up- and down-regulation of RNA's is focused just on the tumor cells through gating of XY

vs XX chromosomes. Total RNA was extracted using Qiagen RNeasy Plus Universal mini kit following manufacturer's instructions (Qiagen, Hilden, Germany). Extracted RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA).

Library Preparation With Poly-A Selection and HiSeq Sequencing

RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented for 15 minutes at 94°C. First strand and second strand cDNAs were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by limited-cycle PCR. The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA). The sequencing libraries were clustered on 1 lane of a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq instrument according to manufacturer's instructions. The samples were then sequenced using a 2x150bp Paired End (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS). Raw sequence data generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

For data analysis, sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic (v.0.36). The trimmed reads were mapped to the *Homo sapiens* reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step. Unique gene hit counts were calculated by using feature Counts from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between the groups of samples was performed. The Wald test was used to generate p-values and Log2 fold changes. Genes with adjusted p-values < 0.05 and absolute log2 fold changes > 1 were called as differentially expressed genes for each comparison. A gene ontology analysis was performed on the statistically significant set of genes by implementing the software GeneSCF. The goa_human (GO) list was used to cluster the set of genes based on their biological process and determine their statistical significance. A PCA analysis was performed using the "plotPCA" function within the DESeq2 R package. The plot shows the samples in a 2D plane spanned by their first two principal components. The top genes, selected by the highest row variance, were used to generate the plot (Genewiz, South Plainfield, NJ).

Construction of Lentiviral Particles and Transduction of ADMC and Adipose Stem Cells

The 293T HEK cell line expanded in 12 x 15 cm dishes until 80-90% confluency and transfected by plasmids containing Green Fluorescent Protein (GFP) (Verma Lab, Salk Institute for Biological Studies) and three additional plasmids (pMDL, pRev and pVSVG) (Invitrogen cat. no. K4975-00) for viral packaging. To make the plasmid mix, 270 μg of transfer vector, 176 μg of pMDL (Gag/Pol), 95 μg of pVSVG (vesicular stomatitis virus glycoprotein) and 68 μg of pREV, were added to 13.5 ml of 0.25M $CaCl_2$ + 13.5 ml 2x BBS solution. The transfection mixture was spread in drops to each plate (2.25 ml/plate) and incubated overnight. Afterward, the media was removed and 15 ml of fresh DMEM + 2% FBS was added to each dish and incubated overnight. The next day, the supernatants were collected, stored at 4°C, and 15 ml fresh media added to the cells. The second harvest of supernatant was collected the next day. To concentrate the viral particles ultracentrifugation was applied at 70,000 *x g* for 2 h at 4°C, which yielded a final concentration of 10^9 particles per ml. MCs were seeded in a 24 well plate (10^5 cells/well) and different amounts of virus (5, 10, 20 and 30 μl) were used to infect the cells.

RESULTS

Peripheral Blood-Derived Human MCs Become Activated Through FcεRI Upon HER2/*neu* -Positive Breast Cancer Cell Binding

It has been reported that HER2/*neu* IgE-sensitized ADMC release anti-tumor mediators upon FcεRI crosslinking when challenged with HER2/*neu*-positive BC cells (SK-BR-3 and BT474-1261) (10). Another autologous source of human MCs that could be utilized for potential cellular-based cancer immunotherapy is the generation of human MCs from precursors in peripheral blood (20). Thus, the ability of BDMC sensitized with the anti-HER2/*neu* IgE to degranulate in the presence of BC cells was investigated. Both SK-BR-3 and BT474-1261 expressed high amounts of HER2/*neu* (**Figure 1A**). The BDMC exhibited significant ($p<0.05$) degranulation and cytokine production through FcεRI when co-incubated with BDMC sensitized with anti-HER2/*neu* IgE (**Figure 1B**). Thus, ADMC, skin-derived, and BDMC respond similarly to BC-induced mediator release (10).

Scanning Electron Microscopy of ADMC Interactions With BT474-1261 Cells

To further investigate the dependence of HER2/*neu* IgE in mediating the binding of MCs to BC cells we performed SEM to examine the nature of this interaction. As seen in **Figure 2A**,

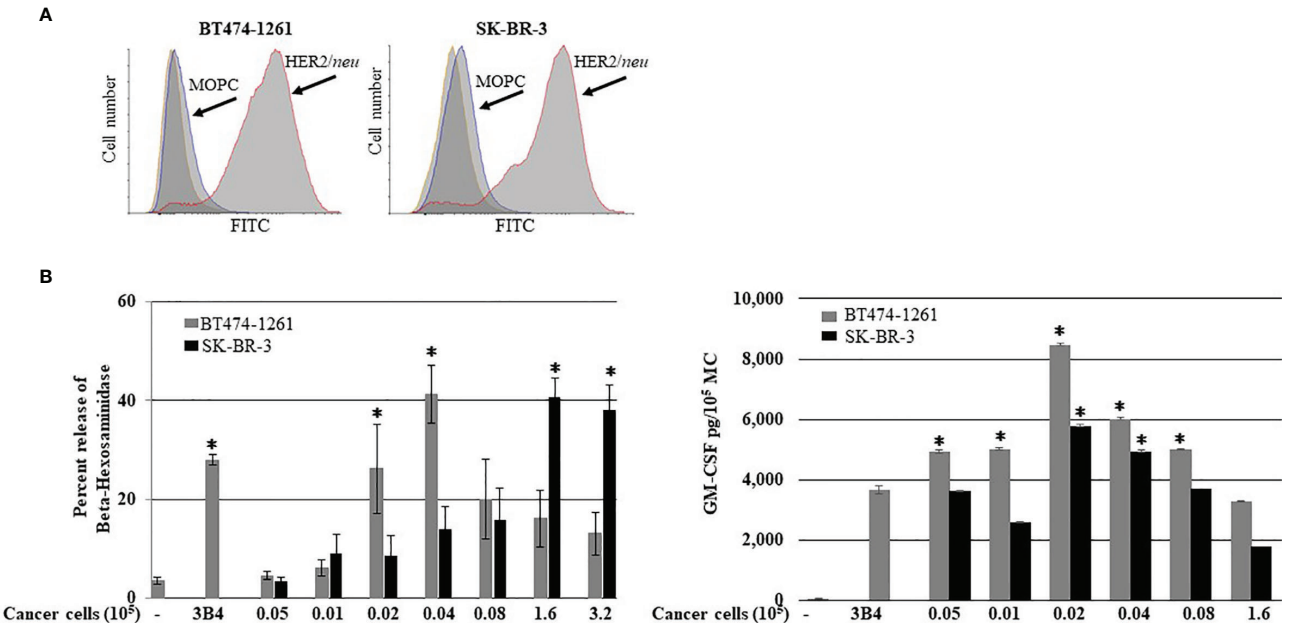


FIGURE 1 | Breast cancer cell-induced BDMC mediator release. **(A)** Expression of HER2/*neu* on BT474-1261 or SK-BR-3 cells using FACS. BDMC were sensitized with 1 μg/ml anti-HER/*neu* IgE washed, and incubated with filtered BT474-1261 or SK-BR-3 cells and degranulation (**B**; left) and cytokine release (**B**; right) assessed. Data are from a single experiment representative of experiments performed on cells derived from three separate donors. Error bars represent ±SD. *$p<0.05$ compared with non-IgE (spontaneous) release.

ADMC sensitized with non-specific IgE did not bind to the BT474-1261 and displayed smooth membranes with few membrane protrusions as determined by SEM. In contrast, the HER2/*neu* IgE-sensitized ADMC bound to HER2/*neu*-positive BT474-1261 cells (**Figure 2B**). Analysis of the specific interaction between the ADMC and BT474-1261 cells indicated the cells were in the process of degranulation (up to 50%; **Figure 1B**) with the appearance of ruffling, lamellipodia-like membrane ridges, and cellular protrusions. Higher magnifications (≤ 2 μm) indicate these protrusions attach to the BC cells through heterocellular, tunneling nanotube (TnT)-like structures. We hypothesized that TnT transport MC-specific molecules into the cancer cells. Further investigation is required to elucidate the role of TnT in cancer cell apoptosis accurately. These results demonstrate that HER2/*neu* IgE regulates MC binding and reveals that this process involves the formation of cellular protrusions between FcεRI-crosslinked MCs with tumor cells. We are currently examining the significance of these TnT to determine if they represent a structure by which mediators and/or organelles could be exchanged.

Time-Lapse Confocal Microscopy of HER2/*neu* IgE Sensitized ADMC Inducing Apoptosis of BT474-1261 Cells

The ability of MCs to induce BC cell death *in vitro* was next investigated. As expected, the BT474-1261 cell killing was dependent on the anti-HER2/*neu* IgE, as non-specific IgE-sensitized ADMC did not bind to or induce significant BT474-1261 apoptosis (**Figure 3A**) compared to those ADMC sensitized with anti-HER2/*neu* IgE (**Figure 3B**). Strikingly, the anti-HER2/*neu* IgE-sensitized ADMC appeared to penetrate and migrate through the cell tumor masses as seen in the time-lapse video using ADMC (**Supplementary Video 1**). The binding of anti-HER2/*neu* IgE-sensitized BDMC to BT474-1261 cells also induced apoptosis of the BC cells (**Figure 3C** and **Supplementary Video 2**). Higher magnifications demonstrated the anti-HER2/*neu* IgE-sensitized ADMC degranulated upon binding and internalization into HER2/*neu* cancer cells as seen by the increase in MC granules and translucent apoptotic bodies within the tumor cells (**Figure 3D**). Quantification of the apoptotic cells from psIgE ADMC challenged BC cells and HER2/*neu* IgE sensitized ADMC and BDMC challenged cells is shown in **Figure 3E**. To further examine these early (<24 h) interactions, we found that non-labelled BT474-1261 cells challenged with non-specific IgE-sensitized ADMC did not bind to or induce BT474-1261 apoptosis (**Figure 3F**). The anti-HER2/*neu* IgE-sensitized ADMC were observed to migrate and be internalized where they appeared to degranulate within the BT474-1261 (**Figure 3G**) and SK-BR-3 (**Figure 3H**) cells. The formation of apoptotic bodies within the cancer cells was evident initially at the point of MC:tumor cell binding which covered the whole inner cell after ∽24 h. Further, the formation of apoptotic bodies appeared at the contact points between the MC and cancer cells, suggesting a bi-directional release of anti-tumor mediators following FcεRI activation (**Figure 3H**). These experiments indicate anti-HER2/*neu*-sensitized MCs bind to, penetrate, degranulate, and induce apoptosis of HER2/*neu*-positive cancer cells and cell masses.

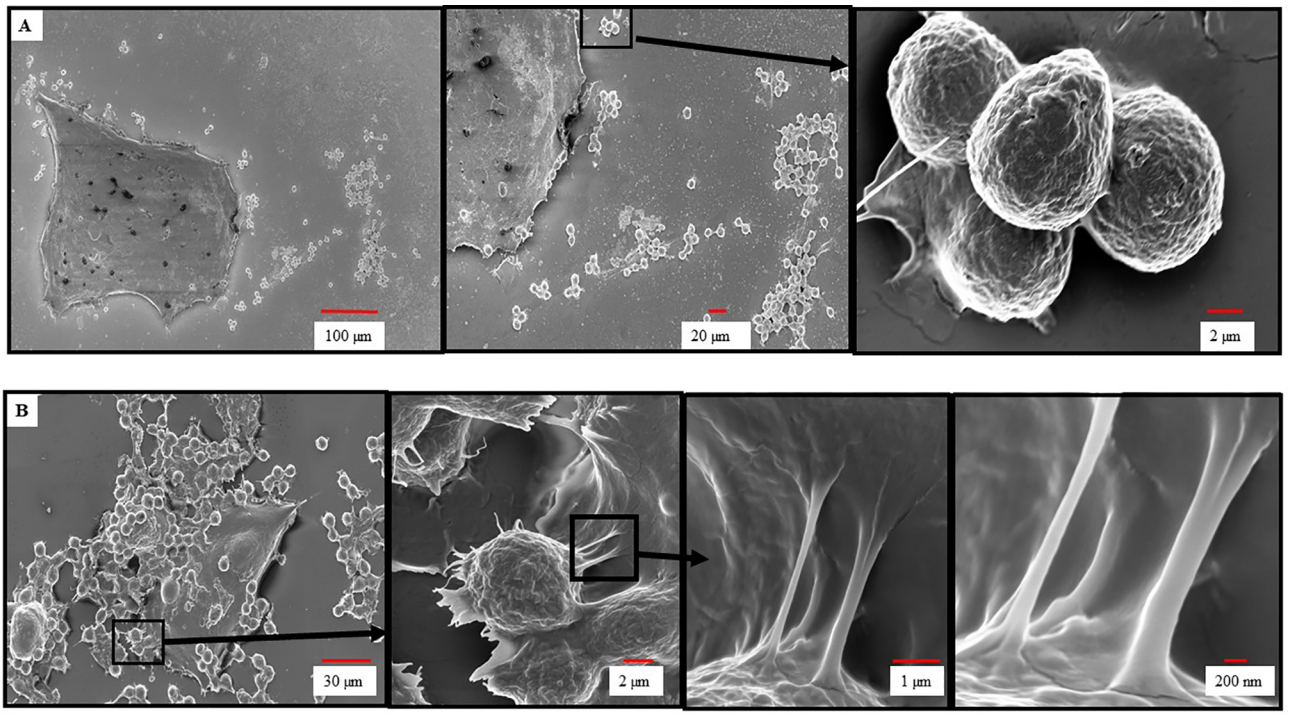


FIGURE 2 | Scanning electron microscopy of ADMC and BC cell interactions. ADMC (1.5×10^5) were sensitized with 1µg/ml of non-specific IgE **(A)** or anti-HER2/*neu* IgE **(B)** for 1 h, washed, and added to HER2/*neu*+ BT474-1261 cells (about 60% confluence) and incubated for 24 h at 37°C. Cells were collected, supernatants removed for β-hexosaminidase release, and samples fixed for SEM as described above. Arrows denote magnified areas.

Mast Cells Induce Cancer Cell Apoptosis Through TNF-α and Apoptosis Related Genes

To further assess the mechanisms of how MCs induce cancer cell death the up- or down-regulation of RNA was assessed in MCs sensitized with tumor-specific vs. non-specific IgE and BT474-1261 cells *in vitro*. As seen in **Figure 4A**, gene expression analysis of tumor cells challenged with tumor-IgE ADMC revealed a significant upregulation of several members of the TNF superfamily (TNFSF). This included the TNF-related apoptosis-inducing ligand (TRAIL/TNFRSF10), which further supports a role for FcεRI-release of TNF-α from MCs that induces apoptosis of cancer cells (10). Additionally, significant upregulation of apoptotic genes such as TNF ligand superfamily members were observed which are targets for clinical development (24). Concomitantly, the FcεRI-induced activation of ADMC induced significant downregulation of several genes shown to be markedly upregulated in certain cancers and metastasis including cystatin 4 (CST4), secretagogin (SCGN), calcitonin receptor (CALCR), hairy and enhancer-of-split related with YRPW motif (HEY2), and the leucine-rich-repeat-containing G protein-coupled receptor 6 (LGR6) (25–30) (**Figure 4B**). Gene ontogeny analysis confirmed significant effects on the cellular response to TNF, apoptosis, and pro-inflammatory pathways (**Figure 4C**).

HER2/*neu* IgE Sensitized ADMC Shrink HER2/*neu*-Positive Tumors *In Vivo*

We next tested the anti-tumor activity of ADMC *in vivo* using a human BC cell xenograft model with BT474-1261. In preparation for *in vivo* studies, experiments were performed to assess the MTD of ADMC. As seen in **Figure 5A**, no toxicity was observed (as assessed by the expected normal >20% change in body weight). Additionally, no other overt signs of distress (e.g. anaphylaxis, death) were observed in mice using up to $6x10^6$ ADMC i.v. To assess the efficacy of ADMC as anti-tumor cells, luciferase-luciferase-expressing HER2/*neu*+ BT474-1261 cells were implanted in immunocompromised mice then injected intratumorally (i.t.) with dye-labelled HER2/*neu* IgE-sensitized ADMC cells and visualized using a whole-body scanner. As seen in **Figure 5B** and **Supplementary Video 3** injecting tumors i.t. demonstrated that ADMC could be visualized and observed to shrink the tumor after 4 days. It is demonstrated for the first time that mice expressing HER2/*neu*+ tumors injected with HER2/*neu* IgE-sensitized ADMC have significantly reduced tumor size and increased survival rates (>30%) compared to those labelled with non-specific IgE (**Figures 5C, D**). Importantly, mice exhibited no change in behavior characteristic of anaphylaxis (**Supplementary Video 4**), respiratory rate or body temperature when injected with HER2/*neu* IgE-sensitized ADMC, consistent with the conclusion that IgE-activation does not induce systemic

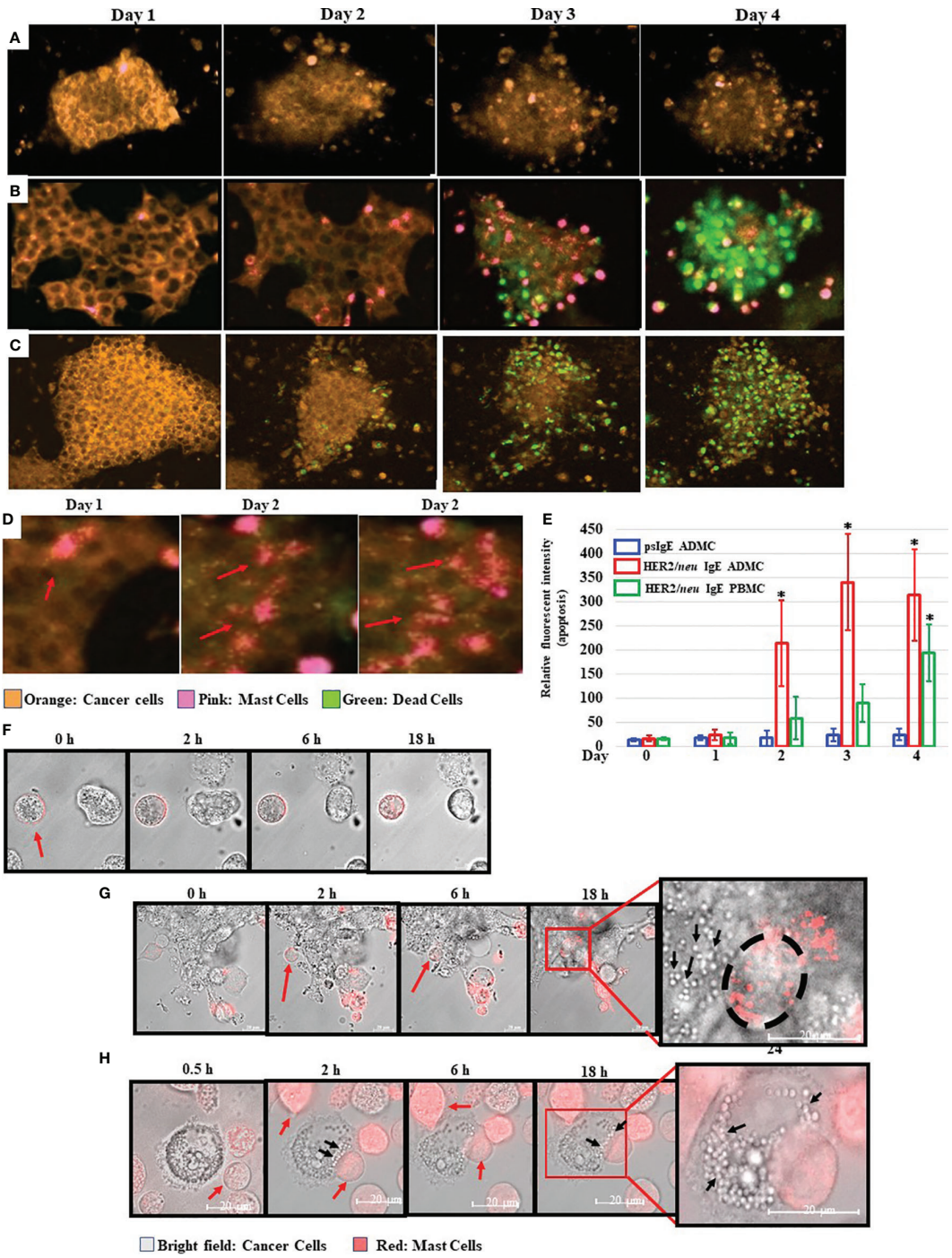
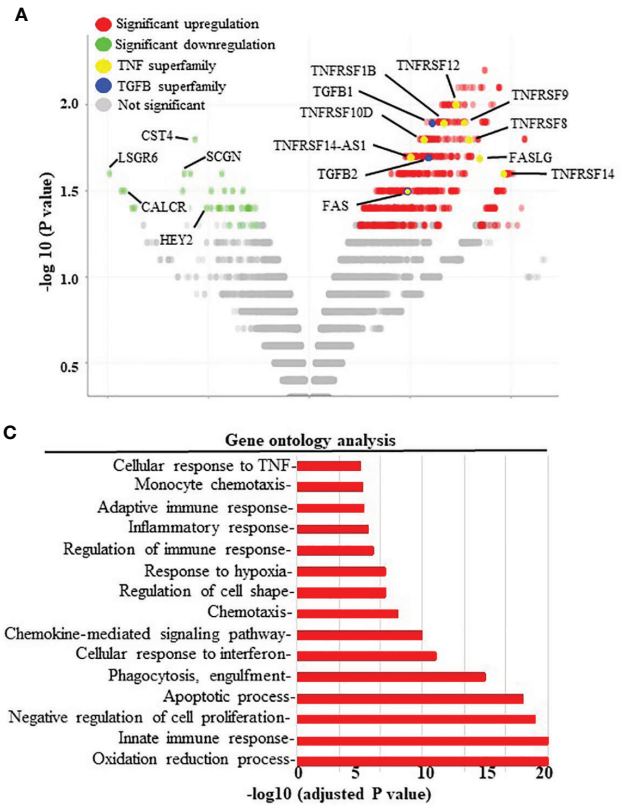


FIGURE 3 | Mast cells bind to, penetrate, degranulate, and induce FcεRI-dependent apoptosis of BC cells. CellTracker™-Deep Red labelled ADMC **(A, B)** or BDMC **(C)** ($7.5x10^4$) were sensitized with 1 μg/ml non-specific IgE **(A)** or anti-HER2/*neu* IgE **(B, C)**, washed, and incubated with MitoTracker™-Red HER2/*neu*+ BT474-1261 in culture medium containing a fluorescent apoptosis detecting dye (CellEvent Caspase 3/7; 1:100). Cells were monitored in a live cell chamber attached to a confocal microscope for up to 4 days. **(D)**. The amount of fluorescent intensity of the apoptosis dye was quantified using Image J of the average of ten fields of view at 63x magnification (±SD, *$P < 0.05$) **(E)**. Higher magnifications of several degranulating MCs is shown to illustrate the release and spreading of MC granules upon cancer cell HER2/*neu*-induced activation of FcεRI. The red arrows indicate spreading MC granules within the BT474-1261 cancer cell mass. **(F–H)**. MitoTracker™-Red labelled MCs ($7.5x10^4$) were sensitized with 1 μg/ml non-specific IgE **(F)** or anti-HER2/*neu* IgE **(G, H)**, washed, and incubated with HER2/*neu*+ BT474-1261 **(F, G)** or SK-BR-3 **(H)** cells and monitored in a live cell chamber for the indicated times. The red arrows indicate the dye-labelled ADMC, black arrows indicate cancer cell apoptotic bodies, and black circle indicates the outline of the ADMC within the tumor cell mass. Experiments are representative of 3 different donors.



B

Top differentially expressed genes

BT-474 cells with HER2/neu IgE MC vs. psIgE MC 4 day

Downregulated			Upregulated		
Gene Name	log2FoldChange	p value	Gene Name	log2FoldChange	p value
LGR6	-9.91	0.02	C1QB	10.57	0.02
CALCR	-9.32	0.03	LUCAT1	10.54	0.02
LINC01621	-9.16	0.04	MEDAG	10.51	0.03
AL807740.1	-8.79	0.04	AP003064.2	10.51	0.03
TRH	-8.66	0.04	CCL19	10.47	0.03
SFTPA2	-6.28	0.03	PCSK1	10.46	0.03
SCGN	-6.2	0.03	IGHG2	10.44	0.02
BRINP3	-5.99	0.04	AQP10	10.43	0.02
CYP1A2	-5.9	0.03	BPIFA1	10.39	0.05
CST4	-5.67	0.02	ADORA3	10.31	0.02
HEY2	-5.11	0.04	WNT2	10.13	0.02
ASCL1	-4.87	0.04	MS4A4A	10.02	0.02
IGSF21	-4.86	0.03	SENCR	9.9	0.02
MUC19	-4.54	0.04	FASLG	9.9	0.02
KLK10	-4.48	0.04	HTR2A	9.89	0.02
TRGC1	-4.38	0.04	LILRB1	9.84	0.02
SCGB2A2	-4.34	0.02	NTRK1	9.83	0.04
SYNPO2L	-4.21	0.03	VSIG4	9.81	0.02
MPPED2	-4.08	0.04	AC002070.1	9.79	0.02
NPY1R	-4.07	0.04	RASAL1	9.77	0.03

FIGURE 4 | Differential gene expression analysis BT474-1261 cells treated of HER2/*neu* IgE-sensitized vs. non-specific IgE-sensitized ADMC. **(A)** Volcano plot of global transcriptional changes of BT474-1261 cells treated with ADMC sensitized HER2/*neu* IgE vs ADMC sensitized psIgE. Each data point in the scatter plot represents a gene. The ADMC were from male donors so that they could be distinguished from BT474-1261 cells by Y-chromosome gene mapping. Red and green indicate significant ($P < 0.05$) differences in control vs. treated cells. The log2 fold change of each gene is represented on the x-axis and the log10 of its adjusted p-value is on the y-axis. Genes with an adjusted p-value less than 0.05 and a log2 fold change greater than 1 are indicated by red dots (upregulated genes). Genes with an adjusted p-value less than 0.05 and a log2 fold change less than -1 are indicated by green dots (downregulated genes). **(B)** Top genes significantly affected by HER2/*neu* vs control ADMC. **(C)** Gene ontology analysis. Significantly (adjusted P-value <=0.05) differentially expressed genes were clustered by their gene ontology and the enrichment of gene ontology terms were tested using Fisher exact test (GeneSCF v1.1-p2).

anaphylaxis. Tumors obtained at day 4 post injection stained with H&E (**Figure 5E**; left) showed clear differences with large areas where apoptotic cells and debris could be observed (**Supplementary Figure 1**) in the HER2/*neu* IgE MCs challenged animals that were not observed in the controls. Immunohistochemistry with anti-human tryptase (**Figure 5E**, right) or control Abs (**Supplementary Figure 2**) revealed HER2/*neu* IgE MCs were retained within tumors. Non-specific IgE sensitized MCs were sparsely found in the primary tumor area but instead were observed around blood vessels in the tumor which we hypothesize is how these cells exit the tumor. It was verified that the psIgE ADMC did not persist within the tumor as did the HER2/*neu* IgE sensitized ADMC as measured by MC signals (**Figure 5F**). Serum histamine and human tryptase were not detected following *in vivo* ADMC challenge and liver function as determined by ALT and AST levels were no different in ADMC challenged and non-challenged mice (**Figure 5G**). These experiments suggest that MCs have anti-tumor activity *in vivo* and promote a localized anti-tumor function in the tumor microenvironment and do not induce anaphylaxis or liver toxicity.

We next used the xenograft tumor model to examine the anti-tumor mechanisms of MCs. The mRNA from tumors obtained from mice treated with HER2/*neu* IgE sensitized MCs was compared to those tumors obtained mice treated with psIgE-sensitized MCs. Overall, there was a downregulation of pro-tumor intermediates and pathways and an upregulation of those pathways involved in apoptosis and tumor inhibition. For example, at one day post MC infusion, gene expression analysis (**Figures 6A–C**) revealed a significant upregulation of genes associated with therapeutic benefits of trastuzumab [e.g. MATK (31)], tumor suppressors [RGS7 (32), MPPED2 (33)], and tumor cell apoptosis [IL-32 (34)]. Similarly, at day 4 MC-treated tumors had significant upregulation of inhibitors of angiogenesis [CNNM1 (35), FILIP1L (36)], tumor suppressors [VWA5A (37), SYNPO2 (38), ALDH1A2 (39)], and those found overexpressed in certain tumors with unknown function [MPV17 (40)] (**Figures 6D–F**). Tumors treated with tumor-

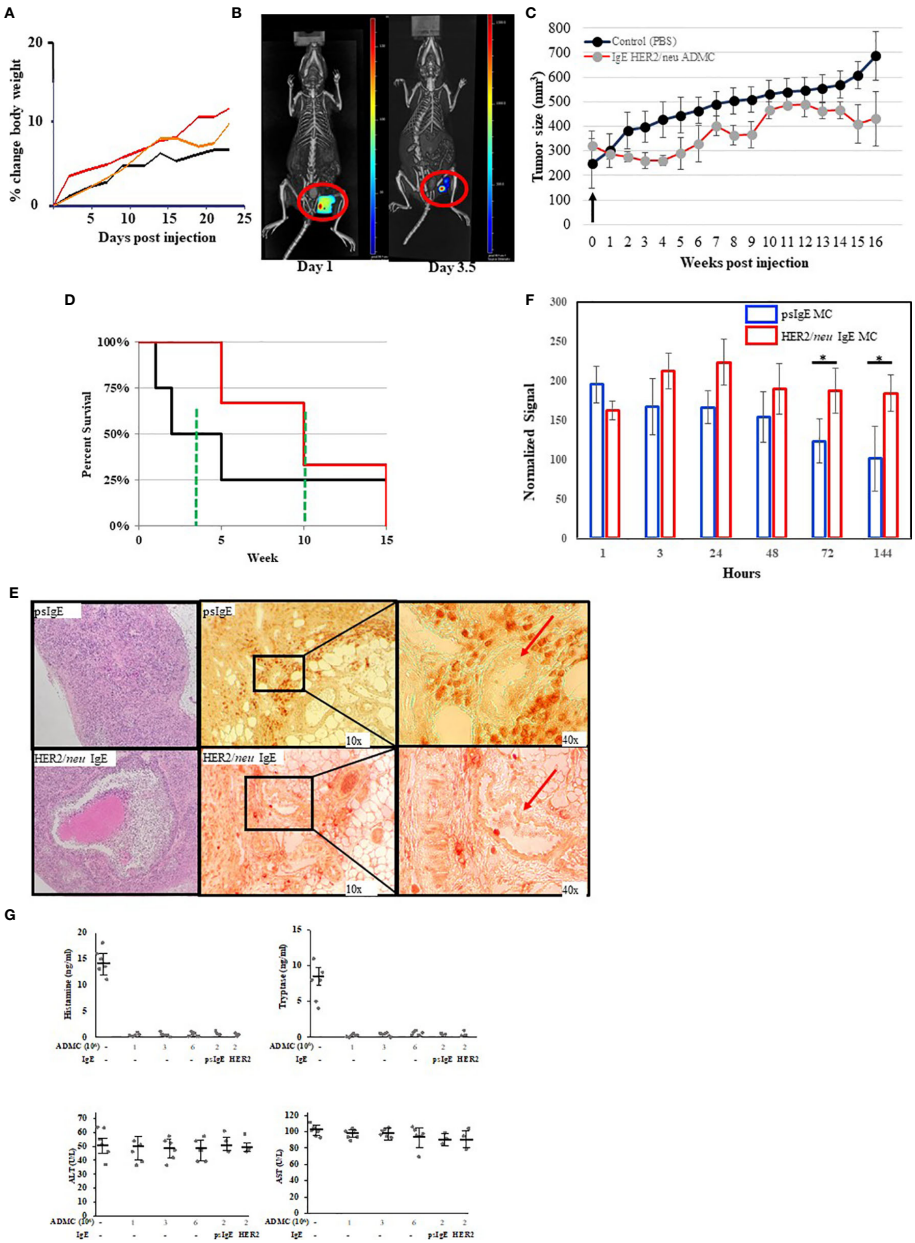


FIGURE 5 | *In vivo* analysis of ADMC. **(A)** Maximum tolerated dose of ADMC *in vivo*. NU/NU mice (female; 8-week-old; 5 per group) injected i.v. with $1x10^6$ (black), $3x10^6$ (orange), or $6x10^6$ (red) ADMC and mouse weights analyzed over time shown. No significant loss in body weight was observed to indicate toxicity. **(B–D)**. Tumor binding and anti-tumor activity of ADMC *in vivo*. Tumors were prepared by injecting Nu/Nu mice with $2x10^6$ HER2/*neu*+ BT474-1261 cells. Mice were injected i.t. with $1x10^6$ HER2/*neu* **(B)** monitored over 7 days using 3D fluorescence imaging with an IVIS-Spectrum imaging system, followed immediately by a CT imaging using an *in vivo* microCT imaging system. **(C, D)** Antitumor activity of ADMC in HER2/*neu* positive human BC tumors in Nu/Nu immunocompromised mice. Tumors were prepared as in **(B)**. When tumors reached ∽200 mm^3, mice (4/group) were injected i.t. with or without (PBS), HER2/*neu* or psIgE sensitized ADMC ($2x10^6$) at day 0 and tumor volume **(C)** and mean survival **(D)** assessed. Points, mean tumor volume (mm^3); bars, ±SD. The green line represents the mean survival for each group. **(E)** Tumors obtained from mice treated as above (day 4) were incubated with H&E (left), mouse anti-human tryptase (middle), or MOPC (**Supplementary Figure 2**) followed by peroxidase anti-mouse Abs. Red arrows are blood vessels. HER2/*neu* IgE-sensitized ADMC are evenly distributed inside the tumor tissue (bottom), while psIgE-sensitized ADMC are sparse inside the tumor tissue (top) and are mostly found in close vicinity of blood vessels, where they flow into the blood stream. **(F)** Quantification of ADMC numbers was assessed by monitoring the fluorescence of the CellBrite signal in the tumors *in vivo* over time. Data are represented as the mean ± s.e.m. of n = 3 technical replicates. *P < 0.05. **(G)** Cytotoxic human MCs do not induce systemic MC release or toxicity. NU/NU mice (no tumors) were injected i.v. with indicated numbers of ADMC. Serum was collected at 1 hour (histamine and tryptase) or after 8 weeks (ALT/AST) and assessed for each mediator using commercially available kits. In last 2 points comparing psIgE vs HER2/*neu* IgE sensitized ADMC injected mice had established (>200 mm^3) HER2/*neu*-positive tumors as above. Spiked mouse serums were used as controls for histamine and tryptase. Data are represented as mean ± s.e.m. No statistical significance change in serum levels was observed using ANOVA between non-ADMC treated animals vs treated.

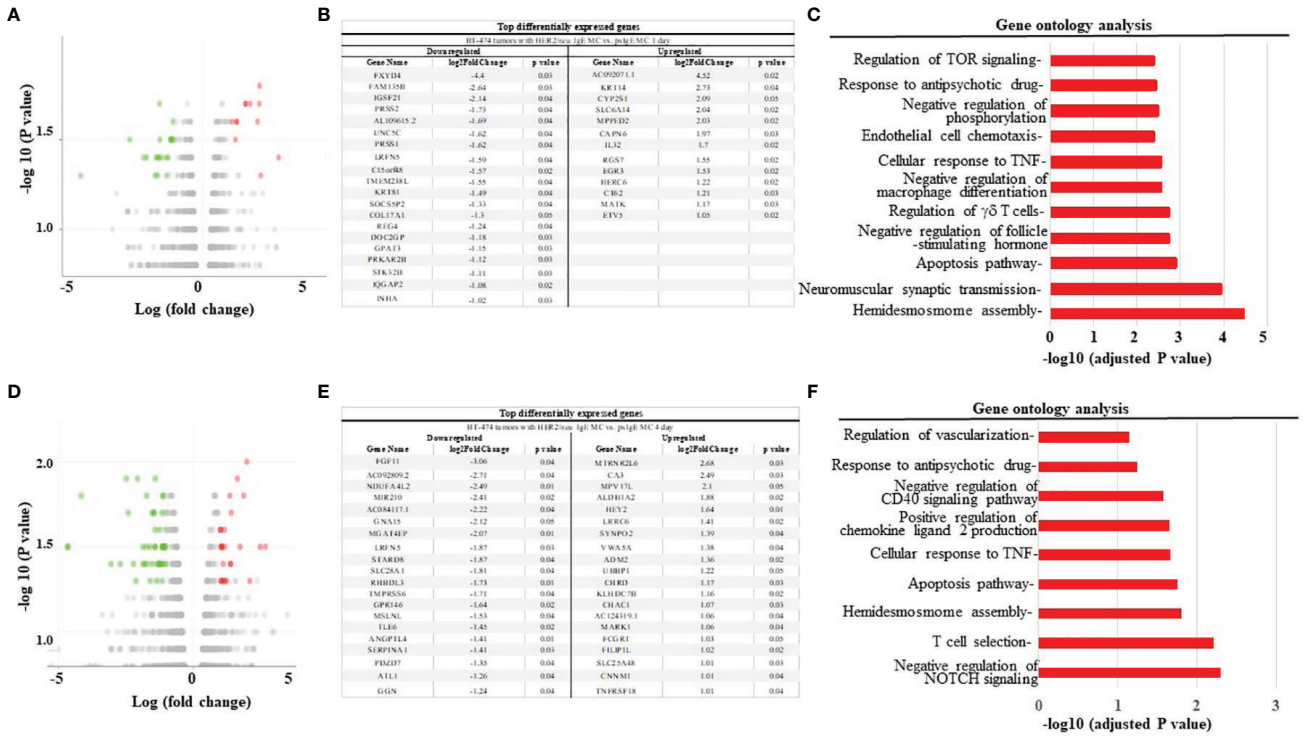


FIGURE 6 | HER2/*neu*-sensitized ADMC induce apoptosis of HER2/*neu*-positive BT474-1261 tumors through a unique set of gene pathways. **(A, D)** Volcano plot of differentially expressed genes in tumors from mice engrafted with human female BT474-1261 cells. When tumors reached 200 mm^3, 1 x 10^6 HER2/*neu* or pslgE sensitized ADMC from a male human donor were injected intratumorally. Tumors were harvested after 1 **(A)** or 4 day **(D)** and scRNAseq was performed on the tumor samples. Adoptively transferred ADMC were distinguished from BT474-1261 tumors by Y-chromosome gene mapping. Red and green indicate significant ($P < 0.05$) differences in pslgE vs HER2/*neu* treated animals. Each data point in the scatter plot represents a gene. The log2 fold change of each gene is represented on the x-axis and the log10 of its p-value is on the y-axis. Genes with a p-value less than 0.05 and a log2 fold change greater than 1 are indicated by red dots (upregulated) and those with less than -1 are indicated by green dots (downregulated). The top genes significantly up or down regulated are shown at days 1 **(B)** and 4 **(E)**. Gene ontology analysis was performed on the statistically significant set of genes by implementing the software GeneSCF v.1.1-p2. The mgi GO list was used to cluster the set of genes based on their biological processes and determine their statistical significance. A list of genes clustered based on their gene ontologies was generated for tumors treated with HER2/*neu* IgE-sensitized vs. pslgE sensitized ADMC at day 1 **(C)** and 4 **(F)**.

specific IgE-sensitized MCs compared to controls also had several downregulated signaling intermediates including those shown to promote tumor growth and progression in tumor microenvironments [PRSS2 (41), PRSS1 (42), REG4 (43), PRKAR2B (44), NDUFA4L2 (45), LRFN5 (46), and ANGPTL4 (47)]. These signaling intermediates and pathways provide a unique overview of the mechanisms of MC-induced killing of tumor cells and work is underway to further validate and delineate the anti-tumor mechanisms involved.

Transduction of ADMC and Adipose Stem Cells With GFP

Little is known about what MC mediators are responsible for the apoptosis of cancer cells upon FcεRI activation and contribute to anti-tumor activity. While we have demonstrated that TNF-α is involved in *in vitro* killing using anti-TNF-α Abs (10), this method is not optimal for *in vivo* assessment. To begin to address the question, we sought to inhibit/knockout selected MC-specific mediators to assess which mediators are anti-tumorigenic. With this information, it might then be possible to polarize and engineer the MCs to remove potential toxic mediators, while retaining (or supplementing) anti-tumor mediators. Our first step was to determine if primary human ADMC could be transduced. As seen in **Figure 7**, it is shown for the first time that primary ADMC (**Figures 7A, B**) and adipose-derived stem cells (which give rise to ADMC; **Figures 7C, D**) maintain viability after being transduced with GFP (under the control of a CMV promoter) *via* a lentivirus system. The transduced ADMC did not lose phenotypic characteristics or FcεRI functional responses over four months (data not shown). Importantly, GFP-transduced adipose stem cells that had been cryopreserved in liquid nitrogen and reconstituted retained GFP fluorescence (**Figures 7E, F**). These same adipose stem cells differentiated into ADMC retained GFP up to four months (**Figures 7G, H**). The observation that adipose stem cells can be cryopreserved without losing viability has important implications for therapeutic applications.

Experiments were performed to assess select inhibitor/knocking down of MC-specific mediators to determine what human MC mediators exert pro- or anti-tumorigenic effects. Tryptase is a MC-specific mediator that has been reported to

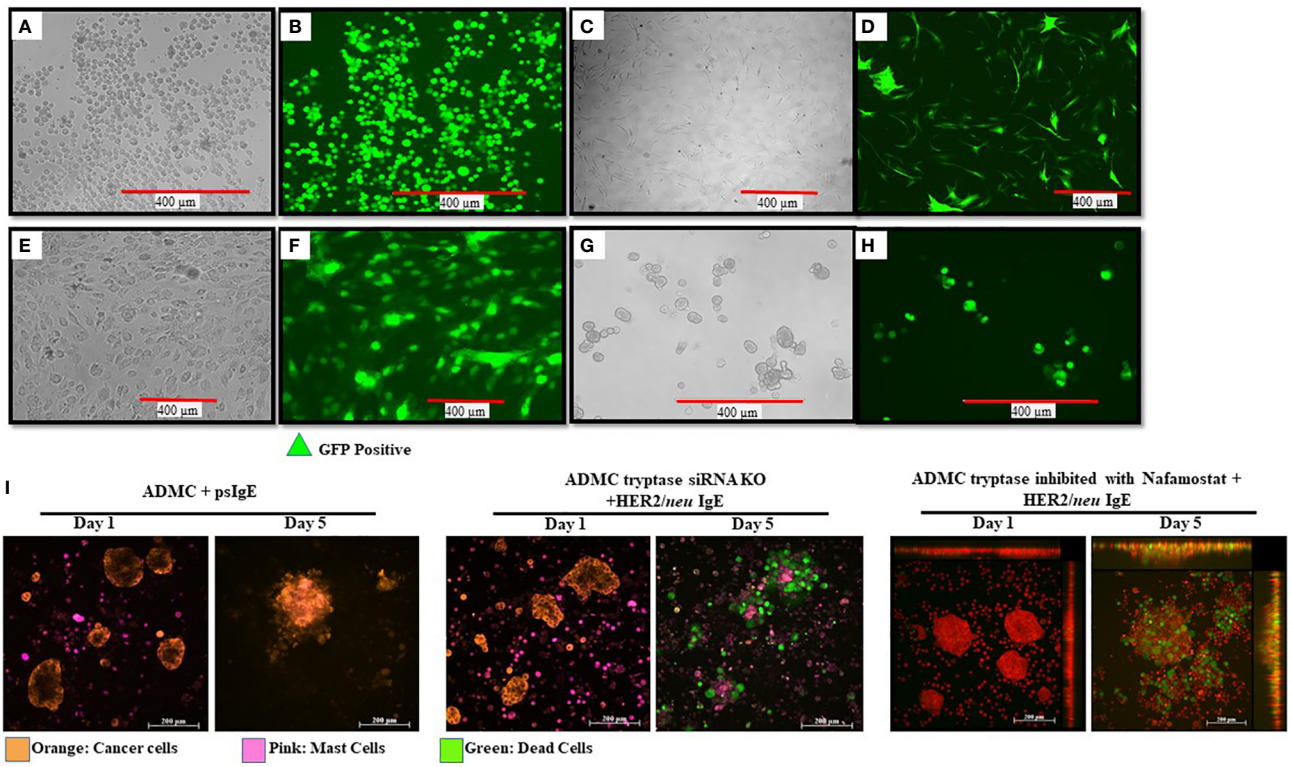


FIGURE 7 | Manipulation of human MCs and adipose stem cells as a platform for increasing safety and cytotoxicity. ADMC (**A, B**; $5x10^6$) or adipose stem cells (**C, D**; $3x10^6$) 3 days after infection by 20 µl of GFP-carrying lentiviral particles ($4x10^8$ Infection Unit). Mag. 20x. Adipose-derived stem cells were transduced with GFP, cryopreserved and reconstituted in stem cell medium **(E, F)** and GFP fluorescence. **(G, H)** Culturing in ADMC medium for 4 months showing ADMC with GFP Mag. 20x. **(I)** MCs ($2x10^5$) were treated with silencer select siRNA targeting the *TPSAB1* gene, sensitized with psIgE or HER2/*neu* IgE followed by staining with CellTracker™ Deep Red. After washing, MCs were added to the pre-stained, adherent BT474 ($1x10^5$ -$8x10^5$) along with caspase 3/7 and the time lapse video was taken over the indicated times. Image colors were adjusted using AxioVision software where, blue is changed to orange (BT474) and red is changed to pink (MCs). Results are representative of three different donors.

have both pro- and anti-tumor effects (48–52). Incubation of MCs with anti-tryptase siRNA was optimal at 20 picomole (**Supplementary Figure 4**). As shown in **Figure 7I** HER2/*neu* IgE-sensitized MCs with tryptase inhibited >95% still exhibited significant apoptosis of BT474-1261 cells. In separate experiments, MCs treated with the tryptase specific inhibitor Nafamostate did not affect their ability to induce apoptosis and also suggested a non-critical role for tryptase in inducing IgE-dependent apoptosis of cancer cells (**Figure 7I**). These studies suggest MC-specific tryptase is not directly involved in tumor cell apoptosis and provide conditions needed so that the role of other MC mediators can be assessed for anti-tumor activity.

DISCUSSION

The number and diversity of autologous cells being explored for their ability to confer anti-tumor properties continues to increase. The most widely recognized and successful strategy of adoptive cellular transfer (ACT) is the use of autologous, peripheral T cells engineered *ex vivo* to express a transmembrane chimeric antigen receptor composed of an extracellular, antigen-specific single-chain antibody and an intracellular T cell signaling domain (CAR T) (53, 54). In addition to other T-cell bases strategies, the use of non-T immune cells are also being investigated for anti-tumor activity. For example, dendritic cells loaded *in vitro* with specific tumor-associated antigens to generate an immune response for cancer-cell elimination has led to clinical trials testing safety and efficacy (55). Natural killer cells that target cancer stem-like cells can skew the immune response toward anti-tumor activity and are an alternative cell type for CAR therapy (56). Peripheral blood eosinophils and neutrophils have demonstrated anti-tumorigenic activity (57, 58). One of the most promising cell types being explored for ACT are, like MCs, resident in tissue. Macrophages polarized using CAR T cell strategies to redirect their phenotype to M1 with phagocytic functions target cancer-specific biomarkers and induce an adaptive immune response against tumor cells (59). In short, most cells being investigated as new platforms for cancer immunotherapy exert both pro- and anti-tumor effects. Therefore, the challenge moving forward is to identify and utilize these cells and take advantage of current technologies that allow for the removal of pro-tumor activity and/or enhance their anti-tumor functions. Here we present evidence that this can be accomplished with *ex vivo* derived MCs.

Our studies demonstrate that human ADMC have anti-tumor properties *in vitro* and *in vivo* and without overt signs of toxicity. These effects are observed even without the need for the anti-cancer adjuvant GM-CSF as human GM-CSF does not bind cognate mouse receptors (60). The studies utilized a single MC injection and the tumor volumes appeared to fluctuate in the HER2/*neu* IgE-sensitized ADMC animals which we hypothesize could be due in part to the well-documented capability of MCs to re-activate following FcεRI activation to levels similar to what is seen in the initial activation (61). Stem cell factor, the ligand for MCs expressing c-kit, is the principal growth signal for MCs. While MCs derived *ex vivo* have a finite lifespan without SCF, they are also capable of autocrine SCF secretion and activation of KIT (62, 63) which may account for the prolonged anti-tumor effect observed with only a single injection. Of course, more frequent injections of ADMC may be needed for this strategy to increase the anti-tumor effects. We are currently investigating the optimal parameters needed to increase efficacy in these *in vivo* models.

One of the most controversial areas of research in MC-tumor biology is the role MCs have in tumor pathogenesis. While MCs may be able to shrink tumors with "natural" anti-tumor mediators (as demonstrated above), our strategy is to be able to increase efficacy by introducing other proven anti-tumor mediators (or decrease potential toxicity). While we reported that TNF-α was involved in *in vitro* killing of tumor cells using anti-TNF-α Abs (10) it is simply not known what the role other mediators elicited from MCs have in apoptosis induction. Knocking out or knocking down histamine would be an obvious choice for selective removal and alleviate the widespread concerns using cytotoxic MCs for cancer therapy. However, an anti-tumor role for histamine has been suggested (8, 64). If "left in" the MCs, a histamine-mediated reaction is immediate and could be dealt with using anti-histamines as is widely prescribed. If "taken out" of MCs, then the anti-tumor effects may be compromised.

We propose a strategy utilizing genetic knockdown of mediators released upon FcεRI-specific activation followed by testing the resulting MC for their ability to induce apoptosis compared to non-manipulated MCs. Primary human MCs are difficult to genetically manipulate with low yields and poor transfection efficiencies. Imaging blood-derived MC degranulation using single cells subjected to shRNA knockdown and high-resolution confocal microscopy was recently described (65) but this procedure results in very low transfection efficiency using ADMC and skin-derived MCs and thus not suitable for numbers needed for therapeutic applications. Our strategy was thus to first determine which MC mediators elicited following tumor cell-induced, FcεRI crosslinking are active in apoptosis. Then rational decisions can be made for more focused efforts at genetic engineering of *ex vivo* MCs for polarization into less toxic, and/or more potent, cytotoxic cells using the lentivirus system described above which is resistant to mobilization (safer for clinical trials), resistant to epigenetic silencing of non-integrating lentiviral vectors, and is a better strategy for delivery of toxic genes (66). Tryptase was initially selected given its MC specificity, availability of inhibitors, and radical conflicting data as to its role in cancer pathogenesis (49, 50, 52). We show tryptase-deficient MCs still exert potent anti-tumor cell apoptosis suggesting this protease does not have a direct effect on tumor cells. Determination of the conditions needed for manipulating primary MC genes, selection of targets, along with the observation that adipose stem cells can be cryopreserved without losing viability (**Figure 7**), has important implications for therapeutic strategies. This opens the possibility to add a wide range of anti-tumor mediators in addition to the "naturally occurring" anti-tumor mediators in MCs (already demonstrated to shrink tumors and extend lifespan) to develop a "super killing" cell for cancer (or other malignancies in which there are IgE targets) immunotherapy.

These are the first reports of primary human FcεRI-activated MC forming TnT with tumor cells in parallel with apoptosis. The TnT have been described in multiple cell types *in vitro* and *in vivo* as a form of cell-to-cell communication in a variety of disease mechanisms, including cancer (67). These thin plasma membrane structures connecting cells can transfer a wide array of molecules, including organelles and small molecules. Previous studies using the MC line LAD reported the emergence of TnT when co-cultured with glioblastoma cells (68). These transformed, immortal cells were not challenged through FcεRI as performed with primary human MCs described here. The significance of the TnT formation between MCs and cancer cells *in vitro* is still unclear. Currently we are investigating the possibility that mediators and/or organelles are transferred between cells, if MCs form TnT *in vitro* using cancer cell spheroids models, and *in vivo* using patient-derived xenograft models in immunocompromised mice to determine any correlations between TnT formation and their ability to induce tumor shrinkage or alter their function and susceptibility to therapeutics.

The induction of MC-induced apoptosis of tumor cells appears to be bi-directional with apoptotic bodies forming at the junctions of the MC and cancer cells (**Figure 3**). Apoptotic bodies are a type of small, membrane-bound extracellular vesicle that range from 50 to 5,000 nm in diameter that are produced from cancer cells undergoing programmed cell death (69). These bodies form during plasma membrane blebbing following an apoptotic signal and can release them as extracellular vesicles that can carry nuclear fragments and cellular organelles such as mitochondria and endoplasmic reticulum for further immune responses (70). In addition, MCs clearly enter and migrate through tumor cell bundles and become activated (**Figure 3** and **Supplementary Video 1**), suggesting they may have the ability to enter tumors to release their mediators. This is intriguing as most ACT strategies are not as effective on solid tumors as they are not able to penetrate the outer cell layer.

TNF-α was investigated early on as a potential anti-tumor therapy based on its ability to necrotize mouse tumors (71). Toxicity associated with systemic TNF administration or release beyond the tumor milieu remains a problem which could be addressed if specific and directed release to cancer cells could be attained (71). Clinical trials utilizing systemic TNF-α

administration have resulted in unacceptable level of toxicities, which blocked its development (72). In contrast, localized administration of TNF-α in the form of isolated limb perfusion have yielded excellent results in soft tissue sarcomas (73). Our results suggest TNF-α plays a significant role in the MC-induced apoptosis of cancer cells and the strategy using cytotoxic MCs with pre-made TNF-α within the granules is exactly what is needed for utilizing TNF-α as an anti-cancer agent; targeted, localized, and controlled release only upon tumor cell engagement. In fact-we provide evidence that this pre-formed mediator is released within the tumor cells further enhancing its effect (**Figure 3** and data not shown) and possibly alleviating the toxicity encountered with systemic release. MCs could provide the exact solution to TNF-α systemic toxicity by selectively releasing it, controlled only by tumor cell engagement, in and around the tumor cells.

The gene analysis of mRNA within tumor cells affected by cytotoxic MCs revealed a plethora of information suggesting a completely new and diverse mechanism of tumor cell killing. In general, there was a significant upregulation of genes previously demonstrated to have anti-tumor activity and downregulation of many having pro-tumor involvement. For example, TNF-related apoptosis-inducing ligand (TRAIL) is a potent stimulator of apoptosis, especially on tumor cells, making them excellent therapeutic targets for cancer (74). Cytotoxic ADMC upregulated several TRAIL receptors (e.g. TNFRSF10) on tumor cells suggesting MCs could provide external triggers for apoptosis. The NADH dehydrogenase 1 alpha subcomplex, 4-like 2 (NDUFA4L2) is important in progression of multiple cancers and educed expression through lentivirus knockdown led to a significant enhancement of tumor cell apoptosis *in vitro* and *in vivo* (45). We show NDUFA4L2 was significantly downregulated (2.5 fold) in tumors challenged with MCs. The serpin family A member 1 (SERPINA1) gene is upregulated and is a marker of poor prognosis for several cancers (75) which was also significantly downregulated in tumor cells following ADMC challenge (1.4 fold). The metallophosphoesterase domain containing 2 protein (MPPED2) was shown to be a potent tumor suppressor involved in the downregulation of breast carcinogenesis (33) and ADMC significantly upregulated (2.0 fold) it in tumor cells. These data suggest MCs induce a unique and potent anti-tumor response through a diversity of anti-tumor pathways leading to apoptosis. We are currently following up on many of these targets affected by cytotoxic MCs to understand their role in tumor cell apoptosis and function.

Similar to those cell types mentioned above being investigated for ACT, MCs have pro- and anti-tumor mediators which has led to speculation and conjecture as to their role in cancer pathogenesis. Depleting or stabilizing MCs has been hypothesized to enhance anti-cancer mechanisms (76). As with MCs, it was hypothesized tumor macrophage depletion and/or inhibition was also a plausible strategy for anti-cancer therapeutics (77). Now, macrophages are at the forefront of translational ACT research and are poised to become another cell in the arsenal of cancer cell immunotherapies (78). Of course, there are a myriad of logical concerns and arguments as to why ACT with MCs is a challenging strategy. Yet, similar arguments against CAR T were also raised even up until clinical trials ensued which resulted in the now manageable cytokine storm driven by IL-6 (79).

Personalized medicine using a cancer patient's own immune system to direct anti-tumor effects is an exciting and growing area of research. MCs possess anti-tumor mediators, can be obtained from autologous sources, and cryopreserved. We exploited the availability of tumor-targeting IgE's that arm the MCs for highly efficient and controlled release upon tumor cell engagement that results in tumor shrinkage and lifespan extension without the systemic release of Type I hypersensitivity mediators. The MCs induce a unique anti-tumor mechanism with upregulation of several anti-tumor genes and downregulation of several pro-tumor genes. Additionally, we have revealed a strategy to identify which MC mediators are responsible for tumor regression and those that are not so that gene transfer methods can polarize MCs to be more potent anti-cancer cells with less potential toxicity potential. The significant anti-tumor activity by MCs revealed a heretofore undescribed effect on increasing tumor cell suppressor genes and decreasing pro-tumor genes. This autologous MC platform has thus the potential to be used against many cancers for which tumor IgE's are available or could be manufactured.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the European Nucleotide Archive (ENA) repository, accession number PRJEB51596.

ETHICS STATEMENT

The animal study was reviewed and approved by UNCG IRB.

AUTHOR CONTRIBUTIONS

Conceptualization: CK. Methodology: MF, EA, PD, MM, KD, YY, SL, AB, TK, and CK. Investigation: MF, EA, MM, KD, YY, SL, AB, TK, DM, and CK. Writing-original draft: MF and CK. Writing-review and editing: MF, EA, MM, KD, AD, YY, SL, AB, TK, DM, and CK. Funding acquisition: CK and TK. Resources: DM, AB, TK, and CK. Supervision: DM, AB, TK, and CK. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.871390/full#supplementary-material

Supplementary Video 1 | Adipose-derived MCs bind to and induce apoptosis of tumor cells. Experiment is described in Figure 3.

Supplementary Video 2 | Peripheral blood-derived MCs bind to and induce apoptosis of tumor cells. Experiment is described in Figure 3.

Supplementary Video 3 | *In vivo* imaging of HER2/*neu* IgE-sensitized MCs binding to and shrinking HER2/*neu* tumors.

Supplementary Video 4 | Representative video of mice injected i.v. with HER2/*neu* IgE-sensitized MCs (1×10^6) into HER2/*neu* tumor engrafted mice. Images were obtained at approximately 20 minutes after tail vein injection and demonstrates no anaphylaxis.

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REVIEW
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Recent Advances in Solid Tumor CAR-T Cell Therapy: Driving Tumor Cells From Hero to Zero?

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Chimeric antigen receptor T-cells (CAR-Ts) are known as revolutionary living drugs that have turned the tables of conventional cancer treatments in certain hematologic malignancies such as B-cell acute lymphoblastic leukemia (B-ALL) and diffuse large B-cell lymphoma (DLBCL) by achieving US Food and Drug Administration (FDA) approval based on their successful clinical outcomes. However, this type of therapy has not seen the light of victory in the fight against solid tumors because of various restricting caveats including heterogeneous tumor antigen expression and the immunosuppressive tumor microenvironments (TME) that negatively affect the tumor-site accessibility, infiltration, stimulation, activation, and persistence of CAR-Ts. In this review, we explore strategic twists including boosting vaccines and designing implementations that can support CAR-T expansion, proliferation, and tumoricidal capacity. We also step further by underscoring novel strategies for triggering endogenous antitumor responses and overcoming the limitation of poor CAR-T tumor-tissue infiltration and the lack of definitive tumor-specific antigens. Ultimately, we highlight how these approaches can address the mentioned arduous hurdles.

Keywords: chimeric antigen receptor, immunotherapy, solid tumors, infiltration, vaccines, tumor microenvironment

1 INTRODUCTION

Immune checkpoints are known as naturally occurring pathways that prevent the immune system from attacking and destroying healthy and domestic cells in the body (1). Cancer cells leverage this immune action mechanism to escape from the harm of the immune system (1). If cancer cells do not use these immune checkpoints as a protection shield, the immune system might attack and destroy them (1). Immune checkpoint inhibitors are a type of solid tumor cancer therapy (1–4). These agents put the brakes on the immune checkpoints and unleash immune responses against tumors (including T-cell responses) (1–4). The mentioned reactions following immune checkpoint blockade therapy can result in effective tumor eradication, as demonstrated in various studies (1–4). However, there are some limitations regarding this type of therapy as it has been evident that it

cannot result in an adequate number of tumor-reactive T cells (1, 5). Moreover, the triggered antitumor responses are often weak and memory T cell formation is not effectively carried out (1, 5).

Adoptive cellular therapy (ACT) is another field of cancer immunotherapy that includes obtaining a patient's or donor's cells, modifying or expanding them in *ex vivo* conditions, and delivering the modified or expanded cells to the patient (6). ACT is mainly categorized into three distinct fields which include tumor-infiltrating lymphocytes (TILs), genetically engineered T-cell receptors (TCRs), and chimeric antigen receptor T cells (CAR-Ts) (7–9). ACT has proven efficient in comparison with immune checkpoint blockade therapy, especially in terms of antitumor T-cell populations and specific responses (7–9). Today's modern CAR-T therapy is based on the experience gained from early ACT. For instance, in the context of TIL therapy, it was discovered that tumor-site T-cell infiltration is observed in various types of solid tumors (including melanoma, head and neck squamous cell carcinoma, cervical cancer, and lung cancer) and this T-cell infiltration has a direct relationship with patients' favorable prognosis (10–13). However, it was elucidated that the antitumor activity of these T cells is significantly hampered by the immunosuppressive tumor microenvironment (TME) (14). Later, isolation, expansion, and adoptive transfer of these infiltrating cells was proposed as a therapeutic solution. This approach proved effective in eradicating various types of tumors both in preclinical and clinical studies (7, 11, 15). Moreover, studies demonstrated that both $CD4^+$ T cells and $CD8^+$ T cells have critical roles in such effective antitumor responses (10, 16–19). In detail, $CD8^+$ T cells are able to produce proinflammatory cytokines once they are activated (10, 16–19). Such proinflammatory cytokines can mediate irreversible tumor cell damage (10, 16–19). Moreover, $CD4^+$ T cells can help B cells produce antibodies once they develop into antibody-producing plasma cells (10, 16–19). In addition, these cells help trigger $CD8^+$ T cell immune responses (10, 16–19).

Moreover, it was also discovered that using interleukin (IL)-2 (its systematic administration or its use for the expansion of TILs) can enhance the antitumor efficacy of *ex vivo*-expanded TILs (15, 20, 21). Such findings implied that the adoptive transfer of *ex vivo*-expanded cells can be both therapeutically safe and beneficial in eliminating solid tumor cells (15, 20, 21). Later, it was revealed that using lymphodepletion chemotherapy alongside ACT can enhance *in vivo* T-cell proliferation and persistence in patients with solid tumors or hematologic malignancies (22, 23). Lymphodepletion chemotherapy is the use of certain chemotherapy agents before the adoptive transfer of *ex vivo*-expanded or modified cells to the recipient. This approach prepares recipients' immune systems for ACT (22, 23). Various studies have demonstrated that this approach results in improved clinical outcomes of ACT (22, 23).

CAR-Ts are T cells engineered to express CARs which are versatile synthetic receptors composed of an antigen-targeting domain, mostly derived from the single-chain variable fragment (scFv) of a monoclonal antibody (mAb), fused to an intracellular T-cell activation signaling domain and one or two co-stimulatory domains (24). CARs have the unique ability to redirect engineered T cells towards cancer cells expressing the target antigen of interest (24). CAR-Ts are activated upon interaction with their target antigen which is in a major histocompatibility complex (MHC)-independent manner (**Figure 1**) (24). It has not been a long time since the first CAR-T product (named *tisagenlecleucel*) received US Food and Drug Administration (FDA) approval in 2017 for medical use in clinics (25, 26). Today, CAR-T therapy is available as an efficient treatment option for the treatment of certain patients with relapsed or refractory (R/R) hematologic malignancies including B-cell acute lymphoblastic leukemia (B-ALL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), and multiple myeloma (MM) (9, 25–35).

Considering the durable clinical responses in the treatment of patients with the mentioned hematological indications (which still has room for improvement), what came out as bad news was the unfavorable efficacy of CAR-T therapy against solid tumors (36–39). These unsuccessful outcomes are mainly due to the lack of suitable CAR-T target antigens in solid tumors and that the targeted antigens are usually found on healthy tissues leading to *"on-target off-tumor"* toxicities. Other important hindrances include the poor accessibility of the target antigen by CAR-Ts that leads to inefficient CAR-T *in vivo* stimulation, activation, and expansion, the heterogeneous pattern of tumor antigen expression and the appliance of escape mechanisms by tumor cells to evade CAR-Ts redirected towards a single tumor antigen, the immunosuppressive nature of the TME rendering CAR-Ts non-responsive or exhausted, insufficient infiltration of CAR-Ts into the TME, and metabolic starvation (40–42). Ever since the remarkable functionality of CAR-Ts was observed in hematologic malignancies, different types of improvement strategies have been developed to at least bring a fraction of the success of hematologic malignancy CAR-T therapy to the table of solid tumor therapy (43). In this review, we explore the most novel strategies for tackling some of the most important and clinical success-limiting challenges of CAR-T therapy in solid tumors.

2 CAR-T THERAPY FUNDAMENTALS

TCRs expressed on the surface of T lymphocytes are responsible for recognizing the peptide antigens presented to them through MHCs by antigen-presenting cells (APCs). The action mechanism of these receptors only enables mediating immune reactions towards peptide antigens that are presented by MHCs. On the other hand, mAbs can recognize and bind cell surface-expressed antigens that are not presented by MHCs. This ability of mAbs has been utilized for redirecting the cytotoxicity of various kinds of immune cells including T cells and natural killer (NK) cells towards tumor surface-expressed antigens of interest which can be either tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs) (44, 45). CAR molecules are made of three key segments. An extracellular domain (consisting of a targeting domain and a spacer), a transmembrane domain, and

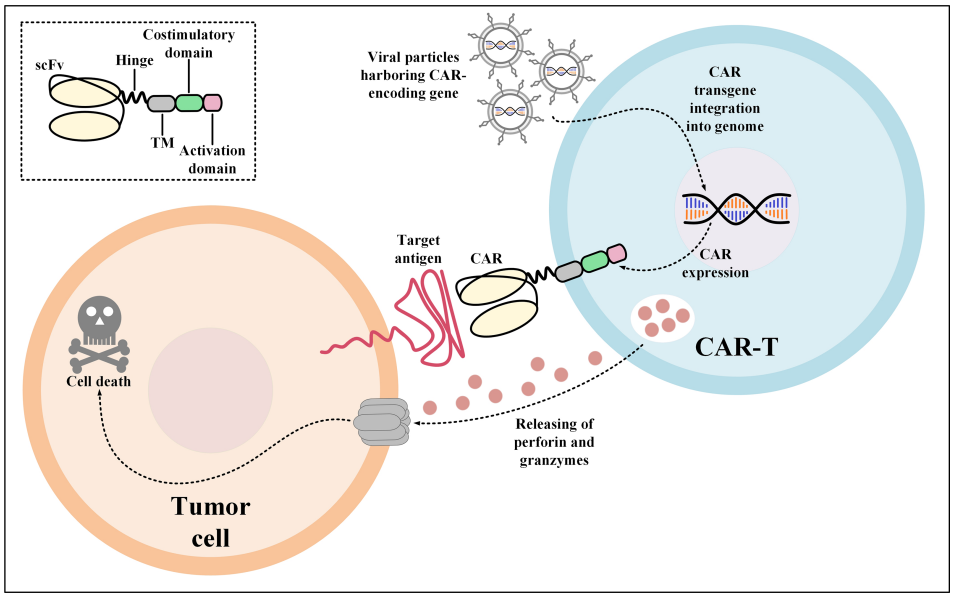

FIGURE 1 | Action mechanism of CAR-Ts for the elimination of target tumor cells expressing the CAR target antigen. Transduction of T cells with viral particles harboring the CAR-encoding transgene leads to the stable expression of CARs on the surface of the transduced T cells. Upon target antigen encountering, CAR-Ts are activated and they release perforin and granzymes leading to tumor cell death. CAR, chimeric antigen receptor; scFv, single-chain variable fragment; TM, transmembrane domain.

an intracellular domain (consisting of one or two costimulatory domains and a primary activation domain) (46, 47).

CAR-T development has experienced a fast-paced journey from its early days that has led to the production of CAR-Ts with versatile capabilities for tackling various CAR-T therapy obstacles (46, 48). In terms of structural designing, CAR-Ts are currently classified into five generations, each with different structural components and rather distinct biological behaviors. The first generation of CAR-Ts expressed extracellular antigen-targeting domains fused to an intracellular domain through a transmembrane domain (49). These CAR-Ts demonstrated insignificant expansion, persistence, and clinical responses in clinical studies (49, 50). Such clinical failures persuaded researchers to develop CAR-Ts capable of overcoming these limitations. In this regard, the second and third generations of CAR-Ts were designed which harbored one and two costimulatory domains in their intracellular domain, respectively (51, 52). These generations of CAR-Ts exhibited significant capabilities in tackling the hurdles of first-generation CAR-Ts and they proved efficient and safe in clinics (9, 27–32, 51, 52). It is worth mentioning that all of the FDA-approved CAR-T products are second-generation CAR-Ts harboring either 4-1BB or CD28 as their costimulatory domains (9, 27–32). Furthermore, researchers used the backbone of second-generation CAR-Ts and modified its intracellular domain by the fusion of additional domains to achieve different goals (53–55). In this regard, the fourth and the fifth generation of CAR-Ts were developed. In detail, the fourth-generation CAR-Ts, alternatively termed as "*T-cell redirected for universal cytokine-mediated killing (TRUCKs)*" or *armored CAR-Ts*, harbor an intracellular expression inducer of a cytokine of interest (which endows them with the capability of targeted delivery of a cytokine of interest) whereas the fifth-generation CAR-Ts have an intracellular fragment of a cytokine receptor (for instance, IL-2Rβ) (53–55). All of these basic CAR designing twists have been carried out on the grounds of achieving superior CAR-T-mediated antitumor responses and enhanced safety profiles. In the upcoming sections, we highlight studies that have specifically aimed to find strategies capable of tackling particular limitations of CAR-T therapy in solid tumors.

3 THE HURDLES OF SOLID TUMOR CAR-T THERAPY

CAR-T therapy of solid tumors has not been very successful in the clinics. Identifying the hurdles of solid tumor CAR-T therapy is the first and the most important step for developing counterstrategies for tackling these limitations. One of the primary limitations of solid tumor CAR-T therapy is target antigen heterogeneity. CAR target antigen heterogeneity results in poor detection of cancer cells by CAR-Ts and leads to inadequate CAR-T-mediated antitumor reactions against cancer cells expressing that particular type of target antigen (**Figure 2A**). Such ineffective antitumor responses can result in

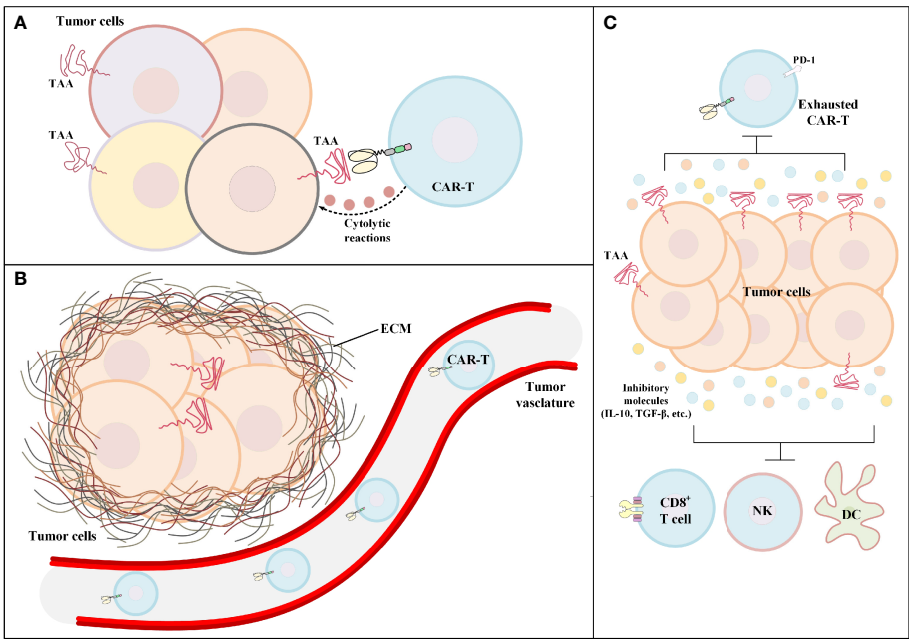

FIGURE 2 | The hurdles of CAR-T therapy in solid tumors. **(A)** Tumor antigen heterogeneity. In such conditions, the tumor bulk consists of tumor cells only a fraction of which express the CAR-redirected TAA on their surface. Other tumor cells may express different TAAs. **(B)** Poor tumor site CAR-T infiltration. In the case of hematologic malignancies, the adoptively transferred CAR-Ts encounter their target cells in the bloodstream or the lymphatic system. On the contrary, in solid tumor CAR-T therapy, CAR-Ts have limited accessibility to the tumor site rendering CAR-T-mediated tumoricidal reactions ineffective and insufficient. **(C)** The immunosuppressive tumor microenvironment. The immunosuppressive characteristics of the tumor microenvironment have negative effects on both CAR-Ts and various immune cells responsible for endogenous antitumor responses. CAR-T, chimeric antigen receptor T cell; DC, dendritic cell; ECM, extracellular matrix; IL-10, interleukin-10; NK, natural killer cell; PD-1, programmed cell death receptor protein 1; TAA, tumor-associated antigen; TGF-β, transforming growth factor-beta.

treatment failure and further tumor outgrowth. Moreover, in solid tumor CAR-T therapy, most targeted antigens are not essential for tumor progression; therefore, the expression of such antigens can be reduced by tumor cells in an intelligent manner (56). Various studies have demonstrated that low-level CAR-T target antigen expression is also an important factor for the ineffectiveness of solid tumor CAR-T therapy and the occurrence of disease relapse (36, 57). For instance, one study reported that low-density expression of target antigen resulted in the inability of CAR-Ts for tumor eradication and also the occurrence of disease relapse in preclinical models of pancreatic and prostate cancer (57). Moreover, according to the results of a clinical study, in some solid tumors, CAR-T-mediated targeting of a particular target antigen may result in its downregulation rendering CAR-T therapy incapable of attacking tumor cells, and leading to treatment failure (36). In detail, O'Rourke et al. reported that in the first-in-human pilot study of epidermal growth factor receptor variant III (EGFRvIII)-redirected CAR-Ts in refractory glioblastoma patients, it was discovered that patient-obtained tumor biopsies following CAR-T therapy had reduced the expression level of EGFRvIII in comparison with those biopsies obtained before CAR-T therapy (36).

In the case of hematologic malignancies, CAR-Ts encounter their target cells in the bloodstream and the lymphatic system, and that is where most of the fight against tumors takes place. But in the case of solid tumors, the story is quite different. There is scientific evidence indicating that different tumor-applied mechanisms result in the declined secretion of various vasculature-related factors rendering CAR-Ts unable to pass through the vascular endothelium and penetrate the tumor tissue (**Figure 2B**) (58). For instance, in a study by Vedvyas et al. investigating the preclinical efficacy of intercellular adhesion molecule-1 (ICAM-1)-redirected CAR-Ts for targeting advanced thyroid cancer, it was elucidated that endothelin B receptors are upregulated in cancer tissues which results in the reduced ICAM-1 expression level rendering CAR-Ts incapable of crossing the blood vessels (59). Additionally, CAR-T migration, penetration, and trafficking into tumor tissues are highly dependent on various types of chemokines (60). It has been evident that such chemokines are downregulated or not expressed in tumor tissues (60). This mechanism results in the reduced migration and penetration of T lymphocytes (as well as CAR-Ts) into the tumor sites (60). Moreover, the presence of a dense extracellular matrix is known as another barrier for T-cell migration and infiltration (61). It has been demonstrated that

controlled degradation of tumor extracellular matrix can result in increased susceptibility of the tumor to CAR-T therapy (62). Other scientific evidence demonstrates that the WNT/β-catenin signaling pathway is responsible for blocking T-cell infiltration in the tumor site in various types of solid tumors (including metastatic melanoma and colorectal cancer) (63–65). This mechanism can result in immune evasion of cancer and resistance to therapy (63–65). In this regard, a deep understanding of solid tumor mechanisms and developing effective strategies to tackle them is an important factor for having an effective CAR-T therapy in solid tumors. In this article, we have tried to discuss some of such strategies.

The territory of solid tumors, known as the immunosuppressive TME, has various characteristics that hamper antitumor immune reactions (including T-cell and CAR-T activity) (**Figure 2C**). An effective solid tumor therapy is based on overcoming the immunosuppressive nature of the TME. There are various types of immune suppressor cells in the tumor environment, including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) (66–69). Alongside immunosuppression, such cells can be beneficial for tumors cells in terms of supporting tumor progression, angiogenesis, and metastasis (66, 68). In detail, these cells assist in such tumor growth-related activities by producing growth factors, chemokines, and cytokines (such as various types of ILs or TGF-β) (66, 68). Studies have demonstrated that immune checkpoint molecules (CTLA-4 and PD-1) may also play important roles in impairing certain antitumor reactions (1, 70–72). Such understanding of the TME and its impact on the CAR-T-mediated antitumor responses has encouraged researchers to find various effective counterstrategies. In the upcoming sections, we mention some of these counterstrategies (some of which have been investigated in clinical trials as summarized in **Table 1**) which might improve the outcomes of solid tumor CAR-T therapy.

4 STRATEGIES FOR OVERCOMING THE HURDLES OF SOLID TUMOR CAR-T THERAPY

4.1 Boosting Vaccines for Overcoming Insufficient CAR-T Stimulation

The robust proliferation signals provided to CAR-Ts by the antigens they are redirected against mainly contribute to the persistence of CAR-Ts; therefore, the target antigen-expressing tumor cells play the role of APCs (73, 74). In hematologic malignancies, these target cells are easily accessible to CAR-Ts but in solid tumors, it is a whole different scenario due to the TME. Therefore, the population of CAR-Ts decreases quickly since there is no proliferation signals provided to them (36, 75, 76). Recently, Reinhard and collaborators developed a nanoparticle-based RNA vaccine for the delivery of the native-conformation CAR target antigen to the APCs of the lymphoid compartments to augment the expansion rate and tumoricidal efficacy of CAR-Ts *in vivo* (77). Their results indicated that this combination strategy is capable of eliminating large tumors in animal models even with doses of CAR-Ts considered below common therapeutic doses (77). Another vaccine-boosting strategy has explored the use of amphiphile CAR target ligands that migrate to lymph nodes following administration and then position themselves into the bilayer membrane of APCs, thus creating an environment for CAR-T priming (78). According to the results observed in various immunocompetent animal tumor models, this vaccine platform might mediate improved antitumor capacity through amplifying CAR-T expansion and donor cell polyfunctionality (78).

Another study has taken a different trajectory by engineering a nanoemulsion-based vaccine [that selectively targets and activates cross-presenting dendritic cells (DCs)] with full-length recombinant ovalbumin to selectively prime DCs to present ovalbumin to CAR-Ts that are genetically manipulated to surface-express transgenic TCRs specific for ovalbumin (79). Alongside favorable *in vitro* results, the investigators also reported augmented tumor-site trafficking and enhanced proliferation of CAR-Ts which strongly correlated with tumor elimination and durable responses in immunocompetent animal models (79). According to another study, Wu et al. generated an epidermal growth factor receptor pathway substrate 8 DC vaccine (Eps8-DC) and reported that this DC vaccination strategy has beneficial effects on CD19-redirected CAR-Ts including the induction of central memory phenotype development, diminishing activation-induced cell death, augmenting proliferation potential, and amplifying cytokine secretion and tumoricidal capacity (80).

The therapeutic benefits of combining adoptive T-cell therapy with cytomegalovirus-based vaccines have also been evident as this combination was effective in delaying the growth of solid skin tumors in a study by Grenier and colleagues (81). One study has developed GD2-redirected varicella-zoster virus-redirected CAR-Ts (GD2-VZV-CAR-T) for the induction of *in vivo* expansion and persistence of which VZV vaccination can be applied (NCT01953900). Despite the possibilities of these CAR-T growing exhausted after tumor cell antigen engagement, vaccination has been effective in partially reversing the immunoinhibitory effects of the TME *via* TCR stimulation (82). Additionally, Slaney et al. engineered T cells to express both a HER2-redirected CAR and a gp100-specific TCR and combined this product with recombinant gp100-expressing vaccinia virus to target large-sized tumors (including liver and breast tumors) (83). Despite negligible neurotoxicity due to the expression of the target antigen by healthy tissues, this combination therapy strategy was able to mediate durable complete remission (CR) in immunocompetent animals with large-sized tumors owing to vaccine-induced pronounced proliferation and facilitated tumor-site colonization of the engineered T cells (83). Overall, based on these preclinical findings, it might be concluded that various types of vaccines can be applied for improving the therapeutic efficacy of CAR-Ts; however, clinical studies are warranted to further answer the remaining questions in this field.

TABLE 1 | A summary of the advances in solid tumor CAR-T therapy investigated in clinical trials.

Clinical trial identifier (Phase)	Notes	Participants	Start – completion date	Indication(s)	Target antigen
NCT01953900 (Phase I)	Varicella zoster virus (VZV) vaccination to enhance the activity of GD2-VZV-CAR-Ts	26	April 2014 - October 31, 2034	Osteosarcoma, Neuroblastoma	GD2
NCT03602157 (Phase I)	$CCR4^+$ CD30-redirected CAR-Ts	59	December 12, 2018 - September 30, 2041	Various lymphomas	CD30
NCT04153799 (Phase I)	CXCR5-expressing EGFR-redirected CAR-Ts	11	November 1, 2019 - December 2022	Non-small cell lung cancer	EGFR
NCT03929107 (Phase II)	IL-7- and CCL19-expressing CAR-Ts	80	March 28, 2019 - April 30, 2022	B-cell lymphoma	CD19
NCT03682744 (Phase I)	Intraperitoneal Infusions of CEA-redirected CAR-Ts for the treatment of peritoneal carcinomatosis	18	September 13, 2018 - March 2021	Peritoneal carcinomatosis	CEA
NCT03389230 (Phase I)	Intratumoral/intracavitary infusion of HER2-redirected CAR-Ts for the treatment of glioma	42	August 14, 2018 - December 15, 2023	R/R malignant glioma	HER2
NCT04661384 (Phase I)	Intracerebroventricular delivery of IL13Rα2-redirected CAR-Ts for the treatment of leptomeningeal metastases	30	March 5, 2021 - December 15, 2023	Leptomeningeal metastases	IL13Rα2
NCT04951141 (Early Phase I)	Intratumoral delivery of GPC3-redirected CAR-Ts for the treatment of hepatocellular carcinoma	10	January 1, 2019 - December 21, 2023	Hepatocellular carcinoma	GPC3
NCT02414269 (Phase I/II)	Intrapleural administration of mesothelin-redirected for the treatment of pleural cancers	113	May 2015 - April 2023	Malignant pleural cancers	Mesothelin
NCT04077866 (Phase I/II)	Intratumoral/intracerebroventricular administration of B7-H3-redirected CAR-Ts	40	May 1, 2022 - July 1, 2024	R/R glioblastoma	B7-H3
NCT04185038 (Phase I)	Ventricular delivery of B7-H3-redirected CAR-Ts	70	December 11, 2019 - May 2041	Central nervous system tumors	B7-H3
NCT03500991 (Phase I)	Delivery of HER2-redirected CAR-Ts into the ventricular system or tumor resection cavity	48	July 26, 2018 - July 26, 2039	R/R pediatric central nervous system tumors	HER2
NCT03638167 (Phase I)	Delivery of EGFR806-redirected CAR-Ts into the ventricular system or tumor cavity	36	March 19, 2019 - March 2040	R/R pediatric central nervous system tumors	EGFR806
NCT05103631 (Phase I)	Transgenic expression of IL-15 by CAR-Ts as an attempt to prolong their persistence in the circulation	27	June 17, 2021 - December 2039	Liver cell carcinoma	GPC3
NCT04684563 (Phase I)	CD19-redirected CAR-Ts engineered to express human IL-18	30	May 6, 2021 - May 2036	Non-Hodgkin lymphoma, chronic lymphocytic leukemia	CD19
NCT03085173 (Phase I)	CD19-redirected CAR-Ts engineered to express the co-stimulatory ligand 4-1BBL	39	March 15, 2017 - March 2022	R/R chronic lymphocytic leukemia	CD19
NCT03774654 (Phase I)	CD19-redirected CAR-NKT cells	48	June 22, 2020 - March 2035	R/R B-cell malignancies	CD19
NCT03294954 (Phase I)	GD2-redirected CAR-NKT cells	24	January 18, 2018 - August 10, 2034	Neuroblastoma	GD2
NCT04814004 (Phase I)	CD19-redirected CAR-iNKT cells	20	March 19, 2021 - April 1, 2024	R/R B-cell malignancies	CD19
NCT04702841 (Early Phase I)	CD7-redirected CAR γδ T cells	8	June 3, 2020 - December 2022	R/R T-cell malignancies	CD7

IL, interleukin; iNKT, invariant natural killer T cells; NKT, natural killer T cells; R/R, relapsed/refractory.

4.2 Strategies for Increasing Poor CAR-T Infiltration Rate

One of the obstacles that weakens the clinical responses of CAR-T therapy in solid tumors is the poor intratumoral CAR-T persistence and trafficking, which can be partly influenced by the cognate receptors on T cells. Recently, attempts have been made to increase the antitumor effects of CAR-Ts through the integration of chemokine receptors into the CAR design (84–86). Moreover, in solid tumors, CAR-T functionality may be deteriorated due to the immunosuppressive characteristics of the TME (87). Such characteristics include the expression of inhibitory ligands such as PD-L1, inhibitory mechanisms applied by regulatory cells, malignancy-associated metabolic dysregulation through various enzymes such as indoleamine-2,3-dioxygenase (IDO), and the presence and suppressive effects of other inhibitory factors such as TGF-β (87). In this section, we review studies that have made elaborate attempts for improving the antitumor functionality and infiltration rate of CAR-Ts in solid tumors.

4.2.1 Engineered Expression of Cytokines/Chemokines or Their Receptors

The tumor-mediated overexpression and secretion of the chemokine IL-8 (CXCL8) have been utilized as leverage to maximize the tumoricidal effects of CAR-Ts in solid tumors by increasing the intratumoral trafficking of these cells (84). In detail, genetic modification of CAR-Ts for the expression of IL-8 receptors (CXCR1 or CXCR2) and the utilization of the tumor-secreted IL-8 to usher the IL-8 receptor-modified CAR-Ts (IL-8R-CAR-Ts) to the tumor foci results in enhanced antitumor activity (84, 86). It has been evident that not only IL-8R modification does not impinge on the ability of the modified T

cells to recognize tumor cells, but it also increases *in vitro* T-cell chemotaxis in the presence of the chemokine (84). Animal studies have indicated that IL-8R-CAR-Ts exhibit superior characteristics in comparison to conventional CAR-Ts (84). In a particular case, deferred migration and low intratumoral presence of CD70-redirected CAR-Ts (CD70-CAR-Ts) have been known to contribute to the formation of an immunosuppressive TME that leads to potential tumor relapse despite the consistent tumor cell CD70 expression and peripheral persistence of the CD70-CAR-Ts (84). It has been evident that reversal of tumor-induced immunosuppression, inhibition of tumor growth, and maximized antitumor responses are achievable through the natural or ionizing radiation-induced secretion of IL-8 that facilitates the intratumoral trafficking of IL-8 receptor-modified CD70-CAR-Ts (IL-8R-CD70-CAR-Ts) (84, 88). According to Jin and collaborators, IL-8R-CD70-CAR-Ts exhibited pronounced presence and persistence in tumor milieus inducing complete tumor elimination and prolonged immunologic memory in glioblastoma, ovarian, and pancreatic cancer animal models (84). Additionally, it has been evident that engineered co-expression of CAR and C-C chemokine receptor 4 (CCR4) by effector T cells can result in enhanced tumor-site trafficking and tumoricidal cytotoxicity (89). In detail, CCR4 is the specific receptor of C-C motif chemokine ligand 17 (CCL17) and C-C motif chemokine ligand 22 (CCL22) which are secreted by DCs and macrophages for the chemotaxis of Tregs and type 2 T helper (Th2) cells (89). Tumor cells have been known to produce these chemokines for the formation of an immunosuppressed TME, a phenomenon that now has been known to be a double-edged sword against them (89). Furthermore, CAR-Ts engineered to express the chemokine receptor CCR2b, which is the receptor of C-C motif chemokine ligand 2 (CCL2), have exhibited more than ten-fold enhanced migration towards CCL2-secreting tumor cells alongside amplified antitumor capacity in comparison with conventional CAR-Ts (90, 91). Such findings highlight the potential of these strategies for overcoming the inadequate tumor localization of CAR-Ts (90, 91). According to another study, intravenous (IV) administration of CXCL11-modified oncolytic vaccinia virus (CXCL11-OVV) in mouse tumor models can be employed to modify tumor cells to secrete CXCL11, thus increasing the intratumoral concentration of CXCL11 (92). This phenomenon has been known to mediate a more pronounced tumor rejection by increasing the intratumoral accumulation of CAR-Ts (92).

4.2.2 Glycoengineering

The vessels of bone marrow and most tumors express endothelial molecules such as the lectin E-selectin which can be utilized as leverage for the extravasation CAR-Ts (93). Sialyl Lewis X (sLeX) acts as the cognate ligand of E-selectin, which is not expressed by T cells (hence not expressed by CAR-Ts either) (93). On the other hand, CAR-Ts exhibit a high profile of sialylated type 2 lactosamine expression that can be doctored to sLeX through α (1, 3)-fucosylation but since culture-dependent expansion downregulates the endogenous fucosylation pathway, engineered fucosylation using α (1, 3)-fucosyltransferase and GDP-fucose can be chosen to carry out this mission (93). According to Mondal and colleagues, the glycoengineered sLeX expression on CAR-Ts increases their infiltration capacity (around 10-fold) into the marrow of mouse models, compared to conventional CAR-Ts, thereby allowing better tissue colonization and obviating the need for high-dose adoptive cell administrations (93). These findings introduce cell surface glycoengineering as an as-is translatable approach for enhancing the homing of CAR-Ts in tissues with E-selectin-expressing endothelial cells. However, more substantiated findings may be required in this regard for such conclusions.

4.2.3 Protein Kinase A (PKA) Blockade

Resistance of CAR-Ts to immunosuppressive molecules such as adenosine or prostaglandin E2 can be also implemented to augment CAR-T efficacy and facilitate their tumor-site trafficking (94). In detail, adenosine or prostaglandin E2 activate PKA which results in its association with *ezrin* leading to T-cell hypofunction (94, 95). In 2016, Newick et al. developed CAR-Ts expressing an inhibitory peptide designated as RAID (regulatory subunit I anchoring disruptor) (94). These researchers demonstrated that RAID nullifies the inhibitory impacts of PKA on TCR activation by disrupting its association with ezrin (94). In comparison with conventional CAR-Ts, RAID-expressing CAR-Ts exhibited better tumoricidal efficacy owing to their better matrix adhesion and augmented trafficking in response to CXCL10 (94). Based on the *in vitro* and *in vivo* findings, Newick et al. suggested that this tactic might have clinical application after passing the necessary evaluations (94).

4.2.4 Photothermal Therapy

Regional hyperthermia of tumors may contribute to the recruitment of bystander immune cells by antigen spreading, diminishing the pressure of interstitial fluids, and disrupting the structural compaction of tumor tissues (96). It has recently been evident that mild hyperthermia of tumor sites can broaden the therapeutic reach of CAR-Ts into the milieu of solid tumors; therefore enhancing their tumoricidal efficacy (96). In detail, Chen et al. reported that the chondroitin sulfate proteoglycan-4 (CSPG4)-redirected CAR-Ts administered into melanoma NOD scid gamma (NSG) mouse models, which were established using the human melanoma cell line WM115, exhibited enhanced tumoricidal activity after tumor ablation using photothermal therapy (96). In addition to this study, Miller et al. evaluated the applicability of synthetic gene switches that mediate the expression of transgenes in specific responses to mild temperature increases (around 40 to 42 °C) (97). These researchers demonstrated that *in vitro* thermal therapy in primary human T cells led to a considerably higher expression level of a reporter transgene without any negative effects on the expansion, migration, and antitumor activity of the T cells (97). Moreover, these researchers also indicated that expression of an IL superatagonist or T-cell-redirecting bispecific antibodies (TRBAs) induced by intratumoral photothermal therapy improved tumoricidal responses and reduced antigen escape in mouse models after the systemic administration of CAR-Ts (97).

However, such ideas cannot be considered as general solid tumor CAR-T therapy solutions until more in-depth preclinical and clinical assessments are carried out.

4.2.5 Application of Docetaxel, Antiangiogenic Drugs, or NEO100

It has been elucidated that docetaxel can also amplify the antitumor activity of CAR-Ts by expanding their action zone into the TME (98). The presence of docetaxel induces a higher expression profile of high mobility group box 1 (HMGB1) from tumor cells which in turn induces the expression of CXCL11 through NF-κB activation (98). CXCL11 upregulation strongly facilitates CAR-T infiltration into the TME and correlates with better therapeutic benefits as reported by Gao and colleagues (98).

Moreover, transient remodeling of tumor-associated vasculature using antiangiogenic molecules may further enhance the therapeutic impact of cancer immunotherapy by expanding the extravasation rate of CAR-Ts into the TME (99, 100). In a neuroblastoma preclinical model, it has been demonstrated that *Bevacizumab* (a clinically approved anti-VEGF-A mAb) can increase the infiltration and tumoricidal capacity of GD2-redirected CAR-Ts (99, 101). Furthermore, CAR-T-mediated secretion of interferon-γ also contributed to the induction of neuroblastoma cell-mediated CXCL10 expression (99). Another study has also reported augmented tumor-site CAR-T accumulation by the help of vascular disrupting agents (100). According to Deng and colleagues, combretastatin A-4 phosphate (CA4P) has been effective in expanding the therapeutic reach of CAR-Ts by enhancing their tumor colonization in preclinical mouse models of ovarian and colon cancer (100).

Furthermore, blood-brain barrier (BBB) impermeability is also considered an obstacle in the case of central nervous system (CNS) tumor CAR-T therapy (102). Intraarterial (IA) administration of NEO100 (which is the purified form of perillyl alcohol (POH)) has been known to safely and reversibly permeabilize the BBB in mice by importing the tight junction-associated endothelial membrane proteins into the cytoplasm (102). This permeabilization paves the way for a more efficient CNS presence of CAR-Ts resulting in more pronounced tumoricidal responses, according to Wang et al. (102).

4.2.6 Regional Delivery of CAR-Ts

Regional delivery of CAR-Ts has recently emerged as a potent strategy for increasing the therapeutic reach of CAR-T therapy in solid tumors (103–109). In particular, Katz et al. reported that regional intraperitoneal (IP) CAR-T delivery leads to a superior tumoricidal capacity against carcinoembryonic antigen (CEA)-positive tumors of colorectal cancer that have metastasized to the peritoneum, compared with systemic administration (105). Moreover, these researchers also added that CAR-Ts delivered regionally to the peritoneum exhibited a rise in effector memory phenotype over time alongside suppressing tumor progression in distant subcutaneous (SC) regions with the involvement of elevated in-serum IFN-γ levels (105). Moreover, Katz et al. also studied IP CAR-T delivery with suppressor cell targeting therapy since MDSCs, with high levels in immunosuppressive PD-L1 expression, and Tregs are highly populated inside IP tumors (105). They also reported that this method resulted in enhanced antitumor activity and suppression of the peritoneum-metastasized tumors (105). In another experiment, Priceman et al. investigated three delivery routes including intravenous, local intratumoral, or regional intraventricular for HER2-redirected CAR-T administration into human xenograft mouse models with leptomeningeal disease and brain-metastasized breast cancer (106). These researchers reported that the regional intraventricular delivery of HER2-redirected CAR-Ts resulted in promising therapeutic responses against leptomeningeal disease and brain-metastasized breast cancer in human xenograft mouse models (106).

IV and regional delivery of HER2-redirected CAR-Ts have also been compared for the elimination of medulloblastoma in animal models (104). It has been evident that the induction of durable antitumor responses *via* the IV route requires a higher dosing scheme in comparison to the regional delivery of CAR-Ts (104). Moreover, it has been concluded that intraventricular delivery of HER2-redirected CAR-Ts in non-human primates does not contribute to the emergence of systemic toxicities and can be considered well-tolerated (104). Based on these outcomes, researchers have proposed that direct delivery of HER2-redirected CAR-Ts into the cerebrospinal fluid (CSF) can be investigated in clinical trials with recurring medulloblastoma patients enrolled (104). Besides the already mentioned investigations, another recent study has also validated the feasibility and safety of intrathecal delivery of EPHA2-, HER2-, and IL-13 receptor α2-redirected CAR-Ts in xenograft mouse models (108). The researchers indicated that this CAR-T administration route is potentially efficacious for the regression of posterior fossa group A ependymomas and medulloblastoma with or without concurrent *azacitidine* administration (a chemical analog of *cytidine*) (108).

Other researchers have also reported that locoregional delivery of B7-H3-redirected CAR-Ts through intracerebroventricular or intratumoral administration has also been correlated with lower levels of systemic inflammatory cytokines and pronounced tumoricidal responses in xenograft mouse models of atypical teratoid/rhabdoid tumors in comparison with intravenously infused B7-H3-redirected CAR-Ts (103). Of note, an ongoing Phase I clinical trial (NCT03500991) is currently investigating the locoregional delivery of HER2-redirected CAR-Ts in children and young adults with R/R tumors of CNS (110). According to a recent report of this trial by Vitanza et al., HER2-redirected CAR-Ts were delivered into the tumor cavity or the ventricular system using a CNS catheter (110). Moreover, no dose-limiting adverse events were reported in the enrolled patients (110). Based on the findings, local CNS immune responses were documented which were associated with significant CCL2 and CXCL10 levels in the CSF (110). Overall, such clinical data propose that repeated delivery of CAR-Ts into the CNS of children and young adults

with R/R tumors of CNS might be well-tolerated and result in local CNS immune reactions (110). However, broader clinical findings in this regard can further validate the strategies discussed in this section.

4.3 Overcoming Low Target Antigen Density or Lack of Definitive Target Antigens Through Induced Expression of CAR Targets in Tumor Cells

CAR-T therapy of solid tumors suffers from poor clinical responses partly due to the lack of definitive or highly expressed tumor antigens. Recently, various strategies have been developed to break the reliance of solid tumor CAR-T therapy on endogenous tumor antigens through the induced expression of CAR target antigens in tumor cells (111–113). A recent study by Park and colleagues has used an oncolytic vaccinia virus equipped with the nucleotide sequence of a truncated form of CD19 (truncCD19OV) to infect tumor cells for the surface expression of truncCD19 enabling their targeting with CD19-redirected CAR-Ts (113). *In vitro* and *in vivo* results indicated that the infected tumor cells exhibited considerable cytolytic sensitivity to CD19-redirected CAR-Ts (113). Furthermore, alongside the induction of endogenous antitumor effects by truncCD19OV, its continuous release from the tumor cells cytolyzed by CD19-redirected CAR-Ts contributed to the promotion of the antigen expression by other tumor cells (113). In 2020, Tang et al. developed recombinant adenoviruses harboring tumor-specific promoters that drive the expression of CD19 in tumor cells rendering them susceptible to CD19-redirected CAR-Ts and later engineered the viruses with replication capability (112). These researchers demonstrated that this oncolytic antigen-labeling strategy was capable of inducing tumor rejection through the formation of cytotoxic immunological synapses between tumor cells and CD19-redirected CAR-Ts in animal models, thus increasing their survival rate (112).

Additionally, another investigation has also developed thymidine kinase-disrupted oncolytic vaccinia viruses to selectively infect cancer cells for CD19 expression (111). In detail, Aalipour et al. demonstrated that utilization of this strategy correlated with considerably prolonged survival of immunocompetent animal models, which was a result of CD19-redirected CAR-T-mediated tumor elimination (**Figure 3**) (111). These novel findings accentuate the importance and feasibility of these strategies for tackling the antigen-related obstacles of solid tumor CAR-T therapy; however, more in-depth investigations are required in this field to better assess the applicability of these tactics.

4.4 Overcoming the Immunosuppressive TME

Cytokines that are capable of boosting the tumoricidal activity of CAR-Ts can be utilized as leverage for overcoming the immunosuppressive nature of TME that contributes to the functional exhaustion and metabolic starvation of CAR-Ts (42, 114, 115). However, constitutive secretion of transgenic cytokines by CAR-Ts and the presence of their corresponding receptors on bystander immune cells, such as T cells and NK cells, contribute to the emergence of life-threatening adverse events to take which under control requires the implementation of safety switches (which are comprehensively discussed elsewhere) (46, 116–118).

Recently, it has been demonstrated that STAT3 signaling can improve the tumoricidal activity of CAR-Ts leading to more favorable antitumor responses (119, 120). In particular, high concentrations of IL-23, achieved through the administration of its recombinant form or its secretion by gene-modified tumor cells, have been associated with antitumor impacts (121, 122). Moreover, this two-subunit STAT3-activating cytokine (composed of IL-23α p19 and IL-12β p40 subunits) has been known to act as a pro-proliferative and a tumoricidal effectivity-enhancing cytokine for memory T cells and T helper 17 (Th_{17}) cells that express its cognate receptor, IL-23R (123–128). Since IL23-R and IL-23α p19 are upregulated upon TCR antigen engagement and the IL-12β p40 subunit is not, Ma et al. demonstrated that engineered expression of the p40 subunit by T cells (p40-Td cells) can lead to their autocrine IL-23 signaling-induced selective proliferation, expansion, and survival upon activation (114). These researchers reported that p40-Td CAR-Ts exhibited augmented tumoricidal functionality characterized by the upregulation and downregulation of granzyme B and PD-1, respectively, compared with conventional CAR-Ts (114). The researchers also added that in mouse models of solid tumors, p40-Td CAR-Ts exhibited superior antitumor activity and decreased adverse complications in comparison with conventional CAR-Ts and IL-18- or IL-15-expressing CAR-Ts, respectively (114). The engineered expression of the p40 subunit can sufficiently mediate the expression and secretion of functional IL-23 exclusively by activated T cells in response to their activation within the TME since IL-23 is only assembled when both of the subunits are upregulated (114). This creates an autocrine action mode for the secreted IL-23 that provides a high-level regulation for the IL-23–IL-23R pathway (114, 121, 122). This prevents cytokine spread and bystander immune cells to be affected by the cytokine secretion while minimizing the side effects observed in other cytokine expression or administration strategies (114, 121, 122).

Additionally, IL-2 is also considered as a cytokine necessary for T-cell expansion, function, and survival modulation (129). However, the pleiotropic characteristics of IL-2 have overshadowed its broad application due to the emergence of toxicities (129). High-dose IL-2 is used in the treatment of patients with renal cell carcinoma and melanoma (130, 131). However, appropriate clinical settings are required for a safe IL-2 utilization and the prevention of treatment-related mortality (130, 131). IL-2 administration-related toxicities can occur in multiple organs including the heart, lungs, and CNS (130, 131). Capillary leak syndrome is known as the most frequent IL-2 administration-related toxicity (130, 131). This toxicity leads to a hypovolemic condition and massive plasma leakage from blood vessels into the extravascular space (130, 132). Capillary leak syndrome can lead to common clinical conditions such as

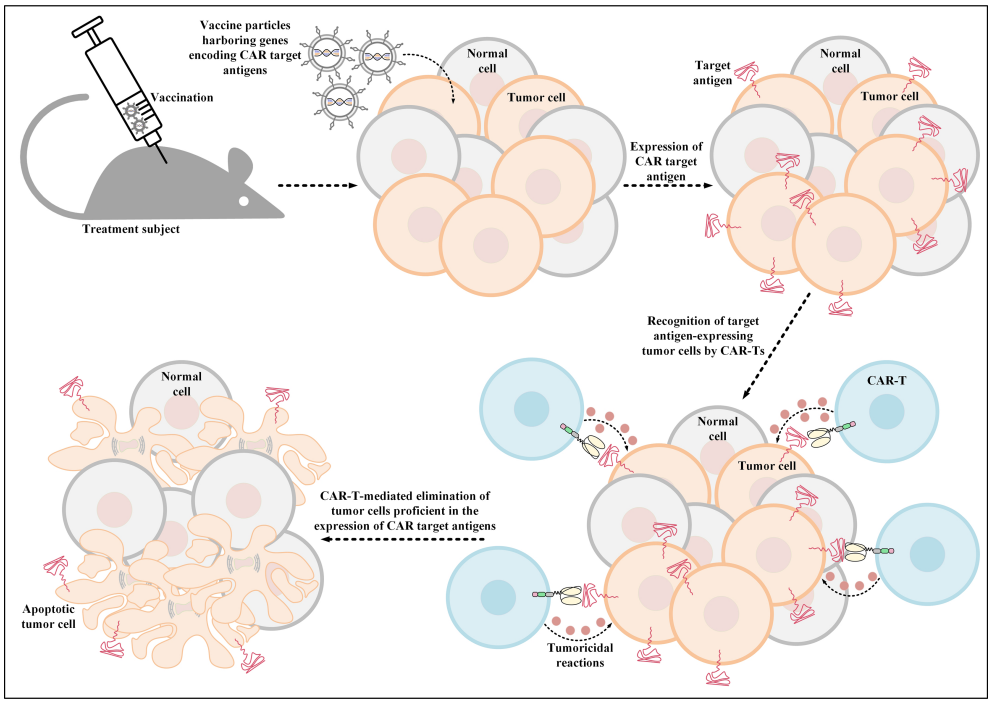

FIGURE 3 | Oncolytic vaccinia virus-mediated induction of CAR target antigen expression. This type of induced CAR target antigen expression in tumor cells results in efficient recognition and elimination of target antigen-expressing tumor cells by CAR-Ts. CAR; chimeric antigen receptor.

oliguria, ischemia, and confusion (130, 132). Even though high-dose IL-2 administration can be accompanied by such severe and life-threatening toxicities, safe and effective high-dose IL-2 administration can be achieved by using highly experienced healthcare professionals and toxicity prevention and controlling approaches (130, 131). A recent study has elaborately designed mutant forms of IL-2 (orthogonal IL-2) and its relative receptor, IL-2R (orthogonal IL-2R), that do not interact with their respective native counterparts, but in the meantime are capable of triggering native IL-2 downstream signaling cascades after specific interaction with each other (129). At-will potentiation of T cells engineered to express the orthogonal IL-2R has been achieved in preclinical animal models by the administration of orthogonal IL-2 without mediating considerable adverse events or complications (129). Such findings highlight the potential of this engineering twist and propose it as a translatable approach in CAR-T therapy; however, careful preclinical and clinical experiments are required in this regard (129).

Recently, one study has discovered that engineered expression of the native form erythropoietin receptor (EpoR) or its truncated form (tEpoR) on the surface of CAR-Ts endows them with the ability to tune up their expansion, survival, and proliferation rate in response to erythropoietin (133). It has been evident that tEpoR exhibits supervisor characteristics, in comparison to EpoR, in terms of T-cell expansion, proliferation, and survival stimulation (133). Vinanica et al. generated tEpoR-expressing CD19-redirected CAR-Ts (tEpoR-CAR-Ts) and reported that these cells demonstrated pronounced *ex vivo* expansion and *in vitro* tumoricidal capacity against leukemic cells in the presence of erythropoietin (133). These results confirmed that the expression of either receptor does not negatively impact the functionality of the other (133). Furthermore, tEpoR-CAR-Ts have been known to require lower cell dosing, in comparison with their conventional counterparts, since physiologic levels of erythropoietin would simply suffice to expand tEpoR-CAR-Ts, thus eliciting an accentuated tumoricidal response in mouse models (133). Of note, erythropoietin and ruxolitinib can be utilized to amplify the effector function of tEpoR-CAR-Ts or to diminish unwanted complications, respectively (133).

5 STRATEGIES FOR AMPLIFYING ANTITUMOR RESPONSES THOUGH THE INDUCTION OF BYSTANDER ANTITUMOR EFFECTS

As mentioned earlier, solid tumor CAR-T therapy suffers from inconsistent clinical responses and tumor escape that are partly resultant from heterogeneous tumor antigen expression and tumors down-regulating or not expressing the antigen targeted by single-antigen targeting CAR-Ts (41). In such cases, the

induction of endogenous immune response against target antigens other than those recognized by the adoptively transferred CAR-Ts can be beneficial. This approach can expand tumor-redirected immune responses *via* the recruitment of a wide range of endogenous T cells infiltrated in the tumor site resulting in more probable prevention of tumor antigen escape (134–137). In the upcoming section, we review strategies that can be exploited to trigger bystander endogenous antitumor responses concurrent with the tumoricidal effects of CAR-Ts.

5.1 TRBA-Secreting CAR-Ts

In the case of glioblastoma (GBM), it has been demonstrated that a single-dose administration of EGFRvIII-redirected CAR-Ts, a GBM-specific tumor antigen, can contribute to the mediation of antigen loss and adaptive resistance in recurrent GBM patients with the tumor retaining high levels of wild-type EGFR expression (36). Recently, an elaborate strategy has been developed to overcome this issue by the incorporation of CARs and TRBAs into a single gene-manipulated T-cell product called CART.TRBA (41). In this strategy, a bicistronic construct encodes an EGFRvIII-specific CAR and TRBAs against the wild-type EGFR; thereby obviating the need for multiple transgene insertions and reducing the risks of insertional mutagenesis (41, 138, 139). TRBAs secreted by CART.TRBA cells not only redirect CAR-Ts to the respective tumor sites, but they can also act in a paracrine manner by recruiting bystander T cells against the tumor (**Figure 4A**) (41). Alongside minimizing the effects of EGFRvIII antigen loss, local TRBA secretion also reduces the risk of on-target toxicity associated with systemic administration of TRBAs (41). Moreover, CART.TRBA-mediated local TRBA secretion also erases the scenario for repeated TRBA infusions since TRBAs are sometimes subject to rapid renal clearance due to their low molecular weight (41). Furthermore, the desired differentiation and phenotype of the T cells can also be simply achieved by their simultaneous redirection using CARs and TRBAs (41). Not only may the CART.TRBA platform serve as a therapeutic option for GBM, but it may also have potent applicability for other types of solid cancers with heterogeneous EGFRvIII expression (including breast cancer, medulloblastoma, and ovarian carcinoma) (41, 140–142). Additionally, this approach may also be utilized for other types of antigen loss- or escape-associated tumors by targeting different combinations of tumor antigens (41).

Other studies have also proposed strategies for tackling antigen heterogeneity using TRBA-expressing oncolytic viruses (143, 144). According to a study by Wing et al., folate receptor-alpha-redirected CAR-Ts (FR-α-CAR-Ts) failed to achieve complete tumor elimination in xenograft tumors due to antigen loss (143). In contrast, these researchers reported that enhanced tumoricidal responses and prolonged survival were observed when anti-EGFR TRBA-expressing oncolytic viruses were combined with FR-α-CAR-Ts (143). The secreted TRBAs redirected FR-α-CAR-Ts and bystander T cells towards the tumor alongside favoring their activation, expansion, cytokine secretion, and tumoricidal effects (143).

Recently, Porter et al. expanded the use of oncolytic viruses for the production of immune checkpoint blockers (anti-PD-L1 antibodies), TRBAs (specific for the variant 6 of CD44), and an immunostimulatory cytokine (namely IL-12) by incorporating the nucleotide sequence of the TRBA into an oncolytic-helper binary adenovirus to simultaneously tackle antigen heterogeneity and TME-mediated immunosuppression (144). It is encouraging to conclude that these researchers reported that the combination of the resultant trio adenovirus with HER2-redirected CAR-Ts resulted in more reliable tumor rejection and survival through the engagement of non-specific immune cells, while compared with the respective monotherapies (144). Such strategies can be applied to overcome the limitation of rapid TRBA renal clearance and obviate the need for its sequential administrations.

5.2 Fms-Like Tyrosine Kinase 3 Ligand (Flt3L) Expression

Recently, it has been demonstrated that induction of pronounced antitumor responses and expanded epitope spreading is achievable through the combination of agonistic anti-4-1BB antibody, poly (I:C), and T cells genetically modified to express Flt3L (145). Flt3L is a DC growth factor and poly (I:C) is a Toll-like receptor 3 agonist that contributes to DC maturation, interferon secretion, and augmentation of T-cell stimulation capability (145, 146). As Lai et al. have reported, this combination therapy is capable of tackling tumor escape variants because of its ability to drive antitumor responses against a broad spectrum of tumor antigens through vast epitope spreading (145). Such antigens include those that are not programmed to be targeted by a particular ACT (145). These researchers have also proposed that utilizing endogenous DCs serves as a potent tactic for tackling the limitation of antigen-negative tumor escape after specific TCR or CAR-T therapies (145).

5.3 Cyclophosphamide Administration

Pretreatment with cyclophosphamide below the lymphodepleting dose has been recently known to induce bystander antitumor effects most likely through endogenous $CD8^+$ T cells (147). It has also been evident that this strategy can mediate TME immunomodulation through the depletion of Tregs (**Figure 4B**) (147). In a particular case, Klampatsa and colleagues reported that mesothelin-redirected CAR-Ts were unable to fully eradicate tumor cells in mouse models when only a small proportion of the tumor cells grew deficient in the expression of the targeted antigen (147). These researchers indicated that co-infusion of IDO inhibitor or PD-1-, CTLA-4-, or TGF-β-specific antibodies did not mediate any bystander antitumor effects (147). In contrast, it was evident that pretreatment with low-dose cyclophosphamide was effective in eliminating a larger proportion of mesothelin-negative tumor cells by inducing bystander antitumor effects (147). It was also reported that these bystander antitumor effects were $CD8^+$ T lymphocyte-dependent, rather than DC-dependent (147).

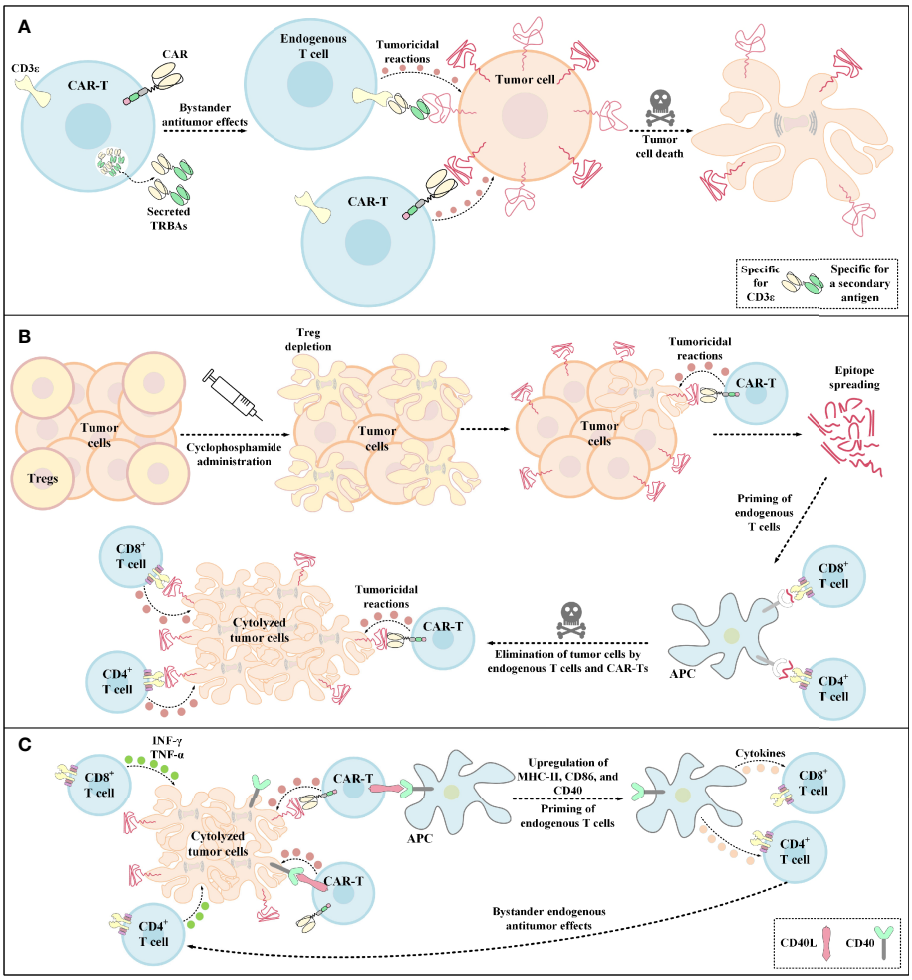

FIGURE 4 | Bystander antitumor effect induction by TRBA-secreting CAR-Ts, cyclophosphamide administration, and CD40L-expressing CAR-Ts. **(A)** TRBA-secreting CAR-Ts. TRBAs are made of two scFvs fused *via* a linker peptide. One of these scFvs targets CD3 (present on the surface of endogenous T cells) and the other one targets a TAA or TSA of interest against which endogenous T-cell responses are intended to be redirected. TRBA-secreting CAR-Ts secrete these bispecific T-cell-redirecting antibodies which results in endogenous T-cell-mediated antitumor reactions against malignant cells alongside CAR-T-mediated tumoricidal responses enabling a more effective tumor cell elimination. **(B)** Cyclophosphamide administration. Cyclophosphamide administration mediates Treg depletion and enables a more efficient CAR-T engagement with its target antigen and the subsequent CAR-T-mediated tumoricidal reactions. Additionally, upon epitope spreading, APCs uptake the released peptide antigens and present them to $CD4^+$ T cells and $CD8^+$ T cells. This mechanism leads to the priming of endogenous T cells and the subsequent elimination of tumor cells through bystander antitumor effects mediated by these endogenous cells. **(C)** The mechanism of action of $CD40L^+$ CAR-Ts. $CD40L^+$ CAR-Ts can mediate tumor cell cytolysis through both their CAR and their CD40L interacting with the CAR target antigen and CD40 on the surface of tumor cells, respectively. Additionally, $CD40L^+$ CAR-Ts mediate DC licensing as indicated by the upregulated level of CD40, CD86, and MHC-II. These APCs in turn recruit other immune effector cells such as endogenous T cells. INF-γ and TNF-α secretion by the recruited endogenous $CD4^+$ T cells and $CD8^+$ T cells also result in tumor cell cytolysis. APC, antigen-presenting cells; CAR, chimeric antigen receptor; CD40L, CD40 ligand; INF-γ, interferon γ; MHC-II, major histocompatibility complex class II; TNF-α, tumor necrosis factor α; TRBAs, T-cell-redirecting bispecific antibodies; Treg, regulatory T cell.

5.4 CD40 Ligand (CD40L)-Expressing CAR-Ts

Constitutive expression of CD40L by genetic engineering of CAR-Ts ($CD40L^+$ CAR-Ts) can be another strategy for tackling antigen loss-related immune escape of tumor cells by operating through direct and indirect tumoricidal responses (148, 149). The direct antitumor effect is established through the CD40/CD40L interaction between $CD40L^+$ CAR-Ts and $CD40^+$ tumor cells (148). On the other hand, the indirect effect arises from $CD40L^+$ CAR-Ts' ability for licensing APCs which results in the recruitment, mobilization, and cytokine production of endogenous effector cells that are capable of tumor recognition (**Figure 4C**) (148). According to one study, Curran et al. reported that $CD40L^+$ CAR-Ts are also capable of increasing the immunogenicity of $CD40^+$ tumor cells by increasing the expression level of HLA molecules, costimulatory molecules, Fas receptor, and adhesion molecules such as CD70 (149). These researchers also added that these CAR-Ts exhibited

boosted expansion and production of proinflammatory TH1 cytokines alongside mediating the maturation of monocyte-derived DCs (Mo-DCs) and inducing the production of IL-12 by them (149). Ultimately, Curran et al. proposed that the clinical application of CD40L+ CAR-Ts might lead to improved outcomes in CAR-T therapy of solid tumors (149).

5.5 C-C Motif Chemokine Ligand 19 (CCL19)- and IL-7-Secreting CAR-Ts

Additionally, it has been demonstrated that fibroblastic reticular cells mediate lymphoid neogenesis in which peripheral lymphocytes and DCs are recruited (150). CCL19 (which act as a chemo-attractant for T cells and DCs) and IL-7 (which is an essential regulator of T-cell proliferation and survival) are crucial homeostatic cytokines produced by fibroblastic reticular cells during the formation of lymphoid organs (151–154). With this in mind, CAR-Ts engineered to produce CCL19 and IL-7 (CCL19-IL-7 CAR-Ts) can act in a similar fashion as fibroblastic reticular cells in terms of T cell and DC recruitment to the desired tumor sites to help amplify tumoricidal effects (150). In 2018, Adachi et al. reported that CCL19-IL-7 CAR-Ts exhibited superior tumoricidal cytotoxicity, in comparison with conventional CAR-Ts, and that these cells benefited from the collaboration of other immune cells in fighting against tumors (150). Moreover, these researchers concluded that not only simultaneous expression of CAR and other immune-regulatory molecules such as IL-7 and CCL19 results in the augmentation of antitumor responses but also contributes to the activation and memory development of conventional T cells (150).

In a recent study, Goto et al. generated CCL19-IL-7 CAR-Ts and reported that these cells mediated effective tumoricidal responses in mouse models of solid tumors with enhanced tumor-site trafficking and expansion even in the immunosuppressive TME (155). In detail, they reported that human mesothelin-redirected CCL19-IL-7 CAR-Ts completely eliminated orthotopic malignant mesothelioma and prevented disease relapse with proportional antigen loss in mouse models (155). Moreover, it was reported that mesothelin-redirected CCL19-IL-7 CAR-Ts mediated meaningful suppression of tumor outgrowth in mice PDX models of mesothelin-expressing pancreatic cancers as compared with conventional CAR-Ts (155). Additionally, these researchers reported that the administration of CCL19-IL-7 CAR-Ts into mouse models also led to an increase in the number of other tumor-site trafficking immune cells alongside suppressing the expression of exhaustion markers (such as PD-1) on the endogenous T lymphocytes (155). Such findings might suggest the suitability of this strategy for the CAR-T therapy of patients with solid tumors; however, after broader preclinical and clinical investigations are carried out successfully (155).

Similarly, Pang et al. also generated GPC3-redirected CCL19-IL-7 CAR-Ts and reported that these cell exhibited improved proliferation and migration functionality according to *in vitro* assessments and more advanced antitumor activity in hepatocellular carcinoma (HCC) and pancreatic carcinoma cell line-established xenograft models (156). These researchers also reported the early results of an ongoing Phase I clinical trial (NCT03198546) in patients with advanced HCC, pancreatic carcinoma, or ovarian carcinoma who have glypican-3 (GPC3) or mesothelin expression (156). According to this report, the intratumor administration of GPC3-redirected CCL19-IL-7 CAR-Ts into a patient with advanced HCC resulted in complete tumor rejection within 30 days following the therapy (156). Moreover, the intravenous (IV) administration of MSLN-redirected CCL19-IL-7 CAR-Ts into a patient with advanced PC led to complete tumor elimination 240 days after the therapy (156). Such preclinical and clinical data support the effectiveness of this strategy for the improvement of CAR-T therapy in solid tumors and pave the way for more clinical studies with broader patient populations (150, 155–157).

5.6 IL-36γ-Secreting CAR-Ts

IL-36 isoforms are vastly expressed by the skin, lung, and gastrointestinal tract epithelial cells in response to damage and they mediate the expression and secretion of more IL-36 through a positive feedback by acting on the IL-36 receptor (IL-36R) on epithelial cells and other immune system cells (such as DCs, monocytes, neutrophils, and T lymphocytes) which triggers inflammatory responses (158–160). Moreover, researchers have demonstrated that IL-36 presence mediates the production and secretion of T lymphocyte-related inflammatory cytokines in a direct manner (158–160). These findings gave rise to the idea that genetically engineering CAR-Ts to deliver IL-36 in a site specific fashion can improve their proliferation and tumoricidal activity alongside triggering bystander antitumor responses (**Figure 5A**) (159, 160). Recently, Li et al. demonstrated that CAR-T-mediated secretion of IL-36γ can also contribute to the mounting of collateral antitumor effects through the activation of APCs and T cells nearly aborting the outgrowth of tumor cells with the loss of CAR-T target antigen (161). Furthermore, the autocrine action mode of IL-36γ has been known to enhance the functionality of these CAR-Ts in terms of proliferation, persistence, and tumor elimination capability in comparison with those of conventional CAR-Ts (161).

5.7 IL-18-Secreting CAR-Ts

IL-18 is a cytokine from the IL-1 cytokine family that is expressed and secreted by macrophages (162). IL-18 induces IFN-γ expression and secretion and demonstrates pleiotropic activity on various cells of the immune system (162). These characteristics of IL-18 have made it a great candidate for improving the tumoricidal activity of genetically manipulated T cell therapies while creating efficient bystander antitumor effects. In 2017, Hu and colleagues generated IL-18-secreting CAR-Ts and demonstrated that these cells were capable of mediating enhanced tumoricidal activity against CD19-expressing xenograft mouse models (163). In the same year, Chmielewski et al. reported that their IL-18-secreting CAR-Ts suppressed the tumor outgrowth in syngeneic pancreatic cancer and xenogeneic lung cancer preclinical mouse models in an enhanced manner in comparison with conventional CAR-Ts without engineered cytokine secretion (164). It is worth mentioning that these researchers indicated that the tumors in

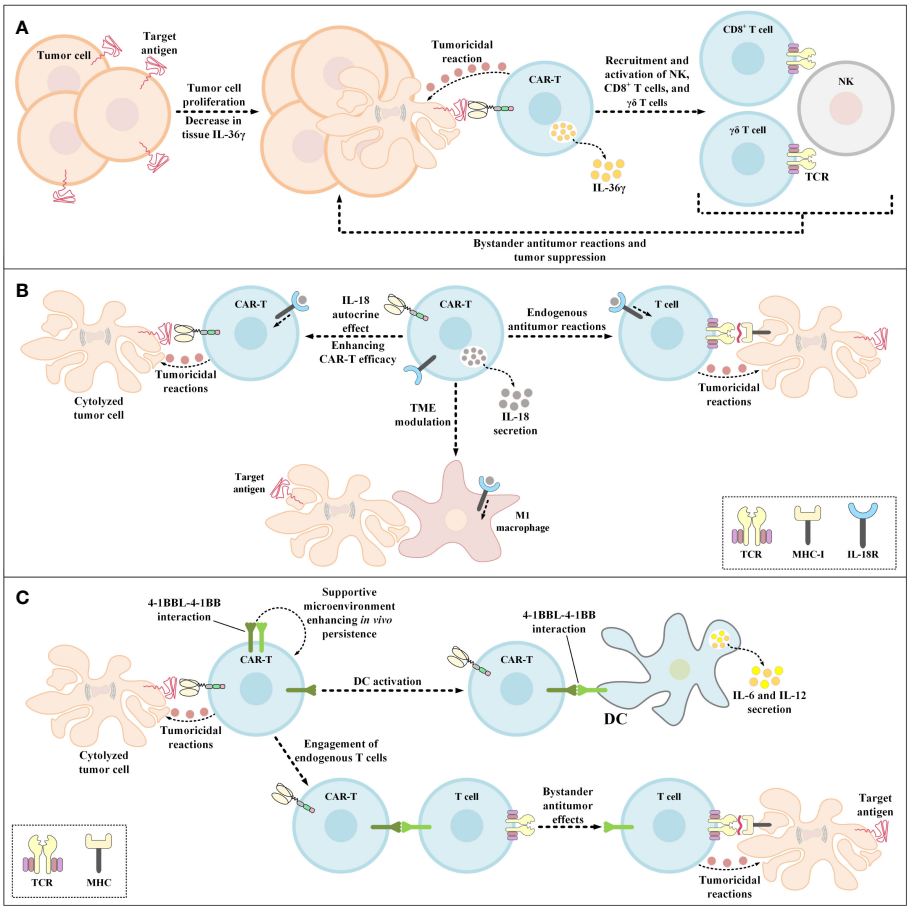

FIGURE 5 | Bystander antitumor effect induction using IL-36γ-secreting CAR-Ts, IL-18-secreting CAR-Ts, and 4-1BBL-expressing CAR-Ts. **(A)** The effects of IL-36γ secreted by IL-36γ-secreting CAR-Ts. IL-36γ secreted by these engineered CAR-Ts results in the recruitment and activation of endogenous immune effector cells, which include type 1 lymphocytes such as $CD8^+$ T cells, NK cells, and γδ T cells. These endogenous immune effector cells can mediate bystander antitumor immune reactions resulting in the suppression of tumor cell proliferation. **(B)** The effects of IL-18 secreted by IL-18-secreting CAR-Ts. CAR-T-secreted IL-18 has autocrine effects on the CAR-Ts themselves enhancing the antitumor activity of these cells. In case of endogenous tumoricidal effects, CAR-T-secreted IL-18 recruits endogenous T cells to the tumor sites and triggers their bystander antitumor reactions. Moreover, CAR-T-secreted IL-18 also modulates the TME and recruits M1 macrophages to the tumor site which results in M1 macrophage-mediated tumor cell cytolysis. **(C)** The beneficial effects of 4-1BBL expression by CAR-Ts. 4-1BBL-expressing CAR-Ts demonstrate enhanced functionality in comparison with their conventional counterparts in three ways. The 4-1BBL expressed on CAR-Ts self-interacts with the 4-1BB on these cells resulting in their enhanced *in vivo* persistence. 4-1BBL expressed on CAR-Ts also interacts with 4-1BB on the surface of DCs, inducing DC-secretion of IL-6 and IL-12. Additionally, CAR-T-expressed 4-1BBL interacts with 4-1BB on the surface of endogenous T cells leading to MHC-mediated bystander tumoricidal reactions. 4-1BBL, 4-1BB ligand; CAR, chimeric antigen receptor; DC, dendritic cell; IL-18R, IL-18 receptor; MHC-I, major histocompatibility complex class I; NK, natural killer cells; TCR, T-cell receptor; TME, tumor microenvironment.

the mentioned preclinical models were refractory to conventional CAR-T therapy (164). Ultimately, these researchers concluded that IL-18-secreting CAR-Ts can be utilized for rendering large solid tumors susceptible to bystander immune system-facilitated antitumor responses based on the finding that IL-18-secreting CAR-T treatment in preclinical models was associated with an increased number of M1 macrophages and $NKG2D^+$ NK cells and reduced number of M2 macrophages, Tregs, and $CD103^+$ DCs (164). According to a study by Avanzi et al., IL-18-secreting CAR-Ts can trigger endogenous antitumor responses alongside being capable of TME modulation and exhibiting pronounced expansion and persistence (**Figure 5B**) (162). In detail, they demonstrated that IL-18-secreting CAR-Ts can meaningfully prolong long-term survival in preclinical mouse models of hematologic and solid tumor neoplasms (162). Additionally, Huang and co-workers investigated the impacts of exogenous IL-18 on CAR-T tumoricidal activity and reported that IL-18 enhances the tumoricidal activity of HER2-redirected CAR-Ts in immunodeficient mice (165). These researchers also indicated that IL-18 demonstrates beneficial characteristics in improving ACT responses based on the finding that IL-18 enhanced the

tumor-targeting capacity of OVA-specific T lymphocytes (165). Moreover, using IL-18 receptor (IL-18R)-knockout condition in immunocompetent mice and CAR-Ts, Huang et al. reported that IL-18R-independent pathways are responsible for antitumor-improving activities of IL-18 (165).

5.8 4-1BB Ligand (4-1BBL)-Expressing CAR-Ts

4-1BBL is a molecule with immune stimulation characteristics interacting with the 4-1BB receptor in the process of antigen presentation in $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes (166). During this process, 4-1BBL activates the downstream signaling cascades of *NF-kB*, *c-Jun*, and *p38* that results in costimulatory signals to T lymphocytes leading to pleiotropic immune system responses (166).

Systemic delivery of ligands that activate costimulatory receptors for the activation of only tumor-reactive T cells may not be feasible since all T cells possess the capability of expressing such costimulatory receptors (167, 168). Moreover, systemic administration of mAbs for this aim may not also be significantly beneficial due to the inability of such therapeutics for targeting these costimulatory receptors on a specific population of T cells (169, 170). Some studies have demonstrated that immunization with tumor cells expressing B7.1, 4-1BBL, or OX40L can mediate robust T cell responses in preclinical mouse models (171–175). Such findings demonstrated that employing costimulation for triggering and amplifying antitumor T cell responses is an important approach.

Engineered expression of CD80 or 4-1BBL by CAR-Ts can support their ability for the activation of DCs and bystander T cells (**Figure 5C**) (176, 177). In particular, Stephan et al. reported that primary human T lymphocytes that expressed CD80 and 4-1BBL showed reactivity towards malignant cells deficient in the expression of costimulatory ligands and triggered robust outgrowth suppression of large and systemic malignancies in preclinical immunodeficient mouse models (176). Also, these researchers demonstrated that CD80- and 4-1BBL-expressing T cells exhibited enhanced expansion, cytokine expression, survival, and persistence *in vitro* and *in vivo* in preclinical mouse models in comparison with T cells without CD80 and 4-1BBL expression (176).

In another study, Zhao et al. reported that $4\text{-}1BBL^+$ CAR-Ts (with CD28 as the costimulatory domain and CD3ζ as the activation domain) exhibited pronounced tumor eradication capacity, which was correlated with the induction of IRF7/IFNβ pathway in these cells (177). Moreover, these researchers reported that $4\text{-}1BBL^+$ CAR-Ts mediated tumor elimination by modulating the TME in two ways (177). First, these CAR-Ts assist the targeted delivery of 4-1BB costimulation leading to trans-costimulation (by presenting the 4-1BBL costimulatory ligand on their surface) (176, 177). Second, they also contribute to the targeted delivery of IFN-β that could improve tumor elimination through various mechanisms including improving DCs' cross-priming functionality, suppressing Treg activation and expansion, and disturbing the microvasculature of the tumor (177). Moreover, Yang et al. studied the delivery IFN-β in a targeted manner using IFN-β-antibody constructs, and reported that this method boosted the cross-presentation of tumor antigens by CD8α DCs, resulted in the activation of $CD8^+$ T lymphocytes, and mediated tumor outgrowth suppression (178). Such data further support that $4\text{-}1BBL^+$ CAR-Ts are capable of mediating endogenous bystander antitumor reactions, expanding the territory of target antigens beyond the CAR-T-targeted antigen, and inducing antitumor immune reactions that surpass CAR-T cytotoxicity in terms of duration (178).

Recently, Park et al. reported the results of a Phase I open label first-in-human clinical trial (NCT03085173) of CD19-redirected $4\text{-}1BBL^+$ CAR-Ts in patients with non-Hodgkin lymphoma and chronic lymphocytic leukemia for investigating the safety profile and overall response (OR) of these "*armored*" CAR-Ts (179). In detail, these researchers reported no severe case of cytokine release syndrome (CRS), and severe neurotoxicity was only documented in 8% of the enrolled patients (179). It was also reported that the CR rate was 57% with 11 out of 12 patients still in CR at the time of the report (179). Such outcomes pave the way for further clinical evaluations.

6 ALTERNATIVE CAR-EXPRESSING EFFECTOR T CELLS

As discussed throughout the article, in recent years, various strategies have been developed for improving the outcomes of CAR-T therapy in solid tumors or to tackle its limitations. Some studies have proposed the employment of alternative effector T cells for CAR expression. Such CAR-expressing effector T cells can be beneficial for the treatment of solid tumors since these cells possess exclusive characteristics over conventional CAR-Ts. In the following section, we will briefly discuss two of these alternative effector T cells.

Natural killer T (NKT) cells are a type of MHC-independent cells originating from T lineage (180). NKT cells harbor morphological and functional features of both T cells and NK cells (180). Invariant natural killer T (iNKT) cells are a particular type of NKT cells that express the invariant TCR of Vα24Vβ11 capable of recognizing CD1d-presented glycolipid antigens (181). These cells act as a link between the innate immune system and the adaptive immune system (181). iNKT cells exhibit strong tumoricidal reactions by attacking and eliminating $CD1d^+$ tumor cells, immunosuppressive TAMs, and MDSCs (67). Similar to NKT cells, iNKT cells are not human leukocyte antigen (HLA)-restricted, rendering them incapable of mediating Graft versus Host Disease (GvHD) (180, 181). Such characteristics make iNKT cells a suitable platform for CAR expression and solid tumor therapy. Of note, studies have demonstrated that there is a direct correlation between the number and proportion of tumor-

infiltrating iNKT cells and the patient's overall survival rate (182). For instance, one study has reported that higher iNKT cell infiltration in colorectal carcinomas is related to a more favorable patient prognosis (183). Another study has reported that the number of circulating iNKT cells can be a prognostic factor predicting the outcome of patients with head and neck squamous cell carcinoma (184).

CAR-modified iNKT (CAR-iNKT) cells have been considered as effector cells for CAR expression since it has been shown that these cells demonstrate exclusive antitumor functionality against various types of tumor cells (for instance in patients with lung cancer, head and neck cancer, or advanced melanoma) (185, 186). CAR-iNKT cells express particular chemokine receptors CCR1, CCR2, CCR4, CCR5, CCR6, and CXCR3 enabling their easy migration to the TME (187). Also, CAR-iNKT cells mediate cytolytic activity against CD1d-expressing TAMs and MDSCs (67). In detail, TAMs and MDSCs stimulate tumor outgrowth and keep tumor cells safe from various immune reactions through developing an immunosuppressive environment (67). Additionally, studies have demonstrated that CAR-iNKT cells coexpressing IL-15 or other cytokines exhibit enhanced survival in a TME with hypoxia, acidic conditions, and nutrient insufficiency (188). Overall, we hypothesize that CAR-iNKT cell therapy alongside immune checkpoint inhibitor therapies or with cytokine- or chemokine-equipped oncolytic virus therapy may result in CAR-iNKT cells with additionally enhanced tumoricidal effects through improved survival and migration.

Gamma delta (γδ) T cells are a specific type of T cells that harbor particular TCRs on their surface. A great proportion of T cells express α (alpha) and β (beta) TCR chains (known as αβ T cells) (189, 190). In contrast, the TCR of γδ T cells is composed of a γ chain and a δ chain (189, 190). These special T cells are about 1-5% of circulating lymphocytes and are the predominant type of lymphocytes in various poorly accessed places including the skin, reproductive system, and the intestine (189, 190). This characteristic of γδ T cells has made them ideal platforms for CAR expression and CAR-T therapy of various types of neoplasms, especially solid tumors (189, 190). γδ T cells express chemokine receptors which respond to chemokines produced and secreted by malignant cells (191). This action mechanism facilitates the migration of these T lymphocytes towards poorly accessed tumor sites (191). Moreover, Vγ9Vδ2 T cells are an important subset of γδ T cells capable of recognizing phosphoantigens (192, 193). This ability of Vγ9Vδ2 T cells is advantageous in targeting various types of tumor cells since tumor cells are known to be accumulators of certain phosphoantigens (192, 193). In regards to GvHD induction, similar to iNKT cell, the activation of γδ T cells is also independent of MHC molecules; therefore, allogeneic γδ T cells can be considered safe in terms of GvHD mediation (194, 195).

As mentioned earlier, the lack of definitive or highly expressed tumor antigens in solid tumor CAR-T therapy results in poor CAR-T stimulation, activation, persistence, and antitumor responses. In such cases, γδ T cells can be beneficial since they can act as proficient antigen-presenting cells following activation resulting in bystander antitumor effects (196). Additionally, γδ T cells can be an ideal type of effector cell for CAR-T therapy since they can be expanded to large numbers during *ex vivo* culture (194, 195). In a nutshell, γδ T cells possess various advantageous features that can benefit solid tumor CAR-T therapy in many ways.

7 CONCLUSION

The use of cutting-edge genetic engineering techniques as well as other combinatorial strategies has changed the face of the back-then novel concept of CAR-Ts which started in the late 1980s. Early CAR-Ts harbored an antibody fragment fused to T-cell activating domains for the redirection of specialized T cells against tumor cells of interest. Newer generations of this concept proved efficient in the treatment of various hematologic malignancies, but in the case of solid tumor treatment, they faced serious obstacles. Even though the unsuccessful outcomes of CAR-T therapy in solid tumors came as discouraging news, they encouraged researchers to develop novel strategies for enhancing CAR-T tumor infiltration rate, inducing cooperative endogenous antitumor effects, induced expression of CAR target antigens in tumor cells, and generating CAR-T boosting vaccines. These developed strategies have demonstrated that it might be feasible to overcome the mentioned biological barriers and to increase the efficacy and safety of CAR-T therapy for the treatment of solid tumors. However, more profound experiments in human trials can further validate the functionality of these strategies and shorten the distance between their preclinical success and their clinical applicability. Furthermore, tearing apart the complex building blocks of the pathophysiology of solid tumors will also give us significant flexibility to use them against the tumors themselves for a more effective cancer immunotherapy. Strategies, such as what we discussed in this review, might serve as a giant leap towards success for CAR-T therapy of patients with solid tumors; however, after broader meticulous preclinical and clinical investigations.

AUTHOR CONTRIBUTIONS

PouSK: Conceptualization, Investigation, Writing - original draft, Writing - review and editing, Validation, Supervision. PooSK: Conceptualization, Investigation, Writing - original draft, Writing - review and editing, Validation, Supervision. MAN: Conceptualization, Writing - review and editing. FY: Conceptualization, Writing - review and editing. SMJM: Conceptualization, Writing - review and editing. FR: Writing - review and editing, Validation. All authors contributed to the article and approved the submitted version.

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Programmable Attenuation of Antigenic Sensitivity for a Nanobody-Based EGFR Chimeric Antigen Receptor Through Hinge Domain Truncation

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Epidermal growth factor family receptor (EGFR) is commonly overexpressed in many solid tumors and an attractive target for chimeric antigen receptor (CAR)-T therapy, but as EGFR is also expressed at lower levels in healthy tissues a therapeutic strategy must balance antigenic responsiveness against the risk of on-target off-tumor toxicity. Herein, we identify several camelid single-domain antibodies (also known as nanobodies) that are effective EGFR targeting moieties for CARs (EGFR-sdCARs) with very strong reactivity to EGFR-high and EGFR-low target cells. As a strategy to attenuate their potent antigenic sensitivity, we performed progressive truncation of the human CD8 hinge commonly used as a spacer domain in many CAR constructs. Single amino acid hinge-domain truncation progressively decreased both EGFR-sdCAR-Jurkat cell binding to EGFR-expressing targets and expression of the CD69 activation marker. Attenuated signaling in hinge-truncated EGFR-sdCAR constructs increased selectivity for antigen-dense EGFR-overexpressing cells over an EGFR-low tumor cell line or healthy donor derived EGFR-positive fibroblasts. We also provide evidence that epitope location is critical for determining hinge-domain requirement for CARs, as hinge truncation similarly decreased antigenic sensitivity of a membrane-proximal epitope targeting HER2-CAR but not a membrane-distal EGFRvIII-specific CAR. Hinge-modified EGFR-sdCAR cells showed clear functional attenuation in Jurkat-CAR-T cells and primary human CAR-T cells from multiple donors *in vitro* and *in vivo*. Overall, these results indicate that hinge length tuning provides a programmable strategy for throttling antigenic sensitivity in CARs targeting membrane-proximal epitopes, and could be employed for CAR-optimization and improved tumor selectivity.

Keywords: cellular immunotherapy, EGFR, CAR optimization, CAR-T, hinge domain, cancer selectivity, cell therapy

INTRODUCTION

Following the remarkable clinical success of CD19-targeted chimeric antigen receptor (CAR) therapies for the treatment of B-cell malignancies, design and development of novel CARs to treat common solid tumors is a highly active area of research and development (1). The first step for CAR development is the identification of an antigen binding domain (ABD) which is functional within a CAR; most typically this domain is composed of an antibody single-chain variable fragment (scFv) (2, 3). Previous work has also demonstrated that camelid single-domain antibodies (sdAbs), also called V_HHs or nanobodies, can also be used as effective CAR ABDs (4, 5), and a sdAb-based BCMA-targeting CAR-T; ciltacabtagene autoleucel (CARVYKTI™) has now gained FDA regulatory approval for treatment of relapsed or refractory multiple myeloma (6, 7). After identification of a functional ABD, molecular optimization can be used to further fine-tune the therapeutic properties of a CAR through alteration of various elements of the CAR molecule including the ABD, hinge, transmembrane domain, and intracellular signaling domains, each of which has been demonstrated to significantly impact the signaling properties and functionality of CARs (2, 3).

For some CARs, it may be desirable to attenuate the magnitude of the signal produced through CAR binding with the target antigen, resulting in lower antigen sensitivity/responsiveness. Such attenuated CARs can provide a more balanced level of signaling to favour long-term T cell persistence and/or to limit the reactivity to off-tumor antigen expression in normal tissues (8). Recent work has shown that decreased CAR signaling in CD19-specific CARs can be achieved by lowering the affinity of the ABD (9), altering the hinge or transmembrane domains (10–12), or engineering signaling domains with reduced activity (13). Intriguingly, these diverse molecular strategies have each been shown to enhance CAR-T persistence and therapeutic benefit in animal models, and shown some promise in at least one clinical trial thus far (9). These observations suggest that an optimal level of signaling may be somewhat lower than that of current generation clinical CD19-targeting CAR-T therapies. Thus, molecular optimization of CARs has emerged as a viable strategy for widening the therapeutic window of novel CAR-T therapies.

Epidermal growth factor receptor (EGFR) is one of the most commonly altered oncogenes in solid cancers, either through a variety of activating mutations or through over-expression of the native receptor (14, 15). EGFR has a relatively large extracellular domain with four subdomains (16), and is a well-established target for monoclonal antibodies and small-molecule inhibitors (17, 18). EGFR has also been explored as a target for CAR-T therapy, and clinical trials have been undertaken using ABDs specific for either the WT (19, 20) or a mutated tumor-specific form of the receptor known as EGFRvIII (21, 22). Recent clinical reports using EGFR-targeted CAR-T therapies in lung, biliary, and pancreatic cancers revealed no unmanageable toxicities and documented partial responses in some patients (23–26). EGFR is also under investigation as a target for bi-specific immune engaging therapy (27).

Although EGFR is attractive as an overexpressed tumor target, it is also expressed in normal tissues, and thus any therapeutic strategy must consider the potential for on-target off-tumor toxicity. In pre-clinical CAR-T work, it has previously been shown that the use of lower affinity EGFR ABDs can improve CAR-T selectivity for overexpressing tumor cells over normal tissues (28). Herein we present new camelid single-domain antibody (sdAb) CARs (sdCARs) with high on-target activity against human EGFR *in vitro* and *in vivo* and demonstrate that truncation of the hinge domain can be used to fine-tune CAR antigenic sensitivity and enhance selectivity for antigen overexpressing tumors. The results presented here extend previously established work examining the CAR hinge domain, demonstrating that for CARs targeting epitopes proximal to the target cell membrane, hinge-truncation offers a powerful and precise means to throttle CAR antigenic sensitivity. Such a strategy could provide a useful tool for optimizing CAR selectivity for tumor-associated antigens, elucidating CAR biology, and developing more complex multi-antigen targeting CAR therapeutics.

RESULTS

High-Affinity EGFR-Specific sdAbs Are Effective Targeting Agents for EGFR-Specific CARs

Generation of camelid sdAbs against EGFR using DNA immunization and phage display was reported in a previous study (29) (see **Supplementary Figure 1** for an overview of the workflow). To assess whether these high-affinity EGFR sdAbs were functional in the context of a CAR we chose three sdAbs with varying affinities and epitopes on EGFR (**Table 1**) (29). EGFR-specific sdAbs were cloned into modular CAR plasmid backbones with a flexible linker-extended human CD8 hinge domain, CD28-transmembrane domain, either CD28 or 41BB co-stimulatory domain, and CD3zeta signaling domain (**Figure 1A**). The resulting EGFR-sdCARs were screened for responses to target cells with varying EGFR expression (**Supplementary Figures 2A, B**) using a high throughput CAR-Jurkat screening assay (32). Jurkat cells electroporated with any of three EGFR-specific sdCAR constructs showed specific upregulation of CD69 following co-culture with EGFR-high H292 or SKOV3 cells (**Figures 1B, C**), with lower but still obvious T-cell activation responses against EGFR-low MCF7 cells (**Figure 1D**). In contrast, EGFR-sdCAR molecules were not activated in response to EGFR-negative Raji or Ramos cells (**Figures 1E, F**), in contrast to CD19-specific CAR-T molecules used as a control. We also observed similar results with a CD28 based co-stimulatory domain containing CAR (**Supplementary Figure 2C**). These data demonstrate that high affinity EGFR sdCARs can induce strong responses to EGFR-expressing target cells, even those with very low apparent EGFR expression.

To investigate whether these EGFR-sdCAR molecules have therapeutic potential, we next produced concentrated lentiviral vectors encoding one of the EGFR-sdCARs (sdAb021-BBz) with co-expression of an EGFP marker to identify CAR-expressing cells. We then transduced polyclonally activated PBMC-derived

TABLE 1 | Antigen-binding domains tested as CAR elements in this study.

Antibody Name	Type	Target	Affinity (K_D)	Cross-Reactivity	Competition	Epitope Location	Ref
sdAb021	sdAb	EGFR (Erbb1)	38.5 nM	Human, Cyno	Competitive with sdAb027, partial with sdAb028	Unknown, likely domain IV	(29)
sdAb027	sdAb	EGFR (Erbb1)	1.6 nM	Human, Cyno	Competitive with sdAb021, partial with sdAb028	Unknown, likely domain IV	(29)
sdAb028	sdAb	EGFR (Erbb1)	9.0 nM	Human, Mouse	Partial competition with sdAb021 and sdAb027	Unknown, likely domain IV	(29)
Trastuzumab	scFv	HER2 (Erbb2)	n.d. (5 nM)[a]	Human, Cyno	Juxtamembrane conformational epitope in domain IV	Very membrane proximal	(30, 31)
F265	scFv	EGFRvIII	n.d. (27.5 nM)[a]	Unknown	Domain I/III neoepitope, non-competitive with F269	Membrane distal	(32) and unpublished
F269	scFv	EGFRvIII	n.d. (31.5 nM)[a]	Unknown	Domain I/III neoepitope of EGFR, non-competitive with F265	Membrane distal	(32) and unpublished

[a]*Binding of scFvs was not evaluated. Monovalent affinity of full-length IgG is shown in parentheses.*
Cyno, cynomolgus monkey.

human donor T cells with EGFR-sdCAR lentivirus and expanded cells for 10 days. To assess EGFR-sdCAR functionality, CAR-T or control untransduced T cells were placed in low density co-culture with red-fluorescently labelled target cells with either high EGFR expression (H292 or SKOV3), low EGFR expression (MCF7), or no EGFR expression (Raji or Ramos). After overnight incubation EGFR-sdCAR cells showed clear upregulation of early T cell activation marker CD69 with EGFR+ target cells but not EGFR-negative target cells (**Supplementary Figure 2D**). We then examined the relative growth of CAR-T and various target cells for 7 days *via* automated fluorescence microscopy. Using automated cell counting to enumerate red target cells, clear target specific killing by EGFR-sdCAR-T cells but not corresponding untransduced T cells was observed (**Figure 1G** and **Supplementary Videos 1–3**). We did not observe similar killing of EGFR-negative Raji and Ramos cells (**Figure 1G**, **Supplementary Videos 5–6**). Similarly, enumeration of GFP+

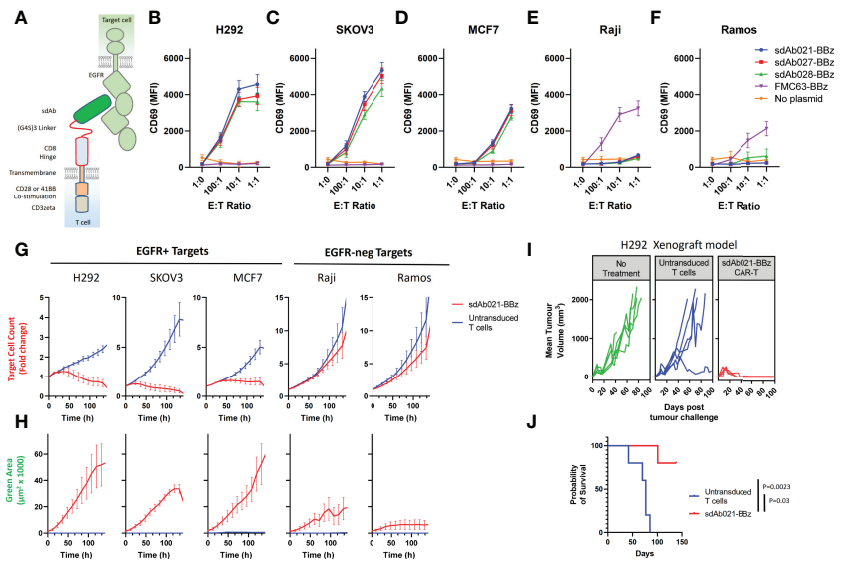

FIGURE 1 | Identification of EGFR-specific sdAbs with CAR functionality. **(A)** The structural elements of human EGFR and anti EGFR-sdAb CAR tested in this study are shown. Three EGFR-specific sdAbs were cloned into a modular CAR backbone with 41BB and CD3z signaling domain *via* golden gate cloning. Jurkat cells were then electroporated with the resulting constructs. Control cells with no plasmid or with a CD19 (FMC63) scFv CAR were also tested here. Jurkat cells (30 000/well) transiently expressing various CAR plasmids as shown were co-cultured with varying doses of **(B, C)** EGFR-high H292 or SKOV3 cells, **(D)** EGFR-low MCF7 cells, **(E, F)** EGFR-negative Raji cells or Ramos Cells and examined for activation *via* staining with APC-labelled anti-human CD69 antibody. CAR-J results show the mean +/- SEM from a three independent experiments performed in duplicate. Primary human T cells were then transduced with lentivirus encoding the sdAb021-EGFR-sdCAR construct and tested for activity against mKate2-expressing target cells with varying EGFR expression. **(G)** Automated fluorescent counting was used to examine CAR-T mediated target cell killing and **(H)** expansion of EGFP-labelled CAR-T cells. Primary CAR-T results show the mean of 3 experiments performed in duplicate. NSG mice were injected with $6x10^6$ H292 human lung cancer cells subcutaneously, then treated with $5x10^6$ sdAb021-BBz CAR-T or untransduced control T cells intravenously (n=5 mice/group). **(I)** Growth of H292 tumors *via* regular caliper measurements is shown. **(J)** Probability of survival throughout the experiment is shown (P values are derived from a Mantel-Cox comparison of treatment groups).

CAR-T cells showed clear expansion in co-cultures with EGFR-positive but not EGFR-negative target cells (**Figure 1H** and **Supplementary Video 1–5**).

To confirm whether these EGFR-sdCAR molecules would have therapeutic activity *in vivo*, we performed a murine xenograft experiment wherein immunodeficient NOD-SCID-IL2Rγ-null (NSG) mice were implanted with 6M H292-lung cancer cells, followed by 5M CAR-T cells delivered intravenously 15 days after tumor injection. In this experiment 5/5 mice treated with EGFR-sdCAR cells showed complete tumor regression (**Figure 1I**) and significantly enhanced survival relative to those receiving untransduced T cells (**Figure 1J**). Overall, these results clearly demonstrate that these high affinity EGFR-sdAbs are functional CAR targeting moieties that result in CAR-T cells with high antigen sensitivity and *in vitro/in vivo* therapeutic activity.

Hinge Truncation Can Be Used to Decrease EGFR-sdCAR Antigenic Sensitivity

Given the high sensitivity of EGFR-sdCAR T cells against even EGFR-low MCF7 target cells, we were concerned regarding possible on-target off-tumor effects. Thus we sought a controlled means by which we could attenuate the EGFR-sdCAR in order to enhance selectivity for EGFR-overexpressing cells. Based on previous work demonstrating that hinge domains of varying size derived from different human proteins can have a strong effect on CAR activity (33, 34), we hypothesized that progressive truncation of the hinge region could be used to more precisely attenuate target sensitivity. We cloned EGFR sdCAR constructs with either a full-length 45 amino acid human CD8 hinge (45CD8h) or N-terminally truncated CD8-hinge domains (34CD8h, 22CD8h, or no hinge; see **Figure 2A**). Jurkat cells transiently expressing the truncated hinge domain containing variants of all three EGFR-sdCARs showed progressively decreasing activation in response to EGFR-high SKOV3 target cells (**Figure 2B**). One possible explanation of the consistent effect of hinge truncation on antigenic sensitivity might be a reduction in surface expression for hinge-truncated CARs. Using a broadly reactive anti-sdAb antibody to label surface sdCAR expression we observed no impact of varying hinge domains on CAR surface expression (**Supplementary Figures 3A, B**). We also confirmed that EGFR sdCAR hinge truncation did not globally inhibit CAR-Jurkat responsiveness, as cells expressing hinge variant sdAb-CARs showed similar T-cell receptor-mediated responses to anti-CD3 antibody (**Supplementary Figures 3C, D**).

Next, we tested the response of EGFR sdCARs with truncated hinge domains against EGFR-low MCF7 cells, where we also observed decreased response with hinge-truncated EGFR-sdCARs (**Figure 2C**). Importantly, responses to EGFR-low MCF7 cells were more dramatically affected by hinge truncation than responses against EGFR-high SKOV3 cells. An analysis of hinge-modified CAR selectivity for SKOV3 vs. MCF7 cells shows the strongest selectivity enhancement with the sdAb021 sdCAR (**Figure 2D** and **Supplementary Figures 3E, F**), thus this construct was selected for further development. Overall, these results indicate that hinge truncation can be an effective tool to adjust the antigen-sensitivity of EGFR-sdCARs and enhance selectivity for EGFR-overexpressing cells.

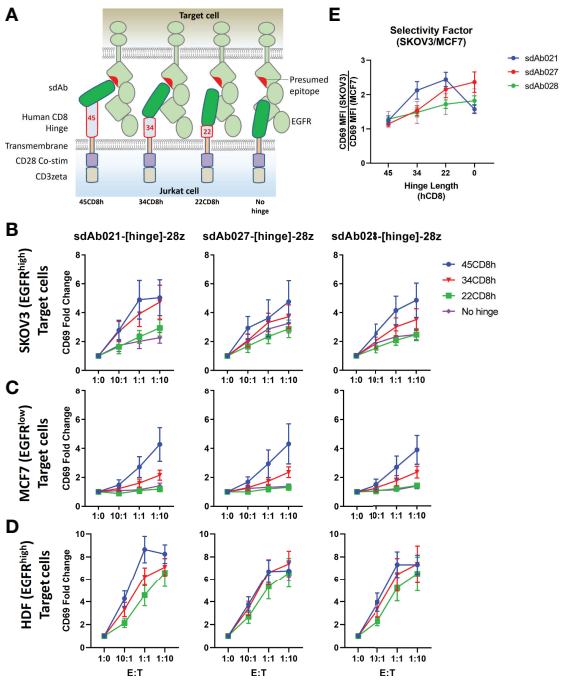

FIGURE 2 | Hinge truncation decreases target response for EGFR-sdAb CAR constructs. **(A)** Structure of single domain antibody based CARs targeting human EGFR were generated with hinge domains of varying length [full length human CD8-hinge (45CD8h), truncated CD8-hinge (34CD8h or 22CD8h), or no hinge element. **(B, C)** Jurkat cells were electroporated with varying CAR plasmid constructs before co-incubation with no target cells (1:0 E:T), EGFR-high SKOV3 cells, or EGFR-low MCF7 cells. 30 000 CAR-Jurkat cells were incubated overnight with varying numbers of target cells before CAR/GFP+ cells were examined for activation *via* staining with APC-labelled anti-human CD69 antibody. **(D)** Shows the relative CAR-J activation of various constructs in response to SKOV3 or MCF7. Results show the mean +/- SEM from three experiments performed in duplicate.

Single Amino Acid Hinge Truncation Provides Fine-Tuned Control of CAR-Antigen Response

Given our observations with truncated forms of the CD8-hinge domain, we wanted to more finely map the effects of progressive hinge truncation on an EGFR sdAb CAR. We also wondered whether hinge extension with an additional N-terminal flexible linker domain could be beneficial for antigen response. Thus, we designed an extended hinge domain with a 17 amino acids flexible linker appended to the N-terminus of the human CD8-hinge sequence [$(GGGGS)_3$GG-45CD8h] within the EGFR-sdCAR construct. We generated an array of sdAb021-CAR constructs with N-terminal single amino acid deletions of the extended human CD8 hinge (every combination between 62 and 1 amino acid). Screening the EGFR-sdCAR single-residue hinge truncation library revealed a clear pattern of CAR activation (**Figure 3A**). CAR constructs containing a full human 45 AA CD8 hinge or longer

produced strong responses to EGFR-high SKOV3 cells and lower but consistent responses to EGFR-low MCF7 cells. While there was some variation, the addition of a flexible linker extension to the CD8-hinge domain did not appear to have a consistent positive or negative effect on EGFR-sdCAR response. In this assay, EGFR-sdCARs with CD8 hinge sizes between 45 and 26 amino acids showed a progressive decrease in CAR activation, while CAR constructs with hinges 26 or less amino acids within their hinge domains showed no response to EGFR-expressing targets over CAR-J cells in the absence of targets (**Figure 3A**). Taken together, these data suggest that hinge truncation can provide precise control over EGFR-sdCAR antigenic sensitivity.

Epitope Location Is a Critical Determinant of CAR Hinge Sensitivity

According to current understanding, the location of the CAR target epitope should be critical in determining the minimal hinge size needed for CAR antigenic responsiveness (35). We do not have definitive data as to the epitope(s) targeted by the EGFR sdAbs used in our sdCAR constructs, though cross-reactivity analysis is suggestive of binding to the membrane proximal domain IV of EGFR (29) (**Table 1**). Thus, to more carefully investigate the role of epitope location in CAR hinge dependence, we generated smaller arrays of truncated hinge CARs with known target epitopes. We selected scFv-CARs based on either trastuzumab, which is known to bind a highly membrane proximal epitope of HER2 (30), or EGFRvIII-specific antibodies we have previously reported to show activity in CAR format (32), which by necessity must bind the membrane distal neo-epitope of EGFRvIII (**Table 1**). The membrane-proximal targeting trastuzumab-derivative scFv-CAR required a very long hinge element; with HER2 scFv CARs losing activity rapidly when hinge domains shorter than a full CD8-hinge were used (**Figure 3B**). In contrast, membrane-distal targeting EGFRvIII CARs maintained full activation with all hinge formats, even when the entire CD8 hinge domain was deleted (**Figure 3C**). These data support a model wherein epitope location is a critical determinant of whether a hinge domain is required for CAR functionality, and thus hinge truncation can only be effectively employed for fine-tuning antigenic sensitivity in membrane-proximal targeting CAR constructs.

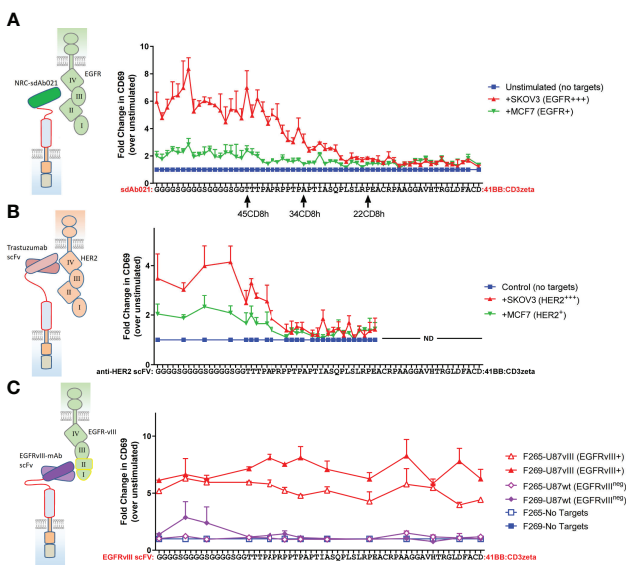

FIGURE 3 | Single amino acid truncations can throttle CAR antigenic sensitivity for membrane proximal epitope targeting CARs. **(A)** A range of sdCAR constructs were produced containing EGFR-specific sdAb021 and N-terminally truncated versions of the linker-extended human CD8 hinge domains ranging between 1 and 62 residues in length. Jurkat cells were electroporated with the resulting constructs and then co-incubated at an effector to target ratio of 1:1 with EGFR-high SKOV3 cells or EGFR-low MCF7 cells. After overnight incubation, CAR/GFP+ cells were examined for activation *via* staining with APC-labelled anti-human CD69 antibody. **(B)** Similarly, HER2-specific trastuzumab-derivative scFv CAR constructs were generated with hinge domains of varying lengths. CAR-expressing Jurkat cells were co-incubated with HER2-high SKOV3 cells or HER2-low MCF7 cells and then CAR-T activation was assessed. **(C)** Similarly, EGFRvIII-specific CAR constructs were generated with hinge domains of varying lengths. CAR-expressing Jurkat cells were co-incubated with EGFRvIII-overexpressing U87vIII cells or EGFRvIII-negative U87wt cells and then CAR-T activation was assessed. All results show means +/- SEMs of three separate experiments.

Hinge-Truncation Progressively Diminished CAR Cell Binding to Target Cells

We next wished to investigate how hinge truncation alters cell-cell interaction between CAR-T cells and target cells. Thus, we generated Jurkat cell lines stably expressing EGFR-targeted CAR constructs with varying hinge sizes and performed cell sorting to isolate pools of EGFR-sdCAR with similar CAR expression level. EGFR-sdCAR cells were then stained with anti-CD45 and mixed at varying effector to target ratios with red-fluorescently labelled EGFR-high SKOV3 cells or EGFR-negative Ramos cells. Co-cultures were then incubated for 30 minutes before using flow cytometry to quantitate the number of CAR-Jurkat cells, target cells, and CAR-target doublets (**Figure 4A**). Examination of cell size and height/width ratio support an interpretation that CAR-target doublets can be quantified *via* this method (**Figures 4B, C** and **Supplementary Figure 4D**). We observed progressively decreasing target cell binding for EGFR-sdCAR cells with truncation of CAR hinge domains (**Figure 4D**). EGFR-sdCAR cells with a hinge length of 22CD8h showed similar binding to control WT-Jurkat cells or CD22-targeted CAR-Jurkat cells. In contrast to SKOV3 binding, interaction with EGFR-negative Ramos cells remained low for all EGFR-sdCAR cells, but was high for a control CD22-targeted CAR (**Figure 4E**). Examining the level of target binding at a fixed ratio across hinge variants tested reveals a clear pattern of decreased target cell binding with hinge truncation (**Figure 4F**). These results indicate that shortening the hinge domain directly limits the ability of EGFR-sdCAR cells to bind to antigen expressing target cells.

Hinge-Truncation Progressively Diminishes CAR Functionality in Primary CAR-T Cells *In Vitro*

We next undertook extensive experiments to confirm whether the attenuation of antigenic sensitivity for hinge-truncated

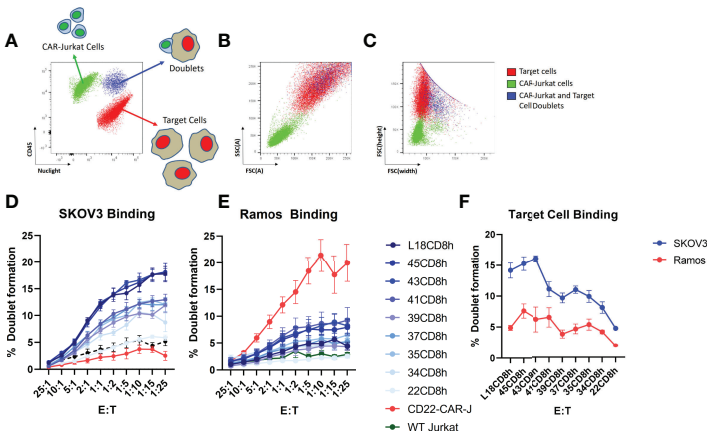

FIGURE 4 | Hinge truncation progressively diminishes CAR-cell binding with target cells. Jurkat cells with stable expression of sdAb021 EGFRsdCAR were generated through lentiviral transduction and cell sorted for similar surface expression. EGFR-sdCAR-Jurkat cells were then mixed at varying doses with mKate2-expressing target cells and co-incubated for 30 minutes at 37°C. **(A)** Flow cytometry was used to assess the number of CAR-Jurkat cells, target cells, and CAR-Jurkat/target doublets. **(B)** Examining the size/granularity parameter shows that doublet cells (blue) appear similar in size to the larger target cell population. **(C)** Examining the height/width parameter indicates that identifies the doublet population as wider than the target cells alone (blue vs red). The proportion of CAR-Jurkat cells engaged in doublet formation with **(D)** EGFR-high SKOV3 cells or **(E)** EGFR-negative Ramos cells were quantified using gating as described in the main text. **(F)** The target cell binding across varying hinge constructs at a fixed effector:target ratio of 1:5 is shown. Graphs show the mean +/- SEM from three experiments performed in duplicate.

EGFR-sdCAR molecules observed in Jurkat cells functioned similarly in primary CAR-T cells. Thus, EGFR-sdCAR-T cells were generated from blood T cells from three healthy human donors using CAR lentivirus encoding three hinge-variant forms of sdAb021-41BB-CD3z sdCAR. As observed in Jurkat cells, CAR-T surface labelling using an anti-single domain antibody did not reveal any effect of hinge truncation on CAR surface expression (**Supplementary Figure 5**). Primary EGFR-sdCAR-T:tumor cell co-cultures were then monitored for tumor cell (red fluorescence) and CAR-T cell (green fluorescence) growth over 7 days without media changes. Consistent with observations in Jurkat cells, hinge truncation progressively diminished the ability of EGFR-sdCAR-T cells to kill SKOV3 tumor cells (**Figure 5A**) and undergo CAR-T cell expansion (**Figure 5B** and **Supplementary Figure 6A**). Despite progressive attenuation of CAR responses, all hinge formats showed more SKOV3 killing and CAR-T expansion than untransduced cells. In contrast to this, only the full-length CD8-hinge domain EGFR-sdCAR-T cells (sdAb021-45CD8h-28z) showed consistent target killing and CAR-T expansion in response to EGFR-low MCF7 cells (**Figures 5C, D** and **Supplementary Figure 6B**). To further investigate the behavior of EGFR-sdCAR-T cells in the context of non-malignant EGFR-expressing cells, we performed a similar co-culture assay using HDF cells, which have a high level of EGFR expression (**Supplementary Figure 2**). Despite high EGFR expression, hinge truncation exerted a more potent effect on EGFR-sdCAR killing against HDF cells and CAR-T expansion, similar to that observed for MCF7 cells (**Figures 5E, F**). These results are consistent with many observations regarding T-cell inhibitory activity of fibroblast cells (36), furthermore these results underline importance of cellular context dependent nature of CAR-T target cell interaction.

To more robustly test EGFR-hinge variant CAR-T responses to target cells, we plated co-culture experiments of CAR-T cells derived from three donors at varying effector:target ratios with each of the three target cells discussed above. These results revealed a highly significant and consistent pattern of enhanced selectivity for EGFR-high targets (SKOV3 or U87vIII) over EGFR-low tumor targets (MCF7) or healthy-donor HDF cells with hinge-truncated EGFR-sdCARs (**Figures 5G, H**). Interestingly, expansion of EGFR-sdCAR-T cells with truncated hinge domains seemed to be somewhat less sensitive to hinge truncation than target killing (**Figure 5H**). Overall these results provided strong evidence that that hinge truncation can be used to attenuate antigenic sensitivity and re-balance CAR selectivity for EGFR-overexpressing tumors.

We also performed an investigation of the effect of hinge truncation on CAR-T killing over an extended period of time. We isolated hinge modified sdCAR-T cells after primary challenge with antigen-overexpressing SKOV3 cells using the low density co-culture assay as described above and re-challenged the sdCAR-T cells by re-plating with additional target cells (**Supplementary Figure 7**). Re-challenged cells maintained similar relative selectivity observed in primary challenge as determined by target killing and CAR-T expansion, which both decreased with hinge length. These results indicate that re-stimulated CAR-T cells show similar or higher antigenic discrimination as observed in primary challenge, with progressively decreasing antigen-induced expansion following serial challenge.

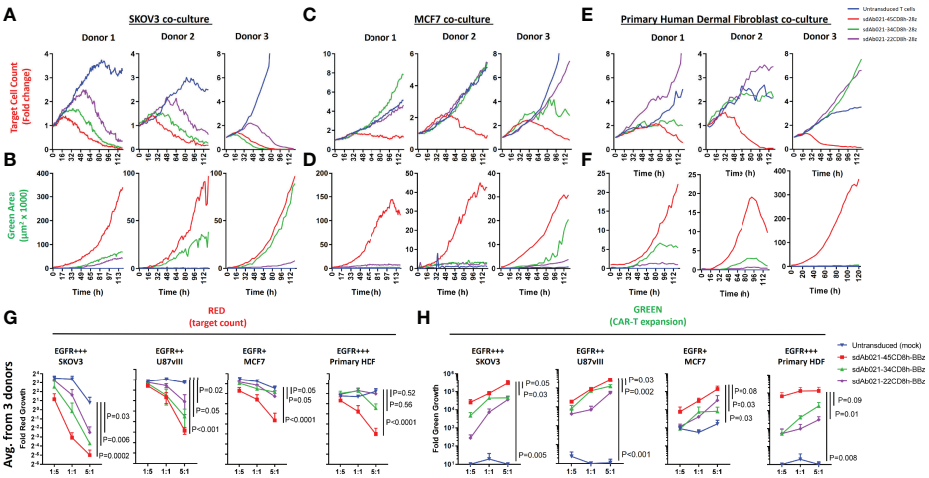

FIGURE 5 | Hinge truncation progressively diminishes tumor cell killing and expansion of primary sdCAR-T cells in response to EGFR expressing target cells. Concentrated lentiviral particles encoding hinge-modified EGFR-specific sdAb021 CARs as well as a GFP marker were generated. Peripheral blood T cells were isolated from 3 independent healthy human donors before polyclonal expansion and lentiviral transduction. Varying doses of sdCAR-T cells or mock transduced cells (empty CAR backbone lentivirus) were placed at an E:T ratio of 1:1 in low density co-culture (2000 sdCAR-T cells and 2000 target cells). Co-cultures were examined over 7 days *via* live fluorescence microscopy (Incucyte) to differentiate red-fluorescent (NLS-mKate2) target cell counts or total area of green-fluorescent (NLS-NanoGreen) CAR-T cells. **(A, B)** Depicts the response to EGFR-high SKOV3 targets, **(C, D)** depicts the response to EGFR-low MCF7 targets, **(E, F)** depicts the response to EGFR-high healthy donor human dermal fibroblast cells. Each graph depicts automated cell counts or fluorescent areas from a single independent experiment. **(G)** Day 5 mean fold change in various target cell growth at varying E:T ratio for hinge-variant EGFR-sdCAR-T cells derived from 3 donors is shown +/- SEM, P values are derived from a two-way ANOVA comparison of response curves for untransduced T cell co-cultures with CAR-T cells expressing hinge-truncated constructs. **(H)** Similarly the mean EGFR-sdCAR-T fold expansion at day 5 of hinge-variant CAR co-cultures from 3 donors is shown +/-SEM is shown. P values are derived from a two-way ANOVA comparison on 45CD8h-hinge containing constructs with other constructs tested in parallel.

Hinge-Truncated EGFR CAR-T Cells Selectively Kill SKOV3 Tumor Cells and Spare Healthy Donor Derived Cells in Triple Co-Cultures

We next tested an *in vitro* model of tumor selectivity wherein both EGFR-overexpressing tumor cells and healthy donor derived HDFs were both present in triple co-culture. We plated hinge variant EGFR sdCAR-T cells and fluorescently labelled SKOV3 tumor cells with or without the addition of unmodified healthy donor HDFs (effector:target:HDF ratio of 1:1 or 1:1:1 respectively). Examining growth kinetics of SKOV3 cells specifically, the presence of HDF cells did not alter SKOV3 growth kinetics with or without CAR-T cells (**Figures 6A, B**, **Supplementary Figure 8A**). The addition of untransduced T cells to the HDF-SKOV3 co-culture slightly slowed SKOV3 growth, but we did not observe SKOV3 killing (**Figures 6A, B** and **Supplementary Video 6**). As observed in dual co-cultures, EGFR sdCAR-T cells mediated strong SKOV3 killing, which was similarly reduced with shorter hinge CAR constructs with or without HDF cells (**Figures 6A, B**). Visual examination of co-cultures revealed complete CAR-T mediated killing of SKOV3 and HDF cells with the long hinge sdAb021-45CD8h sdCAR construct, with progressive sparing of HDF cells in truncated hinge CARs (**Figure 6C**, **Supplementary Videos 7–9**). Results were similar across CAR-T cells derived from three independent blood donors (**Supplementary Figures 8A,B**). Varying E:T within triple co-cultures showed a similar pattern of CAR-T activity to that observed with SKOV3 and CAR-T cells (**Supplementary Figure 8C**).

In order to directly quantitate the relative survival of SKOV3, HDF, and CAR-T cells in this triple co-culture model, we performed a similar experiment wherein 1:1:1 mixture of CAR-T:HDF : SKOV3 from all three donors were cultured for 24 hours and then stained with ZombieViolet fixable viability dye, human CD45, and human CD25, to examine for relative target cell death and T cell activation simultaneously. Using human CD45 staining and red fluorescence to differentiate HDF, CAR-T, and SKOV3 cells (**Supplementary Figure 8D**), we observed preferential killing of SKOV3 cells across all EGFR sdCAR hinge formats (**Figure 6D**). In contrast to SKOV3, where increased target killing was observed for all CAR-T constructs, short hinge EGFR-sdCAR-T cells did not significantly increase HDF cell death relative to untransduced T cells (**Figure 6D**). We finally confirmed that T cell activation was steadily increased for longer-hinge EGFR sdCAR-T cells, as assessed by CAR-T CD69 expression (**Figure 6E**). Overall, these results demonstrate that hinge truncation increases tumor selectivity for EGFR sdCAR-T cells even in simultaneous co-culture with both healthy cells and cancer cells.

In Vivo CAR-T Response Is Progressively Diminished With Hinge Truncation

Finally, we wished to investigate whether our *in vitro* observations regarding the relationship between hinge length and CAR-T activity

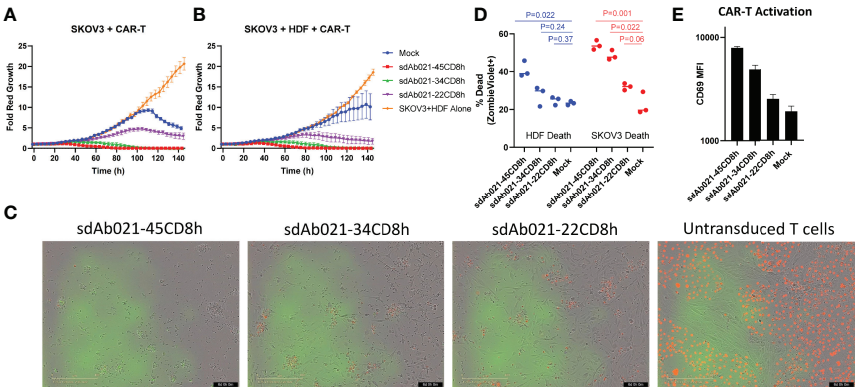

FIGURE 6 | Hinge truncated CAR-T cells maintain enhanced tumor selectivity in triple co-cultures with both healthy donor cells and SKOV3 tumor cells. Hinge variant EGFR sdCAR cells were placed in co-culture with **(A)** equal number of mKate2-expressing SKOV3 cells or **(B)** equal numbers of SKOV3 and unmodified human healthy donor derived dermal fibroblasts (HDF). Graphs depict the average fold change in growth of red fluorescent tumor cells over time from CAR-T cells derived from 3 independent donors. **(C)** Pictures show the state of triple co-cultures after 6 days of incubation. In a similar experiment, triple co-cultures of hinge variant EGFR-sdCAR cells, HDF, and SKOV3 cells were incubated overnight before examining **(D)** HDF or SKOV3 viability and **(E)** T-cell activation marker CD69 expression *via* flow cytometry. Graphs depict the mean results for CAR-T or control T cells from 3 independent blood donors. P values indicate a student T-test comparison for variance between differing hinge constructs, an ANOVA comparison of survival curves is also shown where marked.

were consistent in an *in vivo* xenograft model. Using the relatively slow growing SKOV3 model, mice were challenged with 2×10^6 SKOV3 tumor cells subcutaneously. After allowing 18 days for tumor implantation, all mice formed palpable tumors and were injected with 10 million hinge variant EGFR-sdCAR-T cells or untransduced CAR-T cells. There was a progressive increase in tumor growth in all mice with decreasing therapeutic activity with shorter hinge domain EGFR-sdCARs (**Figure 7A**). While the longest 45CD8-hinge CAR-T cells showed significantly increased survival, those mice treated with hinge-truncated sdCAR-T cells did not have a survival benefit (**Figure 7B**). The progressive effect of hinge truncation can be clearly observed in the final tumor volume measurement taken at day 112 post tumor challenge, although only those mice treated with the longest hinge format sdAb021-45CD8h-BBz showed a significant decrease in tumor volume (**Figure 7C**) which could also be observed by *in vivo* imaging of red-fluorescent tumor cells (**Figure 7D**). Examining CAR-T cells in the blood of treated mice revealed a consistent pattern of increased expansion of sdCAR-T cells with longer hinge regions at 43 and 54 days post-tumor injection (**Figure 7E**). Analysis of T cell differentiation revealed increased numbers of naïve or stem cell memory cells (N/SCM), and decreased effector sdCAR-T cell populations for hinge truncated EGFR-sdCARs (**Figure 7F**), supporting the interpretation that hinge truncation has a predictable and progressive effect on EGFR-sdCAR function *in vivo*.

To expand on these experiments, we also performed a similar xenograft experiment in which wild-type or hinge modified sdCAR-T cells were delivered intratumorally in the relatively more aggressive EGFR-high U87-vIII glioblastoma xenograft model. We observed similar progressive decreases in anti-tumoral effect (**Figures 8A–C**) and CAR-T expansion with truncated hinge EGFR-sdCAR constructs (**Figure 8D**). Furthermore, we observed increased naïve/SCM CAR-T cell populations associated with hinge truncation (**Figure 8E**). Taken together, these results demonstrate that hinge truncation can be used for reprogramming antigenic sensitivity for this EGFR-sdCAR constructs *in vitro* and *in vivo*.

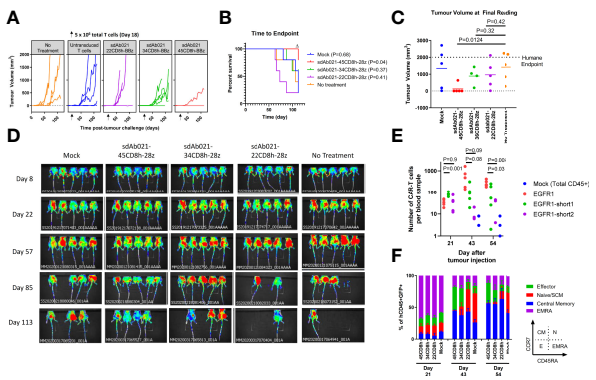

FIGURE 7 | Hinge truncated EGFR sdCAR-T cells show progressively diminished response to human lung cancer xenografts *in vivo*. NOD/SCID/IL2γ-chain-null (NSG) mice were injected subcutaneously with 2×10^6 SKOV3 cells stably expressing mKate2. Mice (N=5 mice/group) were injected with 5×10^6 total T cells (~1×10^6 CAR-T cells) intravenously. **(A)** Tumor volume was assessed using caliper measurements and **(B)** time to defined humane endpoint (tumor volume 2000 mm^3) was assessed. P values are derived from Mantel-Cox test of survival curves in comparison with untreated mice. Δ Note that the experiment was ended early due to non-experimental related animal facility shutdown, and thus the final tumor measurement of day 112 is shown in **(C)**. **(D)** Fluorescence imaging was performed at varying timepoints after tumor cell injection. **(E)** Blood was also collected at selected timepoints after tumor challenge to quantify the proportion of human CD45+ cells that were GFP/CAR+. **(F)** Staining for human CCR7 and CD45RA was used to assess the differentiation status of CAR/GFP+ cells or total T cells for mice treated with mock-transduced CAR-T cells. See inset for gating strategy used to define effector, naïve/SCM, central memory, or effector memory-RA+ (EMRA) cells.

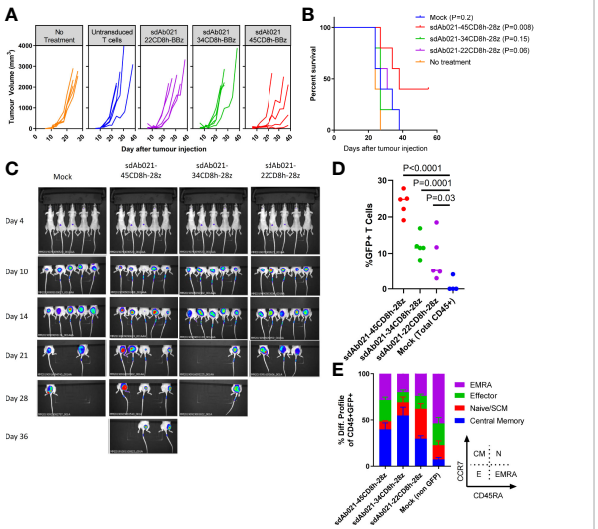

FIGURE 8 | Hinge truncation results in similar progressively diminished EGFR-specific sdCAR response to human glioblastoma xenografts *in vivo*. **(A)** NSG mice were injected (N= 5 mice/group) subcutaneously with 1×10^6 U87vIII cells stably expressing mKate2. At 7 and 14 days post-tumor challenge mice were injected intratumorally with 1×10^7 total T cells (approx. 2.5×10^6 hinge-modified sdCAR-T cells) or with untransduced control T cells (mock). Tumor growth was monitored *via* caliper measurements. N=5 mice per group. **(B)** Mice were sacrificed at pre-determined endpoints based on animal condition or tumor volume >2000 mm^3. P values are derived from Mantel-Cox test of survival curves in comparison with untreated mice. **(C)** *In vivo* imaging was performed to examine the mKate2 fluorescent signal associated with tumor cells. **(D)** Blood was drawn from challenged mice and examined for the proportion of sdCAR-transduced cells (GFP +) within the hCD45+ lymphocyte fraction at the final timepoint where all experimental mice were alive (day 21). **(E)** sdCAR-T cells, or total hCD45+ cells for mock T cell treated mice, were examined for differentiation status using antibody staining for human CCR7 and CD45RA. See inset for gating strategy used to delineate effector, naïve/SCM, central memory, or effector memory-RA+ (EMRA) cells.

DISCUSSION

We sought here to develop a novel EGFR-specific CAR construct that can discriminate between cells with high level expression of EGFR and cells with lower EGFR expression, as found on many non-malignant cells in the body (37). Despite some variation in binding affinity for the three EGFR sdAb moieties tested here (1.6 to 38 nM; **Table 1**), all three sdCARs showed similar strong responses to both EGFR-high SKOV3 and EGFR-low MCF7 target cells. Interestingly, we note EGFR-sdCAR responsiveness to MCF7 cells, despite no apparent reactivity of the purified sdAbs to EGFR-low MCF7 cells which we previously reported (29). These results are consistent with previous observations that EGFR specific CARs are relatively insensitive to ABD affinity up to the micromolar range (28), and underscore the exquisite antigen sensitivity of CAR-T cells to respond to and lyse even very low antigen expressing target cells (38–40). This phenomenon possibly relates to the extreme multi-valency of both the sdCAR and EGFR on their respective cells; increased valency is well known to lead to significant avidity effects that boost the apparent affinity of biological interactions (41–44), and these would presumably apply to CAR-T cells as well. These results can be taken to indicate that lowering ABD affinity may be a somewhat blunt tool for modulation of CAR antigenic sensitivity.

In experiments presented here we did not observe significant differences in antigenic sensitivity for CARs using EGFR-targeting sdAb elements ranging in affinity between 1.6 and 38.5nM, regardless of the hinge domain used. Consistent with this, previous reports using affinity modulation to increase selectivity of CAR constructs for antigen-overexpressing cells required over 1000-fold change in target affinity (28). Based on this previous data and our experience here, it seems that affinity modulation of binding elements is a blunt instrument for modulation of CAR sensitivity and would also carry the risk of generating unpredictable binding behavior, such as unexpected off-target binding, elevated tonic signaling, or poor protein stability., Thus, we wished to pursue an alternate molecular strategy for down-modulating EGFR-sdCAR antigenic sensitivity.

The use of hinge domains derived from various antibody isotypes or receptor ectodomains has been well- documented to have powerful influence on antigenic responsiveness of particular CAR constructs (2), and the strategy to employ different spacer domains of varying length has also been previously explored (3, 33, 45, 46). To our knowledge however, this is the first report of a simple truncation strategy of the human CD8-hinge domain which is most commonly employed in CAR designs. We find that truncations of a human CD8 hinge by as little as a single amino acid can be a remarkably precise throttle mechanism for CAR antigenic sensitivity. For the EGFR sdCAR we tested most extensively here, there was a steady drop-off of antigenic CAR response over a range of 10 to 20 amino acids within the CD8 hinge motif. Our hinge-truncation data using either membrane proximal (trastuzumab) or membrane-distal (anti-EGFRvIII-mAbs) based CAR constructs provides a further molecular demonstration of how epitope location is a determinant of hinge-sensitivity for CAR molecules reacting to tumor cells.

Using Jurkat cells, we were able to demonstrate that truncation of the CAR hinge domain can progressively decrease the upregulation of the early activation marker CD69 following co-incubation with antigen-expressing target cells, but this data does not necessarily provide mechanistic insight into how hinge truncation would affect fundamental processes involved in CAR signaling. With this in mind, we also explored the most upstream process involved in CAR activity, that being binding interaction between CAR antigen binding domain and tumor cells. Thus, we employed a short term co-culture assay to examine the immediate effect of hinge truncation on CAR-target cell interaction, similar to previous work investigating T-cell binding to tumor cells (47). These experiments mirrored results with CD69, with those truncated hinge CAR molecules showing less binding to target cells, despite similar membrane expression levels. These results demonstrate that hinge truncation can be used to selectively limit the ability of CAR cells to bind antigen-expressing target cells. A deeper exploration of how hinge truncation can be used to reprogram

more downstream processes involved in CAR signaling, such as the activation of important transcription factors (NFAT, NFκB, AP1) will be an important area for further study. However, since target interaction is the primary initiator of CAR-T signaling and downstream function; we believe that the evidence of our observations in regards to cell binding provide strong evidence that the strength of CAR binding and signaling can be fine-tuned *via* hinge length modulation.

In contrast to hinge truncation, we found that extension of the CD8 hinge domain does not have a strongly positive or negative effect on CAR response, at least as determined by the CAR-J assay. Similarly, membrane-distal epitope targeting EGFRvIII CARs showed similar responses across all hinge formats tested here. Previous work has indicated that longer hinges can decrease *in vivo* activity for membrane-distal epitope targeting CARs (45), but follow-up studies pinpointed the effect to be related to FC-binding by IgG-hinge motifs rather than hinge length specifically (46). It is also important to note that the multi-[GGGS] linker format used to extend our hinge is a highly flexible sequence typically employed for scFv engineering, and thus may allow for adequate ABD motility and close CAR packing unlike long hinge domains used in previous studies (33, 45). While it is clear that membrane-distal CAR molecules cannot function with a hinge domain which is too short, no firm conclusion on the impact of an excessively long hinge can yet be drawn.

Due to the relatively more demanding technical requirements of testing CAR-T constructs in primary T cells we only tested a limited number of CAR constructs within primary cell assays. Nonetheless, data presented here provide additional evidence that molecular optimization using transient CAR expression in Jurkat cells is predictive of responses in stably transduced primary CAR-T cells, as we have previously reported (32). The wider use of such optimization methodology could lead to improved ABD/hinge design for future CAR products. It may be possible for instance to design CARs with customized signaling for application in CD4, CD8, gamma-delta T cells, or NK cells.

Intriguingly, despite high expression of EGFR on healthy donor HDF cells, we observed obvious and significant decrease in *in vitro* HDF killing by hinge-truncated EGFR-sdCAR-T cells and increased selectivity for SKOV3 tumor cell killing. These results reflect the highly contextual nature of CAR-T activity, and underline that responses are never strictly dependent on CAR-antigen interaction alone. T-cell interaction with target cells involves a great number of cellular receptors, including many positive and negative regulators of T cell function (48), thus it is unsurprising that CAR-T cells should show differential selectivity towards primary versus malignant cells despite similar antigen expression. While it is not clear how specific these results are to HDF cells in particular, the consistency of our observations here clearly indicate that hinge-truncated CARs would have lower overall sensitivity to EGFR in all contexts and thus lower likelihood of on-target off-tumor toxicity.

For the *in vivo* models tested here, only the highest activity/longest hinge format was able to drive significant tumor regression and enhanced mouse survival, underlining the fundamental trade-off between maximizing on-target/on-tumor activity and minimizing on-target/off-tumor responses. It is not clear how predictive results from such xenograft tumor models are to human clinical efficacy, and thus the lack of efficacy results here should be taken with caution. Also, perhaps more extensive dose response *in vivo* studies would be required to demonstrate the differential activity of the hinge truncated CAR constructs. As murine xenograft models are not directly translatable to human clinical efficacy; such studies using large numbers of animals would not be ethically warranted. The data presented here does clearly demonstrate the ability to modulate the activity of the EGFR sdAb CAR with hinge length modulation. Translatability of these findings in human clinical setting would have to be evaluated with appropriately designed clinical trials.

The programmable antigenic sensitivity demonstrated here raises the possibility of a number of novel applications. For example, a clinical strategy wherein first-in-man trials could be performed with high safety/low activity truncated constructs with progressive hinge length escalation in subsequent dosing or patient cohorts may be possible. Alternatively, one could also envision dosing with a longer hinge CAR with an appropriate suicide switch for initial rapid tumor debulking followed by safer/low activity hinge truncated CAR for long term tumor control/surveillance. Finally, it may also be possible to incorporate such reduced sensitivity EGFR-sdCARs in multi-antigen targeting CAR strategy that will ultimately recognize and lyse tumor cells in more selective fashion. The data presented here provide strong evidence that generating similar CAR constructs with partially truncated hinge domains can be a powerful tool to engineer CAR molecules with desired antigenic sensitivity.

MATERIALS AND METHODS

CAR Cloning

Three previously reported EGFR-specific sdAb sequences (29) were cloned into a modular CAR backbone using PCR amplification and single-pot restriction ligation as previously described (32). EGFR-sdCAR constructs bearing either a full-length human 45 amino acid CD8 hinge (45CD8h) or progressively N-terminally truncated hinge variants (34CD8h, 22CD8h, or no hinge) were cloned using Gibson assembly. A library of sdAb021-CAR truncation mutants with single amino acid N terminal truncations of the human CD8 hinge extended with an additional N-terminal flexible linker [$(GGGGS)_3$GG-CD8h] was generated using a modular hinge-CAR with convenient type-IIs restriction sites integrated into the construct 3' of the sdAb coding region. An array of DNA encoding truncated CD8 hinge domains of varying lengths (all possible variants between 60 and 1 amino acid) were synthesized as DNA fragments (Twist Bioscience, San Francisco, CA, USA) and cloned into the sdAb021-modular-hinge-BBz-GFP CAR construct using single-pot restriction ligation. Limited hinge truncation libraries with defined-target CARs were generated by exchanging the sdAb021 sequence with HER2 or EGFRvIII

specific scFv sequences. Trastuzumab derived scFv sequences were generated based on previously reported mutant forms of trastuzumab with enhanced avidity for recognizition of HER2-overexpressing tumor cells (44), whereas EGFRvIII-targeting scFvs were generated as previously reported (32). Both HER2- and EGFRvIII-scFvs were in a VH-$(G4S)_3$-VL format.

Cell Lines and Culture

Other than U87MG and HDF cells, all others were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). The glioblastoma cell line U87 MG-WT and U87 MG-vIII (U87-vIII, expressing EGFRvIII *via* retroviral transduction and sorting) were kindly provided by Professor Cavnee from the Ludwig Institute for Cancer Research, University of California, San Diego (San Diego, CA, USA) (49). HDFs were purchased from Cell Applications (San Diego, CA, USA). The T-cell lines used were Jurkat, and target cells used were SKOV3, MCF7, U87 MG vIII, Raji, and Ramos. Target cells were transduced with lentivirus containing NucLight Red (Sartorius, Essen BioScience, Bohemia, NJ, USA), a third generation HIV-based, VSV-G pseudotyped lentivirus encoding a nuclear-localized mKate2. Nuclight positive cells were obtained by selection with puromycin. HDFs were cultured in all in one ready to use fibroblast growth medium (Cell Applications, Inc). U87 MG, SKOV3, MCF7, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate and 100 µg/mL penicillin/streptomycin. Jurkat, Raji, and Ramos were cultured in RPMI supplemented with 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate and 100 µg/mL penicillin/streptomycin. These cell lines were tested and found negative for the presence of mycoplasma contamination by PCR.

CAR-J Assay

High-throughput assessments of CAR function were performed by CAR-J assay according to a previously outlined protocol (32). Briefly, 5×10^5 cells were suspended in 100 µL of Buffer 1SM (5 mM KCl, 15 mM $MgCl_2$, 120 mM Na_2HPO_4/NaH_2PO_4, 25 mM sodium succinate, and 25 mM mannitol; pH 7.2) and incubated with 2 µg of pSLCAR-CAR plasmids as described in the text or with no plasmid control. Cells and plasmid DNA in solution were transferred into 0.2 cm generic electroporation cuvettes (Biorad Gene Pulser; Bio-Rad, Hercules, CA, USA) and immediately electroporated using a Lonza Nucleofector I (Lonza, Basel, Switzerland) and program X-05 (X-005 on newer Nucleofector models). Cells were cultured in pre-warmed recovery media (RPMI containing 20% FBS, 1 mM sodium pyruvate and 2 mM L-glutamine) for 4 h before being co-cultured with EGFR-expressing target cells U87 MG-vIII, MCF7 and SKOV-3 or negative control Ramos and Raji cells. Electroporated Jurkat cells were added to varying numbers of target cells in round bottom 96-well plates in effector to target (E:T) ratios ranging from 1:10 to 100:1 (effector to target ratio) or with no target cells (or an E:T of 1:0) and cultured overnight before being staining with allophycocyanin (APC)-conjugated anti human-CD69 antibody (BD Biosciences #555533). Flow cytometry was performed using a BD- LSRFortessa (BD Biosciences, San Diego, Ca, USA) and data was analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA) and visualized using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA, USA).

Short Term CAR-Jurkat Target Cell Binding Assay

Jurkat cells stably expressing EGFR-sdCARs with varying hinge domains were generated using lentiviral transduction as described below, using an MOI of 1. CAR-expressing cells were then stained with anti-sdAb antibody described below and subjected to cell sorting using a MoFlo Astrios cell sorting flow cytometer (Beckman Coulter, Mississauga, ON, Canada) in order to isolate populations of EGFR-sdCAR-Jurkat cells with similar levels of CAR surface expression. Healthy cultures of EGFR-sdCAR-Jurkat cells were then stained with anti-human CD45 (BD Bioscience, USA, Cat#563717), then resuspended at $5x10^5$ cells per mL in RPMI-complete media. Healthy cultures of SKOV3 or Ramos target cells were then harvested and resuspended in fresh RPMI-Complete media at $5x10^5$ cells per mL. CAR-Jurkat and target cell cultures were then mixed at varying effector to target ratios in a 96-well plate, followed by incubation at 37°C for 30 minutes and immediate assessment *via* flow cytometry using a BD LSR Fortessa device. The proportion of CAR-target cell doublet formation was assessed as described in the main text. Experiments were repeated 3 times in duplicate.

Plate-Bound Anti-CD3 CAR-J Stimulation Assay

Stable Jurkat cells expressing hinge variant sdAb021-BBz CARs were generated using lentiviral transduction similarly as described below, with cell sorting of GFP+ cells to generate stable CAR-expressing cell lines. OKT-3 stock (BioLegend Inc, San Diego, CA, USA) was diluted to 50 µg/mL, then diluted serially threefold. Plates were left at 4°C for 24 hours to bind. Unbound antibody was washed off the plate using PBS. 10^4 stable Jurkat cells expressing hinge-variant CARs were added to each well; in parallel hinge variant Jurkat-CAR cells were combined with varying doses for SKOV-3 target cells. Plates were incubated at 37°C 5% CO_2 for 24 hours before staining with mouse anti-human CD69-APC and analysis *via* flow cytometry.

Human Peripheral Blood Mononuclear Cell (PBMC) Isolation

Heparinized whole blood was collected from healthy donors under informed consent by venipuncture and transported at room temperature from Ottawa Hospital Research Institute. Blood was diluted 1:1 with Hank's balanced salt solution (HBSS) and PBMCs were isolated by Ficoll-Paque™ density gradient centrifugation. Briefly, samples layered on Ficoll-Paque™ gradient were centrifuged for 20 min at 700 × g without applying a brake. The PBMC interface was carefully removed by pipetting and was washed twice with HBSS by stepwise centrifugation for 15 min at 300 × g. PBMCs were resuspended and counted by mixed 1:1 with Cellometer ViaStain™ acridine orange/propidium iodide (AOPI) staining solution and counted using a Nexcelom Cellometer Auto 2000

(Nexcelom BioScience, Lawrence, MA, USA). T cells from were then activated with Miltenyi MACS GMP T cell TransAct™ CD3/CD28 beads and seeded 1x10^6 T cells/mL in serum-free StemCell Immunocult™-XF media (StemCell Technologies, Vancouver, Canada) with clinical grade 20 U/mL human IL-2 (Novartis, Basel, Switzerland).

Human Primary T Transduction by Spinfection

High concentration lentiviral particles encoding various sdCAR constructs were generated as previously described (32). After 24 h of T cell stimulation with beads, T cells were transduced with sdCAR-GFP lentiviral vectors (multiplicity of infection = 10) by spinfection. Briefly, lentivirus was added to T cells (1×10^6 cells/mL) and the mixture was centrifuged at 850 × g for 2 h at 32°C. After centrifugation, cells were incubated at 37°C for another 2 h. After incubation, cells were plated in a 24 well plate (100,000 cells/mL/well in a total of 1.5 mL) in StemCell Immunocult™-XF supplemented with 20 U/mL IL-2. Media with IL-2 was added at 48 and 72 h post transduction to promote CAR-T cell proliferation without disrupting the cells. Cell number and viability were assessed by AOPI staining and counting using a Nexcelom Cellometer. CAR-T cells were propagated until harvest on days 7, 9, 14, and 21 to assess the efficiency of transduction and to characterize T cell subpopulations by flow cytometry. CAR-T cells that had returned to a resting state (as determined by decreased growth kinetics, day 10 post-T cell activation) were used for assays.

Continuous Live-Cell Imaging Cytotoxicity Assay

Cytotoxicity of the CAR-T cells was assayed using a Sartorius IncuCyte® S3 (Essen Bioscience). Tumor cells, U87-MG-vIII-NucLight, MCF7-NucLight, and SKOV3-NucLight or HDF-NucLight, Raji-NucLight, and H292-Nuclight were resuspended in StemCell ImmunoCult™-XF with 20 U/mL IL-2 and plated in a flat bottom 96-well plate (2000 cells/well). CAR-T cells or control T cells were added into each well in a final volume of 200 μL per well in StemCell ImmunoCult™-XF with 20 U/mL IL-2 at varying effector:target ratios and co-cultured for 7 days at 37°C. In triple co-culture experiments, unmodified HDF and SKOV3-Nuclight cells were first plated at 1000 cells/well each, then additional CAR-T cells were added at varying doses as described in the text. Images were taken at regular intervals in light phase and under red (ex. 565-605 nm; em. 625-705 nm) or green fluorescence (ex. 440-480 nm; em. 504-544 nm). The assays were repeated thrice with T cells derived from independent blood donors. For one donor, CAR-T cells challenged once or twice with EGFR-high SKOV3 cells were rechallenged with various freshly plated target cells after 7 day of co-culture. Automated cell counting of red (target) or green (CAR-T) cells was performed using IncuCyte® analysis software and data were graphed using GraphPad Prism.

Animal Studies

NOD/SCID/IL2Ry$^{-/-}$ (NSG, JAX #005557) mice were purchased from Jackson Laboratories and maintained by the Animal Resource Group at the National Research Council of Canada. Eight-week-old NSG mice were injected with 2×10^6 SKOV3-NucLight in 100 ul of Hanks' Balanced Salt Solution (HBSS) subcutaneously. Eighteen days post tumor injection (when tumor reached approximately 5 mm × 5 mm), mice were retro-orbitally injected with 1×10^7 fresh day 10 post-activation mock T cells or T cells transduced with various CAR-T cells as described in the text. Tumors were measured using calipers twice a week and mice were imaged *via* IVIS *in vivo* imager for red-fluorescence signal (expressed on tumor cells) once a week. For SKOV3 study, eight days after tumor cell injection, cryo-preserved CAR-T cells were thawed, washed with PBS, and 5×10 total T cells (with 20-25% CAR transduction) were immediately delivered intratumorally, ensuring equal distribution of tumor sizes between groups. For the alternative U87 MG vIII model experiments, mice were subcutaneously injected with 1×10^6 fluorescently labelled U87 MG-vIII cells described above, a number we previously determined to consistently produce a palpable tumor within 7 days; mice were then treated at day 15 post-tumor challenge with 10^7 total T cells (approximately 2-3M CAR-T cells). Tumor growth was evaluated three times per week using calipers by trained animal technicians blinded to specific treatment groups. Mice were also assessed for tumor growth using IVIS *in vivo* imaging to examine red fluorescence derived from the NLS-mKate2 marked U87-MG-vIII cells. In order to minimize requirement for animal shaving to the area immediately around the implanted tumor a blanket was used to obscure part of animals during imaging. Primary endpoint was tumor size above 2000 mm^3, with secondary endpoints determined by overall animal health and well-being. Mice were euthanized when they met pre-specified endpoints. The study was approved by the NRC-HHT Institutional Animal Care Committee and was conducted in accordance with Canadian Council on Animal Care (CCAC) guidelines. Tumor growth and survival (humane endpoint) curves were generated using GraphPad Prism.

Generation of a Monoclonal Anti-sdAb Antibody for Assessment of sdCAR Surface Expression

CAR expressing Jurkat cells or primary T cells were generated as indicated elsewhere and assessed for surface expression using an Alexa-Fluor647 labelled murine monoclonal anti-sdAb antibody generated in house. In brief, mice were immunized with sdAb fragments produced in *E. coli* followed by B cell isolation and hybridoma formation as previously reported (50). Clonal hybridoma supernatants were screened for reactivity against a number of plate bound sdAbs to identify broadly sdAb-reactive monoclonal antibodies. Antibodies were then sequenced and transferred to a recombinant murine IgG2a backbone for production in CHO cells. Antibodies were then purified *via* protein-G column before labelling with Alexa-Fluor 647 using a commercial kit. This reagent was confirmed to react broadly against sdAb-CAR expressing cells but not scFv-CAR expressing Jurkat cells. This reagent was then employed through cell staining and flow cytometric assessment as described in the text for quantification of relative sdCAR surface expression.

Flow Cytometry and Antibodies

For *in vitro* studies, cells were stained with antibodies as indicated in the text, incubating the cells for 15 minutes at room temperature before washing with PBS. Cells were then resuspended in PBS and examined using a BD LSR Fortessa Flow cytometer. Staining of human EGFR was performed using anti-human EGFR-PE-CF594 (BD Biosciences, Cat #563431), and anti-sdCAR staining was performed using in house antibody generated as described above. For *in vitro* triple co-cultures were plated within 24-well plates with 50000 SKOV3 and HDF cells and varying CAR-T numbers as described in the text. After overnight incubation, cultures were stained with hCD45 and hCD25 antibodies, washed, stained with ZombieViolet Fixable viability dye (BioLegend USA, Cat #423113), followed by wash and fixation using 2% formalin in PBS. For *in vivo* experiments, blood was obtained from mice at various time points post CAR-T injection. Blood was washed with cold phosphate-buffered saline (PBS) and pelleted at 350 × g for 5 min at 4°C. Red blood cells were lysed using Red Blood Cell Lysing Buffer Hybri-Max (Sigma-Aldrich, St. Louis, MO, USA). Human T cells were identified and analyzed for activation/differentiation status using the following antibodies: hCD45-APC-H7, hCD45RA-BV650, hCD45RO-PE-CF594, hCD27-BUV737, hCCR7-PE, hCD4-BUV395, and hCD8-PerCP-Cy5.5 (all antibodies from BD Biosciences). CAR expression was measured indirectly *via* expression of GFP incorporated in CAR constructs. To evaluate exhaustion, staining by an hPD-1-BV421 antibody was evaluated. T cell activation was detected using hCD25-PE-Cy7 and hCD69-BV786 antibodies. For *in vivo* studies, a BV711-labeled antibody against mouse CD45 was used to identify murine cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Studies involving animals were reviewed and approved by NRC-HHT Institutional Animal Care Committee.

AUTHOR CONTRIBUTIONS

SMc, TN, AM, DB, KH, TS, RG, and RW contributed to conceptualization, experimental design, analysis, and interpretation of results; TN, AS, DB, SMa, CG, RP, AZ, and QZ contributed to technical development, performed experiments, and compiled data; SMc, TN, KH, and RW contributed to writing and editing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.864868/full#supplementary-material

Supplementary Figure 1 | Workflow for discovery and optimization of novel tumor selective sdCAR constructs: Antigen preparation, llama immunization, phage library preparation and panning, cell binding, CAR cloning, high-throughput CAR screening, and fine-tuning of CAR signaling in Jurkat cells through hinge length modulation was performed for several novel EGFR sdCAR constructs. Subsequently, confirmatory experiments were performed to test lead sdCARs *in vitro* and *in vivo* using primary human CAR-T cells.

Supplementary Figure 2 | EGFR-CAR-Jurkat and primary CAR-T cells can respond to EGFR-positive cells with a wide range of antigen expression. Jurkat, Raji, MCF7, U87vIII, SKOV3, and healthy donor derived primary human dermal fibroblast (HDF) cells were stained with varying amounts of commercial PE-CF594 anti-human EGFR antibody and examined by flow cytometry as indicated in **(A)**. **(B)** The mean fluorescence intensity of each cell type is shown. Results demonstrated lack of expression of EGFR in Jurkat and Raji, low expression in MCF7 cells, high expression in U87vIII, and very high expression for SKOV3and HDF cells. Histograms depict the fluorescence signal from a single experiment. **(C)** High throughput CAR-Jurkat screening was performed with CAR constructs composed of EGFR-sdAb binders, an extended hinge domain, CD28-transmembrane domain, CD28 co-stimulatory domain, and CD3zeta signaling domain. CAR-J results show the mean +/- SEM from three independent experiments performed in duplicate. **(D)** Human EGFR-sdCAR-T cells were generated as per description in methods section. CAR-T cells were then co-cultured at E:T of 1:1 with 300 000 cells CAR-T cells and 300 000 target cells with varying EGFR expression in a 96-well plate. After overnight co-incubation, cells were stained with human CD69-APC and examined *via* flow cytometry. Results show the mean of a single duplicate experiment for CAR-T cells from each independent donor.

Supplementary Figure 3 | CAR Surface expression is not altered with hinge domain modification. **(A)** Jurkat cells were electroporated with various EGFR-sdCAR plasmids (GFP expressing) and incubated overnight then stained with an Alexa-Fluor647 labelled murine monoclonal anti-sdAb antibody to examine surface CAR expression. WT Jurkat cells (left panel) show no GFP and no binding to anti-sdAb, whereas all other EGFR-CARs show clear simultaneous GFP expression and binding to anti-sdAb. **(B)** Mean fluorescence intensity for AF647-anti-sdAb stained CAR-expressing Jurkat cells, gated on GFP expression is shown. **(C)** CAR-J CD69 expression of hinge modified EGFR sdCARs stimulated with plate bound OKT3 stimulation overnight are shown in comparison to **(D)** CAR-J cells stimulated with EGFR-high SKOV3 cells at varying dose. Results are representative of 3 repeated experiments and demonstrate modulation of antigen-specific response *via* the truncated hinge. **(E)** Data presented for CAR-J assay against EGFR-high SKOV3 cells as described in were reorganized here to show the activation of EGFR sdCARs over a range of hinge lengths at an effector to target ratio of 1:10, varying from a full 45 amino acid human CD8 hinge to no hinge. **(F)** Data from CAR-J data against EGFR-low MCF7 cells organized *via* hinge length are shown.

Supplementary Figure 4 | Gating scheme for CAR-Jurkat target cell doublet formation assay Flow cytometry was performed to analyze the interaction of EGFR-sdCAR-Jurkat cells with various target cells as described in the main text. To analyze the proportion of CAR cells engaged in binding to target cells the following gating hierarchy was used: size/granularity gating to remove cell debris, followed by gating on total CD45+ population to isolate CAR-Jurkat cells, followed by gating on

GFP+/mKate+ population to isolate CAR-Jurkat target cell doublets. Plots shown are representative of 3 repeated experiments performed in duplicate.

Supplementary Figure 5 | CAR Surface expression in primary CAR-T cells is not changed with hinge length **(A)** Primary T cells from two independent donors were purified, expanded, and transduced with CAR lentivirus as described in the methods section. CAR-T cells were stained with AlexaFluor647 anti-sdAb antibody and examined *via* flow cytometry to assess CAR surface expression. GFP/CAR+ T cells show a clear binding to the sdAb antibody, not seen in untransduced Mock T cells. **(B, C)** Histograms and mean fluorescence intensity for anti-sdAb stained CAR-T cells is shown, with no consistent effect of hinge truncation on CAR surface expression.

Supplementary Figure 6 | Hinge truncation results in reduced response to antigen-low and non-malignant EGFR+ target cells **(A, B)** Primary sdCAR-T cells were generated from two independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth *via* live fluorescence microscopy over 7 days. Graphs display the total areas of GFP+ cells as enumerated through automated cell counting. **(C)** Representative images are provided for various sdCAR-T (GFP/green) or control mock T cells with target cell (mKate2/red) co-cultures 4 days after plating at an effector:target ratio of 5:1. 3 independent experiments were performed with CAR-T cells from independent donors as shown in the text.

Supplementary Figure 7 | Hinge-truncated sdCAR-T cells maintain selectivity for EGFR overexpressing cells following re-challenge. Donor blood derived T cells were transduced with lentiviral particles encoding the EGFR-specific sdAb021 sdCAR with varying hinge domains as described in the text. The resulting sdCAR-T cells were challenged *via* co-culture with EGFR-high SKOV3 cells and examined for target cell expansion or **(D)** sdCAR-T cell expansion as described above. Challenged cells were then diluted 1/10 with fresh media and challenged with **(B, E)** EGFR-high SKOV3 cells, **(G, I)** EGFR-medium U87vIII cells, or **(K, M)** EGFR-low MCF7 cells. Similarly, cells challenged twice with SKOV3 cells were re-challenged with various target cells and examined for **(C, H, L)** target cell expansion and **(F, J, N)** CAR-T cell expansion. Each graph depicts automated cell counts or fluorescent areas from a single well from a single experiment.

Supplementary Figure 8 | Hinge-truncated CARs display enhanced EGFR-high tumor selectivity in triple co-culture of human tumor cells, human healthy donor fibroblasts, and CAR-T cells. **(A)** Unmodified human healthy donor derived dermal fibroblasts (HDF) were placed in co-culture with equal number of mKate2-expressing SKOV3 cells. **(B)** Untransduced (mock) T cells or human T cells expressing hinge variant CAR constructs were added to HDF/SKOV3 co-cultures at equal cell number (1:1:1 ratio of HDF : SKOV3:CAR-T) and incubated with live cell monitoring using the Incucyte device; images show the state of triple co-cultures at day 6 after plating. **(C)** The fold increase of tumor cell growth at day 6 normalized to the initial plating within triple co-cultures with varying CAR-T doses was quantitated using automated cell counting. Graph depicts the average fold change in tumor cells from CAR-T cells generated from 3 independent donors +/- the standard error of the mean. **(D)** Depicts the gating strategy employed to identify CD45+ CAR-T cells, mKate2+ SKOV3 cells, and unlabelled HDF cells and examine their relative activation (CD25 expression on T cells) or viability (ZombieViolet fixability dye). Similar gating/analysis was utilized for triple co-cultures containing CAR-T cells derived from each of 3 independent blood donor samples.

Supplementary Video 1 | EGFR-sdCAR-T Cells with H929 Lung tumour cells Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 2 | EGFR-sdCAR-T Cells with SKOV3 Ovarian tumour cells Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 3 | EGFR-sdCAR-T Cells with MCF7 Breast tumour cells Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 4 | EGFR-sdCAR-T Cells with Raji lymphoma tumour cells Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 5 | EGFR-sdCAR-T Cells with Ramos lymphoma tumour cells Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 6 | Untransduced T Cells (Mock) with SKOV3 Ovarian tumour cells and Primary HDF cells. Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. In parallel, untransduced cells were expanded and treated with a mock transduction procedure with no lentiviral vector. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 7 | sdAb021-45CD8h-BBz CAR-T cells with SKOV3 Ovarian tumour cells and Primary HDF cells. Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 8 | sdAb021-34CD8h-BBz CAR-T cells with SKOV3 Ovarian tumour cells and Primary HDF cells. Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

Supplementary Video 9 | sdAb021-22CD8h-BBz CAR-T cells with SKOV3 Ovarian tumour cells and Primary HDF cells. Primary sdCAR-T cells were generated from 3 independent blood donors as described in the text and placed at low density co-cultures in a 96-well plate. Cells were then monitored for growth via live fluorescence microscopy over 7 days. Images were acquired every 2 hours and assembled into a timelapse video file. Results are representative of 3 similar independent experiments using different donor cells.

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