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# Unveiling the next generation of cancer immunity & immunotherapy

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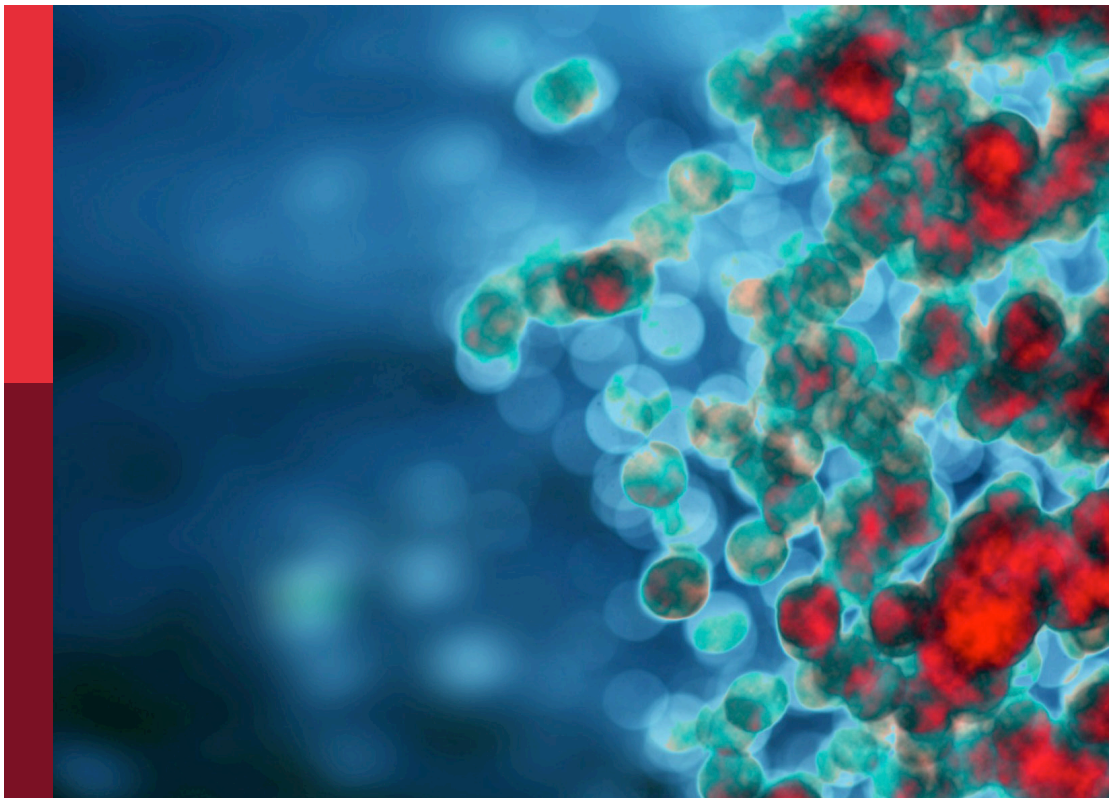

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# Unveiling the next generation of cancer immunity & immunotherapy

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# Editorial: Unveiling the next generation of cancer immunity & immunotherapy

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Editorial on the Research Topic
Unveiling the next generation of cancer immunity & immunotherapy

## Introduction

Over the past two decades, cancer immunology has shifted from a theoretical concept to a central pillar of modern oncology. Immune checkpoint inhibitors, adoptive cell therapies, oncolytic viruses, and personalized vaccines have revolutionized treatment for many malignancies. Yet, despite these remarkable advances, a substantial number of patients still fail to achieve durable responses, highlighting the persistent challenges of tumor heterogeneity, immune evasion, and the complex immunosuppressive tumor microenvironment (TME).

The Research Topic: *Unveiling the Next Generation of Cancer Immunity & Immunotherapy* brings together a collection of articles that address these challenges head-on, offering insights into innovative strategies designed to enhance the effectiveness, safety, and precision of immunotherapies. Collectively, these works showcase the field's dynamic evolution and hint at what the future may hold for cancer treatment.

## Next-generation therapeutic design

A central theme across the Research Topic is the rise of multi-target and next-generation immunotherapies (Figure 1). Traditional monotherapies, while effective for some, often fall short due to tumor escape mechanisms. Dual-target strategies, for instance, seek to overcome this limitation by simultaneously modulating multiple pathways.

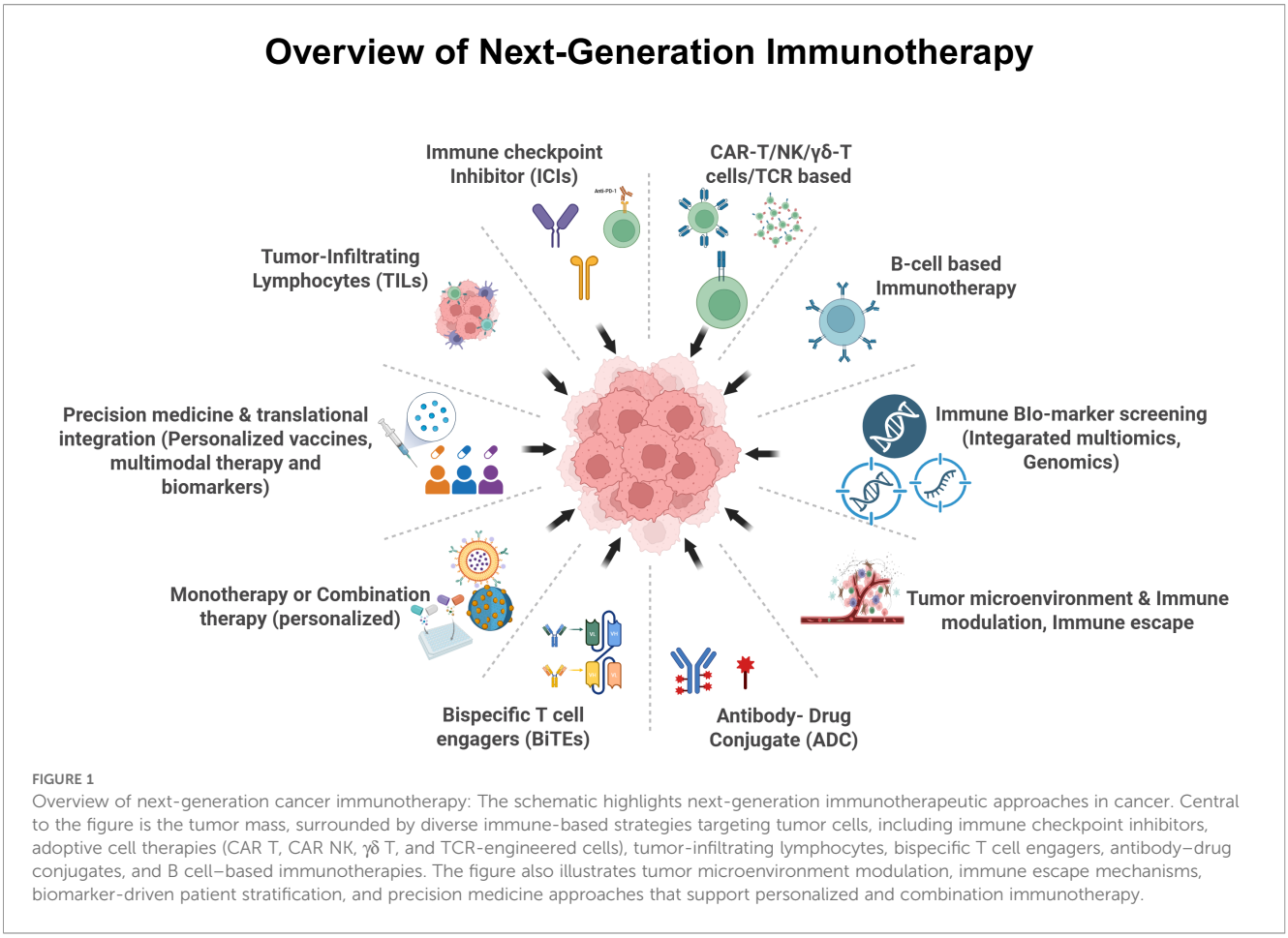


FIGURE 1
Overview of next-generation cancer immunotherapy: The schematic highlights next-generation immunotherapeutic approaches in cancer. Central to the figure is the tumor mass, surrounded by diverse immune-based strategies targeting tumor cells, including immune checkpoint inhibitors, adoptive cell therapies (CAR T, CAR NK, γδ T, and TCR-engineered cells), tumor-infiltrating lymphocytes, bispecific T cell engagers, antibody–drug conjugates, and B cell–based immunotherapies. The figure also illustrates tumor microenvironment modulation, immune escape mechanisms, biomarker-driven patient stratification, and precision medicine approaches that support personalized and combination immunotherapy.

The review '*Unlocking new horizons in oncology: Ivonescimab's dual-target approach to anti-VEGF/PD-1(L1) therapy*' exemplifies this approach. By targeting both angiogenesis and immune checkpoints, ivonescimab addresses the structural and immunological barriers that tumors employ to resist therapy. This dual-target strategy illustrates the power of rationally engineered biologics in maximizing immune responses while reducing the likelihood of resistance.

Similarly, '*Dual targeting of BCMA and SLAMF7 with the CARtein system: chimeric antigen receptors with intein-mediated splicing elicit specific T cell activation against multiple myeloma*' describes an advanced CAR T-cell platform in which intein-mediated protein splicing enables simultaneous recognition of two tumor antigens, resulting in enhanced and specific T-cell activation against multiple myeloma. This strategy aligns with the broader development of dual-target CAR approaches, such as CD19/CD22 and HER2/IL13Rα2 CARs, which are designed to improve antitumor efficacy and reduce antigen escape. Together, these innovations enhance target specificity, mitigate antigen escape, and exemplify the increasing sophistication of engineered cellular therapies, highlighting how advanced design principles can enable highly personalized, tumor-specific immunotherapeutic interventions.

The development of multi-functional antibodies further expands the therapeutic toolkit. '*Discovery and preclinical evaluation of BPB-101: a novel triple-functional bispecific antibody targeting GARP-TGF-β complex/SLC, free TGF-β and PD-L1*' highlights how one molecule can simultaneously disrupt several immunosuppressive pathways. By integrating checkpoint inhibition with modulation of TGF-β signaling, these approaches address multiple layers of tumor immune evasion in a single intervention.

## Reimagining the tumor microenvironment

Equally important are studies focusing on the tumor microenvironment, which plays a decisive role in shaping immunotherapy outcomes. The study '*Effect of bispecific recombinant oncolytic adenovirus carrying apoptin on apoptosis of MCF-7 cells*' demonstrates how engineered viruses can selectively eliminate tumor cells while simultaneously stimulating immune recognition, effectively converting "cold" tumors into "hot" ones more amenable to immune attack.

The review '*Revisiting the role of cancer-associated fibroblasts in tumor microenvironment*' reminds us that stromal cells are not passive bystanders. Cancer-associated fibroblasts actively shape immune cell infiltration, secrete regulatory cytokines, and

influence extracellular matrix composition. Therapeutic strategies targeting or reprogramming these fibroblasts may enhance responses to both cellular and humoral immunotherapies.

Lymphoid structures also play a critical role, as highlighted in '*Immune microenvironment of tumor-draining lymph nodes: insights for immunotherapy*'. Tumor-draining lymph nodes orchestrate systemic immune responses, and their manipulation can significantly influence therapy outcomes. Understanding these hubs may allow us to improve T-cell priming, enhance trafficking, and ultimately improve clinical efficacy.

## Translational and clinical perspectives

Several contributions underscore the translation of mechanistic discoveries into clinical application. '*Personalized peptide-based immunization in an advanced-stage prostate cancer patient with bone metastasis*' illustrates how individualized immunotherapy can be practically implemented, even in late-stage disease. By designing vaccines tailored to a patient's unique antigenic landscape, this work emphasizes the promise of precision medicine.

Additionally, '*Local radiotherapy in extensive-stage small-cell lung cancer sustainably boosts the clinical benefit of first-line immunotherapy*' highlights the power of combination strategies. Radiotherapy, traditionally seen as cytotoxic, can act synergistically with immunotherapy, promoting antigen release and immune activation. These studies collectively argue that next-generation immunotherapy is most effective when combined with other modalities.

## Emerging frontiers

Immunotherapy is undergoing a significant evolution, extending beyond its established role in oncology. CAR T-cell therapy, which has achieved notable clinical success in hematologic malignancies, is now being actively investigated for solid tumors as well as autoimmune and inflammatory diseases, reflecting growing confidence in the flexibility and translational potential of this platform. Although CD19 and BCMA remain the only approved CAR T-cell targets, an expanding repertoire of emerging antigens—including CD70, CD123, IL13Rα2, MUC1, NCAM, GD2, B7-H3, HER2, EGFRvIII, and mesothelin—is broadening the therapeutic landscape and enabling applications across solid and other non-hematologic tumors. The article '*Expanding the horizon of CAR T cell therapy: from cancer treatment to autoimmune diseases and beyond*' highlights how mechanistic and clinical insights gained in oncology can inform CAR T-cell strategies for autoimmune and inflammatory conditions, while '*B7-H3 in glioblastoma and beyond: significance and therapeutic strategies*' underscores the promise of alternative immune checkpoints as therapeutic targets for tumors resistant to conventional PD-1/PD-L1–based therapies. These insights point to a future where immunotherapy is not only more effective but also broader in scope and applicability.

## Synthesis and future directions

Across these studies, four themes emerge:

1. Innovative therapeutic design — the development of multi-target biologics, CAR-T/NK cells, and oncolytic viruses.
2. Tumor microenvironment modulation — understanding and manipulating stromal and lymphoid components to enhance immune response.
3. Precision immunotherapy — leveraging biomarkers, patient-specific vaccines, and adaptive strategies to maximize benefit.
4. Translational and clinical integration — combining conventional therapies, like radiotherapy, with advanced immunotherapies to achieve durable responses.

The field is moving from single-agent interventions toward multidimensional strategies that address tumor biology and immune contexture holistically. While challenges such as heterogeneity, resistance, toxicity, and access remain, the integration of mechanistic insights with translational innovation promises to deliver more durable, effective, and personalized therapies.

## Concluding remarks

The Research Topic '*Unveiling the Next Generation of Cancer Immunity & Immunotherapy*' provides a panoramic view of a rapidly evolving field. From cutting-edge therapeutic design and sophisticated cellular platforms to a nuanced understanding of the tumor microenvironment and clinical translation, these articles collectively chart the trajectory of next-generation immunotherapy.

By integrating molecular, cellular, and systemic perspectives, this Research Topic offers both a snapshot of current progress and a roadmap for future innovation. The challenge ahead is to translate these discoveries into durable, broadly applicable treatments that improve patient outcomes. These insights collectively indicate that, with ongoing cross-disciplinary integration, the field is well positioned to advance toward this outcome.

## Author contributions

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# Intratumoral injection and retention hold promise to improve cytokine therapies for cancer

Karsten Sauer[1]*, Kavya Rakhra[1], Kaida Wu[1], Naveen K. Mehta[1], Jennifer S. Michaelson[1] and Patrick A. Baeuerle[1,2]

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As powerful activators of the immune system, cytokines have been extensively explored for treating various cancers. But despite encouraging advances and some drug approvals, the broad adoption of cytokine therapies in the clinic has been limited by low response rates and sometimes severe toxicities. This in part reflects an inefficient biodistribution to tumors or a pleiotropic action on bystander cells and tissues. Here, we first review these issues and then argue for the intratumoral delivery of engineered cytokine fusion proteins that have been optimized for tumor retention as a potential solution to overcome these limitations and realize the potential of cytokines as highly effective therapeutics for cancer.

KEYWORDS

cytokine, intratumoral, IL-2, IL-12, collagen, CLN-617, immunotherapy, cancer

## Introduction

In this perspective, we advocate for the intratumoral (i.t.) injection of engineered cytokine molecules with optimized tumor retention as a potential solution to overcome the limitations which have thus far hindered the broad adoption of cytokines as safe and effective cancer immuno-therapeutics. The promise of this strategy is increasingly being recognized (1, 2).

As soluble and potent immune activators, cytokines play key roles in orchestrating productive anti-tumor immune responses (3). For this reason, several cytokines have been explored as cancer immunotherapeutics. The currently most pursued ones include interleukin 2 (IL-2), IL-12, IL-15, IL-18, interferons (IFNα, β and γ), tumor necrosis factor α (TNFα), and granulocyte-monocyte colony-stimulating factor (GM-CSF). But despite encouraging examples of efficacy, the clinical use of cytokines is relatively rare, and approved products are limited to IL-2 (Proleukin), IFNα (Besremi, Pegasys, PegIntron, Intron A) and GM-CSF (Leukine). Additionally approved viral products include the

IFNα-2b expressing adenovirus Nadofaragene firadenovec-vncg (Adstiladrin), and the GM-CSF expressing oncolytic virus (OV) Talimogene laherparepvec (T-VEC, product name Imlygic). However, these products have only been approved in a narrow set of indications (4–6).

The paucity of approved cytokine products may reflect dose-limiting toxicities (DLT) and low response rates of systemically administered cytokines (2, 3, 5). The toxicity of pro-inflammatory cytokines primarily comes from their pleiotropic action on bystander cells, along with a fundamental difference in how endogenous and exogenously administered cytokines are regulated. Endogenous cytokines are produced locally at sites of inflammation, act in an auto- or paracrine fashion and are quickly consumed by their target cells. This limits systemic cytokine exposure, which if dysregulated can cause severe toxicities such as cytokine release syndrome (CRS) - prominently seen in COVID-19 patients (7, 8). For cancer therapy, cytokines are typically administered systemically at high doses and repeatedly to ensure sustained engagement of the targeted immune cells in tumors. This will however expose unintended target cells and tissues expressing the respective cytokine receptor, causing toxicities (2). A well-documented example are the vascular leak syndrome (VLS) and pulmonary edema caused by IL-2 binding to receptors on lung endothelial cells (2, 5, 9).

Low cytokine efficacy can be caused by short serum half-lives and an inefficient biodistribution to tumors and tumor-draining lymph nodes (tdLN). Moreover, wildtype IL-2 can engage both desired effector T cells and NK cells, and undesired immune-suppressive $T_{reg}$ cells (5, 10). These factors limit the activation of immune cells by the administered cytokine, particularly when given at sub-efficacious concentrations due to low maximum tolerated doses (MTD). Moreover, feedback-inhibition (tachyphylaxis) can limit the efficacy of repeatedly administered cytokines such as IL-12 (11).

## Limitations of current cytokine modalities

Multiple approaches have been explored to improve the safety and efficacy of cytokine therapeutics for cancer. Broadly, these can be categorized as systemically delivered modalities or as modalities that are i.t.-injected directly into their desired sites of action.

### Systemically delivered cytokines

Since this perspective focuses on i.t. administered cytokines, we only briefly discuss systemically administered modalities here and refer to excellent recent literature for more details (2, 3, 5, 12–15). Advantages of systemic cytokine delivery include simple administration and predictable pharmacokinetics (PK) in serum. Recent approaches for systemic delivery aim to minimize toxic "off-tumor" activity, increase activity within tumors and prolong cytokine exposure. Methods include (i) altering cytokine specificity for receptor subunits, (ii) engineering cytokines for increased stability, (iii) masking cytokines in circulation, (iv) fusing cytokines with tumor- or effector cell-targeting moieties, (v) embedding cytokines in biomaterials which accumulate in tumors, and (vi) expressing cytokines only within tumors (10).

All these approaches have distinct advantages and limitations, which may explain their limited success in the clinic to date. Receptor-biased cytokine 'muteins' are designed for reduced binding to target cells mediating toxicities or tachyphylaxis. For example, so-called non-alpha IL-2 variants avoid binding to the high-affinity IL-2R α-subunit (CD25) expressed on lung epithelia, NK cells and $T_{reg}$ cells (3). However, CD25 is also expressed and upregulated on activated T cells and important for effector responses and IL-2 synergy with PD-1 blockade (16). The lack of CD25 binding might explain why neither non-alpha muteins nor similar IL-15 variants have succeeded in patients yet (16). Likewise, cytokines fused to albumin, immunoglobulin Fc domains or polyethylene glycol (PEG) polymers for half-life extension have not yet borne out in the clinic. This might reflect limited tumor penetration, toxicities or, possibly, exacerbated tachyphylaxis due to prolonged systemic exposure of the cytokine. Similar concerns apply to masked cytokine prodrugs that are activated by tumor-resident proteases, ATP or the low intratumoral pH. Here, heterogeneous or insufficient presence of the activating mechanisms in tumors or tdLN may limit efficacy, and drainage of the activated cytokine from tumors might limit efficacy or cause toxicities (2, 5).

So-called immuno-cytokines and other modalities incorporating tumor-targeting moieties are designed to enrich cytokines in tumors while limiting systemic exposure. However, much of the biodistribution is governed by binding of the cytokine moiety to its receptors on peripheral immune cells rather than tumor cells. This causes cytokine-related toxicities and limits tumor exposure (10). An alternative strategy is to selectively deliver cytokines *in cis* to targeted immune cells (e.g., CD8 T cells) in the periphery via immuno-cytokines or cytokine-releasing nanoparticles. Although effector cells are targeted in the periphery in this case, the cytokines are expected to be maximally active only in the tumor and tdLNs, where the respective high-affinity cytokine receptors are preferentially upregulated. Whether these approaches, or the adoptive transfer of tumor-specific T cells loaded with immuno-cytokines or cytokine-releasing nanoparticles, increase the so far limited success of immuno-cytokines in cancer patients remains to be shown (2, 15, 17). The conceptually related infusion of tumor-specific T cells engineered to express cytokine genes may be limited by toxicities due to variable cytokine expression and short durability of engraftment, and by the high cost and challenging logistics of engineered cell therapies (18, 19).

Finally, the systemic administration of OVs engineered to express cytokines such as GM-CSF has so far been safe in patients but elicited lower response rates than i.t. delivery. This approach is further challenged by complicated logistics and biosafety considerations, uncertainty about how much cytokines versus direct tumor cell lysis contribute to efficacy, unclear optimal doses, and a need for better understanding of PK and neutralization by anti-OV immune responses (4). Altogether, even advanced modalities have not yet led to a broad clinical success of systemically delivered cytokines.

## Intra-tumorally delivered cytokines

A conceptually attractive alternative approach to maximize "on-tumor" exposure and minimize "off-tumor" systemic exposure is to directly inject cytokine therapeutics into tumors. Initially, i.t. delivery was limited to easily accessible body surface-located tumors such as melanoma, but advances in image-guided delivery and robotic endoscopy now allow treating lesions deeper in the body. Many more cancer indications can now be addressed, including breast, lung, head and neck, cervical, pancreatic, prostate, colorectal, liver, ovarian and kidney cancer, sarcoma and glioblastoma (Table 1, Supplementary Table 1) (1, 10, 46–48). Modalities for i.t. cytokine delivery include cytokine-encoding mRNAs or DNAs alone or contained in lipid nanoparticles (LNP), OVs or other viruses encoding cytokines, cytokine-expressing transgenic cells, immuno-cytokines, recombinant cytokines and biomaterial-anchored cytokines (Figure 1) (1, 2, 5, 46, 48–52).

In comparing key features, i.t. delivered modalities fare favorably over systemically administered modalities, in particular when the i.t. modalities are engineered for tumor retention (1, 5, 10). Moreover, i.t. injection of exogenous cytokines mimics the local production and auto-/paracrine mode of action of endogenous cytokines in diseased tissues with its associated advantages. In particular, the high cytokine doses in tumors achieved by i.t. injection enable saturated receptor occupancy, followed by a slow and reduced systemic distribution - conditions that are generally unachievable with systemically delivered cytokines at the MTD. The improved control of exposure and PK in tumors can allow efficacious dosing without major systemic toxicity. Importantly, i.t. delivery provides cytokines immediate access to tertiary lymphoid structures (TLS) in tumors and to tdLNs, both important sites for initiation, priming and maintenance of anti-tumor T cell immunity (5). This is critical because the therapeutic efficacy of i.t. administered cytokines relies on an abscopal effect, where local injection into one or a few tumors triggers a systemic anti-tumor immune response that eliminates non-injected tumors as well. Rather than systemic cytokine exposure, the mechanism involves T cell priming and activation in tumors and tdLNs (1, 53).

A considerable number of clinical trials have investigated i.t. administered cytokine modalities [Tables 1, 2, Supplementary Table 1 and (1, 48)]. Most are still ongoing, but several have reported initial results. I.t- delivered cytokines are generally well tolerated. Several examples of significant single-agent efficacy in solid tumor indications have been reported. They include overall response rates (ORR) of up to 31.4% for the GM-CSF expressing OV Imlygic (Talimogene laherparepvec/T-VEC, the first approved OV and i.t. modality) (5, 20, 21), 28.6% for the GM-CSF expressing OV OrienX010 (23–25), and 25% for the OV VG2025 expressing both IL-12 and IL-15 (26). Other examples include ORRs of 50% for the tumor matrix-binding IL-2 + TNFα immuno-cytokine mix Nidlegy/Daromun (which also improved recurrence-free survival for patients with locally advanced fully resectable melanoma) or single-agent matrix-binding IL-2 Darleukin (which also yielded a median survival of 905 days) (21, 30–33, 48), a median OS of 61.57 months for the GD2-targeted immuno-cytokine Hu14.18-IL2 which exceeded that achieved by i.v. administration (34–37), variable ORRs of 25-99.5% for i.t. injected IL-2, and ORR of up to 25% for recombinant IFNα. There are multiple reports of abscopal effects or other evidence of systemic immune activation. Efficacy is often increased by combination with checkpoint blockade or other treatments (Tables 1, 2, Supplementary Table 1). Notably, these reports of efficacy of i.t. modalities extend beyond OVs – where the cytokine may function together with other viral mechanisms of action – to include tumor-targeted immuno-cytokines and naked cytokine proteins, thus far primarily focusing on IL-2 and IFNα. However, many more drug candidates are under investigation (5, 21, 48).

A comparison of clinical data for IL-2 modalities that have been well studied in both i.v. and i.t. administration settings indicates that either mode of administration can elicit significant anti-tumor efficacy at overlapping doses (Table 2). However, efficacy tended to be higher after i.t. administration, even at lower doses. Moreover, i.v. administration caused severe toxicities which were not seen upon i.t. administration of the same modality, which was usually well tolerated. This holds true for recombinant human IL-2 (Proleukin) as well as for immunocytokines including Hu14.18-IL2. Although there are caveats due to differences in the precise doses, treatment regimen, trial designs and patient populations between the different studies, these findings do point to notable advantages of i.t. administration.

Among modalities, i.t.-injected or electroporated cytokine-encoding cDNAs, mRNAs and viruses have in particular been widely explored in clinical trials (Figures 1A, B; Table 1, Supplementary Table 1). Here, transfected or transduced cells in the tumor produce the cytokine and other payloads. OVs preferentially propagate in and kill tumor cells through additional mechanisms. The promise of localized cytokine production in tumors, at least when combined with other OV mechanisms, is illustrated by the safety and efficacy of the OVs discussed above, and by the FDA approval of Imlygic. Yet, complex biosafety requirements and logistics limit OV application, and efficacy upon injection into large tumors can be limited to areas near the needle track, as seen for the TNF-producing virus TNFerade (4, 5, 48, 50, 60). Highlighting another limitation, infrequent responses of visceral metastases in patients indicate an insufficient abscopal effect of i.t. administered Imlygic in Phase 3 studies (20).

While usually safe, the clinical efficacy of non-OV DNA and RNA modalities so far has been variable, being sometimes significant but in other cases not, particularly as a monotherapy (Table 1, Supplementary Table 1) (2, 21, 48). This likely reflects difficulties in achieving consistent expression inside injected tumors, reaching effective cytokine doses and sufficiently controlled exposure, along with cytokine leakage out of tumors and peripheral turnover. Remarkably, the four cytokine-encoding mRNA combination BNT131/SAR441000 had low efficacy and its trial was discontinued (61–63). Tavokinogene telseplasmid (TAVO) did not meet its ORR endpoint in PD-1 refractory melanoma when combined with pembrolizumab but has yielded significant response rates in checkpoint combinations in certain patient populations (Table 1) (27, 28). Clearly, approaches employing nucleic acids and

TABLE 1 I.t. injected cytokines that have reached clinical trials.

| Modality | Asset | Cytokine(s) | Phase | Indication(s) | Comments* | References |
|---|---|---|---|---|---|---|
| OV | T-VEC (Talimogene laherparepvec, Imlygic/Amgen) | GM-CSF | 2 & 3 | Melanoma | - *First approved OV and IT asset*<br>- Single agent activity with ORR up to 31.5%, higher in sub-patient populations<br>- Low response rate in patients with visceral metastases<br>- ORR 39% in combo with ipilimumab<br>- ORR 42-67% in combo with pembrolizumab, 3 year OS 71%<br>- All treatment well tolerated | (5, 20–22) *(Reviews of multiple studies)* |
| | OrienX010 (OrienGene Biotechnology) | GM-CSF (±αPD-1) | 1/2 | Melanoma | - ORR generally ≤28.6%<br>- ORR 20.7% in combo with toripalimab for stage IV (M1c) liver metastases<br>- Showed abscopal effects<br>- Treatment tolerated | (22–25) |
| | VG2025 (Virogin Biotech) | IL-12 + IL-15 | 1 | Solid Tumors | - ORR 25% (n=4)<br>- No DLT, acceptable safety | (22, 26) |
| cDNA Plasmid | Tavokinogene telseplasmid (TAVO, Oncosec Medical/Merck) | IL-12 | 2 (+ Pembrolizumab or Nivolumab) | Melanoma, TNBC | - Pembro combo showed ORR 10.2% (did not meet ORR endpoint in PD-1 refractory melanoma); also showed ORR 41% in advanced melanoma with low PD-1$^{high}$ CTLA-4$^{high}$ CD8$^{+}$ CTL (n=22)<br>- Neoadjuvant combo with Nivolumab: Pre-operative response rate 77.8% (n=9)<br>- No tumor retention of the drug<br>- Showed systemic immune responses<br>- Well tolerated | (22, 27–29) |
| Tumor-matrix binding immuno-cytokine | Mixture of L19 –IL2 + L19 –TNFα (Nidlegy/ Daromun, Philogen) | IL-2 + TNFα (Fibronectin ED-B domain-targeted) | 2/3 (pivotal) | Melanoma, skin cancers | *NCT02938299 Ph 3:*<br>- Neoadjuvant Nidlegy + surgery improved relapse-free survival (RFS) vs. surgery alone (HR = 0.59)<br>- Median RFS 16.7 vs. 6.9 months<br>- 21% complete pathological responses<br>- Manageable TRAE<br>*NCT02076633 Ph 2, completed:*<br>- In melanoma, ORR 50% (week 12)<br>- Robust abscopal effects<br>- Well tolerated | (21, 22, 30–32) |
| | L19–IL2 (Darleukin, Philogen) | IL-2 (Fibronectin ED-B domain-targeted) | 2 | Melanoma | - Stage IIIB/IIIC (n=24): ORR 53.9%, CR 25% (6/24, 5 patients with DOR >24 months)<br>- Median survival 905 days<br>- Well tolerated<br>- Decreased MDSC and transiently increased CD4 $T_{reg}$ cell proportions in blood samples. Transiently increased total NK cells and CD8 T cells in blood. | (22, 33) and Table 2 |
| Immuno-cytokine | Hu14.18-IL2/ (APN301/ EMD273063, EMD/Apeiron Biologics/Lexigen) | IL-2 (GD2-targeted) | 2 | Melanoma, neuroblastoma | - *Completed*<br>- mOS in resectable stage III/IV melanoma 61.6 months (18 patients)<br>- Reversible toxicities<br>- TIL observed in on treatment biopsy associated with efficacy | (22, 34–37) and Table 2 |
| Recombinant or natural cytokine | Proleukin | IL-2 | 2 | Melanoma | - *Completed*<br>- Favorable 2 year OS (95.5% stage | (38) and Table 2 |

*(Continued)*

TABLE 1 Continued

| Modality | Asset | Cytokine(s) | Phase | Indication(s) | Comments* | References |
|---|---|---|---|---|---|---|
| | | | | | IIIB, 72% IIIC, 66.7% IV M1a)<br>- Well tolerated | |
| | IL-2 | IL-2 | | Melanoma | - *Comparison of 5 studies in 2001-2011*<br>- Variable ORR of 25% to 99.5%<br>- Well tolerated | (39) and therein |
| | Natural IFNα or recombinant IFNα 2b | IFNα | | Melanoma | - ORR 18% (9/51) | (40) |
| | Recombinant IFNα | IFNα | 2 | Melanoma | - ORR 25%<br>- Well tolerated | (41) |
| | Recombinant IFNα 2b | IFNα | 2 | Melanoma | - ORR 14.3% | (42) |
| Tumor-retained IL-2-IL-12 fusion protein | CLN-617 (Cullinan Therapeutics) | IL-2 + IL-12 | 1 | Solid tumors | - *Clinical study initiated Q4, 2023* | (43, 44) |
| Cytokine anchored to exogenous biomaterial deposits | ANK-101 | IL-12 | 1 | Solid tumors | - *Clinical study initiated Q1, 2024* | (2, 22, 45) |

Listed here are clinical i.t. cytokines discussed in the text. Additional examples are listed in Supplementary Table 1. *Monotherapy unless indicated otherwise. Chemo, chemotherapy; CPI, checkpoint inhibitor/blocker; CR, complete response; CTCL, cutaneous T-cell lymphoma; CTG, ClinicalTrials.gov; DLT, dose-limiting toxicity; DOR, duration of response; GBM, glioblastoma; HR, hazard ratio; MKI, multi-kinase inhibitor; mOS, median OS; MSC, mesenchymal stem cell; MSS, microsatellite-stable; MTD, maximum tolerated dose; NMIBC, Non-Muscle Invasive Bladder Cancer; ORR, overall response rate; OS, overall survival; P/C, pemetrexed/cisplatin; Pembro, pembrolizumab; PR, partial response; RFS, relapse-free survival; RT, radiation therapy; SAE, serious adverse events; SD, stable disease; SOC, standard of care; TRAE, treatment-related adverse effects.
Continued in Supplementary Table 1.

particularly OVs have promise, but more work is needed to optimize them for indirect cytokine delivery. The same is true for i.t. injection of cytokine gene-engineered cells (Figure 1C), which so far had limited efficacy in initial trials (5).

Altogether, notwithstanding some promising examples of efficacy, multiple trials of i.t.-delivered cytokine therapeutics reported little or no efficacy and have been terminated or discontinued (Supplementary Table 1). The reasons likely depend on the modality, mechanism of action, and the specific design and/or patient population studied. One major problem is that i.t. injected or intratumorally produced cytokines can quickly diffuse into circulation, in particular, when initial i.t. concentrations are high and saturate binding sites within tumors, or when cytokine release rates exceed rates of uptake by target cells in tumors. This leakage from injected tumors then causes systemic exposure to the cytokine and greater than expected toxicities (2, 10, 48, 64, 65).

## Promising new developments

One solution to avoid leakage from tumors is to endow i.t.-delivered cytokines with moieties that anchor them to the tumor microenvironment or limit diffusion out of tumors. This has been achieved in various ways. For example, cytokine retention in tumors can be achieved via fusion to antibodies specific for tumor antigens (Figure 1D). However, downregulation of a targeted tumor antigen on therapy or its heterogeneous expression on tumors could diminish tumor retention of immuno-cytokines. Nevertheless, the clinical safety and efficacy of the immuno-cytokines Daromun, Darleukin and Hu14.18-IL2 highlight the promise of designed tumor-retention following i.t.-delivery. Retention of cytokines in the tumor microenvironment may also enable the delivery of cytokine combinations that would otherwise be intractable.

Cytokine retention in tumors can also be achieved by targeting collagen, an abundant component of nearly all tumors, via fusion to collagen-binding proteins such as lumican or LAIR2, or via fusion to bulky moieties such as human serum albumin (HSA) (Figures 1E, F) (2, 10, 66, 67). One example is CLN-617, a fully human fusion protein comprising IL-2, IL-12, LAIR2 and HSA (Figure 1G) (43). To our knowledge, CLN-617 is the first clinical modality that co-delivers IL-2 and IL-12 on a single molecule. It builds on the promising safety and efficacy observed with i.t. co-administered collagen-binding IL-2 and IL-12 combined with radiation therapy in spontaneous canine metastatic melanoma (68). To enhance tumor retention, CLN-617 leverages both LAIR2 and HSA. Employing LAIR2 for collagen-binding has two advantages: First, its ability to bind multiple types of collagen may mitigate potential challenges due to heterogeneous collagen expression among tumors and metastases (69). Second, LAIR2 might block immune-inhibition by the immune cell-expressed "checkpoint" receptor LAIR1, which binds collagen with lower affinity (70). Delivery via i.t. injection limits potentially toxic retention in collagen-rich normal kidney or liver tissues. The HSA moiety provides a mechanistically distinct, complementary means of tumor retention: reduced diffusion of bulky payloads out of tumors

TABLE 2 Juxtaposition of clinical data for i.t. versus i.v. administered IL-2 modalities.

| Modality | Asset | Indication | Route | Application Schedule & Dose | Pharmacodynamics | Toxicity Profile | Efficacy | References |
|---|---|---|---|---|---|---|---|---|
| Recombinant human IL-2 | Proleukin | Melanoma | i.v. | - Every 8 hours for up to 14 consecutive doses over 5 days<br>- 600,000 or 720,000 IU/kg<br>- Second identical treatment cycle scheduled after 6 to 9 days of rest<br>- courses could be repeated every 6-12 weeks | - Systemic IL-2 can cause transient increases in CD4 $T_{reg}$ cells | - Severe toxicities, reversed after treatment termination<br>- 6 patients (2%) died from adverse events, all related to sepsis | - *8 clinical trials, n=270*<br>- ORR 16%<br>- 6% CR<br>- >50% of CR progression-free after 5 years<br>- No progression in patients who had responded for >30 months. | (54, 55) |
| | | | i.t. | - 3 x weekly, individually escalated doses<br>- Median duration 6.5 weeks<br>- Median total IL-2 dose 72 million IU<br>- Median 10 injected metastases | - A dose-dependent inflammatory reaction at site of injection induced selective necrosis of tumor tissue associated with an intra- and peritumorous lymphocytic infiltrate mainly of $CD3^+$ T cells and some $CD3^-CD56^+$ NK cells | - Well tolerated<br>- Adverse events mainly grade 1-2<br>- Most common: local erythema and slight local swelling | - n=72<br>- 25% recurrence-free<br>- Up to 11 years of follow-up<br>- Favorable 2 year OS (95.5% stage IIIB, 72% IIIC, 66.7% IV M1a)<br>- 36.7% response rate to subsequent chemotherapy | (38, 56) |
| | | | | - Biweekly with goal of 4 sessions<br>- Mean 5 sessions<br>- Average dose 10.4 million IU | | - Well tolerated<br>- Minor discomfort<br>- 85% flu-like symptoms, resolved in 24-48 hr | - n=39<br>- ORR 82%<br>- 51% CR (eventually relapsed in 20%), 31% PR<br>- 80% 5-year survival of CRs, 33% of PRs | (39) |
| | | | | - Twice weekly<br>- 3-18 million IU | | - Well tolerated<br>- Only few mild side effects (Grade 1-2) | - n=7<br>- ORR 99.5%<br>- 96% CR, 3.5% PR | (57) |
| IL-2 Immuno-cytokine | L19–IL2 (Darleukin, Philogen) | Melanoma, stage IIIB/IIIC | i.t. | - Weekly for 4 weeks<br>- Maximum dose 10 million IU | - In 76% of patients, inflammatory injection site reaction limited to tumor tissue, followed by selective tumor necrosis<br>- Decreased MDSC and transiently increased CD4 $T_{reg}$ cell proportions in blood samples<br>- Transiently increased total NK cells and CD8 T cells in blood<br>- Sustained increase in frequency and absolute count of lymphocytes (mainly CD4 T cells) | - Well tolerated<br>- Mostly grade 1-2 toxicities<br>- No SAE | - n=24<br>- ORR 53.9%, CR 25%<br>- 5 patients with DOR >24 months<br>- Median survival 905 days | (22, 33) |

*(Continued)*





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TABLE 2 Continued

| Modality | Asset | Indication | Route | Application Schedule & Dose | Pharmacodynamics | Toxicity Profile | Efficacy | References |
|---|---|---|---|---|---|---|---|---|
| | Hu14.18-IL2 (APN301/ EMD273063, EMD/Apeiron Biologics/ Lexigen) | Melanoma, neuroblastoma | i.t. | - 3 courses of 6 mg/m$^2$/day on days 1, 2 and 3 of each 28-day course<br>- Corresponds to about 18 million IU/m$^2$/day of IL-2<br>- Treatment was neoadjuvant (Group A) or post resection (Group B) | - TIL and immune signatures in on treatment biopsy associated with efficacy in Group A (Neoadjuvant)<br>- All 18 patients developed anti-drug antibodies<br>- Treatment induced transient lymphopenia on day 3 with subsequent rebound lymphocytosis<br>- Increased levels of soluble IL-2Rα and CRP suggesting immune activation | - Reversible and manageable toxicities, including IL-2 constitutional symptoms, grade 1-2 laboratory changes, hypotension and pain<br>- Dose reductions required for several patients<br>- MTD 7.5 mg/m$^2$/day (Phase 1 trial) | - n=18<br>- mOS in resectable stage III/ IV melanoma 61.6 months<br>- No difference by GD2 status | (22, 34–37) |
| | | Melanoma | i.v. | - 6 mg/m$^2$/day on days 1, 2 and 3 of each 28-day cycle<br>- Corresponds to about 18 million IU/m$^2$/day of IL-2<br>- 2-4 cycles | - Peripheral blood lymphopenia on day 3 followed by lymphocytosis on Day 8 and increased CRP<br>- Transiently increased serum sIL-2Rα<br>- No correlation between peak drug level on Day 1 and toxicity or response<br>- 13 patients developed anti-drug antibodies (93%) | - Reversible toxicities, including grade 3 thrombocytopenia and blood chemistry, and one transient grade 4 lymphopenia<br>- Grade 3 hypotension (n=2) and grade 2 renal insufficiency (n=1) required dose reductions in 3 patients who had a PR or SD | - n=14<br>- 1 transient PR (7.1%) | (58) |
| | | Neuroblastoma | | - 12 mg/m$^2$/day for 3 days every 28 days<br>- Corresponds to about 36 million IU/m$^2$/day of IL-2 | - Multi-modal mechanism of action where Fc-portion mediates ADCC and CDC while IL-2 moiety activates NK cells and T cells<br>- hu14.18-IL2 peak serum levels similar for responders and nonresponders<br>- Transient lymphopenia followed by lymphocytosis consistent with immune activation<br>- Transiently increased serum sIL2R, no association with DLT<br>- 13-16 patients developed anti-drug antibodies, not associated with drug serum levels or responses<br>- No association between factors at diagnosis and responses | - n=38<br>- Phase 2 DLT included vascular leak, hypotension, hypoxia, pain, allergic reactions, transaminitis, hyperbilirubinemia<br>- Most toxicities reversible | - No responses in measurable/bulky disease (n=13)<br>- 21.7% CR with 9 to >35 month durability in patients with non-measurable disease (n=23)<br>- Overall 63% 1-year OS | (59) |





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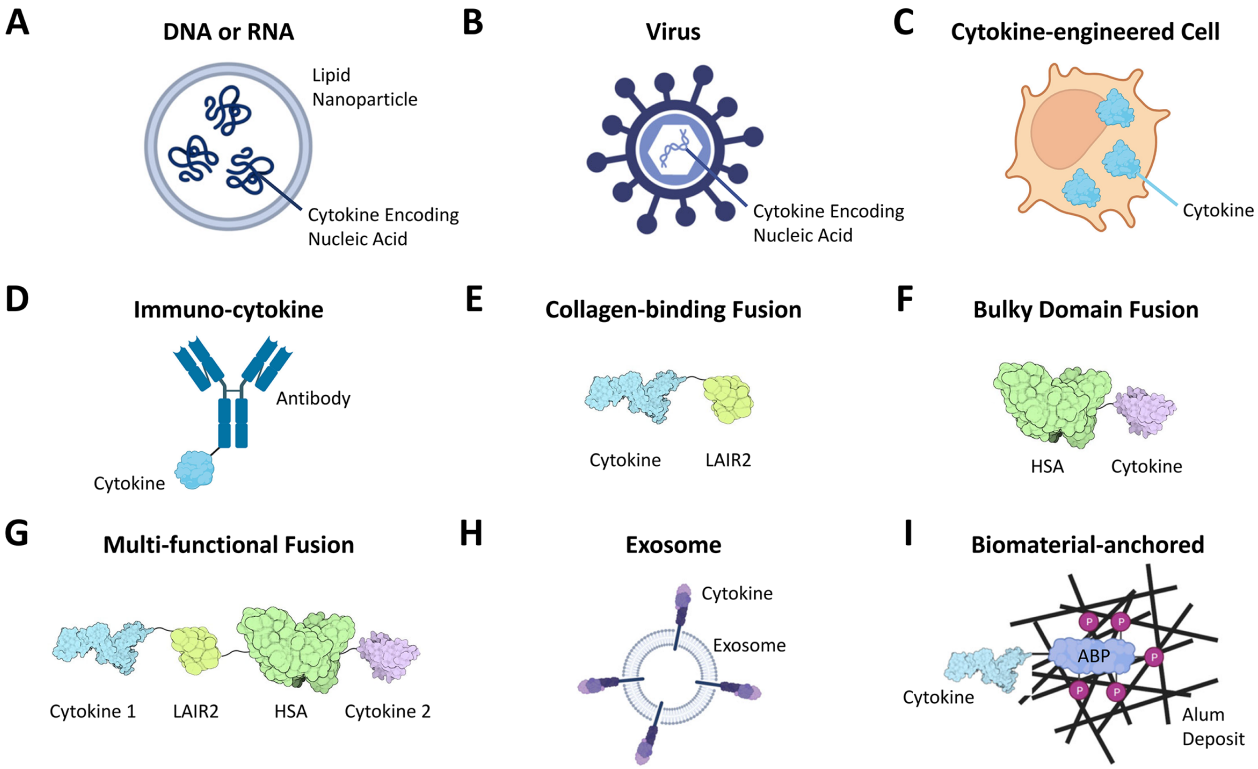


FIGURE 1
Cytokine modalities for i.t. administration include free or lipid nanoparticle-loaded DNAs or RNAs encoding cytokines **(A)**, cytokine expressing OV or other viruses **(B)**, and cells engineered to express exogenous cytokines **(C)**. Additional modalities engineered for cytokine stabilization and retention at sites of injection include immuno-cytokines containing both cytokines and tumor antigen-binding moieties (typically an antibody, **D**), cytokine fusions with tumor matrix/collagen-binding domains such as LAIR2 **(E)** or with bulky domains such as human serum albumin (HSA, **F**) or multi-functional fusions of several cytokines with both collagen-binding and bulky domains (**G**, ref. 43), liposomes or exosomes "presenting" cytokines on their surface **(H)** and cytokines anchored to exogenous biomaterial deposits in tumors (**I**, shown is a cytokine fused to a phosphorylated alum-binding peptide [ABP] whose phosphate residues [P] undergo ligand-exchange reactions with alum deposits (49)). For details, see text. Created with BioRender.com.

(2, 10, 66, 67). CLN-617 has additional beneficial properties such as encoding wildtype cytokines, and by co-delivering IL-2 and IL-12, mimicking a natural immune response where multiple cytokines typically act in concert in a local manner. This is exemplified by the known synergy of IL-2 and IL-12 in enhancing T cell and NK cell responses and anti-tumor immunity through mechanisms which include mutual receptor-upregulation (43). An i.t.-delivered murine surrogate of CLN-617 exhibited compelling and safe single-agent anti-tumor efficacy dependent on its retention in tumor tissue, strong abscopal effects and over 10-fold higher tumor than systemic exposure in preclinical models. It also synergized with systemically delivered PD-1 blockade (43). CLN-617 is currently in a Phase I clinical trial both as a monotherapy and in combination with PD-1 blockade (NCT06035744).

Another approach for prolonging tumor-retention is embedding cytokines on the surface of liposomes or exosomes before i.t. injection (Figure 1H) (2, 10, 51, 66). However, cytokine-containing liposomes are compromised by rapid endocytic clearance or biodegradation, and encapsulation in hydrogels or chitosan minimizes cytokine bioavailability (10). It remains to be investigated whether such issues contribute to the so far limited clinical efficacy of IL-12 displaying exosomes (CDK-003/ExoIL-12) and mRNA lipid nanoparticles (LNP, mRNA-2752, Supplementary Table 1) (71).

A final approach to prolong tumor-retention is embedding cytokines in depots of co-injected synthetic biomaterials like aluminum hydroxide (alum). Alum aggregates persist for weeks at the site of injection. This has led to a broad use of alum as a safe and effective vaccine adjuvant (2). A recent novel application are cytokine therapeutics that bind to alum deposits via phosphorylated peptide tags (Figure 1I) (2). This can restrict cytokine exposure to the injected site and limit cytokine dissemination into circulation. An exciting example is ANK-101, an alum-anchored IL-12 in Phase 1 clinical trials (NCT06171750) (2). The canine surrogate cANK-101 thus far appears safe and tolerable, has shown immune activation and elicited an objective response in a Phase 1 trial in canine melanoma subjects (72). In murine tumor models, alum-bound IL-12 could be detected up to 3 weeks after a single i.t. injection, indicating tumor retention and prolonged exposure (49). Alum-anchoring has also been used preclinically to prolong tumor-retention of i.t.-injected type 1 interferons (52). One theoretical concern is that alum-anchoring

might increase the immunogenicity of recombinant cytokines and promote the development of anti-drug antibodies which eventually limit exposure and efficacy. Whether this occurs in patients remains to be shown.

## Conclusions

We consider i.t. administration of cytokines to be more favorable than systemic administration because it can widen the therapeutic index. This is critical to leverage the well-established potency of cytokines as cancer therapeutics, while mitigating their often dose-limiting toxicities, which has prevented a broader utility of cytokines in the clinic.

In our opinion, i.t. delivered cytokines engineered to be retained and stabilized for prolonged periods in tumors are superior therapeutics because they maximize target exposure while minimizing toxic systemic exposure. They also avoid the potential complications of nucleic acids, cells and OVs, particularly related to the control of cytokine exposure and PK. In particular, i.t. injected proteins avoid the liability of excessive and uncontrolled expression of cytokine-encoding nucleic acids or viruses. To achieve optimal tumor retention, bulky moieties such as albumin (Figure 1F) or anchoring to synthetic biomaterials (Figure 1I) can further improve other retention approaches, including tumor antigen-binding immuno-cytokines or cytokines fused to collagen-binding domains (10, 43, 49, 52, 66). The combination of a collagen-binding moiety with albumin, as realized in CLN-617 (Figure 1G), appears particularly powerful because it avoids the need for co-administration of biomaterials such as alum and the theoretical associated risk of eliciting anti-drug antibodies. Nevertheless, both fusion to retention-domains or anchoring to alum combine excellent tumor retention, long PK and high but well controlled tumor exposure with low systemic exposure to achieve high anti-tumor efficacy and safety in preclinical studies. It will be interesting to see how they compare in the clinic.

Because i.t. injection can usually not access all lesions in a patient, ensuring robust abscopal effects is key for success. We believe that this is achievable by combining adaptive and innate immunomodulators with checkpoint blockade. Additionally, properly sequenced combination with T cell engagers, or with antigen-releasing gamma-irradiation or chemotherapy, may be beneficial (10, 53).

For the specific future evolution of i.t. cytokine delivery, we consider co-delivery of synergistic cytokines which activate different arms of adaptive immunity (e.g., IL-2 and IL-12 as in CLN-617), or of cytokines which activate both adaptive and innate immune cells, a particularly promising avenue. We believe that multi-modal molecules containing several cytokines and possibly other immune modulators can facilitate co-delivery. Effective tumor retention will be critical to avoid the increased toxicity potential of cytokine combinations upon systemic exposure. Co-delivery will also require innovative ways to ensure proper exposure of each cytokine to its respective target cells, which may be spatially separated. Finally, different cytokines may act optimally at different times post-delivery and depending on the microenvironment in a given tumor. In one example, alum-bound IFNα and IFNβ had differential efficacies depending on the syngeneic tumor model used (52). These issues may necessitate modifications such as a sequenced delivery or a patient-optimized composition of combination agents for optimal efficacy. Determining the optimal timing and composition of sequenced therapeutics remain considerable challenges, as relevant mechanisms need to be identified and translated into patients, and the required logistics need to be implemented. Without doubt, i.t.-delivered cytokines will provide prospect for innovation for years to come.

## 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.

## Author contributions

KS: Conceptualization, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing. KR: Writing – original draft, Writing – review & editing. KW: Writing – original draft, Writing – review & editing. NM: Conceptualization, Writing – original draft, Writing – review & editing. JM: Conceptualization, Writing – original draft, Writing – review & editing. PB: Conceptualization, Writing – original draft, Writing – review & editing.

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## Conflict of interest

All authors are current or former paid full-time employees of, and own shares and/or stock options of Cullinan Therapeutics, Inc. Cullinan develops CLN-617, one of the modalities discussed in this perspective.

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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.2024.1456658/full#supplementary-material

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# Unlocking the potential of iPSC-derived immune cells: engineering iNK and iT cells for cutting-edge immunotherapy

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Induced pluripotent stem cells (iPSCs) have emerged as a revolutionary tool in cell therapies due to their ability to differentiate into various cell types, unlimited supply, and potential as off-the-shelf cell products. New advances in iPSC-derived immune cells have generated potent iNK and iT cells which showed robust killing of cancer cells in animal models and clinical trials. With the advent of advanced genome editing technologies that enable the development of highly engineered cells, here we outline 12 strategies to engineer iPSCs to overcome limitations and challenges of current cell-based immunotherapies, including safety switches, stealth edits, avoiding graft-versus-host disease (GvHD), targeting, reduced lymphodepletion, efficient differentiation, increased *in vivo* persistence, stemness, metabolic fitness, homing/trafficking, and overcoming suppressive tumor microenvironment and stromal cell barrier. With the development of advanced genome editing techniques, it is now possible to insert large DNA sequences into precise genomic locations without the need for DNA double strand breaks, enabling the potential for multiplexed knock out and insertion. These technological breakthroughs have made it possible to engineer complex cell therapy products at unprecedented speed and efficiency. The combination of iPSC derived iNK, iT and advanced gene editing techniques provides new opportunities and could lead to a new era for next generation of cell immunotherapies.

KEYWORDS

**iPSC, iNK, iT, next generation immunotherapy, engineering strategies, differentiation**

## 1 Introduction to immunotherapy

In the past decade, the field of cancer therapy has been significantly transformed by the advent of CAR-T cell immunotherapy, which has emerged as a pivotal treatment modality alongside traditional methods such as surgery, radiation, and chemotherapy. This innovative approach of engineering T cells with chimeric antigen receptors (CARs)

to redirect T cells to recognize antigens such as CD19 or BCMA have shown remarkable efficacy against certain types of leukemia, lymphoma, and multiple myeloma (1). Since 2017, six CAR-T therapies have been approved by the U.S. Food and Drug Administration (FDA), with many more products at various stages of clinical development (2). CAR-T cells are also being explored for a multitude of nononcologic indications, including transplant rejection, infection, autoimmunity, cardiovascular disease, fibrosis, and senescence (3–6). However, the limitations of autologous FDA approved CAR-T therapies, such as cost, donor variability and time required for manufacturing, are widely recognized barriers to the wide adoption of CAR-T therapy (7–9). iPSCs have the potential to serve as a lower-cost source of high-quality engineered, off-the-shelf therapy, with scalable manufacturing and consistent product quality. To realize this vision and enable large-scale manufacturing, researchers have worked to develop and improve iPSC-to-T cell differentiation protocols.

Natural killer (NK) cells are another type of immune cell that can kill target cells via cytotoxic mechanisms. CAR-NK cells may have some notable benefits over CAR-T cells, such as: (1) increased safety (no neurotoxicity or cytokine release syndrome, and avoidance of GvHD), (2) several ways to trigger cytotoxic action, and (3) great likelihood of "off-the-shelf" production (7, 10). CAR-NK cells could be engineered to target a broad range of antigens, with the potential to deliver potent responses for cancer and autoimmune conditions (11, 12). The development of CAR-NK cells represents an exciting frontier in immunotherapy, with the potential to overcome the limitations of current CAR-T cell therapies and provide patients with more accessible and effective treatment options (7, 13).

Other immune cell types being studied for their potential in immune cell therapy are phagocytic cells, such as macrophages and dendritic cells. They patrol the body and assist in cleaning up infection and activating other immune cells. For solid tumors, macrophages can efficiently infiltrate into tumors and are abundantly present in tumor microenvironment (TME). As major immune regulators, CAR macrophage can turn cold TME (absence of T cells and pro-inflammatory cytokines) to hot (presence of T cells and pro-inflammatory cytokines) and attract and activate adaptive immune cells, in addition to directly killing tumor cells (14). As a result, there is a great interest in creating CAR macrophages for cancer immunotherapy in order to get around some of the problems that CAR T/NK treatment has, particularly with solid tumors (7, 14). Dendritic cells are a specialized type of phagocytic cell presenting antigen to bridges innate and adaptive immunity. Dendritic cells are crucial in the induction of immune responses to pathogens and tumors as well as for the maintenance of self-tolerance. Understanding the strengths and limitations of different cell types is highly needed to engineer cell therapies. The traits of CAR T, NK and macrophage are summarized in Table 1. Given that the current focus of cell-based immunotherapy are T cells and NK cells, we have focused the rest of this review on those two cell types.

TABLE 1 Characterization and comparison of current CAR T, NK and macrophage immunotherapies.

| | CAR T | CAR NK | CAR Macrophage |
|---|---|---|---|
| Cell source | auto, allo, iPSC | allo, auto, iPSC | auto, iPSC |
| Off-the-shelf | no | yes | yes |
| Toxicities | CRS, ICANS common | less common | no clinical data |
| Cytotoxicity mechanisms | CAR-dependent | CAR-dependent and-independent | multiple, immunostimulatory TME |
| Infiltration into tumors | poor | poor | abundant |
| Clinical experience | Proven efficacy, 6 FDA approved | Limited but promising | very limited clinical experience |

CAR, chimeric antigen receptor; auto, autologous; allo, allogenic; CRS, cytokine release syndrome; ICANS, Immune effector cell-associated neurotoxicity syndrome.

# 2 Generation and engineering of iPSC-derived iNK and iT cells

## 2.1 Overview of the generation process of iPSCs differentiation into iNK and iT cells

iPSCs are a type of stem cell derived from somatic cells, usually skin fibroblast or white blood cells by expression of Yamanaka factors (15, 16). Due to their unique features: unlimited expansion, the ability to differentiate into different cell types, and ease of editing, iPSCs have been developed as a new method to generate transplantable immune cells.

The differentiation of iPSCs to immune cells is a multiple step process. First, iPSCs undergo mesoderm induction in embryonic bodies (EB) upon the loss of pluripotency-related gene expression as well as increased expression of mesodermal genes to form multipotent progenitor cells. Second, hematopoietic progenitor cells (HPC) are induced and produce a population of cells capable of committing to various cell lineages (Figure 1). HPC are guided toward common lymphoid progenitors (CLP) cells, which subsequently develop into iNK or iT cells with a particular set of factors and extracellular matrix supports. As the focus cell type of this review, the procedures of iNK, iT generation are discussed in detail in following sections. In order to differentiate into iPSC-derived macrophages (iMACs), floating EBs are reseeded for attachment to culture vessels. This creates myeloid factories that give rise to progenitor macrophages, which then mature into M1 or M2 macrophages after incubation with specific cytokines (17, 18). For iPSC-derived dendritic cells (iDC), HPC are directed to CD11c+ DCs through exposure to a cocktail of growth factors (19, 20). These cell types are at different stages of research in preclinical and clinical settings and iNK cells are the most advanced and have demonstrated clinical efficacy in hematological malignancies.

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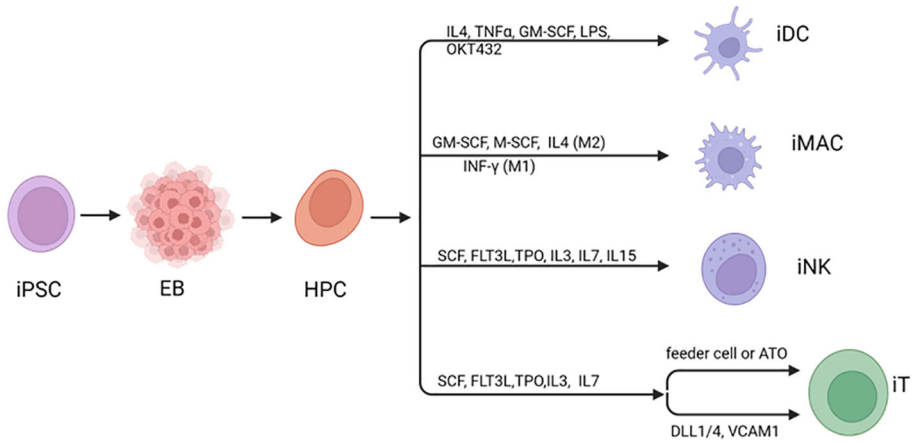


FIGURE 1
Differentiation of iPSC to immune cells. Human iPSC can be induced to differentiate to hematopoietic cells usually through generation of mesoderm in embryoid bodies (EBs). During differentiation to mature blood cells, hematopoietic progenitor cells (HPCs) are generated, leading to a diverse group of cells that can differentiate into various specialized cell types. Under specific cytokines and stimulating factors, HPC can be directed various immune cell types. Created with BioRender.com.

## 2.2 Methods of iNK differentiation from iPSC

The differentiation of iNK cells from iPSCs is a complex but fascinating process which has evolved in the past 20 years. As was recently and excellently reviewed (21–23), a number of research groups have developed methods for producing NK cells from iPSC and embryonic stem cells (ESCs), including stromal cells and stroma-free protocols, or 2D and 3D differentiations.

In initial 2D studies from the Kaufman group, human ESCs were co-cultured with murine bone marrow (BM) stomal cells in cytokine-enriched media with BMP4, FGF2, VEGF, and SCF (24, 25). During the first step in differentiating iPSCs into iNK cells, hematopoietic lineage is induced to form hemogenic endothelium (HE). From the HE, CD34+ CD45+ hematopoietic progenitors are generated, then sorted and co-cultured with a second stromal feeder cells in medium containing interleukin IL-3 (during the first week), IL-7, IL-15, SCF, IL-2, and Flt3L (24, 26). Although Notch signaling is required for the stimulation of T cell development, it also plays a role in the effective development of NK cells in humans (27–29).

This 2D protocol was then modified to 3D, the "spin EB (embryoid bodies)" method. This method capitalizes on the fact that when iPSCs are cultured in suspension without feeder layers (stroma and serum free), they spontaneously form aggregates known as EB, before differentiation toward the NK-cell lineage (30–32). The "spin EB" protocol produces hematopoietic organoids that contain HPC, as well as endothelial and mesenchymal cells. These HPC then differentiate into NK cells under defined conditions (31, 32). This approach enhances the reproducibility and uniformity of the resulting iNK cells. IL-3 and IL-7 are commonly used to promote CLP which are a precursor to NK cells. From CLPs NK cell precursors begin to form in response to IL-15, which is required for proper differentiation and maintenance of NK cells. The emerging iNK cells are expanded and matured in culture. During this phase, they acquire the necessary receptors and functional capabilities that characterize mature NK cells, such as the ability to recognize and kill tumor cells. Feeder cells, such as K562 or EBV-LCL cell which are susceptible targets for NK cells, have been engineered with membrane bound IL-21 and 4-1BBL to improve expansion and cytotoxicity (33–36). Similar to PB-NK and UCB-NK cells, hESC/iPSC-derived NK cells express both activating and inhibitory receptors, including TNF-a, CD16a, NKG2D, TRAIL, NCR receptors (NKp44, NKp46), IFN-g, and NKG2D. The interplay of different cytokine combinations on NK cell differentiation and maturation are still being explored and warrant further research.

The differentiation of iNK cells from iPSCs represents a significant achievement in the field of regenerative medicine and immunotherapy. By leveraging the plasticity of iPSCs and the power of modern cell culture techniques, scientists are able to produce iNK cells that could potentially be used to treat a variety of cancers and autoimmune conditions. While still in its infancy, iPSC derived iNK cells from multiple groups are already showing promise in the clinic, where they have demonstrated deep depletion of target B cells in oncology, and currently being evaluated in autoimmune diseases (12, 37).

## 2.3 Methods of iT differentiation from iPSC

The stem cells used to make iPSC-derived T cells can come from different types of somatic cells. The iPSC derived T cells express random TCR due to rearrangement after differentiation from non-T cells that contain germline TCR genes. Cell therapy can be accomplished by introducing the exogenous TCR into iPSCs. Transgenic TCRs generate the CD3 signal during T cell differentiation, which inhibits the rearrangement of endogenous TCRs, allowing T cells derived from iPSCs to target specific antigens (38). Studies to engineer exogenous TCRs to generate potent T cells from iPSCs have produced exciting results (38, 39). Reprogramming of iPSCs can also be derived from peripheral blood T cells. In this case, the rearranged TCR gene is retained in

iT cells. Antigen-specific T cell clones can be reprogrammed to produce antigen-specific T cells, or T-iPSCs can be engineered with a CAR to improve tumor specificity for use in cell therapy. The only iT cell candidate in clinical trials is FT819, derived from T-iPSC cells, where TRAC is knocked out and a CD19 CAR is inserted into iPSC before differentiation into iT cells (40). The results of phase 1 clinical trial are promising: no dose related toxicity, no ICANS or GvHD, no grade 3 CRS, and evidence of anti-tumor activity, with 3/15 patients demonstrating complete response (41).

iT cell differentiation from iPSCs has been historically challenging. The most challenging part of iPSC derived T cells is how to generate potent iT cells from iPSC in serum and stromal cell free conditions. For the natural T cell development process *in vivo*, CD34+CD43- hemogenic epithelial (HE) cells at AGM (Aorta-ganod-mesonephros) undergoes endothelial-to-hematopoietic transition (EHT), in which the HE is rounded up and releases the floating cells with HPC markers CD34 (42, 43). In a process dependent on Notch signaling, HPC differentiate into CD5/CD7 double positive T cell progenitor cells. Under reduced Notch signaling and increased TCR signaling, CD4/CD8 double positive cells are produced and mature to single positive T cells (44–46). This understanding of endogenous T cell differentiation has informed multiple efforts (reviewed below) to recreate the T cell differentiation *in vitro*.

The Zuniga-Pflucker group at Toronto University has done seminal work to recreate T cell development *in vitro*, by expressing Notch ligand DL1, DL4 in thymic stromal cells (47). Subsequent feeder-based systems, such as the artificial thymic organoid (ATO) platform, have been developed to better replicate the three-dimensional structure of the thymus (39, 48). The latest breakthrough is chemically defined differentiation with DL4 and VCAM1 in feeder cell free condition (44, 45, 49, 50). The stromal cell-free, DL4 microbead-based approach that supports efficient *in vitro* development of human progenitor T cells from pluripotent stem cells (PSCs), provides a simple, robust and potentially scalable platform to both study human T cell development and facilitate the development of engineered T cell therapies from renewable sources (50).

All the pioneering groups started the same iT differentiation process from iPSCs with EB formation and isolated CD34 HE cells from EB. The major difference was the different coating matrix. Kaneko group seed HPCs on to plates coated with recombinant DL4 and retronectin, a fragment of the fibronectin protein that can also bind to integrin as VCAM1. This process yielded CD8 single positive (CD8SP) cells that expressed the original TCR, but they failed to generate iT cells from non-T iPSC lines where the TCR locus is unrearranged (49). This limitation indicates that this protocol fails to capture some key aspects of T cell development. A major milestone was from the Zandstra group, which showed synergistic effect of VCAM1 and DL4 to enhance Notch signaling and progenitor T cell differentiation (51). This group demonstrated that primary human HPC from CB could be directed to become T cell progenitors on DL4, VCAM1 coated plates and mature T cells *in vivo* with T cell progenitor transplantation. VCAM1 is a stromal matrix protein and a ligand for integrin which is highly expressed in HPC cells. Closely following Kaneko's work, the Zandstra and Daley group finally differentiate iPSCs to mature T cells in a feeder free system in 2022. They isolated CD34 positive HE cells from EB, seeded them on DL4+VCAM1 coated plates, grew them with SCF, TPO, IL-3, IL-7 and TNFa, for another 14 days, resulting in CD5CD7 double positive cells. The addition of VCAM1 significantly increased the percentage of CD5CD7 double positive progenitor cells, and TCRαβ, γδT cells were generated with diverse TCR repertoire from TCR locus sequencing (44, 45).

## 2.4 Limitations of iPSC derived therapeutics

While iPSCs hold great promise for regenerative medicine, there are a few potential limitations that need to be addressed. First, iPSCs can be prone to genetic instability, including point mutations, copy number variations, and chromosomal rearrangements that can result from donor somatic cells, during reprogramming, or during extended cell culture (52, 53). Residual undifferentiated iPSCs have the potential to form teratomas or other types of tumors if transplanted into patients (54). During the iPSC creation and differentiation process, routine genetic screening and monitoring are carried out utilizing high-throughput sequencing and other cutting-edge genomic technologies to identify and remove cells with genetic abnormalities. Advanced differentiation techniques can be developed to ensure that all iPSCs fully differentiate into the intended cell types, and the final differentiated cell products can be purified with selection in order to remove residual iPSCs. Furthermore, as discussed in section 3.1, suicide genes/safety switches can be inserted into iPSCs and activated to eradicate any undifferentiated iPSCs that may still exist, further improving the safety profile of iPSC derived cells.

# 3 iPSC engineering for next generation iNK, iT cells

FDA approved T cell therapies have had a remarkable impact on patient care for a subset of hematological malignancies. This foundation has motivated the development of off-the-shelf engineered T and NK cell therapies for a broad range of indications. Achieving this vision will require cost-effective manufacturing of precision cell products capable of addressing multiple process and clinical-design challenges. In addition, expanding the breadth of indications possible with cell therapy and ensuring highly effective and safe therapies will require sophisticated cell engineering. For next generation iT, iNK cell therapies, the strategies outlined below could overcome current challenges and limitations of iPSC derived therapies and lead to better success of immune cell therapies, summarized in Figure 2 and Table 2.

## 3.1 Safety switches

Safety switches provide a mechanism to eliminate the infused cells in case of adverse events, thus increasing the safety of these

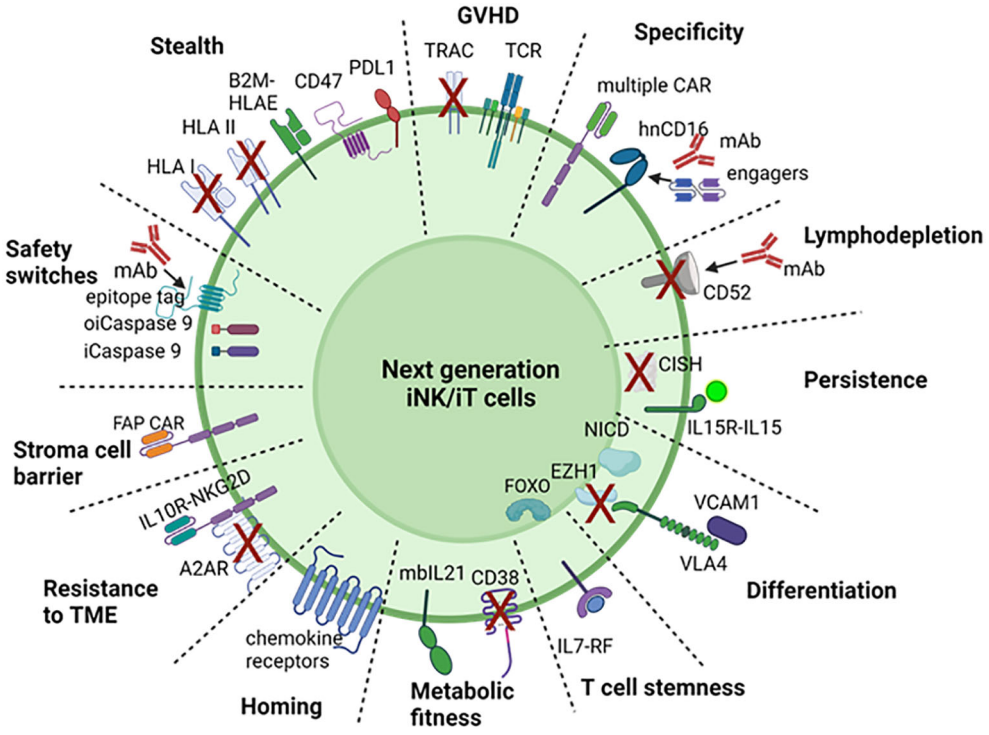


FIGURE 2
Engineering strategies for next generation iNK, iT cell. To overcome the challenges and limitation of CAR T or NK cell for immunotherapy, 12 strategies are proposed to engineer iPSC for with safety switches, stealth edits, specific targeting, avoiding GvHD, lymphodepletion, *in vivo* persistence, efficient differentiation, T cell stemness, metabolic fitness, homing/trafficking, overcoming suppressive tumor microenvironment and stromal cell barrier. OiCaspase 9, orthogonal inducible caspase 9; iCaspase 9, inducible caspase 9; GvHD, graft versus host disease; KO, knockout; KI, knockin; hnCD16, high affinity non-cleavable CD16; mIL-15RF, membrane bound IL15-receptor fusion; NICD, notch intracellular domain; Flt3L, FMS-like tyrosine kinase 3 ligand; IL-7RF, IL-7 receptor fusion; ICI, immune checkpoint inhibitor; DNTGFbR, dominant negative TGFβ receptor; IL10DR, IL-10 decoy receptor; FAP, fibroblast activated protein. Created with BioRender.com.

therapies. There are several types of safety switches used in cell therapy as summarized in Table 3.

Inducible apoptosis: This method involves a safety switch based on the fusion of human caspase 9 to a modified FK-binding protein, which can be activated by a synthetic dimerizing drug (55, 56). When the drug is administered, it induces apoptosis in the cells expressing this construct, effectively eliminating them. This approach has been clinically validated, where the Inducible Caspase 9 (iCaspase-9) safety switch allowed for the rapid elimination of more than 90% of the modified T cells within 30 minutes after administration of a dimerizing drug, effectively ending GvHD without recurrence (57). The iCaspase-9 system is advantageous because it's derived from human proteins, reducing the potential for immunogenicity. It also doesn't rely on DNA synthesis for its activity, meaning it can control both dividing and non-dividing cells. This makes it a valuable tool for improving the safety of various cell therapies.

Antibody-mediated cytotoxicity: In order to destroy the therapeutic cells, monoclonal antibodies are used to target particular antigens on their surface. Unlike small-molecule targeting, T cell expression of cell surface markers allows for targeted elimination through the administration of target-specific antibodies, which also allows for monitoring by flow cytometry. This strategy includes the use of truncated human epidermal growth factor receptors (58, 59), RQR8 (60), CD20 (61), and permits the clearance of specific cell populations (expressing the specific receptors) for elimination by administration of FDA-approved antibodies like cetuximab or rituximab. In preclinical models this approach has the potential drawback of slow and insufficient cell ablation that have poor receptor expression (62).

HSV-TK suicide gene: HSV-TK (herpes simplex virus thymidine kinase) based suicide switch is a safety feature that has been previously used in cell therapy for control of GvHD in patients (63), particularly in the context of adoptive cell transfer like T-cell. HSV-TK is a cell cycle-dependent suicide gene, that catalyzes the generation of triphosphate ganciclovir (GCV), which is toxic to proliferating cells by inhibiting DNA chain elongation (64–66). In addition to cell-cycle dependence, another limitation of HSV-TK is the immunogenicity of the viral TK protein. Various clinical studies with HSV-TK transduced donor lymphocyte have been conducted, confirming its safety and efficacy (63, 66).

These safety switches are designed to manage potential side effects associated with advanced cellular therapies and expand their clinical applications by providing a controlled way to eliminate therapeutic cells if necessary.

TABLE 2 Summary of edits for engineering iNK, iT cells.

| Strategies | Edits | Examples |
|---|---|---|
| Safety switches | OiCaspase 9, iCaspase 9, epitope-Ab targeting | iCaspase 9 (55, 56) |
| Stealth edits | B2M, CIITA DKO +B2M-HLAE, CD47 KI | B2M KO (68–73) |
| GvHD | TRAC KO or antigen specific TCR KI | TRAC KO (98–100) |
| Specificity | Multiple CAR, hnCD16 KI | CAR (101–103) |
| Lymphodepletion | CD52 KO for CD52 Ab conditioning | Not available |
| In vivo persistence | mIL-15RF, CISH KO | IL-15 KI (121) CISH KO (127) |
| Differentiation/ expansion | NICD, EZH1 KO, Flt3L, BMP4 | EZH1 KO (45) |
| T cell stemness | IL-7RF KI, FOXO KI | FOXO KI (114) |
| Metabolic fitness | CD38 KO, mbIL-21 | CD38 KO (23, 130) |
| Homing/ infiltration | Chemokine receptors KI | CXCR4 KI (147) |
| Suppressive TME | ICI PD1, CTLA4, LAG3 decoy receptor, DNTGFbR, IL10DR KI | DNTGFbR (154–156) |
| Stromal cell barrier | FAP CAR | FAP CAR (169) |

OiCaspase 9, orthogonal inducible caspase 9; iCaspase 9, inducible caspase 9; GvHD, graft versus host disease; KO, knockout; KI, knockin; hnCD16, high affinity non-cleavable CD16; mIL-15RF, membrane bound IL-15-IL-15 receptor fusion; NICD, notch intracellular domain; Flt3L, FMS-like tyrosine kinase 3 ligand; IL-7RF, IL-7-IL-7 receptor fusion; ICI, immune checkpoint inhibitor; DNTGFbR, dominant negative TGFβ receptor; IL10DR, IL-10 decoy receptor; FAP, fibroblast activated protein.

## 3.2 Stealth edits

Because of its potential cost-effectiveness, scalability, and on-demand availability, allogeneic cellular immunotherapies hold considerable promise for the treatment of cancer and autoimmunity. A significant barrier to achieving therapeutic responses similar to those seen with existing autologous CAR-T cell treatments is immunological rejection of adoptively transferred allogeneic T and NK cells. For immunotherapy, four different kinds of genetic modifications for immune evasion have been investigated (Table 4). Human Leukocyte Antigens (HLA) genes play a crucial role in graft rejection. Differences between the recipient's and graft's HLA class I and II genes can lead to activation of host CD8+ and CD4+ T cells, respectively, resulting in direct killing of graft cells. Researchers are investigating ways to modify HLA expression on T or NK cells to be immunologically silent and evade the host versus graft (HvG) response, sometimes termed "stealth".

### 3.2.1 Global disruption of HLA molecules: B2M KO, TAPi

HLA class I contains the polymorphic HLA-A, HLA-B, and HLA-C surface proteins. These molecules are heterodimers that consist of two polypeptide chains: the polymorphic HLA-encoded alpha chain and B2-microglobulin (B2M). HLA class I molecules are expressed in all nucleated cells and are the anchors to present intracellular peptides to CD8+ T cells (20). Direct targeting of the specific alpha chain HLA-A evades the immune response (67). The knockout of B2M to achieve reduced allorecognition by CD8+ T cells is by far the most common strategy in allogeneic cell transplantation and was first described in the stem cells (68–71), a finding which has been shown in iPSC derived cells (72, 73) In T cell therapies, HLA class I disruption is often combined with disruption of the TRAC locus to prevent surface expression of the T cell receptor (TCR) and preclude GvHD (74, 75).

Reducing HLA class I expression (via B2M KO) is not sufficient for full stealth, as HLA class I molecules have an important function as inhibitory ligands for NK cells. HLA-C and certain HLA-A and HLA-B alleles are ligands for Killer-cell immunoglobulin-like receptors (KIRs). HLA class I interactions with KIRs and NKG2A/CD94 play a major role in self-tolerance of NK cells, such that when these interactions are lost, the balance between activating and inhibitory signals on NK cells is shifted towards activation, resulting in a "missing-self" lysis of target cells (76). Expression of synthetic HLA-E–or HLA-G- β2m fusion proteins on target cells suppresses activation of NK cells that express the inhibitory receptor heterodimer NKG2A–CD94 (77, 78). An alternative approach to avoid CD8+ T cell HvG while minimizing the induction of NK "missing-self" is to specifically delete the HLA-A, HLA-B, and HLA-C genes while leaving B2M and HLA-E intact to engage NKG2A on patient NK cells (72).

HLA class II is frequently knocked out on the surface of adoptive cells in order to elude recipient CD4+ T cells. Major or

TABLE 3 Summary of different safety switch types.

| Type | Characterization | Advantage | Disadvantage | References |
|---|---|---|---|---|
| HSV-TK | Express HSV-TK kinase and in combination with antiviral drug ganciclovir | Ganciclovir clinical available; confirmed safety | Only work in dividing cells; immunogenicity; Not suitable to CMV patients | (63, 66) |
| Antibody-based targeting | Express an epitope at the engrafted cell surface in combination with antibody targeting | Clinical available antibodies | Slow and incomplete ablation with low epitope expression | (58–61) |
| iCaspase 9 | Inducible caspase suicide gene combination with bio-inert small molecule | Rapid; Human protein no immunogenicity | Potential iCasp9 resistance | (55–57) |

HSV-TK, herpes simplex virus thymidine kinase; iCaspase 9, inducible caspase 9.

TABLE 4 Features of stealth editing strategies in cell therapy.

| Type | Characterization | Advantage | Disadvantage | References |
|---|---|---|---|---|
| HLA depletionand HLA-E/G-B2M KI | Direct remove the major HLA mismatch | Reduced CD8, CD4 T and NK cell recognition | None reported | (68–73) |
| Immune checkpoint inhibitor | Overexpress ICI at cell surface | Simple and easy engineering | None reported | (81, 88, 89) |
| ADR | CAR targeting activated host T, NK cells | Only target activated lymphocyte, spare resting cells | Potential resistant | (90, 91) |
| Immune synapse disruption | Dissemble of immune synapse | New layer of stealth | None reported | (93, 95) |

HLA, human leukocyte antigen; ICI, immune checkpoint inhibitor; ADR, alloimmune Defense Receptor.

small mismatches with HLA class II during an allogeneic encounter activate CD4+ T cells, which then promote alloreactive CD8+ T cells and cause direct cytotoxicity from CD4+ T cells. A master regulator of MHC-II (major histocompatibility complex II) expression, the transcription factor class II major histocompatibility complex transactivator (CIITA) decreases surface expression of HLA class II when CIITA is knocked down (79). CIITA-KO hypoimmunogenic iPSC lines have been produced by a number of research groups employing CRISPR technology, either alone or in conjunction with B2M-KO (72, 73, 80, 81). A cell therapy lacking both HLA class I and II (B2M KO plus CIITA KO) will be required to prevent host CD8+ and CD4+ T cell responses and avoid any potential donor HLA antibodies the patient may produce or have already developed.

Another strategy for avoiding immune recognition is based on decreasing expression of MHC molecules and the antigen processing and presentation machinery (APM), including latent membrane protein (LMP) 2 and LMP7, transporter associated with antigen processing (TAP) protein. This is a mechanism by which malignancies are known to escape immune recognition (82, 83). These molecules mediate and regulate efficient antigen processing and presentation; subsequent T-cell responses have been abolished by shRNA-mediated TAP1 knockdown in combination with CIITA depletion in engineered adoptive cells (84). In addition, genetic disruption of TAPBP1 significantly reduced immunological rejection in mice (85).

### 3.2.2 Harnessing immune checkpoints: CD47, PD-L1, CTLA4

Another strategy to prevent HvG is to overexpress CD47 (80). CD47 is a transmembrane protein with a well-described role as a "don't eat me" signal due to its binding to signal regulatory protein a (SIRPa) on myeloid cells (86) and high CD47 expression on tumor cells is thought to protect tumor cells from immune responses (87). Recently, it was found that IL-2 stimulated NK cells upregulate SIRPa and can be inhibited through high levels of CD47 expression on B2M-KO target cells (81). PD-L1 overexpression alone or in combination with CTLA-4 was shown to improve iPSC-derived islet function and persistence of in a humanized mouse (88, 89). Altogether, the concept to express ligands for inhibitory receptors is a promising strategy to evade patient NK cells for T cell-based therapies, however for NK cell therapies, a careful evaluation is necessary to ensure that trans inhibition does not limit their function.

### 3.2.3 Targeted killing of alloreactive cells: alloimmune defense receptor

Following initial stimulation, T cells and NK cells upregulate surface expression of several costimulatory molecules of the tumor necrosis factor receptor (TNFR) family, including CD27, 4-1BB, OX40 and CD30. These TNF family receptors are markers for activated lymphocyte populations, distinguishing them from naive populations (90). A new synthetic receptor called alloimmune defense receptor (ADR) selectively recognizes 4-1BB. The ADR-expressing T cells resist cellular rejection by targeting alloreactive lymphocytes *in vitro* and *in vivo*, while sparing resting lymphocytes. Cells co-expressing CAR and ADR persisted in mice and produced sustained tumor eradication in two mouse models of allogeneic T-cell therapy of hematopoietic malignancies and solid tumors. This approach enables generation of rejection-resistant "off-the-shelf" allogeneic T-cell products to produce long-term therapeutic benefit in immunocompetent recipients (91, 92).

### 3.2.4 Disruption of immune synapse

Immune synapse formation is required for NK cell and T cell cytolytic function and enables the precise delivery of lytic-granule contents onto a susceptible target cell. Immune synapse formation is mediated by binding of the adhesion molecules CD54 (also known as ICAM-1) and CD58 (also known as LFA-3) on target cells to their cognate receptors, the integrin CD11a/CD18 (also known as LFA-1 or ITGAL) and adhesion and costimulatory receptor CD2 on immune cells (93, 94). Recent combined genetic deletion of CD54 and CD58 had shown significantly better *in vivo* persistence compared to both B2M -/- CAR T cells and HLA-E +B2M -/- CAR T cells in the presence of PBMC from healthy donors (95).

Overall, there are many proposed strategies to avoid the HvG response, and their efficacy will need to be determined in clinical trials. Importantly, better characterization of patient immune responses against administered allogeneic NK or T cell therapies will facilitate improved stealth approaches in the future.

## 3.3 Avoid GvHD: TRAC KO or choose an antigen specific TCR

Following allogeneic T cell transplantation, GvHD still presents a challenge for successful treatment. When immunocompetent donor T cells identify the recipient host as alien, they initiate an immune response against allogeneic antigen-bearing cells, which results in the

death of host tissues and the development of GvHD. Even with current preventive measures, GvHD patients still have high rates of morbidity and mortality, with only about 40% of patients having a durable response to corticosteroid therapy (96, 97). Knocking out the TCR alpha chain is a strategy that has been explored to prevent GvHD. This approach involves genetically modifying donor T cells so they lack the alpha chain of the TCR, which is necessary for the recognition of host antigens and the subsequent immune response that leads to GvHD. Briefly, T cell activation is dependent on antigen recognition by the TCR. The TCR is composed of multiple subunits, including the alpha and beta chains. By knocking out the alpha chain, the TCR is rendered non-functional, which means the T cells cannot effectively recognize and attack the host tissues. This could potentially reduce the incidence and severity of GvHD after allogeneic cell transplantation. Multiple studies have generated CAR T cells with disrupted endogenous TCR to avoid the risk of GvHD inherent in allogeneic T cell therapy (98–100).

## 3.4 CAR insertion for specific targeting and hnCD16

CARs are synthetic receptors that redirect lymphocytes to recognize and kill cells expressing the target ligand. CARs have a modular design with four major components: an extracellular antigen-binding domain, a hinge, a transmembrane domain and an intracellular signaling domain (101). Variation of each of these component parts of CAR constructs enables fine tuning of the functionality and anti-tumor activity of the resultant CAR T cell product and has the potential to improve the safety and efficacy of CAR-T-cell therapy (102). For this purpose, several CAR generations have been generated, and the fifth generation is currently being tested in clinical trials (103). Recently, CAR gene constructs have been modified to express an 'armoring' protein, such as IL-12 or IL-15, to enhance T or NK cell function (101).

CAR-engineered T cell therapeutics were the first to emerge, demonstrating impressive clinical results, resulting in FDA approvals for hematological malignancies (1). CARs conventionally designed for T cells with CD3 zeta and T cell co-stimulatory signals have also been used for generation of CAR NK cells, and studies have demonstrated that these cells can effectively and specifically target tumors, while maintaining a desirable safety (11). Despite intensive research efforts to define optimal CAR design for both T and NK cells, a universally improved CAR structure has not yet been identified. As of now, each CAR construct needs empirical testing for evaluation, and several studies indicate that small modifications can have major consequences on the therapeutic outcome.

NK cells express the activating immunoglobulin gamma Fc receptor CD16a, which identifies the Fc portion of IgG antibodies attached to target cells. Patients treated with the high affinity CD16 variant (F158V) and monoclonal antibody have demonstrated enhanced antitumor responses (104). A second Fc receptor, CD64 binds to IgG with 30-fold greater affinity than CD16. iNKs expressing the fusion receptor of CD64 extracellular binding region and CD16a transmembrane, intracellular domain killed cancer cells effectively when combined with anti-HER2 trastuzumab or anti-EGFR1 cetuximab antibody. The higher affinity of CD64 allowed for monoclonal antibodies to be pre-adsorbed to the NK cells expressing the recombinant CD64 and improved tumor targeting without additional antibody (105).

## 3.5 lymphodepletion conditioning: CD52 KO for CD52 Ab

Lymphodepletion is a necessary process for patients undergoing hematopoietic stem cell transplants in order to make space for the transplanted cells. They currently need intense, non-specific lymphodepletion either using ionizing radiation or cytotoxic agents such as fludarabine and cyclophosphamide (flu/cy). Replacing non-specific lymphodepletion with targeted antibody-based conditioning could avoid harming mature hematopoietic cells and result in significantly less inflammation and unintended collateral organ damage than existing conditioning regimens. One way to improve therapeutic efficacy is through recurrent antibody-based infusion and conditioning after transplanted cells' cell surface antigens have been selectively eliminated.

CD52 is highly expressed on mature T and B lymphocytes, with lower expression on other blood cells, such on monocytes, macrophages, and natural killer cells. Importantly, CD52 is not expressed on CD34+ HSCs. Therefore, the use of a depleting anti-CD52 antibody would specifically target lymphocytes of interest (B and T cells), while sparing the critical HSCs, required to repopulate the blood compartment. In support of this, a homozygous CD52 knockout mouse revealed no significant difference on lymphocyte populations, including resting T- and B-cell numbers (106). Thus, CD52 is an ideal target to knockout in donor cells in combination with antibody conditioning. Alemtuzumab is an FDA-approved, humanized mAb against CD52, which can effectively deplete T- and B-cell lymphocytes (107). The knockout of non-essential gene CD52 in combination with an FDA approved antibody is expected to be an effective combination to avoid current non-specific lymphodepletion conditioning regimens.

## 3.6 Novel strategies for efficient iT, iNK differentiation: inducible NICD, EZH1 KD, Flt3L and BMP4 KI

Current manufacturing of autologous CAR-T cell therapies transforms the patients' own T cells, and this personalized manufacturing process adds significant cost, time and variability in producing the final T cell product. Building more robust, scalable, and reproducible manufacturing workflows for T cell therapies will help improve product safety and efficacy and will expand access to these life changing therapies. A major bottleneck on the path to T cell production is to efficiently differentiate hPSCs into definitive hematopoietic progenitors with T cell potential. Definitive hematopoietic stem cells (HSCs) arise from a cell type known as HE in a process called the endothelial-to-hematopoietic transition (EHT).

Prior approaches to generate T lineage-competent HPCs from hPSCs include the use of DL4-expressing immortalized stromal cell lines, or more recently developed cultures were coated with DL4, VCAM1, or DL4 coated microbeads (44, 45, 50). Notch signaling plays a pivotal role in the differentiation and development of lymphocytes, which are critical components of the immune system. There are four Notch receptors (Notch1–4) that interact with various ligands like Delta-like and Jagged proteins. These interactions initiate the Notch signaling cascade. Notch signaling is essential for T cell development in the thymus. It influences the fate of thymocytes, guiding them to differentiate into various T cell subsets. Dependent on Notch signaling, HPC differentiate into CD5CD7 double positive T cell progenitor cells. Under reduced Notch signaling and increased TCR signaling, CD4CD8 double positive cells are produced and mature to single positive T cells. To differentiate iT cells without notch ligand expressing feeder or DL4-VCAM1 coating and simplify the manufacturing process, an inducible expression of notch intracellular domain and VCAM1-VLA4 fusion can be knocked into the iPSC genome (Figure 2), which is turned on during the transition of CD34+CD45+ HE to CD5+CD7+ T cell progenitor cells and turned off after for T cell maturation.

Recent studies have revealed key roles for epigenetic regulators during definitive hematopoiesis and lymphoid development. The Daley group discovered that during embryonic hematopoietic development, EZH1, a part of polycomb repressive complex 2 (PRC2), is a crucial negative regulator of definitive lymphoid commitment (108). The production of CD3+ T cells was significantly increased in a subsequent study that used shRNA-mediated EZH1 knockdown or a doxycycline-inducible CRISPR interference (CRISPRi) construct into iPSC-derived CD34+ HE cells to transcriptionally repress EZH1 expression. This was due to the depletion of EZH1 during T cell specification (week 0-2) (45). EZH1 knockdown T cells displayed a significant increase in CD3+TCRαβ+ and decrease in CD3+TCRγδ+ T cells, indicating that EZH1 knockdown promotes differentiation towards αβ T cell fate rather than γδ T cells and exhibits a more mature T cell phenotype, highly diverse T-cell receptor (TCR) repertoire and enhanced antitumor activities. EZH1 is a positive regulator of Notch signaling, and this study further emphasizes the importance of timing and strength of notch signaling during T cell development (45). Knockin of the tunable EZH1 expression elements in iPSC could further improve the potency and yield of iT.

Further an elegant study from Zandsra group demonstrated the engineering of iPSCs with tunable cytokine signaling (109). The result was the precise control of the differentiation outcome and complete elimination of the need for exogenous BMP4 by engineering stem cells to express and secrete BMP4, a factor that is typically added exogenously to promote germ-layer differentiation or by using synthetic microRNA to fine-tune BMP4 expression level (110, 111). FLT3L is also essential for T cell development and commonly supplemented in the differentiation process. Integration of genetic codes to control BMP4 and FLT3L expression will likely be the subject of future engineering.

## 3.7 Increase iT stemness: IL-7RF, FOXO KI

The effectiveness of T cell therapy is limited by the fast exhaustion and death of T cells. Therefore, one strategy to improve the efficacy of T cell therapies is to increase the cells' "stemness," or their capacity to self-renew and differentiate into distinct kinds of T cells. T cell stemness is heavily dependent on the interaction between IL-7 and its receptor IL-7R. In fact, according to analyses using the PanCancer TCGA and iATLAS datasets, RNAseq of bulk tumors and scRNAseq of TILs, gene expression of IL-7R pathway components on tumor bulk is strongly connected with improved overall survival (OS) or progression-free survival (PFS) across various malignancies (112). In addition, expression of IL-7R and/or the IL-7R pathway is significantly greater in immune checkpoint inhibitors (ICI) responders versus non-responders in melanoma, NSCLC, ovarian, TNBC, HNSCC, and/or kidney malignancies (112). Genes associated with stemness are upregulated in TILs overexpressing IL7R, while genes associated with exhaustion are downregulated. Furthermore, IL-7R high TILs express a significantly higher quantity of BCL2, an anti-apoptotic molecule, and are less apoptotic.

Another major limitation of CAR T cell therapies is the poor persistence of these cells *in vivo*. The expression of memory-associated genes in CAR T cells is linked to their long-term persistence in patients and clinical efficacy. Recently, two separate research teams have found another way to rejuvenate these cells: make them more like stem cells. Taking into account that IL-15 improves CAR T cell persistence and metabolism, Chan et al. set out to discover important transcription factors that are elevated by this treatment. A Foxo1 gene signature was found to be significantly enriched when the epigenome and transcriptome of CAR T cells grown in the presence of IL-15 were analyzed (113). In CAR T cells generated from either healthy human donors or patients, overexpression of a constitutively active form of FOXO1 (FOXO1-ADA) promotes a stem-like phenotype, which corresponds with increased mitochondrial fitness, durability, and therapeutic efficacy *in vivo*. Doan et al. discovered that endogenous FOXO1 gene editing or pharmacological suppression reduced memory-associated gene expression, induced a state resembling tiredness, and decreased CAR T cell antitumor activity. In environments of prolonged stimulation, CAR T cells that overexpressed FOXO1 maintained their functionality, memory capacity, and metabolic fitness. They also demonstrated improved durability and tumor control *in vivo* (114). Thus, these findings provide an engineering strategy with translational potential to enhance the effectiveness of CAR T cells against solid tumors by genetically enforcing a favorable metabolic phenotype.

Numerous other strategies have been investigated to positively influence the development of CAR T cells. These include the overexpression of additional transcriptional regulators IRF4, c-Jun (115, 116), the application of homeostatic cytokines by tethered IL-15 (117), and epigenetic regulation with BET bromodomain inhibitors (118), which has emerged relatively recently.

## 3.8 Increase iNK *in vivo* persistence: IL-15RF KI, CISH KO

IL-15 plays a crucial role in the differentiation and survival of NK, it promotes the survival of these cells by maintaining the expression of anti-apoptotic factors like Bcl-2 (119). Furthermore, NK cells require IL-15 to mature in order to become responsive to this cytokine and reach functional maturity (120). Greater *in vivo* expansion and longer-term persistence are induced in CB-NK cells transduced with a fourth-generation vector encoding anti-CD19 CAR and IL-15 compared to nontransduced (NT) NK cells (121).

A key negative regulator of IL-15 signaling belongs to the suppressor-of-cytokine signaling (SOCS) family of proteins, known to play a significant role in NK cell biology (122, 123). One of its components, the CISH gene, is a key negative regulator of IL-15 signaling; it encodes the cytokine-inducible Src homology 2-containing protein (CIS) (124, 125). CISH knockout iPSC-NK cells have improved expansion, enhanced anti-tumor activity, and persistence *in vitro* and *in vivo*, and have exhibited improved metabolic fitness, which is mediated by the mTOR signaling pathway (126). CISH gene knockout in IL-15–secreting CAR-NK cells could improve their metabolic fitness, permitting greater *in vivo* persistence and cytotoxic function (127). When IL-15 expression is coupled with the disruption of the cytokine checkpoint gene CISH, CAR-NK cells' therapeutic potential may be significantly amplified in the clinic.

## 3.9 Increase metabolic fitness: CD38, mbIL21 KI

CD38 is an enzyme with NAD+ glycohydrolase and its expression can lead to NAD+ depletion and immune cell exhaustion. CD38 is a multifunctional ecto-enzyme that plays a role in metabolism by metabolizing NAD+ and mediating nicotinamide dinucleotide (NAD+) and extracellular nucleotide homeostasis, as well as intracellular calcium signaling (128). CD38 knockout has been studied for its potential to increase metabolic fitness. Knocking out CD38 in mice can lead to increased longevity and protection against the development of cancers, especially under high metabolic pressure, such as high-fat diets (129). Additionally, CD38 knockout NK cells have shown increased resistance to oxidative stress-induced death and enhanced metabolic fitness due to a reduction in reactive oxygen species (23, 130). This suggests that CD38 knockout can be beneficial in improving metabolic fitness and may have therapeutic potential in conditions associated with metabolic dysfunction of NK or T cell therapy (131).

Members of the common gamma-chain receptor family, IL-15 and IL-21, have all been shown to have well-documented effects on NK cells; IL-15 is essential for NK cell development but IL-21 can improve NK cell viability and functions with increased metabolic fitness (33, 132). While IL-15 primarily signals through STAT5, IL-21 is known to largely signal through the STAT3 component of the JAK/STAT pathway with minimal participation from STAT5. Human telomerase reverse transcriptase (hTERT) is known to be activated by STAT3 (133). NK-cell senescence from mbIL15-mediated expansion can be reverted through hTERT gene editing (134). NK cells expanded with membrane bound (mbIL-21) have longer telomeres and less senescence than those expanded with mbIL-15, mbIL-21 promotes improved proliferation of human NK cells (33). IL-21-expanded TILs exhibited a 'young' phenotype with longer telomeres and higher expression of CD27 and CD28, and this phenotype was linked to stem-cell-like improved lifespan (132, 135). mbIL-21 increased NK cell metabolism with a shift towards aerobic glycolysis, induced robust and sustained proliferation of highly cytotoxic NK cells which exhibit increased cytotoxic function against various cancer cells (34). Inducible expression of mbIL-21 at expansion stage of iNK production may provide large quantities and mature, potent cell products for clinical applications.

## 3.10 Increase homing/infiltration: chemokine receptor KI

One limitation on the efficacy of CAR T or NK cell therapy is insufficient homing to or infiltration of the relevant sites (e.g. tumor, BM, secondary lymphoid tissues). NK cells develop mainly in the BM and egress into the blood circulation when they mature. They then migrate to peripheral tissues, though some special subsets home back into the BM or secondary lymphoid organs (136–138). NK cell lineage comprises of remarkably diverse population, two major PB NK cell subsets are $CD56^{bright}$ and $CD56^{dim}$ (139, 140). Normally, BM, lung, spleen, subcutaneous adipose tissue, and breast tissue are dominated by $CD56^{dim}$ NK cells. In contrast, the proportion of total NK lineage cells in the stomach and intestinal mucosa, liver, uterus, visceral adipose tissue, adrenal gland, and kidney is significantly enriched in $CD56^{bright}$ NK cells (141, 142). Accordingly, these distinct patterns of tissue localization correspond with markedly different patterns of chemokine receptor expression. While both subsets of NK cells express CXCR4, PB $CD56^{bright}$ NK cells express CCR7, CXCR3, high levels of L-selectin (CD62L), for homing and/or entry into tissues that are secondary lymphoid or expresses the reciprocal ligands. Conversely, PB $CD56^{dim}$ NK cells express sphingosine-1-phosphate receptor (S1PR5), and CXCR1, CXCR2, and CX3CR1 (141, 143, 144).

CAR T or NK cell trafficking and retention within tumor sites is essential for optimal anti-tumor efficacy. NK cells' migration and homing to the lymph node-associated chemokine CCL19 against hematological malignancies was improved by NK cells' induction of CCR7 expression (145, 146). Enhancing CAR-NK cells targeting the glioma antigen epidermal growth factor variant III (EGFRvIII) with CXCR4 expression led to better chemotaxis for U87-MG glioblastoma cells. These cells release CXCL12/SDF-1α, a CXC chemokine that interacts with receptors CXCR4 and CXCR7 (147). A novel strategy to improve homing and target NK cell-based immunotherapies to the BM (critical for ablation of auto-reactive plasma cells or hematological malignancies) is the ectopic expression of CXCR4 gain of function mutant R334X on expanded NK cells, which led to significantly greater BM homing after adoptive transfer into NSG mice compared to non-transfected NK cell controls. Additionally, BM migration of CXCR4 knockin iNK cells targeting various antigens results in superior tumor cell killing in the marrow in aggressive disseminated heme xenograft

models (148, 149). Furthermore, inducing expression of CXCR1 in NK cells with a NKG2D CAR were shown to significantly increase anti-tumor responses in subcutaneous and intraperitoneal xenograft models along with an intravenous injection model against established peritoneal ovarian cancer xenografts (150).

### 3.11 Overcome suppressive TME: decoy receptors KI, A2AR KO

The TME is made up of a harsh metabolic environment that is characterized by a variety of immunosuppressive metabolites, hypoxia, acidity, upregulation of transforming growth factor beta (TGFβ), and glucose and amino acid deprivation (151). These factors work together to impede efficient antitumor immunity and are likely responsible for the challenges in treating solid tumors with cell therapies.

Because of its suppressive role in the TME, TGFβ has been targeted to boost cell therapy anti-tumor response. TGFβ mediates downregulation of NKG2D, NKp30, TRAIL, and DNAM1 receptors on activated NK cells (152, 153). To shield adoptive NK cell therapies from the suppressive effects of TGFβ, introduction of a dominant negative form of TGFβ type II receptor (TGFβRII) efficiently blocked TGFβ signaling and maintained cell surface expression of receptors and cytotoxicity in NK and T cells (154–156). Elegant strategies embracing the inhibitory cytokine and converting it into a potent stimulatory signaling have been created by rewiring the recognition domain into a second-generation CAR-T cell to orchestrate upregulation of cytokine production (157). Similarly, expressing a CAR with a TGFβRII extracellular and transmembrane domains combined with the intracellular domain of NKG2D on NK-92 cells converted the immunosuppressive signal into increased cytotoxicity while preventing downregulation of NKG2D surface expression (158). This strategy has also been applied to other inhibitory receptors such as PD-1, generating a PD-1 CAR with NK tailored endodomains such as NKG2D or DAP10/NKG2D to mediate cytotoxicity by NK cells against solid malignancies in the TME (159, 160).

The A2AR (adenosine A2A receptor) knockout and its role in the suppressive TME is a significant area of research in cancer immunotherapy. CD39 and CD73, which are highly expressed in a variety of TME cell types, regulate the extracellular environment's metabolism of ATP and adenosine (161). Tumor cell immune evasion and inhibition of antitumor immune responses are facilitated by the CD39/CD73/A2AR pathway, which plays a critical role in the formation of an immunosuppressive TME (162). Targeting the A2AR, either through genetic knockout or pharmacological inhibition, can improve the function of antitumor immune cells (163, 164).

### 3.12 Overcome the physical barrier of stromal cells

The extracellular matrix (ECM) of tumor stroma creates a physical barrier to cancer therapies by preventing infiltration of therapeutic agents into tumors. ECM is made up of various structural molecules such as fibrous proteins, glycosaminoglycans, and proteoglycans. These are produced by tumors and cancer-associated fibroblasts (CAFs) that contribute to tumorigenesis (144). Normal tissue has thin fibroblasts in elongated spindle shape. Fibroblasts are thought to be in a resting state most of the time, but they can become activated in response to stimuli including stress, hypoxia, and cytokines as well as tissue injury (165). Following the wound healing, the quantity of activated fibroblasts decreases, and they most likely return to their resting state (166). However, in tumors, fibroblasts are often hyper-activated through mediating factors such as TGF-β, platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2). CAFs are therefore considered as an irreversibly activated heterogeneous population of fibroblasts with distinct functions (165). They greatly contribute to the TME's immunosuppression by secreting various chemokines and cytokines, including TGF-β, IL-6, IL-8, IL-13, CXCL12, and VEGF (167).

Fibroblast activation protein (FAP) is a membrane protease that is highly expressed CAFs. By altering the ECM, FAP can modify the TME, and its overexpression on cancer reduces the effectiveness of CAR-T cell therapy in solid tumors and is linked to a poor prognosis in a number of malignancies (168). One tactic being investigated to overcome barrier stroma cells is targeting FAP using CAR-T cells. FAP-targeting CAR-T cells have been engineered to target CAFs in various solid cancers, such as mesothelioma, lung and pancreatic cancers (169). A number of studies have shown anti-tumor activity in preclinical models (170, 171). Anti-FAP CAR-T cell treatments have been tested in clinical settings. The effectiveness of CAR T cell treatment may be increased by targeting FAP in addition to cancer antigen targets.

## 4 New gene editing technologies make engineering of complex cell therapies possible

In the past decade CRISPR–Cas9-based technologies have revolutionized basic and applied research in biology. However current gene integration approaches require DNA double-strand breaks and rely on repair pathways such as homology directed repair (HDR) that are inactive in terminally differentiated non-dividing cells. Programmable and multiplexed genome integration of multi-kilobase DNA cargo is still challenging. Together with the new families of editing enzymes, which include transposases, integrases, recombinases and single-stranded DNA-annealing proteins, new CRISPR/Cas-based long sequence integration technologies have been developed. This discipline is rapidly evolving, and it has the potential to spark a new wave of ground-breaking biomedical applications. These new technologies are critical to the advancement of cellular therapies given the multitude of edits mentioned above that are needed to ensure safe and effective treatments.

Traditional CRISPR/Cas9 systems exploit one of three types of DNA repair mechanisms: HDR, nonhomologous end joining (NHEJ), and microhomology-mediated end joining (MMEJ).

These strategies may result in imprecise insertions or deletions with substantial indel errors, and the efficiencies vary greatly depending on cell type. Recent new CRISPR techniques for multi-kilobase DNA cargo insertion have been developed to help overcome some of the limitations of previous editing technologies, including: (1) the transposon-encoded CRISPR/Cas system;(2) recombinase/integrase with CRISPR/nCas9; (3) single-stranded DNA-annealing protein (SSAP) editor coupled with CRISPR/dCas9 (Table 5).

Among the instruments discussed above, CRISPR-associated transposons have mostly been examined in a limited range of prokaryotes (172–174). Recombination independent, multi-kilobase DNA insertions at RNA-programmed genomic sites are made possible by CRISPR-associated transposases (CASTs). However, substantial off-target integration and a transposition mechanism that produces a mixture of acceptable simple cargo insertions and undesirable plasmid cointegrate products limit the usefulness of type V-K CASTs (172–174). Another programmable RNA-guided transposon system, called Insert Transposable Elements by Guide RNA-Assisted TargEting (INTEGRATE), is capable of transposing cargo genes up to 10 kb in length to the human genome, however it lacks control over the orientation of insertion (173). Recently, the 5′ nicking capability required for cargo excision on the DNA donor was restored by engineering a nicking homing endonuclease fusion to TnsB (dubbed HELIX). HELIX allows for cut-and-paste DNA insertion with high homogenous insertion product purity and significantly higher on-target specificity than canonical CASTs. However, HELIX has very low efficiencies (<0.1%) in mammalian cells and requires at least 4 protein co-factors, indicating that more work is needed to fully realize their potential in genome engineering applications (175). While HELIX has been examined in several mammalian cell types, there isn't any assessment yet available in iPSC.

A new cleavage-free gene editor dCas9-SSAP, which utilizes microbial single-strand annealing proteins (SSAPs) with catalytically inactive dCas9, promotes the integration of long sequences in mammalian cells (176). With an efficiency of up to 20%, it works well for inserting kilobase-scale sequences into human cell lines in therapeutic places like AAVS1. When compared to previous editing techniques that rely on single-strand nicks or DNA double stranded breaks, dCas9–SSAP produces nearly zero off-target mistakes while facilitating homology-mediated transgene insertion via non-cutting Cas9s (176, 177).

Programmable Addition via Site Specific Targeting Elements (PASTE) is a novel method that combines the programmability of Cas9 nickase, the writing ability of reverse transcriptase, and the size-agnostic DNA integration capacity of large serine integrases (LSI) to enable the integration of large pieces of DNA into specific genomic locations (178). This method avoids the generation of DNA double stranded breaks, which allows for efficient multiplexing. A similar method has been developed using an engineered integrase (eePASSIGE) that has shown low efficiency integration in iPSCs (~4% using 5kb cargo) (179).

Recently, Tome has developed an improved version of the PASTE technology, Integrase-mediated Programmable Genomic Integration (I-PGI). I-PGI is capable of efficient gene insertion in iPSCs cells, achieving >50% integration using nanoplasmid cargos. Tome has demonstrated the ability to multiplex integration of up to 4 inserts, and have integrated very large cargo (>31 kb) using adenovirus templates. For one of its iPSC derived therapeutic programs, Tome has demonstrated to ability to generate 3 knock outs and insert 12 kb of code in a single process (180). This capability to create complex cell therapies with relative ease and high efficiency opens the door to the development of highly editing cell therapies, including multiple CARs, safety switches, stealth, and other edits to increase *in vivo* persistence, cell fitness, and antibody mediated lymphodepletion.

In conclusion, the development of sophisticated iPSC-derived cell therapies involving extensive engineering has been made possible by these novel long-sequence integration technologies.

## 5 Future directions and concluding remarks

The field of immunotherapy is on the verge of a revolution, thanks to the advent of iPSC-derived immune cells and genome editing technologies. These cells have the potential to become the cornerstone of treatment for myriad diseases, heralding a new era in cell therapies. However, before this potential can be fully realized, several significant challenges must be addressed.

TABLE 5 Summary of new gene editing technologies for precise large DNA insertion in human genome.

| Enzymes | Technologies | Insert size | Efficiency | References |
|---|---|---|---|---|
| Transposases | INTEGRATE | >10kb | 1% (HEK293T) | (173) |
| | HELIX | >10kb | 0.04%(HEK293T) | (175) |
| SSAP | REDIT | >2 kb | 5% (ESC) | (176) |
| | dCas9-SSAP | >2kb | 4% (ESC) | (177) |
| Recombinase/Integrase | eePASSIGE (PASSIGE/TwinPE) | <6kb | <4% (iPSC) | (179) |
| | I-PGI (PASTE) | Up to 36 kb | 50-60% (iPSC) | (178, 180) |

shCAST, CRISPR-associated transposase from cyanobacteria *Scytonema hofmanni*; INTEGRATE, Insert Transposable Elements by Guide RNA-Assisted TargEting; HELIX, HE-assisted Large-sequence Integrating CAST-compleX; SSAP, single-stranded DNA-annealing proteins; REDIT, RecT Editor via Designer-Cas9-Initiated Targeting; TwinPE, twin prime editing; eePASSIGE, evolved and engineered prime-editing-assisted site-specific integrase gene editing; PASTE, programmable addition via site-specifc targeting elements; I-PGI, integrase-mediated programmable genomic integration.

Optimization of Differentiation Protocols. The journey of an iPSC to a mature and functional immune cell is complex and requires precise control over the differentiation process. Current protocols need refinement to ensure that the resulting cells are not only mature but also possess the functional capabilities necessary to combat diseases effectively. This optimization is crucial for the cells to perform their intended therapeutic roles once administered to patients.

Minimizing Immunogenicity and GvHD. One of the primary concerns with off-the-shelf cell products is their potential to elicit an immune response in the recipient, leading to rejection or other adverse effects. Researchers are working to minimize the immunogenicity of iPSC-derived cells to ensure they can be used widely and safely across different patient populations.

Scalable Manufacturing Processes. To bring iPSC-derived immune cell therapies to the masses, it is imperative to develop manufacturing processes that can produce these cells in large quantities without compromising quality. Scalability is key to making these therapies affordable and accessible to all who need them.

Overcoming *In Vivo* Hurdles. Once inside the body, iPSC-derived immune cells face numerous challenges, including a suppressive TME, insufficient trafficking and infiltration, and the need for enhanced persistence and fitness to maintain efficacy. Researchers are exploring various strategies discussed in this review to help these cells overcome these hurdles and perform optimally *in vivo*.

Mitigating Safety Concerns. The potential for iPSC-derived cells to cause tumors or other safety issues is a significant concern that must be addressed. Ensuring the safety of these therapies is paramount to their clinical success and widespread adoption.

Advancements in Genome Editing Technologies. The use of advanced editing technologies offers the ability to integrate large DNA sequences at specific genomic locations in a multiplex fashion. This precision engineering of iPSCs and their derivatives at unprecedented speed and efficiency opens up the possibility of creating advanced immune cells obviating the obstacles of current immunotherapies.

Conclusion. iPSC-derived iNK and iT cells stand at the forefront of a promising new avenue in immunotherapy. These cells offer hope for treatment that could be applied to a wide range of diseases, from cancer to autoimmune disorders. As research continues and technology advances, iPSC-based approaches are set to revolutionize oncology and regenerative medicine, offering the potential for improved patient outcomes and a new standard of care in the years ahead.

## Author contributions

MF: Conceptualization, Writing – original draft, Writing – review & editing. AA: Writing – review & editing. CL: Writing – review & editing. JF: Writing – review & editing.

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MF, AA, CL and JF are current employees at Tome Biosciences.

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# High specificity of engineered T cells with third generation CAR (CD28-4-1BB-CD3-ζ) based on biotin-bound monomeric streptavidin for potential tumor immunotherapy

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**Introduction:** Immunotherapy has revolutionized cancer treatment, and Chimeric Antigen Receptor T cell therapy (CAR-T) is a groundbreaking approach. Traditional second-generation CAR-T therapies have achieved remarkable success in hematological malignancies, but there is still room for improvement, particularly in developing new targeting strategies. To address this limitation, engineering T cells with multi-target universal CARs (UniCARs) based on monomeric streptavidin has emerged as a versatile approach in the field of anti-tumor immunotherapy. However, no studies have been conducted on the importance of the intracellular signaling domains of such CARs and their impact on efficiency and specificity

**Method:** Here, we developed second-generation and third-generation UniCARs based on an extracellular domain comprising an affinity-enhanced monomeric streptavidin, in addition to CD28 and 4-1BB co-stimulatory intracellular domains. These UniCAR structures rely on a biotinylated intermediary, such as an antibody, for recognizing target antigens. In co-culture assays, we performed a functional comparison between the third-generation UniCAR construct and two second-generation UniCAR variants, each incorporating either the CD28 or 4-1BB as co-stimulatory domain

**Results:** We observed that components in culture media could inhibit the binding of biotinylated antibodies to monomeric streptavidin-CARs, potentially compromising their efficacy. Furthermore, third-generation UniCAR-T cells showed robust cytolytic activity against cancer cell lines upon exposure to specific biotinylated antibodies like anti-CD19 and anti-CD20, underscoring their capability for multi-targeting. Importantly, when assessing engineered UniCAR-T cell activation upon encountering their target cells, third-generation

UniCAR-T cells exhibited significantly enhanced specificity compared to second-generation CAR-T cells

**Discussion:** First, optimizing culture conditions would be essential before deploying UniCAR-T cells clinically. Moreover, we propose that third-generation UniCAR-T cells are excellent candidates for preclinical research due to their high specificity and multi-target anti-tumor cytotoxicity

KEYWORDS

**engineered cells, immunosuppression, Treg, Chimeric Antigen Receptor, streptavidin-based CAR**

# 1 Introduction

Malignant tumors pose a significant global health concern due to their increasing annual incidence, some coupled with a still insufficient response rate to available therapies for achieving long-term remission (1). One of the most successful strategies that have emerged in recent years, particularly in the treatment of hematological malignancies, is adoptive immunotherapy based on Chimeric Antigen Receptor T (CAR-T) cells (2–4). Engineered CAR-T cells recognize and target tumor-associated antigens (Ags) on cancer cells.

Conventional CARs are synthetic proteins formed by an extracellular domain that binds to a specific Ag on the surface of cancer cells, a hinge domain, a transmembrane domain and intracellular signaling domains (5). The CAR extracellular domain is often a single-chain variable fragment (ScFv) that maintains the original affinity and specificity for an Ag, such as CD19 on malignant B cells (6, 7). The intracellular region is the one that provides the necessary signals for CAR-bearing T cell activation. Depending on the domains of the intracellular region, several CAR generations have been developed. First generation CAR is solely composed of the CD3-ζ chain, which is responsible for the primary T cell activation signaling (8). To mimic the physiological T cell activation, the intracellular domain of second-generation CARs combined the CD3-ζ chain with an additional co-stimulation signal provided by CD28, 4-1BB, CD27, OX40 or ICOS proteins, among others (9). The third generation CAR is composed of the CD3-ζ chain next to two co-stimulatory domains. This combination can induce a higher rate of proliferation and greater long-term survival *in vivo*, while causing less cellular exhaustion in engineered T cells compared to second-generation CARs (10).

Although CAR-T therapy has shown remarkable success, some drawbacks still limit its global implementation. Significant toxicities, such as cytokine release syndrome and neurotoxicity, can be life-threatening in severe cases. Additionally, CAR-T therapy is currently only feasible for certain types of cancer and has shown limited efficiency against solid tumors, restricting its availability to a broader patient population (2, 11–13). Addressing these challenges requires further research to optimize this technology and expand its applicability to a wider range of cancer patients.

To overcome some of the limitations outlined for CAR-T cell technology, various approaches have been developed. Universal CARs (UniCARs) stem from the modification of conventional CARs, utilizing small molecules as extracellular intermediaries that recognize the desired target antigen (14). The main advantage of UniCARs, through this intermediary approach, is their ability to use a single CAR construct against multiple therapeutic targets by simply changing the intermediary, making it a flexible tool. This approach has the potential to reduce treatment costs and enhance scalability (15). Moreover, because UniCARs depend on an intermediary to achieve target recognition, this technology offers the possibility to modulate CAR-mediated T cell activation and function by regulating the availability of the intermediary. This approach holds potential for mitigating conventional CAR-related toxicity (16).

One of the most promising approaches for UniCARs is based on the interaction between biotin and biotin-binding proteins (BBP) (15). Lohmueller et al. developed two second-generation UniCARs with extracellular domains containing monomeric streptavidin 2 (mSA2)-UniCAR (17). This mSA2-UniCAR structure addresses both the immunogenicity and non-specificity limitations observed in other BBP-based UniCARs, while retaining remarkable biotin affinity (Kd= 5.5 x $10^{9}$at 37°C) (18). In our study, we tested the extracellular domain mSA2-UniCAR generated by Lohmueller et al. (17) in a third-generation construct combining the co-stimulatory domains 4-1BB and CD28. The anti-tumor functionality of this combination was compared against academic conventional anti-CD19 CAR (CAR19; ARI-0001 CAR-T) (19–21) and second-generation mSA2-UniCARs based on 4-1BB or CD28. We demonstrated that the combination of these co-stimulatory domains positively influenced the specificity of mSA2-UniCAR-T cells. Third-generation mSA2-UniCAR-28-BB-T cells exhibited greater specificity compared to both second-generation mSA2-UniCAR-T cells, while retaining the elevated anti-tumor activity characteristic of conventional CAR19-T cells. This suggests that the third-generation mSA2-UniCAR-28-BB-T cells are a promising

therapeutic approach, combining high specificity and potent anti-tumor activity, thus addressing some limitations of previous CAR-T cell technologies.

# 2 Materials and methods

## 2.1 Primary human T cell culture and lentiviral transduction

Peripheral Blood Mononuclear Cells (PBMC) were isolated from deidentified human Buffy Coats from healthy volunteer donors obtained from the Madrid Transfusion Center. PBMC were isolated using Ficoll centrifugation and cultured at 37°C and 5% of CO2 in X-vivo15 (Lonza, Walkersville, MD, USA) supplemented with 5% of human AB serum (ABS, Sigma-Aldrich, St. Louis, MO, USA) and 300 U/ml IL-2 (ImmunoTools, Friesoythe, Germany). PBMCs were stimulated and expanded using αCD3/αCD28 Dynabeads Human T-Activator (Invitrogen, Waltham, MA, USA) for 48 hours; then, activated cells were transduced with lentivectors (100 to 300 ng of p24gag per 1.5 million cells). Cells were then washed and cultured for 3 additional days for their expansion. Every UniCAR+ cells (2nd or 3rd generation) expressing Green Fluorescent Protein (eGFP), were flow-sorted by MACSQuant®Tyto®(Miltenyi Biotec, Bergisch Gladbach, Germany) and then cultured at 37°C and 5% of CO2 in RPMI 1640 + 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and 1% antibiotic mix (125 µg/ml of ampicillin, 125 µg/ml of cloxacillin and 40 µg/ml of gentamicin, Merck Life Science, Madrid, Spain), supplemented with 300 U/ml IL-2. 24 hours after sorting, cells were analyzed for viability and purity by flow cytometry.

## 2.2 Cell line culture

Human tumor cell lines Jurkat Clone E6–1 (TIB-152, RRID: CVCL_0367) and Raji (CCL-86, RRID: CVCL_0511) were obtained from Reagent Program (NIH, Bethesda, MD, USA); and K562 (CCL-243, RRID: CVCL_0004) were obtained from European Collection of Authenticated Cell Cultures (Salisbury, UK). These cell lines were cultured at 37°C and 5% of CO2 in RPMI 1640 (Biochrome, Holliston, MA, USA) medium supplemented with 10% FBS and 1% antibiotic mix. UniCAR-28-BB+ Jurkat cells were generated by transducing Jurkat cells with the third-generation UniCAR-28-BB expressing-lentivirus. Transduced UniCAR-28-BB + Jurkat were enriched by sorting cells for positive eGFP expression. CD19+ K562 cells (K562-CD19) were generated by transducing K562 cells with full-length CD19 expressing-lentivirus (VectorBuilder GmbH, Neu-Isenburg, Germany). Transduced K562-CD19 were enriched by sorting CD19+ cells. Human embryonic kidney (HEK)-293T cells (American Type Culture Collection, ATCC; RRID: CVCL_0063, LGC Standards S.L.U., Barcelona, Spain) were cultured at 37°C and 5% of CO2 in high glucose-DMEM (Gibco, ThermoFisher, Waltham, MA, USA) supplemented with 10% FBS, and 1% antibiotic mix.

## 2.3 Lentiviral vector construction and virus production

Driven by the EF1a promoter, UniCAR extracellular recognition domain consists in the mSA2 sequence (17) followed by the CD8a-hinge spacer domain, the CD28 transmembrane domain, either CD28, 4-1BB or both cytoplasmic domains and, finally, the CD3ζ cytoplasmic domain (Figure 1A). eGFP gene was encoded in the same lecture frame, but it was separated from UniCAR by the T2A co-translation peptide sequence (Figure 1A). The structure of the resulting protein is shown in Figure 1B. Once the constructs were transduced into the desired cells, mSA2-UniCAR-T cells need a biotinylated intermediary, such as an Ab, to recognize the target cell (Figure 1C).

The third-generation pUniCAR-mSA2-CD28-41BB-CD3ζ-eGFP was provided by Creative Biolabs (Frankfurt, Germany), based on the published sequences (17). Second-generation pUniCAR-mSA2-CD28-CD3ζ-eGFP and pUniCAR-mSA2-41BB-CD3ζ-eGFP were synthetized by the deletion of 41BB and CD28 regions, respectively, from the third-generation pUniCAR-mSA2-41BB-CD28-CD3ζ construct. To accomplish these depletions, Q5®Site-Directed Mutagenesis Kit (New England Biolabs, NEB, Ipswich, MA, USA) was used, following manufacturer instructions and the corresponded primers (Supplementary Figures S1A, B).

The second-generation anti-CD19 CAR (CAR19) T cells, which consist of the A3B1 scFv, CD8 transmembrane domain, 4-1BB co-stimulatory domain, and CD3ζ stimulatory domain, was used as a positive control for CD19 target recognition and anti-tumor functionality of T cells (19, 21).

Lentiviral vectors were generated through the co-transfection of HEK-293T cells with various plasmids:pUniCAR-mSA2-41BB-CD28-CD3ζ, pUniCAR-mSA2-CD28-CD3ζ, pUniCAR-mSA3-41BB-CD3ζ, pCD19 (containing the full-length sequence of CD19) or pCAR-CD19, in combination with psPAX2 (RRID: Addgene_12260), pRSV-Rev (RRID: Addgene_12253), and pMD2.G (RRID: Addgene_12259, all plasmids were a gift from Prof. Dr. Trono, Addgene, Watertown, MA, USA). Transfection was performed using a calcium phosphate transfection kit (Sigma-Aldrich). The physical titers of the vectors were assessed after 0.45 µm filtration (Corning, Corning, NY, USA) by quantifying HIV-1-p24gag levels using an ELISA kit (Abcam, Cambridge, UK). The resulting lentivectors were named UniCAR-28-BB (3rd generation), UniCARCD28, UniCAR41BB, CD19, and CAR19, respectively. For lentivectors encoding eGFP, transduced cells were assessed by flow cytometry and sorted based on their eGFP expression.

## 2.4 UniCAR neutralization assay

To test de biotin accessibility of mSA2-UniCAR-28-BB, human Jurkat and Jurkat-UniCAR-28-BB cell lines were cultured in different culture media, such as RPMI 1640 medium or in X-vivo15 medium, both media supplemented with either 10% FBS + 1% antibiotic mix or with 5% of human ABS. The fluorochrome Atto-655 bound to a Biotin (Atto-655-Biotin, Sigma-Aldrich) was

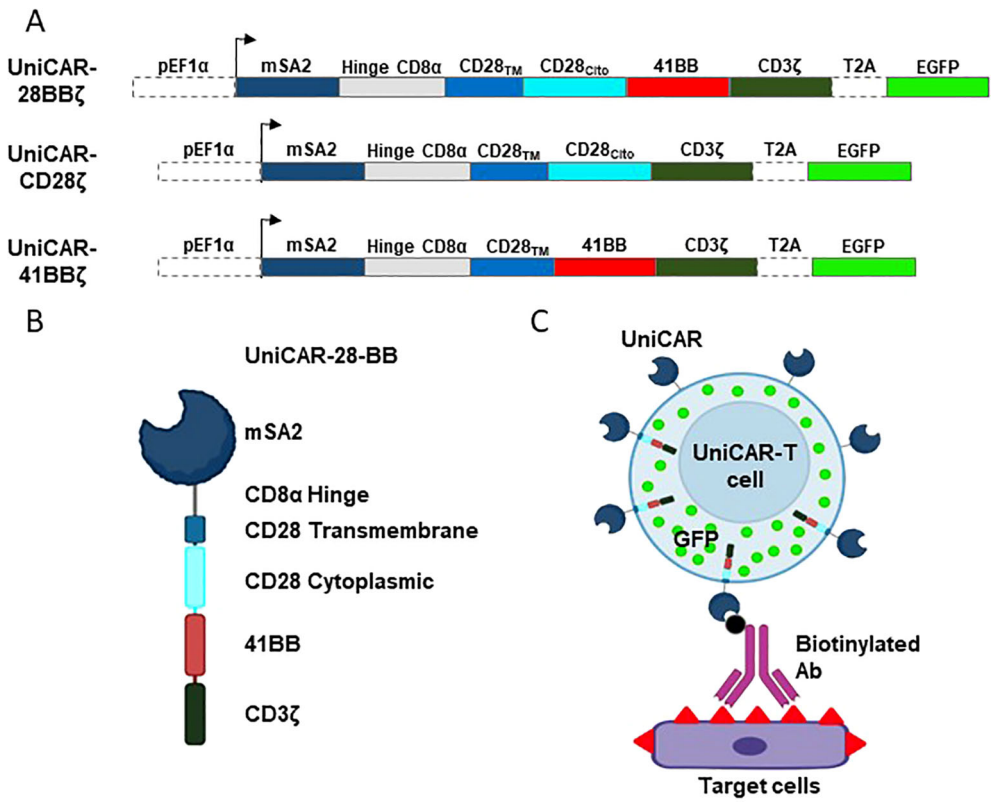


FIGURE 1
Schematic design of the vectors, UniCARs and their recognition system. **(A)** Design of the lentiviral expression construct encoding the 3rd generation UniCAR-28-BB, both 2nd generation UniCARCD28 and UniCAR41BB, and the eGFP. **(B)** Representation of the extracellular, transmembrane and intracellular elements that compose the 3rd generation UniCAR-28-BB. **(C)** Representation of a transduced cell expressing eGFP (green dots) and 3rd generation UniCAR-28-BB, which is able to recognize and bind to a biotinylated intermediate, such as a biotinylated monoclonal antibody. The biotinylated intermediate recognizes the antigens at the surface of the target cell. Figure created with BioRender.

used to specifically label the extracellular mSA2 of the UniCAR expressed by Jurkat- UniCAR-28-BB cells.

## 2.5 *In vitro* activation and co-culture experiments

P96 well U-bottom plate was set-up with 20,000 non-engineered or engineered primary T cells cultured either alone or co-cultured with 20,000 target cells (K562, K562-CD19 or Raji cell lines) in complete RPMI 1640 medium and with 1.5 μg/ml of anti (α)-CD19 biotinylated Ab (Miltenyi Biotec Cat# 130-113-644, RRID: AB_2726197) or αCD20 biotinylated Ab (Miltenyi Biotec Cat# 130-111-336, RRID: AB_2656082). Non-biotinylated αCD19 Ab (Miltenyi Biotec Cat# 130-122-301, RRID: AB_2801882) and non-specific IgG biotinylated Ab (Miltenyi Biotec Cat# 130-119-877, RRID: AB_2751902) were used as control conditions, and the negative control condition was performed without any antibodies. Target cells were previously stained with Cell Trace Violet (CTvio) following the manufacturer's recommendations (Invitrogen) to facilitate their visualization by flow cytometry.

After 72 hours of co-culture, cells were washed and stained with fluorochrome-bound antibodies against T cells and activation markers (CD3, CD4, CD8, CD25, CD71, ICOS and PD-1) to assess their expression by flow cytometry. Following surface labeling, effector engineered T cells were identified as CTvio-negative cells, and these activation markers were measured in CTvio-negative living cells, which were identified by the absence of staining with 7-Amino Actinomycin D (7-AAD, Invitrogen).

Additionally, to assess the cytotoxic activity performed by effector cells on target cells, co-cultured cells were directly stained with 7-AAD in the co-culture well. The viability of target CTvio-positive cells was measured by flow cytometry, with dead cells identified by positive 7-AAD staining. Cytotoxicity was calculated using the equation: 100 * (% of dead cells of experimental condition – % of dead cells of Cytotoxicity Negative control)/(% of dead cells of Cytotoxicity Positive control – % of dead cells of Cytotoxicity Negative control) (17). The Cytotoxicity Positive Control was K562, K562-CD19 or Raji cultured alone + 5% DMSO, and the Cytotoxicity Negative Control was K562, K562-CD19 or Raji cultured alone or with intermediary antibodies (depending on the culture condition).

## 2.6 Flow cytometry staining

As previously commented, cells were stained using anti-CD3 VioGreen®(Miltenyi Biotec Cat# 130-115-972, RRID:

AB_2751292), anti-CD8 BV570 (BioLegend Cat# 100740 (also 100739), RRID: AB_2563055), anti-PD-1 PE (Miltenyi Biotec Cat# 130-120-382, RRID: AB_2752069), anti-CD4 ECD (Miltenyi Biotec Cat# 130-113-226, RRID: AB_2726037), anti-CD25 PE-Cy7 (Thermo Fisher Scientific Cat# 25-0259-42, RRID: AB_1257140), anti-ICOS BV650 (Biolegend Cat# 313550, RRID: AB_2749929) and anti-CD71 APC-Vio770 (Miltenyi Biotec Cat# 130-115-032, RRID: AB_2726862) Abs for 30 minutes at 4°C in staining buffer (PBS + 2% FBS). For the UniCAR labeling with Atto-655-Biotin, cells were incubated for 1 h at room temperature and in the dark in staining buffer. For the frequency of the CARCD19+ cells, we used FITC-Labeled Human CD19 (ACROBiosystems, Basel, Switzerland). Finally, 5-10 minutes before flow cytometry acquisition, cells were incubated with 7-AAD. Flow cytometry acquisition was done by MACSQuant Analyzer 16 - Flow Cytometer (Miltenyi Biotec) and the resulting data were analyzed using the Kaluza software (2.1 Version, Beckman Coulter, Brea, CA, USA, RRID: SCR_016182).

## 2.7 Statistics

Statistical analysis of the data was performed using GraphPad Prism 8 software (Dotmatics, Boston, MA, USA, RRID: SCR_002798) with the support of the Biostatistics Unit of the IiSGM. The measurements, their error deviations and the types of statistical methods used are specified in their respective figure captions. Statistical results with a $p < 0.05$ were considered statistically significant.

# 3 Results

## 3.1 Neutralization of UniCAR/biotin recognition by culture media

Due to the extracellular exposure of the mSA2, components of the culture medium could potentially block the biotin binding of intermediaries to UniCAR (22). Since the aim of the adoptive cell immunotherapy is to be used under physiological conditions, we tested how two commonly used culture media (RPMI 1640 and Xvivo15) supplemented with bovine or human serum affected the ability of the 3rd generation UniCAR-28-BB construct expressed on Jurkat cells to bind the biotin.

Transduced Jurkat cells with the UniCAR-28-BB were sorted and detected by flow cytometry as eGFP+ cells. They also can be distinguished by the positive expression of Atto-655-Biotin binding to the mSA2 domain (Figure 2A). Sorted cells showed high eGFP expression, which was maintained independently of the culture media (Figure 2B). When cells were cultured in RPMI 1640, independently of its serum supplementation, the biotin union with the eGFP+ Jurkat-UniCAR-28-BB cells was maximum (Figure 2C). Notwithstanding, this ability to bind biotin was significantly compromised when cells were cultured in Xvivo15, independently of the serum source (Figure 2C). This result led us to infer that the Xvivo15 culture medium contains components unrelated to human serum, which hinder the binding.

Finally, to test if the biotin access to the UniCAR-28-BB could be restored, cells were moved from one medium to the other. While cells transferred from RPMI 1640 + FBS to Xvivo15 + ABS reduced their union ability to fluorescent biotin, cells previously cultured in Xvivo15 + ABS and subsequently cultured in RPMI 1640 + FBS significantly restored their Atto-655-biotin recognition capacity within 24h (Figure 2D). In summary, selecting the appropriate complete culture medium is crucial for studying streptavidin-based UniCAR cells. Based on these results, all subsequent experiments were performed using RPMI 1640 supplemented with 10% FBS. Additionally, the translation of UniCAR for potential human treatment should remain uncompromised, as the mSA2-biotin binding was not affected by the presence of human serum.

## 3.2 No alteration of the T cell phenotype after UniCARs transduction

After detecting the pivotal role of the culture medium, we transduced human PBMCs with UniCARs and CAR19 structures. These genetic modifications did not alter the cellular viability (Figures 3A, B). Following transduction, the expression of the three UniCARs structures was assessed via flow cytometry, with cells being identified as positive for UniCAR expression if they exhibited both eGFP+ and Atto-655-Biotin+ signals (Figure 3C). Subsequently, after sorting eGFP+ cells, the frequency of eGFP+ and UniCAR+ expressing cells exceeded 90% in every condition (Figures 3D, E). Since CAR19 construct did not have a selection marker, CAR19 transduced T cells could not be sorted without compromising its functionality. Nonetheless, 60.56 ± 2,89% (Mean ± SEM) of cells expressed CAR19 (Figures 3F, G).

As genetic modifications could potentially induce phenotypic changes in cells, we studied the phenotype of engineered T cells. In every UniCAR or CAR19 condition, the expression of the CD3 marker was found to be greater than 99% of living cells (Supplementary Figure S2A). Furthermore, we observed that genetic modification had no discernible effect on the frequency of CD4+, CD8+, double positive (DP, CD4+/CD8+), and double negative (DN, CD4-/CD8-) T cells (Supplementary Figures S2B, C). These frequencies remained consistent across all conditions, which is crucial as both CD4 and CD8 T cells contribute to anti-tumor activity (23).

## 3.3 Multi target anti-tumor efficacy of UniCARs

Once the viability and phenotype of the engineered T cells were verified, the effector cell cytotoxicity was assessed by co-culturing engineered T cells with K562 and K562-CD19 cells, after evaluating the CD19 expression on modified K562-CD19 cells (Supplementary Figure S3A). As CAR19-T cells did not need any intermediary to exert their function, their elevated ability to kill is solely triggered by

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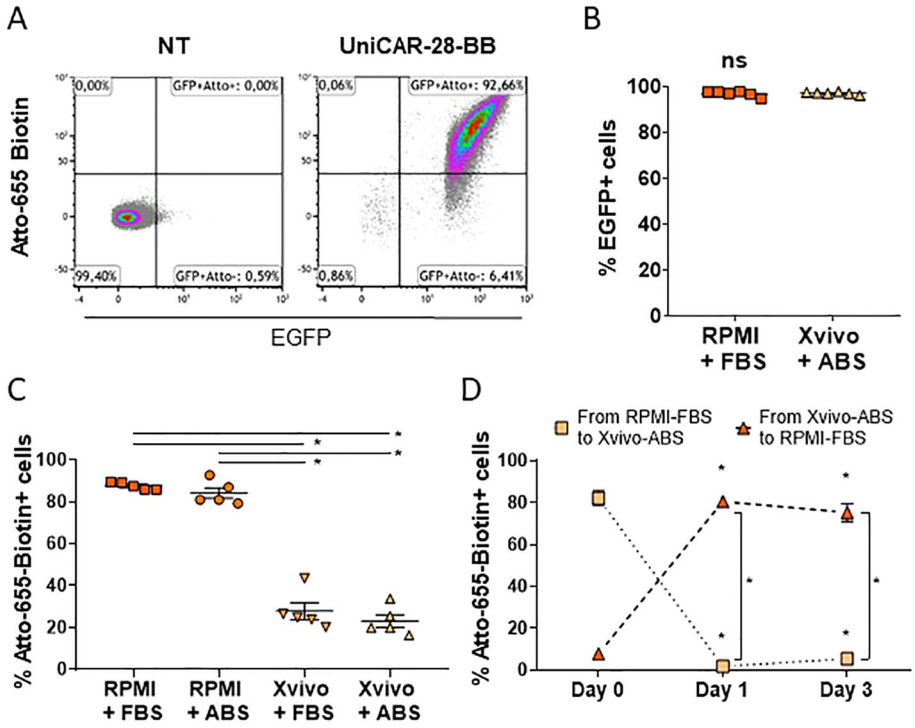


FIGURE 2
Components of culture media can neutralize the binding capacity of UniCAR to biotin. **(A)** Flow cytometry dot plots depicting non-transduced (NT) and transduced Jurkat cells with lentivectors encoding 3rd generation UniCAR-28-BB. Cells cultured in RPMI 1640 + 10% FBS were labeled with Atto-655 and followed by eGFP expression. These dot plots serve as a representative example from n=11. **(B)** Graph illustrating eGFP expression in UniCAR-28-BB Jurkat cells cultured in RPMI 1640 + 10% FBS and in Xvivo15 + 5% ABS, along with their mean + SEM. Ns= non-significant difference as determined by the Mann-Whitney comparison test. **(C)** Graph depicting the frequencies of Atto-655-Biotin-labeled UniCAR-28-BB-Jurkat cells when cultured in RPMI 1640 + 10% FBS or + 5% ABS and in Xvivo15 + 10% FBS or + 5% ABS, with their mean ± SEM. *Significant difference between conditions with $p<0.05$, as determined by the one-factor ANOVA multiple comparisons test. **(D)** Graph depicting the change in frequencies of Atto-655-Biotin-labeled UniCAR-28-BB Jurkat cells which had been cultured in RPMI 1640 + 10% FBS and moved to Xvivo15 + 10% FBS or had been cultured in Xvivo15 + 10% FBS and moved to RPMI 1640 + 10% FBS, with their mean ± SEM. *Significant difference between conditions with $p<0.05$, as determined by the 2way ANOVA multiple comparisons test.

the direct recognition of the CD19 target protein on K562-CD19 cells, while maintaining low cytotoxicity against K562 negative for CD19, as expected (Figure 4A). There were also no differences in the low cytotoxic capacities of any of the UniCAR-T cells when they were co-cultured with K562 and K562-CD19 in the absence of any intermediary. However, when all UniCAR-T cells were co-cultured with K562-CD19 and αCD19 biotinylated Ab, their cytotoxicity was significantly increased, being comparable to CAR19-T cells (Figure 4A).

To test the multi target capacity of the UniCAR, we co-cultured engineered T cells with Raji cell line, which equally expressed CD19 and CD20 markers (Supplementary Figures S3A, B). CAR19-T cells showed the same cytotoxic ability on Raji as on K562-CD19, as expected (Figure 4B). When αCD19 biotinylated Ab was added, UniCAR-28-BB and UniCARCD28-T cells exhibited a slight, but significant, increase in their cytotoxicity towards Raji cells compared to non-transduced (NT) T cells. On the other hand, UniCAR41BB-T cell condition did not show any clear cytotoxicity increase. Furthermore, each UniCAR-T subset demonstrated significantly greater cytotoxic capacities when utilizing αCD20 biotinylated antibody compared to NT-T cells when co-cultured with Raji cells. The cytotoxicity observed in Raji + anti-CD20-Biotin + UniCAR-T cells reached the level of cytotoxicity observed in Raji + CAR19-T cells (Figure 4B). To validate the specificity of UniCAR-T cell cytotoxicity dependent on the UniCAR and the correct biotinylated intermediary, we co-cultured engineered T cells with K562 cells lacking CD19 expression, supplemented with αCD19 biotinylated antibody. Results showed that although UniCAR41BB-T cells induced a slight increase in cytotoxicity, it was not significant. However, UniCARCD28-T cells induced toxicity in K562 cells despite their lack of CD19 on their surface, upon addition of the antibody (Figure 4C, Supplementary Figure S4 for the statistical representation of inter-condition differences). Conversely, none of the UniCAR-T cell subsets induced death in K562-CD19 cells when co-cultured with non-biotinylated αCD19 antibody (Figure 4D). Thus, these findings suggest that the presence of biotin alone was sufficient to activate the cytotoxic activity of UniCARCD28-T cells, independently of specific target antigen engagement. To further explore this non-specificity phenomenon, UniCAR-T cells were co-cultured with K562-CD19 or Raji cells in the presence of a non-specific biotinylated IgG, which does not recognize either CD19 or CD20 markers. The presence of non-specific biotinylated IgG increased significantly the UniCARCD28-T cell cytotoxic activity compared with the NT condition in Raji cell

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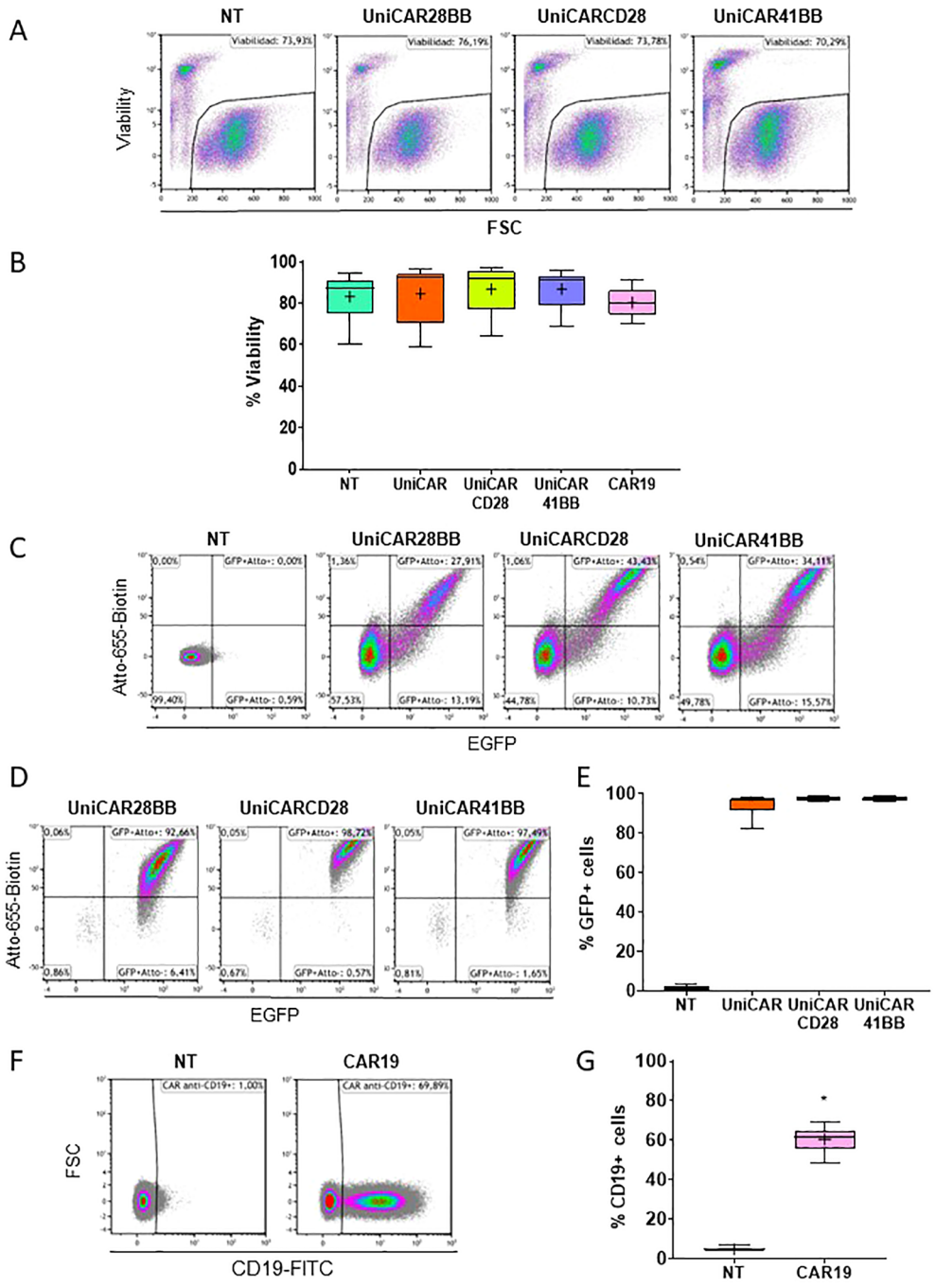


FIGURE 3
Viability and frequency of effector T cell transduction. **(A)** Flow cytometry dot plots representing the viability (visualized by the 7AAD negative signal) of Non-transduced (NT), UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 transduced T cells. These dot plots are a representative example of n=6. **(B)** Box and whiskers representing the frequency of viability of NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells, n=6. **(C)** Flow cytometry dot plots representing the frequencies of the eGFP and Atto-655 detection of NT, UniCAR-28-BB, UniCARCD28, and UniCAR41BB T cells before sorting. **(D)** Flow cytometry dot plots representing the frequencies of the eGFP and Atto-655 detection on NT, UniCAR-28-BB, UniCARCD28, and UniCAR41BB T cells after sorting. These dot plots are a representative example of n=6. **(E)** Box and whiskers representing the frequency of eGFP expression on NT, UniCAR-28-BB, UniCARCD28, and UniCAR41BB T cells, n=6. **(F)** Flow cytometry dot plots representing the frequency of CD19-FITC expression on NT and CAR19 T cells. These dot plots are a representative example of n=6. **(G)** Box and whiskers (10-90 percentile, the + symbol is the mean) representing the frequency expression of CD19-FITC on NT and CAR19 T cells, n=6.

cocultures, but not with K562-CD19 cells (Figures 4E, F, Supplementary Figure S4 for the statistical representation of inter-condition differences). The co-culture of K562-CD19 or Raji cells with non-specific biotinylated IgG did not significantly increase the cytotoxicity of UniCAR-28-BB-T and UniCAR41BB-T cells (Supplementary Figure S4).

In summary, UniCAR-T cells showed efficacy in inducing cell death in two different target cell lines, achieving cytotoxicity levels

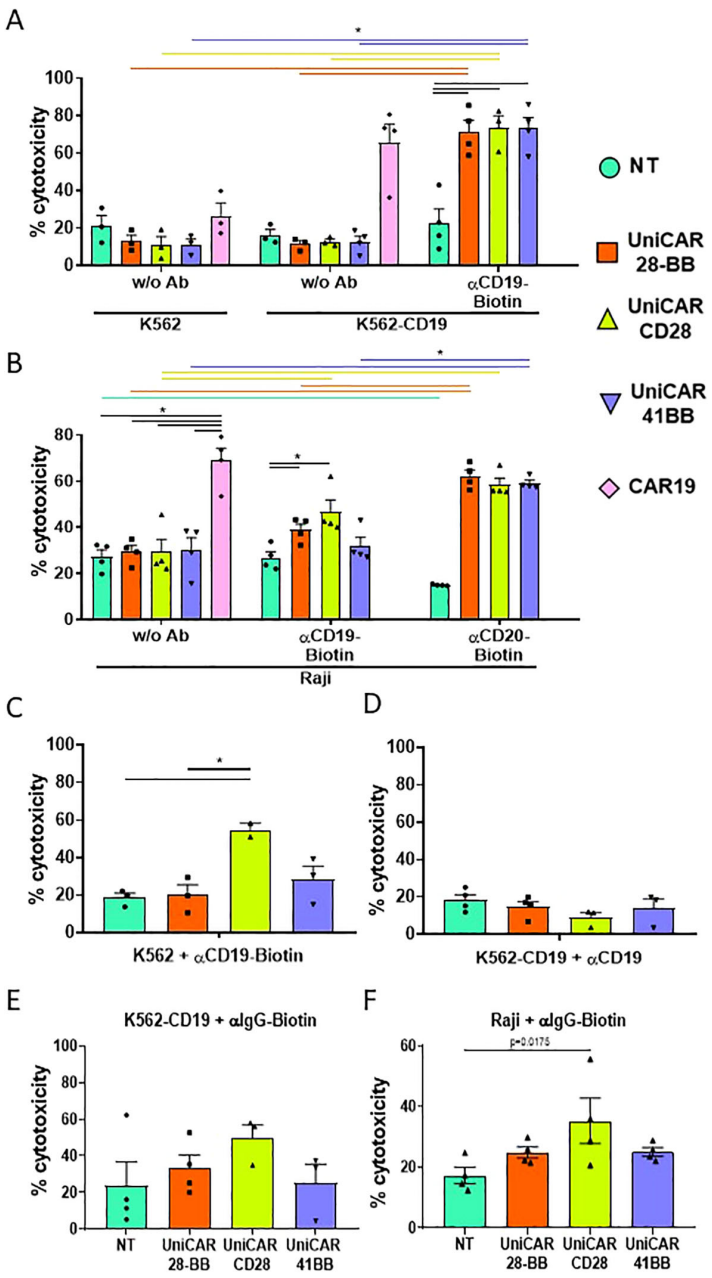


FIGURE 4
Cytotoxicity assay on target cell lines. **(A)** Histogram representing the frequencies of cell death (referred to as cytotoxicity, mean ± SEM) of CTVio+ K562 and K562-CD19 cells after 72 h of co-culture with Non-transduced T (NT), UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells, both in the absence of intermediate antibody (w/o Ab) or with biotinylated αCD19 Ab. Significant differences were determined using a 2way ANOVA multiple comparisons, corrected with the Tukey's test. **(B)** Histogram representing the cytotoxicity of CTVio+ Raji cells after 72 h of co-culture with NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells, in absence of intermediate antibody, with biotinylated αCD19 Ab, or with biotinylated αCD20 Ab (mean ± SEM). Significant differences were determined using a 2way ANOVA multiple comparisons, corrected with the Dunn's test. **(C)** Histogram representing the frequencies of cell death (mean ± SEM) of CTVio+ K562 cells (which do not express CD19) after 72 h of co-culture with NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells, stimulated with biotinylated αCD19 Ab. Significant differences were determined using a 1way ANOVA multiple comparisons (Kruskal-Wallis's test). **(D)** Histogram representing the frequencies of cell death (mean ± SEM) of CTVio+ K562-CD19 cells after 72 h of co-culture with NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells, stimulated with the non-biotinylated αCD19 Ab. Significant differences were determined using a 1way ANOVA multiple comparisons (Kruskal-Wallis's test). **(E)** Histogram representing the frequencies of cell death (mean ± SEM) of CTVio+ K562-CD19 cells after 72 h of co-culture with NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells, stimulated with the non-specific biotinylated αIgG Ab. Significant differences were determined using a 1way ANOVA multiple comparisons (Kruskal-Wallis's test). **(F)** Histogram representing the frequencies of cell death (mean ± SEM) of CTVio+ Raji cells after 72 h of co-culture with NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells, stimulated with the non-specific biotinylated αIgG Ab. Significant differences were determined using a 1way ANOVA multiple comparisons (Kruskal-Wallis's test). All co-culture conditions were performed with a ratio 1:1 (effector T cell: Target cell). Each data point represents one experiment. *: Significant differences between indicated conditions are depicted by black lines, and significant differences inter-conditions are indicated by colored lines which correspond to the histogram's color code. Inter-conditions statistics for **(C–F)** were depicted on Supplementary Figure S4. Significant differences when $p<0.05$.

nearly comparable to classical CAR19-T cells, depending on the target antigen. However, second-generation UniCARCD28-T cells displayed non-specific cytotoxicity when exposed to any biotinylated antibodies, regardless of the presence of the target marker on the cell surface.

## 3.4 Third-generation UniCAR-28-BB-T cell activation was more specific than both second-generation UniCAR-T cells

CAR-T cell activity is related to the ability to recognize their specific targets and subsequently to be activated through CAR intracellular signaling domains (24). Given that UniCARCD28-T cells exhibited non-specific cytolytic activity, understanding their activation patterns becomes crucial. We first observed the expression of the CD25 marker on effector UniCAR-T cells when co-cultured with K562 or K562-CD19 target cell lines (Supplementary Figure S5). As it was expected, the frequency of CD25+ in conventional CAR19-T cells increased when they were in contact with K562-CD19 (69.8 ± 6.5%, % ± SEM) but not with K562 (24.6 ± 7.4%, % ± SEM, Figure 5A, Supplementary Figure S5, and Supplementary Figure S6A for the statistical representation of inter-condition differences). UniCAR-28-BB-T cells exhibited a basal activation level, as evidenced by CD25 expression without Ab (in K562: 14.1 ± 2.7%, in K562-CD19: 35.5 ± 13.8%, % ± SEM), similar to NT-T cell co-culture conditions with K562 (14.1 ± 3.7%), or K562-CD19 (31.8 ± 12.0%, % ± SEM). However, when biotinylated αCD19 Ab was added in the culture medium with the specific target K562-CD19 cells, frequency of CD25+ UniCAR-28-BB-T was significantly higher than the CD25 level in the NT control condition (86.8 ± 6.0% and 22.8 ± 4.6%, % ± SEM, respectively, Figure 5A).

Although the frequencies of CD25 expression levels on UniCAR41BB (85.8 ± 5.8%) and UniCARCD28-T cells (89.1 ± 4.9%) were similar to UniCAR-28-BB-T cells under the experimental condition of K562-CD19 + biotinylated-αCD19 antibody, they also exhibited higher CD25 expression in several control conditions, especially UniCARCD28-T cells. Thus, UniCARCD28-T cells showed a nonspecific CD25 expression in the co-culture with K562 + biotinylated αCD19 Ab (65.6 ± 10.4%), K562-CD19 without Ab (51.1 ± 15.5) and K562-CD19 + biotinylated IgG (75.0 ± 10.3%, % ± SEM), while CD25 expression of UniCAR41BB-T cells was only elevated in K562 + biotinylated αCD19 Ab condition (50.3 ± 9.8%, % ± SEM, Figure 5A). The expression level of CD25 was also measured on CD25+ living effector cells using Mean Fluorescence Intensity (MFI, see Supplementary Figure S7A). Although there were non-specific increases in the frequency of CD25+ cells in some culture control conditions (Figure 5A), only the K562-CD19 + αCD19-Biotin condition induced a significant increase in CD25 MFI on effector cells.

Similar results were also observed when UniCAR and CAR19-T cells were co-cultured with the Raji cell line. It is notable that the frequency of CD25+ cells bearing every UniCAR construct was increased, not only with biotinylated αCD19 antibody but also with biotinylated αCD20 antibody, confirming their multi-target recognition ability (Figure 5B, Supplementary Figure S6B for the statistical representation of inter-condition differences). In summary, 2nd generation UniCARCD28 and, to a lesser extent, UniCAR41BB-T cells exhibited nonspecific activity in the presence of their biotinylated ligand, regardless of whether the target marker was present on the objective cells (Figure 5B).

In patients, ideally, UniCAR-T cells should not be activated once the tumoral target cells have been eliminated, as sustained cellular activation could lead to hyper-activation of the immune system and exhaustion of the therapeutic cells. Therefore, we also studied the frequency of CD25+ effector T cells when cultured alone (with or without antibody intermediaries) to test if this phenomenon was also observable in the absence of target cells. It was seen that CD25+ frequency expression on UniCAR-28-BB-T cells remained at low levels, regardless of the antibody with which they were cultured or in the absence of antibody (with biotinylated αCD19 Ab; 28.5 ± 9.2%, with αCD19 non biotinylated; 15.8 ± 5.4%, with biotinylated αIgG; 26.8 ± 7.9%, and without Ab; 16.5 ± 6.0%, % ± SEM), as the NT negative control (with biotinylated αCD19 Ab; 12.1 ± 2.7%, with αCD19 non biotinylated; 10.2 ± 2.6%, with biotinylated αIgG; 10.7 ± 2.3% and without Ab; 11.5 ± 2.9%, % ± SEM, Figure 5C, Supplementary Figure S6C for the statistical representation of inter-condition differences). Therefore, in the absence of target cells, even in the presence of the specific biotinylated antibodies, UniCAR-28-BB-T cells did not exhibit significant activation. On the other hand, frequencies of CD25+ UniCAR41BB-T cells and, especially, CD25+ UniCARCD28-T cells exhibited significantly increases when cultured with biotinylated αCD19 antibody (52.8 ± 8.8% and 70.5 ± 8.0%, % ± SEM, respectively) or non-specific biotinylated IgG (38.3 ± 6.8% and 62.1 ± 6.0%, % ± SEM, respectively), even in the absence of any target cell, compared to NT and UniCAR-28-BB-T cells (Figure 5C).

All these results were confirmed by observing a similar expression pattern for other activation markers, such as ICOS (Figure 6). ICOS is an activation marker and has already been identified as a potential indicator of CAR-T immunotherapy success (25). The frequency of cells expressing ICOS, like CD25, increased when all UniCARs T-cells were co-cultured with K562-CD19 + biotinylated-αCD19 (Figure 6A), although their MFI did not change (Figure 6B). Notably, the frequency of ICOS+ UniCARCD28-T cells exhibited significant non-specific increases when cultured with K562 and biotinylated-αCD19 (50.34 ± 9.5%, % ± SEM), or with K562-CD19 and non-specific biotinylated-IgG (54.35 ± 6.5%), compared to the NT condition with the biotinylated-IgG (21.70 ± 3.7%, % ± SEM, Figure 6A). Other activation markers, CD71 and PD-1, were analyzed and showed a similar pattern to CD25 on UniCAR-T cells and NT-T cells when exposed to K562, K562-CD19 or Raji cells (Supplementary Figure S8) or when cultured alone without target cells (Supplementary Figure S9). However, their MFI was significantly increased only with the specific combination of K562-CD19 + biotinylated-αCD19 (Supplementary Figures S7B, C).

Finally, the significant correlation and linear regressions between effector T cell activation (CD25 expression) and their

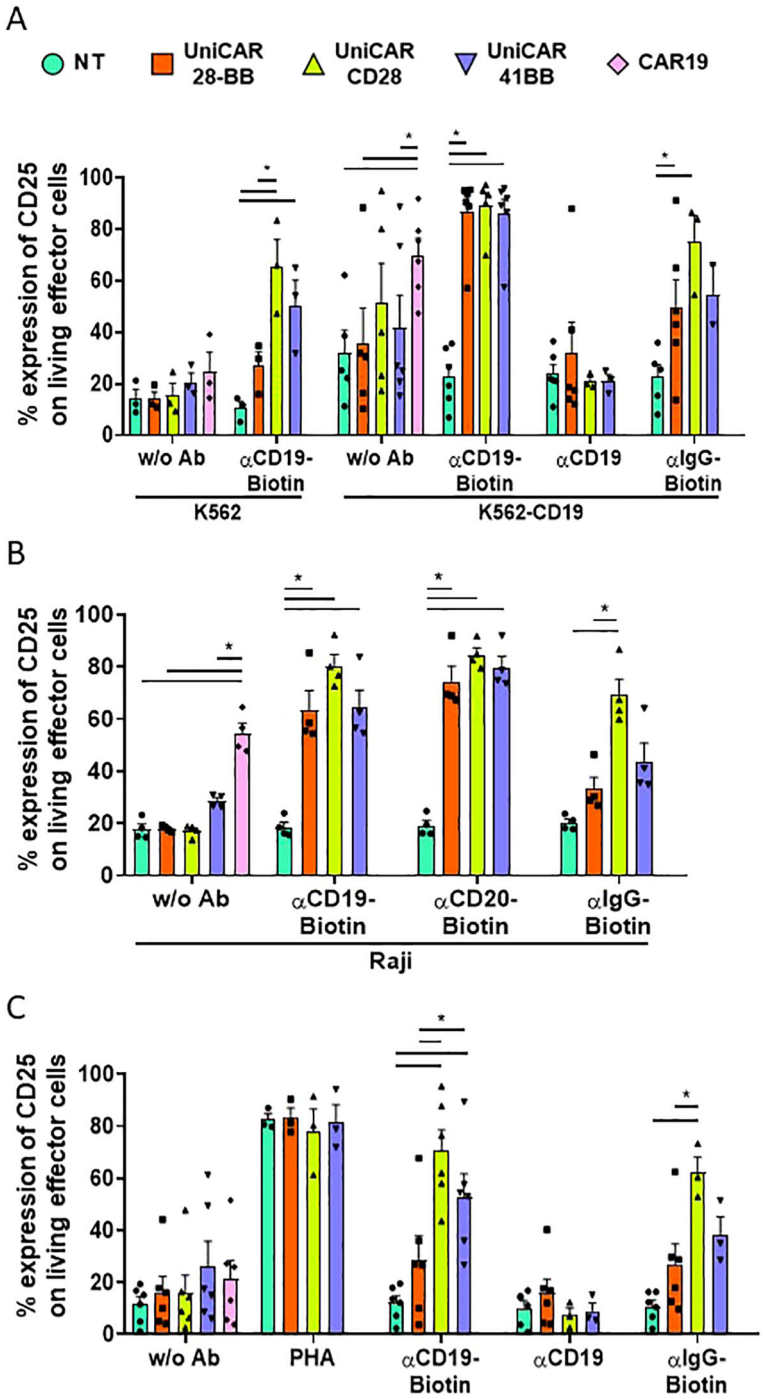


FIGURE 5
Activation of effector T cell subsets measured by CD25 expression. **(A)** Histogram representing the frequencies of CD25+ cells on NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells (gated on living cells, mean ± SEM) after 72 h of co-culture with CTVio+ K562 and CTVio+ K562-CD19 cells, in the absence of antibody intermediate, or with biotinylated αCD19 Ab, non-biotinylated αCD19 Ab or with biotinylated IgG. *Significant differences between indicated conditions. Inter-conditions statistics for **(A)** were depicted on Supplementary Figure S6A. **(B)** Histogram representing the frequencies of CD25+ cells on NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells (gated on living cells, mean ± SEM) after 72 h of co-culture with CTVio+ Raji cells, in the absence of intermediate antibody, or with biotinylated αCD19 Ab, biotinylated αCD20 Ab or with biotinylated IgG. *Significant differences between indicated conditions. Inter-conditions statistics for **(B)** were depicted on Supplementary Figure S6B. **(C)** Histogram representing the frequencies of CD25+ cells on NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells (gated on living cells, mean ± SEM) after 72 h of culture alone in the absence of intermediate antibody, or with PHA, biotinylated αCD19 Ab, biotinylated αCD20 Ab or biotinylated IgG. *Significant differences between indicated conditions. Inter-conditions statistics for **(C)** were depicted on Supplementary Figure S6C. Significant differences when $p<0.05$, as determined by the by 2way ANOVA multiple comparisons test, corrected with the Tukey's test. All co-culture conditions were performed with a ratio 1:1 (effector T cell: Target cell). Each data point represents one experiment.

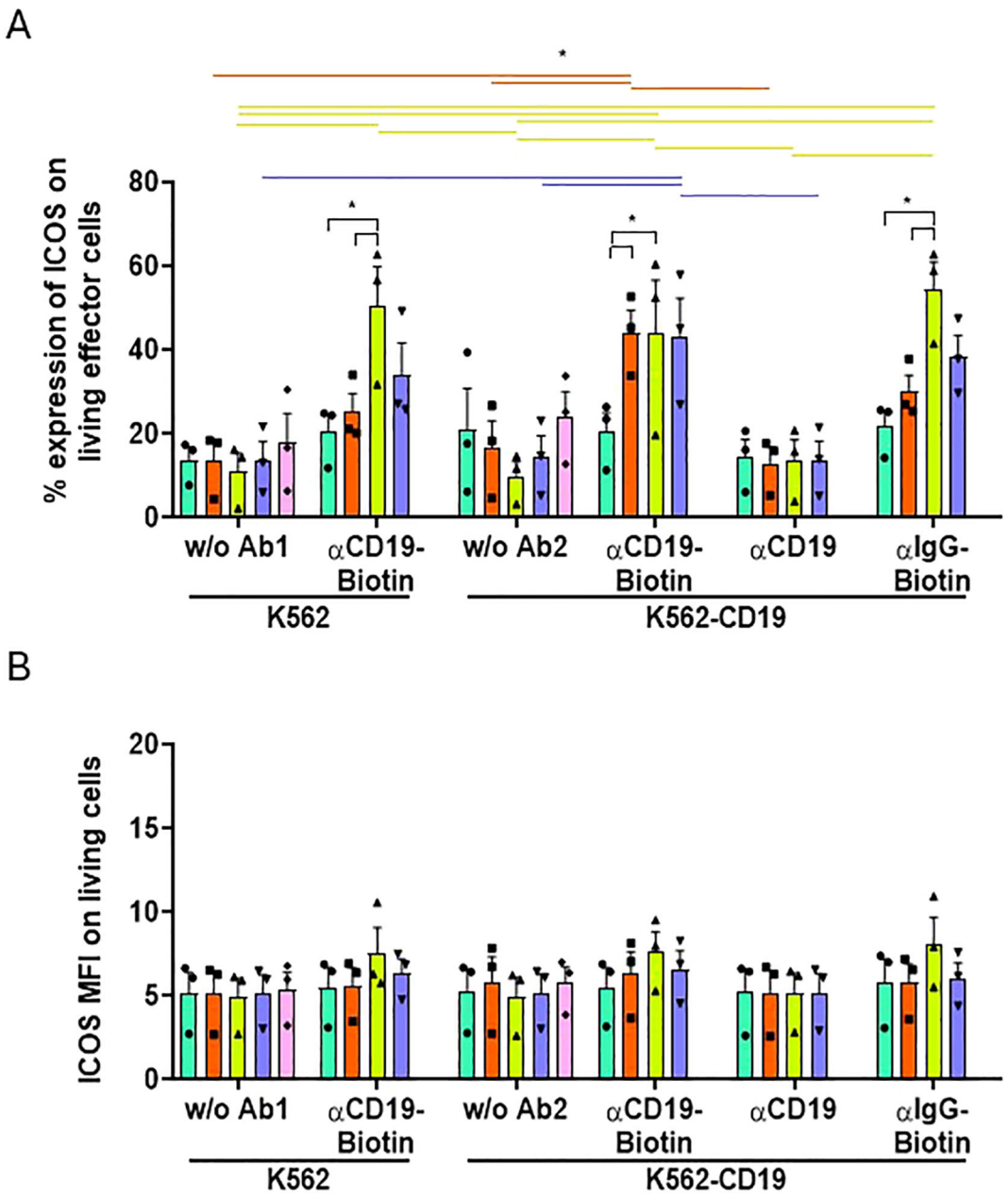


FIGURE 6
Activation of effector T cell subsets measured by ICOS expression. **(A)** Histogram representing the frequencies of ICOS+ cells on NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells (gated on living cells, mean ± SEM) after 72 h of co-culture with CTVio+ K562 and CTVio+ K562-CD19 cells, in the absence of antibody intermediate, or with biotinylated αCD19 Ab, non-biotinylated αCD19 Ab or with biotinylated IgG. *: Significant differences between indicated conditions are depicted by black brackets, and significant differences inter-conditions are indicated by colored lines which correspond to the histogram's color code. **(B)** Histogram representing the Mean of Fluorescent Intensity (MFI) of ICOS+ cells on NT, UniCAR-28-BB, UniCARCD28, UniCAR41BB and CAR19 T cells (gated on living cells, mean ± SEM) after 72 h of co-culture with CTVio+ K562 and CTVio+ K562-CD19 cells, in the absence of antibody intermediate, or with biotinylated αCD19 Ab, non-biotinylated αCD19 Ab or with biotinylated IgG. Significant differences when $p<0.05$, as determined by the by 2way ANOVA multiple comparisons test, corrected with the Tukey's test. All co-culture conditions were performed with a ratio 1:1 (effector T cell: Target cell). Each data point represents one experiment.

cytolytic activity over K562, K562-CD19 ($r=0.5141$; $p<0.0001$) and Raji ($r=0.5351$, $p<0.0001$) target cells showed that the higher the activation induced by each UniCAR or CAR19 on T cells, the higher the cytotoxic ability against both cell lines used in this study (Figure 7). Therefore, the specific activation of UniCAR-28-BB-T cells and the non-specific activation of UniCARCD28- and UniCAR41BB-T cells could preclude their cytotoxic capacity.

## 4 Discussion

The use of mSA2-UniCAR structures may overcome some limitations observed in classical antigen-specific CAR structures, such as modulation of functionality based on the presence or absence of an intermediate. However, incorporating a third element, such as biotinylated intermediates, introduces new

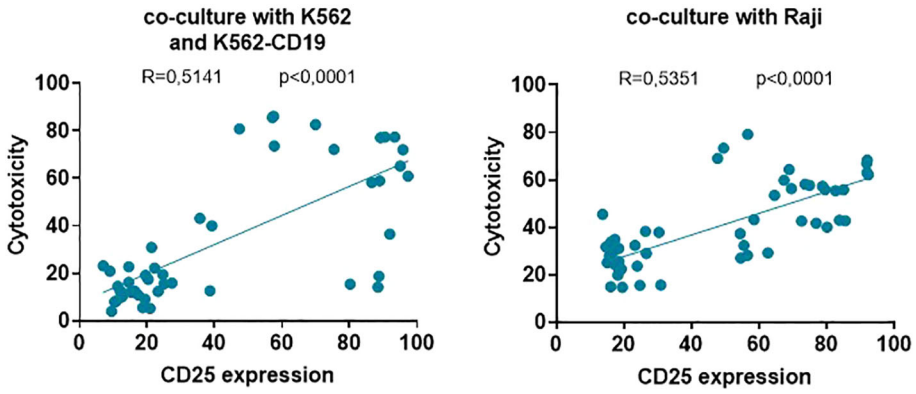


FIGURE 7
Correlations between effector T cell activation and cytotoxicity of target cell lines. Correlations and linear regression between the frequency of effector T cells positive for the CD25 marker and the percentage of cytotoxicity of K562 and K562-CD19 cells (left) and Raji cells (right). Correlations were determined by Pearson's rank correlation and considered statistically significant when $p < 0.05$. Each symbol corresponds to one culture condition.

potential impediments. Indeed, as we have observed, culture conditions could impair the availability of biotin recognition. While the classical complete medium culture RPMI 1640 supplemented with human serum did not inhibit the mSA2/Biotin interaction, another commonly used culture medium containing components not defined by the manufacturer abolished this union. The presence of biotin in culture media might affect the binding of UniCAR-cells to their biotinylated intermediates. However, according to the manufacturer's information, the biotin concentration in the RPMI 1640 medium used in this study is 200 ng/mL, whereas circulating serum concentrations of biotin in the general population typically range from 0.1 ng/mL to 0.8 ng/mL (26). Therefore, it is unlikely that this biotin concentration would hinder the proper mSA2-UniCAR/biotin binding. Therefore, as this phenomenon is independent of serum supplementation, we do not foresee any difficulties in future animal models or in its translation to human applications.

Other essential factor to consider is that, for successful therapeutic approaches using CARs, the appropriate co-stimulatory domain, or combination thereof, must be selected for each strategy (10). In fact, understanding how the structure of a UniCAR affects the T cell function is critical to guide their clinical implementation. In this study, second generation UniCARCD28- and UniCAR41BB-T cells presented a nonspecific activity when in contact with a biotinylated intermediate, regardless of their antigen specificity or the presence of target cells, while third generation UniCAR-28-BB-T cells were only activated by the correct combination of the target cell and the specific biotinylated intermediary.

The difference in the combination of the UniCAR's co-stimulation domains likely explains the differences in recognition specificity, which in turn influence the cellular activation pattern and cytotoxic ability. In T cells, conventional CARs based on CD28 are characterized by intense cellular activation, consequently leading to rapid cytolytic capacity and tumor elimination, both of which are significantly higher than those exerted by CAR41BB (27–31). Conversely, CAR structures based on 4-1BB mediate a more sustained cellular activation, enhancing proliferation and mitigating exhaustion of effector cells compared to CAR-T cells based on CD28 (9, 28, 31–33). In non-conventional CAR-T approach such as UniCAR-T cells, which need intermediaries to be activated, the correlation between specificity for the intermediaries and the cellular functionality must be extensively studied to avoid non desired cellular hyper-activation. Sun et al. described that the origin of the differences between these two co-stimulatory signals could be attributed to the kinase LCK, recruited by the joint action of the CAR and the co-receptors CD4 or CD8. This group reported that the phosphorylation sites that this LCK kinase has on the CD3-ζ chain are basally phosphorylated in CAR constructs with the CD28 sequence, triggering a highly elevated and permanent tonic activation signal promoted by an increase in the glycolysis metabolic pathway (34).

We hypothesized that the tonic basal activation of CARCD28-T cells could contribute to their non-specificity. Indeed, transmembrane and intracellular CD28 CAR domains are known to induce a heterodimerization with endogenous CD28 and increase homodimers of endogenous CD28, which may induce off-target activation (35). Additionally, the formation of CAR clusters can lead to antigen-independent tonic signaling and subsequent cellular activation (9, 36). In our study, we observed by flow cytometry that median fluorescence intensity of UniCARCD28 and UniCAR41BB structures expression (subset eGFP+ Atto-655-Biotin+) was higher than that of UniCAR-28-BB expression in T cells. The increased presence of UniCAR proteins on the cell surface may promote the formation of such UniCAR clusters, potentially leading to nonspecific subsequent activation (Figures 3C, D). This hypothesis is supported by a comparative study of CD28, 4-1BB or CD28 + 4-1BB based CAR-T cells that showed that the CAR-CD28 structures generate large clusters with high CAR dimers density at the surface of the cells and hence participate in the elevated cellular activation, possibly lowering the activation threshold (9, 37). Moreover, the UniCARCD28 clusters may increase UniCAR sensitivity for low abundant antigens or, in our case, for biotin regardless of the target cells presence (37, 38). Therefore, this enhanced tonic activation state could explain why

the intense cellular activation cascade is induced after contact only with the biotinylated antibody in UniCARCD28-T cells, while UniCAR41BB-T cells showed lower nonspecific activation. Since there was correlation between the cellular activation and the cytotoxic capacity, the tonic stimulation of UniCARCD28-T cells is likely associated with their non-specific ability to kill co-cultured cells. On the other hand, we cannot exclude the possibility that the lower MFI of UniCAR-28-BB on the surface of effector cells, likely due to the size of the construct affecting its transduction ability, could explain the lack of non-specific activity observed with this construct compared to the second-generation UniCARs. The lower presence of UniCAR-28-BB may decrease its sensitivity to low-abundance antigens, such as biotin in our case, and consequently increase the minimum threshold required for activation signals. Tonic activation could potentially lead to cytokine release syndrome or immune cell-associated neurotoxicity syndrome in patients undergoing adaptive CAR-T therapy. These results are partially contradictory with those shown by Lohmueller et al. (17). Although they demonstrated that their mSA2-UniCAR-T cells with CD28 or 4-1BB increased their expression of CD69 when cultured with specific biotinylated Ab, they did not observe activation of UniCARCD28 or UniCAR41BB-T cells in the presence of a biotinylated antibody for a non-relevant target. This difference with our second generation UniCAR-T cells cannot be explained by the structure of the UniCAR since they have the same CAR sequences under the same promoter. On the other hand, the backbone vectors differ, and it is known that vector structures can influence the transduction efficiency. Our vectors may allow for an increased number of integrated molecules, thereby increasing the number of UniCARs on the cell surface. As previously mentioned, the quantity of these molecules might affect the response to the target antigen by either lowering or raising the activation threshold. Without comparing the vector structures directly, we cannot fully explain the observed differences in specificity for activation. However, our findings may be pivotal and could justify a more in-depth analysis.

In our efforts to combine the potent cytotoxicity of CD28-based CARs with the reduced exhaustion and long-term persistence of 4-1BB-based CARs, we observed that third generation UniCAR-28-BB-T cells exhibited significant activation only in the presence of the correct biotinylated intermediary antibody and the appropriate target cells. However, in this study, the exhaustion of effector cells was not examined and should be addressed in future research, given its significance in CAR-T immunotherapy approaches.

Furthermore, this specific cytolytic capacity was observed with two different tumor cell lines, K562-CD19 and Raji, targeting two different antigens, CD19 and CD20. The activation of UniCAR-T cells, as assessed by measuring CD25, ICOS, CD71, and PD-1 using flow cytometry, was specific to co-culturing with Raji cells and the specific biotinylated antibodies. However, it is important to note that the cytotoxic activity of all UniCAR-T cells combined with biotinylated-αCD19 was lower against Raji cells compared to that observed with biotinylated-αCD20. This discrepancy may be explained by the downregulation of CD19 that might impede subsequent cell death. To support this hypothesis, previous studies have shown that in co-culture experiments with Raji cells and classical αCD19 or αCD20 CAR-T cells, there is a rapid down-modulation of full-length CD19 expression, and to a lesser extent, CD20 (39). Furthermore, when anti-CD19 or anti-CD20 antibodies were associated with liposomes to develop an intracellular drug delivery system targeting B cells, anti-CD19 was more effective, as it was internalized more rapidly than anti-CD20, which was considered to be a non-internalizing antibody (40). The rapid and extensive down-modulation of CD19 expression at the cell surface could impair target cell recognition by effector cells, allowing Raji cells to evade cell death and continue replicating. However, the cytotoxic capacity of UniCAR-28-BB-T cells did not surpass that exerted by both second generation UniCARs-T cells, as reported by some authors in their animal model studies (41–44) or even in clinical trials (45). These studies suggested that the accumulation of multiple additional co-stimulatory signals might not induce enhanced cytotoxic capacity and could even lead to a detriment in cellular functionality. However, those studies primarily utilized conventional CAR structures, and the requirement for a biotinylated intermediary could potentially introduce differential flexibility to the entire UniCAR structure. It has already been demonstrated that each element of the CAR structure, not limited to their intracellular domains, can influence CAR signaling, thereby affecting activation and functionality. Hence, a comprehensive analysis of the individual components of the structure could aid in the design of future CAR structures that are both highly specific and functional.

Due to their therapeutic potential, two clinical trials (NCT03190278 and NCT04633148) based on a switchable universal CAR-T platform, where UniCAR-T cell activity depends on the presence of a soluble adapter, will start soon (46). These clinical trials demonstrate the significant interest in a flexible CAR structure for future immunotherapies. Our results suggest that UniCAR-28-BB-T cells could represent a highly specific therapeutic approach with potent anti-tumor capabilities, particularly highlighting their ability to target multiple antigens.

## 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

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

## Author contributions

JG-V: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft,

Writing – review & editing. VP-F: Data curation, Investigation, Methodology, Validation, Writing – review & editing. JR-M: Data curation, Investigation, Methodology, Writing – review & editing. SG-M: Data curation, Investigation, Validation, Writing – review & editing. MC: Methodology, Resources, Writing – review & editing. EG-N: Methodology, Resources, Writing – review & editing. RC-R: Funding acquisition, Methodology, Resources, Writing – review & editing. MJ: Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing. MP: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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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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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1448752/full#supplementary-material

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# Oncolytic viruses: a potential breakthrough immunotherapy for multiple myeloma patients

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Oncolytic virotherapy represents an innovative and promising approach for the treatment of cancer, including multiple myeloma (MM), a currently incurable plasma cell (PC) neoplasm. Despite the advances that new therapies, particularly immunotherapy, have been made, relapses still occur in MM patients, highlighting the medical need for new treatment options. Oncolytic viruses (OVs) preferentially infect and destroy cancer cells, exerting a direct and/or indirect cytopathic effect, combined with a modulation of the tumor microenvironment leading to an activation of the immune system. Both naturally occurring and genetically modified viruses have demonstrated significant preclinical effects against MM cells. Currently, the OVs genetically modified measles virus strains, reovirus, and vesicular stomatitis virus are employed in clinical trials for MM. Nevertheless, significant challenges remain, including the efficiency of the virus delivery to the tumor, overcoming antiviral immune responses, and the specificity of the virus for MM cells. Different strategies are being explored to optimize OV therapy, including combining it with standard treatments and targeted therapies to enhance efficacy. This review will provide a comprehensive analysis of the mechanism of action of the different OVs, and preclinical and clinical evidence, focusing on the role of oncolytic virotherapy as a new possible immunotherapeutic approach also in combination with the current therapeutic armamentarium and underlying the future directions in the context of MM treatments.

KEYWORDS

**oncolytic viruses, multiple myeloma, immunotherapy, microenvironment, antitumor immunity**

# 1 Introduction

Multiple myeloma (MM) is a hematological malignancy that represents a significant therapeutic challenge due to its biological complexity, heterogeneity, and propensity to develop drug resistance (1, 2). Despite advances in therapeutic strategies, MM remains largely incurable, with frequent relapses and refractory disease (3).

In this context, the introduction of several immunotherapeutic approaches, such as monoclonal antibodies, bispecific antibodies, and CAR-T cell therapy, has changed the treatment landscape of MM. By harnessing the immune system's ability to recognize and eliminate malignant cells, immunotherapy offers a promising way to overcome treatment resistance and improve patient outcomes (4–6).

Among the new emerging anti-cancer treatments, oncolytic virotherapy has gained considerable attention as a complementary and synergistic therapeutic modality within the MM treatment paradigm (7). Oncolytic virotherapy utilizes genetically engineered or naturally occurring viruses that selectively infect and replicate within neoplastic cells, leading to their lytic destruction while sparing normal tissues (8). This orchestrated cascade of events triggers stronger immunogenic responses, including activating innate and adaptive immune effectors against the tumor or reversing immunologically exhausted compartments (8, 9). Indeed, oncolytic viruses (OVs) exert profound immunomodulatory effects within the tumor microenvironment, reshaping the balance between pro-inflammatory and immunosuppressive signals (10). By targeting key immunoregulatory cell populations and promoting the recruitment and activation of cytotoxic lymphocytes, oncolytic virotherapy enhances the local and systemic antitumor immune response, thereby increasing therapeutic efficacy and durability (10); OVs also could sensitize refractory tumors to subsequent therapeutic interventions (11).

Nevertheless, despite the considerable therapeutic potential of oncolytic virotherapy, there are still significant challenges that require further investigation and clinical evaluations to demonstrate its efficacy and safety. These include optimizing viral delivery and dissemination strategies, enhancing tumor specificity and minimizing off-target effects, addressing immune evasion mechanisms related to pre-existing immunity, and developing combination approaches with novel immunotherapeutic strategies to enhance therapeutic synergy and overcome resistance to conventional treatments (12).

# 2 Oncolytic virus direct mechanisms and immunogenic effects in multiple myeloma

A well-known antitumor mechanism employed by OVs involves replicating within cancer cells and subsequently causing their direct lysis. This process is referred to as direct virus-induced oncolysis.

The aberrant molecular landscape and disrupted cellular homeostasis that characterize MM are closely related to the preferential targeting of OVs towards neoplastic plasma cells (PCs) (13).

OVs leverage the overexpression of specific surface receptors on cancer cells as a route of targeted cell entry. Several of these receptors, including membrane cofactor protein (CD46), junctional adhesion molecule-A (JAM-A), intercellular adhesion molecule-1 (ICAM-1), and decay-accelerating factor (DAF) are frequently upregulated in MM cells (14–17). These receptors serve as molecular gateways for OVs, facilitating viral attachment, entry, and subsequent oncolysis (14–17).

OVs also take advantage of other molecular alterations that are commonly found in MM cells. These alterations enable the replication of the viruses selectively, thus enhancing their efficacy.

A pivotal player in this context is the dysregulated RAS signaling pathway (13, 18). RAS-transformed cancer cells often exhibit defects in the innate antiviral defense mediated by the double-stranded RNA (dsRNA)-activated protein kinase (PKR) pathway. This compromised antiviral response makes cancer cells more susceptible to viral infection and facilitates unrestrained viral replication, leading to enhanced oncolysis (19–21). Similarly, OVs take advantage of the dysregulation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) axis. The activation of this axis enhances viral internalization and endosomal sorting, facilitating viral propagation within host cells (22, 23).

MM cells often exhibit defects in the interferon (IFN) pathway, which they exploit to evade immune surveillance, and consequently, OVs replicate in tumor tissues without interference from the antiviral effects of interferons (24, 25).

The therapeutic efficacy of OVs also depends on the indirect activation of the immune system against tumor cells.

Indeed, following viral infection and tumor cell lysis, OVs induce immunogenic cell death (ICD) pathways within MM cells, triggering the release of cytokines, tumor-associated antigens (TAAs), and other danger signals, including damage-associated molecular pattern (DAMPs) and pathogen-associated molecular pattern (PAMPs). These are a potent stimulus for the maturation and activation of antigen-presenting cells (APCs), particularly dendritic cells, initiating a robust adaptive immune response against the tumor (24, 26–29). Additionally, virus-infected MM cells activate pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), thereby amplifying the immune response within the MM microenvironment (30). OVs also play a pivotal role in modulating the tumor microenvironment by polarizing infiltrating monocyte-derived macrophages from an M2 phenotype, associated with tumor-promoting activities, to an M1 phenotype, characterized by enhanced antitumor immune responses. This polarization is facilitated by the release of pro-inflammatory cytokines and chemokines from infected MM cells, reshaping the immune landscape to favor tumor suppression (28). Finally, some OV antigens or specific antigens loaded on genetically engineered OVs are implicated in reversing the exhausted T cell phenotype present in the tumor microenvironment. OVs can act as agnostic antigen vaccines, expanding the repertoire of cancer-specific

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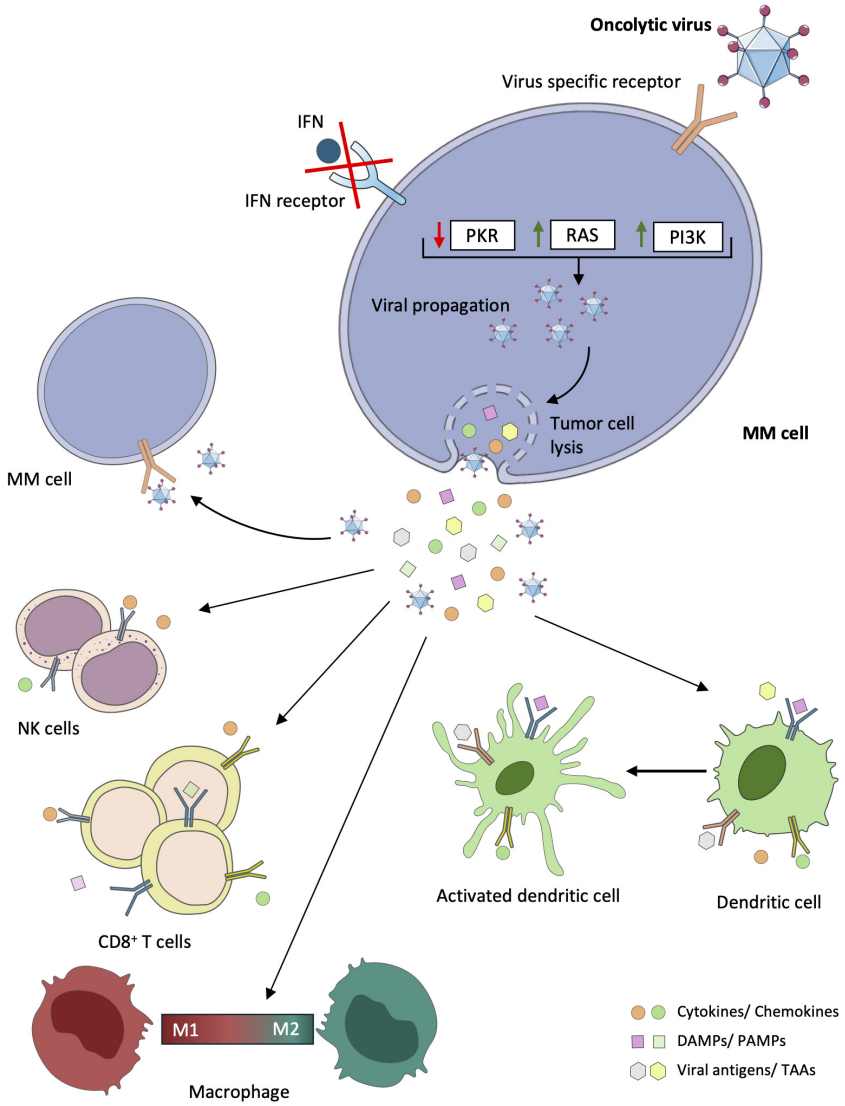


FIGURE 1
Direct and indirect antitumor effects of oncolytic viruses in multiple myeloma. In multiple myeloma (MM) cells, mutations or deletions are present in genes coding for key proteins of antiviral signaling pathways, including the interferon (IFN) pathway, the RAS pathway, the double-stranded RNA-activated protein kinase (PKR) pathway, and the phosphatidylinositol 3-kinase (PI3K) pathway. Consequently, oncolytic viruses (Ovs) can exploit these dysregulated signaling pathways in tumor cells to promote replication, infection, virus spread, and consequently lysis of tumor cells. Following viral infection, OVs can also induce tumor cell death through the mediation of immunogenic cells. Cytokines, viral elements (tumor-associated antigens (TAAs), viral pathogen-associated molecular patterns (PAMPs)), and cell damage-associated molecular patterns (DAMPs) can be released. These stimuli play a critical role in the recruitment and activation of immune cells, such as dendritic cells (DCs), natural killer (NK) cells, macrophages, and $CD8^+$ T cells, which can reverse the immunosuppressive microenvironment that characterizes MM.

neoantigens (29). Figure 1 illustrates the various mechanisms through which OVs exert their anti-MM cell effects.

## 3 Oncolytic virotherapy for multiple myeloma: preclinical and clinical evidence

Preclinical and clinical studies have examined the use of human and non-human viruses in the treatment of MM. Specifically, five RNA viruses (Measles virus, Reovirus, Coxsackievirus A21, Vesicular stomatitis virus, and Bovine viral diarrhea virus) and four DNA viruses (Adenovirus, Herpes simplex virus type 1, Vaccinia virus, and Myxoma virus) have been studied. These viruses have been evaluated in both monotherapy and combination therapy associated with chemotherapy and/or radiotherapy, as well as purging agents during autologous stem cell transplantation. Although these therapeutic viruses are derived from naturally occurring viral strains, they have been modified to increase their selectivity toward cancer cells or to improve their efficacy in eradicating MM. For instance, OVs have been engineered by integrating targeting ligands or peptides into their capsids to recognize MM-specific antigens or surface receptors, as well as

utilizing tissue-specific promoters or enhancers to restrict viral gene expression within MM cells (24, 28, 31–34). Similarly, the integration of therapeutic transgenes into OVs facilitates targeted delivery of cytotoxic agents, immune modulators, or proapoptotic factors specifically to MM cells, thereby enhancing the oncolytic effect and potentiating antitumor immune responses within the MM microenvironment (23, 28, 35, 36). Figure 2 illustrates the main receptors through which individual viruses enter MM cells, while Table 1 lists the clinical studies conducted to date. The following sections will provide a detailed overview of all viruses studied for the treatment of MM, with an analysis of the underlying molecular mechanisms and preclinical and clinical results up to this point.

## 3.1 Measles virus

Measles virus (MV) is a negative single-stranded RNA virus of the Paramyxovirus family. Its genome encodes six proteins, three of which are essential for viral envelope formation: matrix protein (M), hemagglutinin (H), and fusion protein (F) (37). The hemagglutinin facilitates entry into target cells by binding to cellular receptors such as CD46 and Signaling Lymphocytic Activation Molecule Family Member 1 (SLAMF1), which are particularly expressed in MM PCs, and Nectin-4 (14, 37). The vaccine strain currently used is the Edmonston strain (MV-Edm), which was first isolated from a patient in 1954. An important distinction among MV strains is their preference for specific host cell receptors: wild-type strains tend to prefer the SLAMF1 receptor, whereas it is known that MV-Edm selectively targets the CD46 receptor (37).

*In vitro* studies have shown excellent replication of MV-Edm in several MM cell lines and primary MM cells isolated from patients' bone marrow (BM), and a limited replication in phytohaemagglutinin (PHA)-stimulated peripheral lymphocytes (38). MV-Edm infection induces significant cytopathic effects in MM cells, leading to the formation of multinucleated syncytia and subsequent cell death (38).

Moreover, MV-Edm demonstrated antitumor efficacy by inhibiting MM cell engraftment in xenografts in immunocompromised mice (38).

Recently, a correlation between CD46 receptor expression and the tumor suppressor gene Tumor Protein P53 (TP53) has been highlighted. Lok et al. showed that TP53-deficient MM cells exhibit increased CD46 expression, with an increased susceptibility to MV infection compared to cells with functional TP53 (39).

To enhance the oncolytic efficiency of the MV-Edm virus and facilitate non-invasive imaging of infected tissues, a recombinant version expressing human sodium iodide symporter (NIS) was developed (40). This modified virus, designated MV-NIS, exhibited a replication rate comparable to MV-Edm (40). MM cells infected with MV-NIS have been shown to efficiently incorporate radioiodine. Serial gamma chamber imaging showed the intratumorally spread of the virus and the uptake of iodine-123 ($^{123}$I) in both MV-sensitive tumors that responded positively to MV-NIS treatment and non-responsive tumors. Notably, complete regression of MV-resistant tumors was observed when radioactive iodine, $^{131}$I, was administered 9 days after a single intravenous injection of MV-NIS (40).

To further optimize therapeutic efficacy, retargeted viruses derived from MV-Edm were developed. These viruses were engineered to selectively target MM cells, reducing side effects on healthy tissues. The incorporation of antibody fragments specific for PCs markers, such as CD38 or Wue-1, into the H envelope protein of the MV-Edm virus resulted in enhanced directional ability, contributing significantly to tumor growth inhibition and increased survival in animal models (31, 41).

In addition to direct strategies for MV treatment, alternative approaches have been developed involving the use of Cytokine-Induced Killer (CIK) cells as vectors for oncolytic therapy. CIK cells represent a heterogeneous subset of *ex vivo* expanded T lymphocytes that exhibit phenotypic and functional properties of both natural killer (NK) cells and T lymphocytes (42). The infected cells exhibited a high capacity to eradicate MM cells in both culture and animal models, significantly outperforming the efficacy of uninfected CIK cells (43).

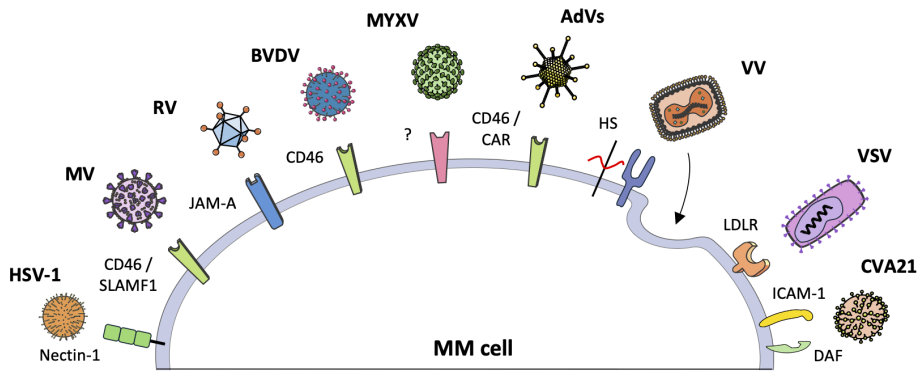


FIGURE 2
The interaction between oncolytic viruses and specific receptors in myeloma cells. Oncolytic viruses (OVs) can recognize and penetrate multiple myeloma (MM) cells through interaction with specific receptors on the cell surface. The figure illustrates the various OVs and the respective receptors with which they interact. Vaccinia virus and myxoma virus represent two distinct examples. Vaccinia virus employs endocytosis and membrane fusion to enter cells, forming syncytia. Myxoma virus has demonstrated efficacy against MM cells; however, its specific surface receptor remains unidentified.

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TABLE 1 List of oncolytic viruses currently being tested in myeloma clinical trials.

| Virus | Name | Phase | Combination | Trial No. | Status | References |
|---|---|---|---|---|---|---|
| Measles (MV) | MV-NIS | I/II | ± Cyclophosphamide | NCT00450814 | Completed | (29, 44) |
| | | II | Cyclophosphamide | NCT02192775 | Completed | |
| | | I | None | NCT03456908 | Completed | |
| Reovirus (RV) | Reolysin | I | None | NCT01533194 | Completed | (61) |
| | | I | Lenalidomide or pomalidomide | NCT03015922 | Unknown status | |
| | | I | Bortezomib + dexamethasone | NCT02514382 | Completed | (63, 64) |
| | | I | Carfilzomib + dexamethasone | NCT02101944 | Completed | (62) |
| | | I/II | Bortezomib + pembrolizumab | NCT05514990 | Recruiting | |
| | | I | Dexamethasone + carfilzomib + nivolumab | NCT03605719 | Completed | |
| Vesicular stomatitis virus (VSV) | VSV-IFNβ-NIS | I | ± Cyclophosphamide | NCT03017820 | Recruiting | (110) |

In parallel, clinical trials, such as the Phase I/II trial NCT00450814, evaluated the efficacy of MV-NIS in the treatment of MM (29, 44). A total of 32 patients were enrolled in this study. The 29 evaluable patients had a median age of 62 years and a median of 5 prior therapies. In Phase I, 13 patients were initially enrolled to receive various doses of MV-NIS. Although some patients experienced adverse reactions, including severe neutropenia, the maximum tolerated dose was not reached, and $TCID_{50}$ $10^{11}$ was established as the treatment dose for the Phase II trial (44). After confirming the safety of the initial doses, Phase 2 was designed with the addition of cyclophosphamide before MV-NIS treatment.

The study reported significant clinical improvement after MV-NIS treatment. One patient treated with $TCID_{50}$ $10^{11}$ achieved a complete response lasting 9 months with an isolated relapse in the skull without recurrent BM involvement. Four other patients showed a transient reduction in serum immunoglobulin-free light chains of at least 25% during the first 4 weeks of therapy, indicating a possible response to therapy. One patient had a subjective reduction and shrinkage of his extramedullary plasmacytomas on his back and thighs (44).

A subsequent study based on the same trial suggests that MV-NIS boosts anti-MM T cell responses in MM patients (29). Before virotherapy, more than 50% of patients showed T cell responses against multiple tumor-associated antigens, indicating existing immune activity against cancer cells. After MV-NIS treatment, T lymphocyte responses against specific antigens such as MAGE-A3 and MAGE-C1 were significantly enhanced. Particularly encouraging was the case of a patient enrolled in the study with high levels of cytotoxic T lymphocytes reactive to MV and tumor antigens. This patient achieved long-term complete remission, highlighting the potential of MV-NIS in combination with other immunomodulatory agents to support durable tumor remission in MM patients (29).

## 3.2 Reovirus

Reovirus (RV), also known as respiratory enteric orphan virus, got its name because it was initially not associated with any known disease. The Dearing strain of reovirus, classified as serotype 3 and commercialized as Reolysin for therapeutic purposes, is a ubiquitous, non-enveloped human virus with a genome of 10 double-stranded RNA segments (45). RV employs the JAM-A as a means of entering tumor cells (46). MM cells exhibit high expression of JAM-A, particularly in advanced stages of the disease or in the presence of treatment resistance (15). The replication is facilitated by the MM cells' suppression of the antiviral protein PKR, which allows for increased viral production (47). Altered PKR allows RV to circumvent this cellular defense mechanism, replicate efficiently, and cause lytic infection (30).

Several studies have demonstrated that RV induces endoplasmic reticulum (ER) stress and the expression of the pro-apoptotic protein NOXA, resulting in the apoptosis of MM cells (48, 49). RV also promotes autophagy in MM cells, contributing to reduced cell viability (50). In mouse models, the combination of RV with bortezomib has been shown to potentiate apoptotic activity, increasing ER stress and NOXA expression, while reducing MM tumor burden without significant adverse effects (48). Kennedy et al. identified nicotinamide adenine dinucleotide ($NAD^+$) as a critical factor in the susceptibility of MM cells to reovirus-induced oncolysis. Pharmacological inhibition of nicotinamide phosphoribosyl transferase (NAMPT), a key enzyme in the $NAD^+$ synthesis pathway, with FK866 sensitized MM cells to RV oncolysis, causing mitochondrial dysfunction and promoting autophagy and cell death (51). In addition, the concomitant administration of RV with histone deacetylase inhibitors (HDACi) resulted in an increased expression of JAM-A, rendering MM cells more susceptible to oncolytic action (52). Furthermore, RV demonstrated efficacy in the *ex vivo* purging of autologous stem cell transplantation, selectively killing MM cells while sparing healthy ones, thus improving therapeutic outcomes (53, 54).

Besides direct effects, it has been observed that RV exerts indirect effects on the immune system. In mouse models, RV has demonstrated the ability to significantly reduce tumor burden and MM-induced bone disease, which correlates with an increase in NK cells and effector memory $CD8^+$ T cells (55). The combination of

RV with lenalidomide or bortezomib has been shown to stimulate a robust antitumor immune response in preclinical studies (56–58).

The rationale for combining OV with bortezomib is based on the latter's ability to also induce the ICD. This process is characterized by the exposure of calreticulin on dying MM cells, their phagocytosis by dendritic cells, and the induction of a specific immune response against MM (59).

The use of RV together with lenalidomide and dexamethasone has been demonstrated to overcome the resistance of MM cells to direct viral death by activation of NK cells (56). RV also reduces the protection offered by BM stromal cells, thereby improving the overall efficacy of the treatment by lenalidomide and dexamethasone (56).

Moreover, Thirukkumaran et al. observed that co-treatment with bortezomib reduces regulatory T cells (Tregs) and suppressive myeloid cells (MDSCs), thereby increasing the activity of immune effector cells (57). Specifically, the RV-bortezomib combination stimulates the production of pro-inflammatory cytokines such as interferon-gamma (IFN-γ), creating an inflammatory environment that potentiates the activity of NK and $CD8^+$ T cells.

The increased expression of immune markers such as PD-1 and PD-L1 observed following RV treatment suggests that this approach could be particularly effective when combined with targeted therapies such as PD-1/PD-L1 inhibitors (60).

The clinical effects of RV in the treatment of MM patients have been studied in various clinical trials, demonstrating an acceptable safety profile but limited efficacy results (61–64).

In clinical trial NCT01533194, patients received two doses of Reolysin ($3\times10^9$ $TCID_{50}$/day or $3\times10^{10}$ $TCID_{50}$/day) without experiencing dose-limiting toxicities (DLTs). Nevertheless, no significant objective responses were observed, with only a few patients achieving disease stability for up to eight months (61). Analyses indicated that viral resistance, limited antitumor immune response, and inadequate viral dosing may have reduced treatment efficacy. A critical factor that emerged was the lack of JAM-A receptor expression in the patient's cells, which may have limited viral infection of MM cells, reducing treatment efficacy. Otherwise, RAS mutations, which are prevalent in patients with relapsed MM, did not demonstrate a significant correlation with treatment efficacy (61).

In a different trial (NCT02101944), the efficacy of a combined treatment regimen comprising Reolysin, carfilzomib, and dexamethasone was evaluated in MM patients who had demonstrated resistance to carfilzomib (62). Six patients completed 28-day cycles, during which reovirus infection was observed in the BM on day 9 of the first cycle. Two patients demonstrated partial responses; however, one of them developed a cytokine storm with severe symptoms, and the other one discontinued treatment due to fever and severe thrombocytopenia. This cytokine storm, the first observed in blood cancer patients treated with OVs, was associated with T-cell activation due to combination therapy. The observed clinical responses were attributed to the infection of MM cells, the recruitment of $CD8^+$ and NK cells, the increased expression of activated PD-L1 and caspase-3, and the viral protein production in MM cells (61).

NCT02514382 trial evaluated the safety and efficacy of RV combined with bortezomib and dexamethasone in patients with relapsed and refractory MM who had previously undergone at least one course of therapy (63, 64). The combination was well tolerated, with most toxicities presenting as transient flu-like symptoms that could be managed with acetaminophen, antiemetics, or antidiarrheals. No DLTs were observed, indicating that the maximum tolerated dose was not reached. The $3\times10^{10}$ $TCID_{50}$ dose of Reolysin was administered for five consecutive days in 21- and 28-day treatment cycles. Six of eleven evaluable patients (55%) demonstrated a reduction in paraprotein levels. In patients who responded to the treatment, there was an association between viral proliferation and increased apoptosis, as indicated by the increase in cleaved caspase-3-positive cells. Immunohistochemical analysis revealed a significant increase in cytotoxic T cells in responders, suggesting that these cells cluster around MM cells. This spatial change in the tumor microenvironment could contribute to the efficacy of the treatment (64).

## 3.3 Adenovirus

Adenoviruses (AdVs) are non-enveloped, double-stranded DNA viruses with an icosahedral capsid primarily composed of hexon, penton, and fiber proteins, belonging to the Adenoviridae family. In humans, over 100 AdV types have been identified, and classified into seven genetically distinct species (A–G) based on phylogenetic analysis of their genomic sequences, pathobiology, and immunological and tumorigenic properties (65). Although human AdVs cause significant numbers of respiratory, ocular, and gastrointestinal diseases, severe AdV-associated illness occurs predominantly in immunocompromised individuals. In the general population, AdV infections are typically self-limiting and lead to lifelong immunity (66).

Among AdV types, Ad5 (species C) is the most widely used vector for oncolytic virotherapy, having demonstrated success in both preclinical and clinical trials across various cancers (67). The infection of tumor cells by Ad5 begins with viral fiber knob attachment to receptors on the surface of malignant cells. The receptor specificity differs according to the viral serotype. For instance, Ad5 binds preferentially to the Coxsackie and Adenovirus Receptor (CAR), while Ad3 binds to desmoglein-2, CD46, or CD80/86 (68). This receptor diversity is particularly relevant to MM, where CD46 and CAR are variably expressed on malignant cells (69).

Preclinical studies have demonstrated the therapeutic potential of AdVs in MM. In one investigation, AdVs were employed as a therapeutic tool for purging MM cells, showing their ability to deliver the TK gene into MM cells using the DF3/MUC1 tumor promoter, with tumor cell transduction observed to be highly efficient (>80%) (34). Treatment with ganciclovir selectively eliminated MM cells without affecting normal progenitor cells (34). Senac et al. demonstrated that distinct AdV serotypes, including Ad5, Ad6, Ad26, and Ad48, can effectively infect and destroy MM cells while exhibiting minimal cytotoxicity against $CD138^-$ cells. Ad5 and Ad6 exhibited a high capacity to infect MM cells through CAR and integrin receptors, while Ad26 and Ad48 utilized alternative receptors, such as CD46 and sialic acid (69).

Innovative strategies have been developed to enhance the therapeutic efficacy of Ad5 in MM treatment, taking advantage of its amenability to genetic modification. Through targeted genetic engineering, Ad5 can be optimized to improve tumor specificity and enhance its oncolytic efficiency, thereby increasing selectivity for malignant cells while reducing off-target cytotoxicity. Fernandes et al. developed an oncolytic AdV, AdEHCD40L, which expresses CD40 ligand (CD40L) under the control of hypoxia-specific promoters (32). This vector has been demonstrated to effectively inhibit the growth of MM cell lines *in vitro* and to significantly reduce tumor volume in mouse models. The therapeutic efficacy of AdEHCD40L has been attributed to both direct viral lysis and CD40L-mediated induction of apoptosis, suggesting a dual mechanism of action (32). Wenthe et al. investigated the use of AdVs LOAd700 and LOAd703, which had been modified to express trimerized CD40 ligand (CD40L) and 4-1BB ligand (4-1BBL), respectively (28). The viruses demonstrated potent oncolytic activity against MM cell lines and the activation of antitumor immune responses. LOAd703 demonstrated superior efficacy in controlling tumor growth in xenograft models, as evidenced by its ability to stimulate cytotoxic T cells and increase the expression of death receptors such as Fas (28).

Further therapeutic approaches have also demonstrated significant potential. Tong et al. combined an oncolytic AdV expressing TRAIL (ZD55-TRAIL) with the PI3K inhibitor LY294002 (23). This combination enhanced the cytotoxicity of the virus toward MM cells by inhibiting the Akt/mTOR survival pathway and enhancing the induction of apoptosis in tumor cells. Furthermore, the addition of the proteasome inhibitor MG132 resulted in a further increase in the expression of Death Receptor 5 (DR5), thereby sensitizing MM cells to ZD55-TRAIL-induced apoptosis (23).

Stewart et al. developed an oncolytic AdV, ADCE1A, which employs the MM-specific CS1 promoter to regulate E1A gene expression. This AdV demonstrated selective infection and replication in MM cell lines and induced oncolysis in $CD138^+$ cells of MM patients, without affecting non-tumor cells (70).

Moreover, the combined use of a recombinant p53 AdV (rAd-p53) and bortezomib showed synergistic inhibition of proliferation and induction of apoptosis in MM cells. rAd-p53 enhanced the expression of p21, arresting the cell cycle and reducing the expression of cyclin B1, thus improving the efficacy of bortezomib treatment (71).

However, there are significant limitations to the use of Ad5 as a vector. First, an estimated 50–90% of the adult population is seropositive for pre-existing anti-Ad5 neutralizing antibodies (72, 73). These neutralizing antibodies have been shown to limit the antitumor efficacy of Ad5, particularly during systemic intravenous delivery (74–76). Second, intravenous administration of Ad5 can lead to liver toxicity due to significant liver sequestration, driven by Ad5 hexon binding to coagulation factor X (FX) (77, 78). In response to this, Alba et al. developed FX-binding-ablated Ad5 hexon vectors to mitigate this side effect (79).

To circumvent these limitations, researchers have begun exploring alternative AdV serotypes. For example, the low seroprevalence of antibodies against species D AdVs, coupled with their lack of FX binding, makes them promising candidates for further exploration as oncolytic agents (80). Additionally, chimeric AdVs have been developed to evade neutralizing antibodies (81).

## 3.4 Herpes simplex virus type 1

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus with icosahedral symmetry, belonging to the family Herpesviridae. It is primarily known to cause oral infections such as cold sores (82). Recently, oncolytic versions of HSV-1 (oHSV-1) have shown a promising ability to selectively infect tumor cells (83). This specificity is defined by the surface glycoproteins of individual virions, which interact with cell surface receptors, particularly Nectin-1 and Herpes Virus Entry Mediator (HVEM) (82). These receptors are highly expressed in MM cells, contributing to the selectivity of oHSV-1 infection (84).

Ghose et al. demonstrated that oHSV-1 effectively infected MM cells *in vitro*, causing apoptosis through cleavage of caspase-3. In murine models, infection with oHSV-1 led to a significant reduction in tumor volume (84). Furthermore, the combination of oHSV-1 with NK cells immunotherapy has been demonstrated to enhance therapeutic efficacy through the activation of NK cells and the subsequent increased release of cytokines and cytotoxic capacity (85).

Additional therapeutic combinations including oHSV-1 with bortezomib or lenalidomide have shown synergistic effects (25, 86). Specifically *in vitro*, HSV1716 (SEPREHVIR®), when combined with bortezomib, prevented MM cell regrowth for up to 25 days (86); at the same time, third-generation HSV-1 T-01, when used together with lenalidomide, increased the cytotoxic effect and enhanced antitumor activity through the activation of NK cells and the modulation of the immune environment (25).

## 3.5 Coxsackievirus A21

Coxsackievirus A21 (CVA21) is a member of the Picornaviridae family and is a non-enveloped virus with an icosahedral structure and a genome consisting of a single strand of positive-sense RNA. In humans, natural infections of CVA21 are generally asymptomatic and not associated with severe disease (87). ICAM-1 and/or DAF cell surface receptors are responsible for the specific adhesion of CVA21 and subsequent infection of the host cell (88). CVA21 can bind to DAF expressed on the cell membrane but is unable to infect a cell unless ICAM-1 is co-expressed on the cell surface. Consequently, ICAM-1 is considered a pivotal factor in CVA21 entry, uncoating, and replication under normal infection conditions (88). In comparison to most non-malignant cells, MM cells express ICAM-1 and DAF at relatively high levels, which allows for selective oncolysis by CVA21 (16). The elevated level of ICAM-1 expression in MM cells can be attributed to the constitutive activation of the transcription factor NF-κB, which is present in numerous cell lines and patient biopsies (89).

*In vitro* studies have revealed that MM cell lines incubated with different concentrations of CVA21 exhibit a rapid cytopathic effect, even at low doses (16, 90). The oncolytic ability of CVA21 was confirmed in MM patients' BM mononuclear cells, demonstrating that this effect is not limited to laboratory-adapted MM cell lines but is also effective in primary tumor samples infected *ex vivo*. Following infection with high levels of CVA21, a substantial clearance of tumor cells was observed, with minimal effects on non-malignant cells (16).

In SCID mice with human MM xenografts, a study demonstrated that the tumors exhibited rapid and complete responses to treatment with CVA21, either by intravenous or intratumorally administration (90). However, once the tumors regressed, the mice developed hind-limb paralysis and died rapidly. Pathological analysis revealed the complete ablation of tumor tissue, accompanied by the presence of diffuse myositis in the muscle tissues. CVA21 virus was recovered from muscle biopsies, but no evidence of central nervous system infection was found. Toxicity was observed in tumor-bearing animals with a dose of CVA21 up to 560 $TCID_{50}$. To mitigate myositis, adenoviral vectors encoding for mouse IFN-α were administered before CVA21 therapy. However, the impact on tumor response or survival was minimal (16). Ongoing studies aim to find potential alternatives to reduce off-target effects.

## 3.6 Vaccinia virus

Vaccinia virus (VV) is a double-stranded DNA virus belonging to the Poxviridae family, best known for its use in the eradication of smallpox in the 1970s (91). This virus employs several mechanisms to enter host cells. Once attached to the cell surface via specific receptors, such as heparan sulfates, VV exploits the process of endocytosis mediated by macropinocytosis to penetrate the cytoplasm (91). Although the mechanism of high selectivity of VV for MM cells is not fully elucidated, it is postulated that aberrant signaling pathways through the RAS/Mitogen-Activated Protein Kinase (MAPK) pathways may contribute to this effect (91).

Attenuated VV variants with specific genetic deletions have been developed to selectively infect and replicate in malignant PCs, minimizing toxicity in normal tissues. A modified VV with double genetic deletion and insertion of a reporter gene has been shown to effectively infect several MM cell lines, inducing apoptosis and reducing cell viability *in vitro*, as well as slowing tumor growth and improving survival in MM mouse models without causing significant damage to healthy tissues (92). Another approach involved the use of a VV regulated by let-7a microRNA and with a deletion of the thymidine kinase gene (ΔTK), which demonstrated preferential localization of the virus in MM cells and reduced systemic toxicity. This engineered virus showed significant antitumor effects and improved survival in SCID mice (93). A further study explored the use of two novel VVs (TK-deletions) as vectors for anti-cancer gene delivery, miR-34a and Smac, respectively (36). The results demonstrated that the novel oncolytic VVs can effectively infect MM cell lines and significantly enhance exogenous gene expression. Furthermore, the combined use of VV-miR-34a and VV-Smac exhibited a synergistic effect by inhibiting tumor growth and inducing apoptosis *in vitro* and *in vivo*. The proposed underlying mechanism is that blockade of Bcl-2 by VV-miR-34a increases cytochrome c release from mitochondria and thus synergistically amplifies the antitumor effects of Smac-induced cell apoptosis (36). Finally, a VV modified to express Beclin-1 protein demonstrated the ability to induce significant autophagic cell death in MM cells by activating Sirtuin 1 (SIRT1) protein and promoting deacetylation and transfer of Microtubule-Associated Protein 1A/1B-light chain 3 (LC3) from the nucleus to the cytoplasm. This suggests a novel mechanism of action that could overcome the resistance of cancer cells to apoptosis (35).

Preclinical data regarding the use of VV in the treatment of MM find confirmation in a significant clinical case involving a 67-year-old patient with IgA-type MM (94). The patient received intravenous injections of a specific variant of VV, known as the AS strain, which resulted in a substantial reduction in monoclonal IgA levels and an increase in NK cell activity. It is noteworthy that no significant adverse effects were observed throughout treatment, which suggests a favorable safety profile for VV (94). This clinical case not only demonstrates the efficacy of VV in reducing disease biomarkers but also its potential to improve the patient's immune response.

## 3.7 Myxoma virus

Myxoma virus (MYXV) belongs to the genus Leporipoxvirus of the family Poxviridae. It possesses a large linear double-stranded DNA genome enclosed in a brick-shaped virion (95). The entire MYXV replication cycle takes place in the cytoplasm of infected cells, where the virus produces a variety of immunomodulatory proteins that interact with the host. In the wild, MYXV exclusively infects rabbits and European brown hares and is not pathogenic to other hosts (95). Nevertheless, MYXV is capable of replicating in human tumor cell cultures, which exhibit a particular degree of permissiveness towards this virus. This permissiveness is attributed to its interaction with deregulated cellular pathways, particularly the Akt pathway (96). MM cells exhibit alterations in this pathway, rendering MYXV a potential oncolytic agent for the treatment of this tumor type (13).

The mechanism of action of MYXV differs from traditional oncolytic approaches, as it induces apoptosis in MM cells through the activation of caspase-8. This process is due to the depletion of apoptosis inhibitory proteins (cIAPs) caused by virus-mediated translational arrest (97). Additionally, MYXV induces autophagy, as evidenced by increased expression of the key proteins ATG-5, Beclin-1, and LC3B, and the presence of autophagosomes (98). Furthermore, the virus is capable of inhibiting the Activating Transcription Factor 4 (ATF4) expression and reducing the Myeloid Cell Leukemia 1 (Mcl1) levels, thereby overcoming resistance to proteasome inhibitors (99). A recent study investigated the combination of MYXV with lenalidomide and bortezomib, revealing a significant reduction in MM cell viability and an increase in early apoptosis. This synergistic effect is mediated

by increased caspase-9 expression (100). In mouse models, the systemic administration of MYXV demonstrated efficacy in eliminating MM cells by inducing robust $CD8^+$ T antitumor immune responses, suggesting the possibility of its use as a systemic therapy in patients (101).

De Matos et al. investigated the therapeutic potential of armed MYXV, engineered to express immunomodulatory proteins such as IL-12 and decorin. The authors reported significant oncolytic effects and transgene expression in MM cell lines, suggesting that this new approach could be applied in patients resistant to other immunotherapy strategies (102).

As with other previously treated viruses, MYXV has been demonstrated to be effective in purging *ex vivo* autologous hematopoietic stem cells (HSPCs) contaminated with MM cells, while preserving normal HSPCs and reducing post-transplant recurrence (103). Furthermore, *ex vivo* virotherapy with MYXV demonstrated encouraging outcomes in optimizing allogeneic hematopoietic cell transplantation. This approach significantly reduced the proliferation of alloreactive T cells and prevented graft-versus-host disease (GVHD) without compromising the antitumor effect (27). Finally, infusion of autologous leukocytes preloaded *ex vivo* with MYXV revealed remarkable efficacy in targeting minimal residual disease (MRD) of MM, suggesting a promising approach to overcome drug resistance and improve survival rates (104).

## 3.8 Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is an enveloped, single-stranded, negative-sense RNA virus belonging to the Rhabdoviridae family (105). It employs surface molecules such as the low-density lipoprotein receptor (LDLR) to target cells. LDLRs, due to their ubiquitous expression, enable VSV to infect a diverse range of cell types. However, VSV infection is typically inhibited by the activation of PKR and the production of IFN. Given that the PKR system is defective in cancer cells, VSV exhibits high selectivity (106).

In MM, preclinical studies have demonstrated promising results for the use of VSV both *in vitro* and *in vivo* (107). An attenuated variant, VSV(Δ51)-NIS, with a deletion of methionine 51 in the matrix protein and expression of the NIS gene, demonstrated specific oncolytic activity against MM cell lines and primary MM cells, without causing neurotoxicity in mouse models (108). Infection was monitored noninvasively by serial gamma camera imaging of radioactive iodine biodistribution. The combination of VSV(Δ51)-NIS with $^{131}I$ further enhanced the efficacy of tumor regression and survival in immunocompetent mice (108). In addition, another study demonstrated that a VSV-IFNβ construct, which expresses IFN-β, significantly prolonged the survival of mice with disseminated MM (24). This was achieved through the combined action of the oncolytic activity of VSV and the immunomodulatory properties of IFN-β. VSV-IFNβ demonstrated specific oncolytic activity against human MM cells and primary patient samples, although with variable susceptibility (24). Moreover, the combination of VSV with bortezomib demonstrated synergistic effects *in vivo*, despite the antagonism observed *in vitro*. This suggests that the enhanced therapeutic efficacy observed may be mediated by host immune responses (109).

These promising results led to the design of a phase I clinical trial (NCT03017820) to assess the safety and optimal dosing of VSV-IFNβ-NIS in patients with relapsed or refractory MM. The study included 15 patients with relapsed/refractory hematologic malignancies, of whom 7 had MM (110). Patients received a single intravenous infusion of VSV-IFNβ-NIS across four dose levels (DL), with the highest dose being $1.7 \times 10^{11}$ $TCID_{50}$ (DL4). No dose-limiting toxicities were observed, although 1 of 2 MM patients treated at DL4 experienced grade 2 cytokine release syndrome, which was transient and resolved within 24 to 48 hours (110). Overall, MM patients did not show significant clinical responses, with disease stabilization being the best outcome observed. Notably, one MM patient had an osteolytic lesion in the right ilium that showed increased $^{99m}Tc$-pertechnetate uptake on days 1 and 5 post-treatment, indicating viral presence. This lesion also demonstrated reduced [$^{18}F$]Fluorodeoxyglucose activity on PET/CT, suggesting an initial response to therapy, although the patient experienced overall disease progression. A separate lesion in the left acetabulum showed no changes post-treatment, with increased size and bone destruction observed at the 6-month follow-up (110). Additional study arms have been added to this ongoing phase I trial to explore the safety and efficacy of combining VSV-IFNβ-NIS with drugs that modulate antiviral or antitumor immune responses.

## 3.9 Bovine viral diarrhea virus

Bovine viral diarrhea virus (BVDV) is a small, enveloped RNA virus belonging to the genus Pestivirus of the family Flaviviridae (111). BVDV is a significant pathogen of cattle, causing syndromes affecting the intestinal, respiratory, and reproductive systems. It is important to note that BVDV is not pathogenic to humans (111). It has been demonstrated that BVDV utilizes CD46 as a receptor for entry into host cells, a process analogous to that observed with the MV (112). Furthermore, several studies have shown that members of the heparan sulfate family, including the CD138 molecule (a distinctive marker of MM), act as cellular receptors for BVDV binding to host cells (113).

Marchica et al. investigated the efficacy of BVDV in the treatment of MM cells, emphasizing its potential as a novel therapeutic strategy (17). Specifically, BVDV demonstrated a selective cytotoxic effect on MM cells, with a significant increase in cell death and activation of apoptotic markers. In *ex vivo* experiments, BVDV treatment significantly reduced the percentage of viable $CD138^+$ cells within mononuclear cells isolated from BM aspirates of MM patients, without altering the viability of other cell populations. Moreover, pretreatment with bortezomib markedly augmented the cytotoxic impact of BVDV on MM cell lines, indicating a synergistic effect resulting from the activation of the caspase-3-mediated apoptotic pathway (17).

Finally, in mice injected subcutaneously with MM cells, BVDV treatment significantly reduced tumor burden without evidence of toxicity to vital organs such as the heart and lungs (17).

## 4 Challenges and optimization strategies in myeloma oncolytic virotherapy

Oncolytic virotherapy presents a promising avenue for treating MM, but it faces significant challenges related to delivery, immune evasion, and biosafety that must be meticulously addressed (114).

In diseases such as MM, characterized by systemic involvement, intravenous (i.v.) delivery of OVs provides a feasible strategy to target malignant cells residing in the BM and other extramedullary sites (114). However, systemic delivery introduces several obstacles, including poor extravasation from tumor vasculature, non-specific sequestration in the liver, and rapid neutralization by pre-existing antiviral antibodies or treatment-induced neutralizing anti-viral antibodies. Neutralization by the immune system, especially in immunocompetent hosts, limits the therapeutic potential of OVs by reducing their circulation time and preventing effective tumor targeting (115). Various strategies have been developed to circumvent these issues. For instance, PEGylation, a polymer-based technique, has been shown to shield OVs from antibody-mediated neutralization and prevent nonspecific accumulation in the liver, thereby prolonging their systemic half-life (116). Similarly, encapsulation in nanoparticles, including graphene, has demonstrated efficacy in protecting viral particles during systemic circulation (117). In addition, "stealth" viruses, such as MeV-Stealth, have been engineered to evade pre-existing immunity (118). MeV-Stealth, specifically re-engineered to evade neutralizing antibodies in measles-immune patients, has demonstrated promising results in targeting CD46-expressing MM cells (118). An equally significant challenge is the rapid clearance and liver trapping observed particularly with AdV (119). Ad5, as described above, binds to coagulation FX, which mediates liver transduction, leading to off-target effects and reduced therapeutic efficacy (79). To mitigate this, researchers have genetically modified Ad5 to remove its FX-binding domain, preventing sequestration in the liver without compromising its oncolytic function (79). Moreover, CAR-independent infection mechanisms have been developed to enhance tumor-specific targeting while avoiding liver trapping, a critical advancement for systemic virotherapy (120).

To further improve the efficacy of OVs, immunosuppressive agents such as cyclophosphamide have been co-administered to transiently suppress both innate and adaptive antiviral responses. Cyclophosphamide not only enhances viral replication but also depletes regulatory T cells, rebooting the immune system and promoting an enhanced antitumor response (121). Ruxolitinib, a JAK/STAT inhibitor, has also been employed to enhance viral replication by inhibiting interferon responses in IFN-competent tumor cells, allowing for improved OV propagation while mitigating the risk of excessive cytokine release or rapid tumor lysis syndrome (122). In addition to these pharmacological interventions, the use of non-human or rare viruses presents a promising alternative to circumvent the issue of pre-existing immunity. These viruses, being non-pathogenic to humans, are unlikely to encounter neutralizing antibodies in the human population (123). However, they do pose biosafety risks, including potential shedding or recombination with wild-type viruses, underscoring the need for stringent safety protocols in clinical settings (114). While clinical trials involving OVs such as MV-NIS, Reolysin, and VSV-IFNβ-NIS in MM have shown acceptable safety profiles, with responses ranging from partial remission to disease stabilization, the risk of latent viral infections or recombination remains a concern (29, 44, 61–64, 110). For instance, HSV-1, which serves as the backbone for T-VEC, the first FDA-approved oncolytic virus, has been associated with latent infections, highlighting the importance of long-term safety surveillance (124). In the case of the MV-NIS trial specifically, viral shedding in sputum and urine was typically limited to the first 8–15 days following therapy, suggesting a controlled window of viral presence post-treatment (44). Nonetheless, the potential for reactivation or recombination, as seen with other viral platforms, warrants close surveillance even after the acute phase has passed.

A critical advantage of OVs is their potential to synergize with immune-based therapies, such as bispecific T-cell engagers (BiTEs) and CAR-T cell therapies (125, 126). OVs can serve as genetic engineering platforms to express BiTEs, which can redirect T cells to target tumor-specific antigens without relying on MHC-I antigen presentation (125). However, due to their small molecular weight and short half-life, BiTEs typically require continuous infusion, which increases the risk of systemic adverse effects (127). The selective replication of OVs in tumor cells provides a means to restrict BiTE expression to the tumor microenvironment, reducing off-target effects and enhancing therapeutic specificity (125). Moreover, OVs can reshape the tumor microenvironment by inducing the production of pro-inflammatory cytokines and chemokines, which promote the infiltration and activation of CAR-T cells (126). This ability to reprogram the immunosuppressive tumor niche is particularly valuable in MM, where the tumor microenvironment often limits the efficacy of CAR-T cell therapy alone (126).

Genetic modifications of OVs to enhance tumor tropism are also crucial. The heterogeneity of MM, along with the variability in viral receptor expression across patients, complicates the implementation of uniform treatments. Investigating differentially expressed genes in MM cells and designing ligand-pseudotyped OVs that target these specific markers could significantly enhance tumor selectivity (11).

Overall, continued research is needed to optimize safety and efficacy of OVs in MM, including monitoring for long-term risks such as viral persistence or recombination. Nevertheless, the integration of OVs with immunotherapies holds significant promise for overcoming the current barriers in MM treatment.

## 5 Conclusions

Over the past decade, in the field of oncolytic virotherapy, significant progress has been made, in exploring the use of different viruses, both natural, engineered, and of non-human origin. This innovative approach is particularly promising in the context of immunotherapy for the possible capacity to potentiate the effect of other drugs and to potentiate immune-mediated MM cell death.

Oncolytic virotherapy may also represent an innovative strategy for a possible personalized approach due to the specific characteristics of tumoral cells and their microenvironment that make them susceptible to viral therapy. While OVs show potent anti-MM activity both *in vitro* and *in vivo* in pre-clinical mouse models, their clinical use as monotherapy has limitations. To maximize therapeutic efficacy, it is critical to combine OVs with other therapeutic agents including proteasome inhibitors and immunotherapeutic agents such as immunomodulatory imide drugs, monoclonal antibodies, and T cell therapy.

In conclusion, the future success of oncolytic virotherapy in the treatment of MM will depend on key factors such as the identification of biomarkers predictive of tumor response, the improvement of administration strategies, and the enhancement of the immune response through combination approaches. It is hopeful that through continued research and the integration of innovative therapeutic strategies the full potential of oncolytic virotherapy can be realized with an appropriate clinical development for MM patients.

## Author contributions

VR: Writing – review & editing, Writing – original draft, Conceptualization, Visualization. RV: Writing – review & editing, Writing – original draft. MD: Writing – review & editing, Visualization. GD: Writing – review & editing, Supervision. PS: Writing – review & editing, Supervision, Conceptualization. NG: Writing – review & editing, Supervision, Conceptualization.

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# Local radiotherapy in extensive-stage small-cell lung cancer sustainably boosts the clinical benefit of first-line immunotherapy: a case report

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**Background:** Extensive-stage small-cell lung cancer (ES-SCLC) has a dismal prognosis owing to its high aggressiveness, rapid drug resistance, and early metastasis. ES-SCLC responds well to first-line chemotherapy, and chemotherapy coupled with immunotherapy can further improve overall survival. However, the long-term survival of patients remains unsatisfactory because of its high recurrence rate and the poor efficacy of second-line treatment. Although local radiotherapy is an important component of the overall treatment for ES-SCLC, its value in the age of immunotherapy remains controversial.

**Case description:** A 54-year-old male with ES-SCLC achieved a complete response (CR), as determined using enhanced computed tomography (CT) after four cycles of immunochemotherapy (serplulimab, carboplatin, and etoposide). Whole-body positron emission tomography-CT was performed during maintenance treatment with serplulimab, which showed primary lung, liver, and bone metastatic lesions with CR. However, several mediastinal lymph nodes exhibited glucose metabolism uptake, and new lesions appeared on the head. The patient underwent palliative radiotherapy of the head and consolidative thoracic radiotherapy of the chest and continued maintenance treatment with serplulimab. Subsequent magnetic resonance imaging of the head suggested good control of metastatic lesions (CR). The patient received first-line immunotherapy for approximately 20 months.

**Conclusions:** This report presents a patient with ES-SCLC who underwent local radiotherapy in addition to serplulimab as maintenance therapy. Although the

programmed death-ligand 1 (PD-L1) expression level was negative and a PD-1 inhibitor instead of a PD-L1 inhibitor was used, the patient did not experience significant pneumonia during treatment, and the efficacy of the current treatment was evident. This treatment model warrants further clinical investigation.

KEYWORDS

**local radiotherapy, extensive-stage small-cell lung cancer, clinical benefit, immunotherapy, case report**

## 1 Introduction

Small-cell lung cancer (SCLC) is the most aggressive subtype of lung cancer, accounting for approximately 15% of all cases. It is a highly malignant neuroendocrine tumor, and smoking has been identified as the biggest risk factor. Surgery, radiotherapy, and chemotherapy are the three main treatments for SCLC; however, extensive-stage SCLC (ES-SCLC) is not eligible for radical surgery and concurrent chemoradiotherapy because of late staging. The median overall survival (mOS) of patients with SCLC without active treatment is only 2–4 months (1, 2). Over the past few decades, platinum-based chemotherapy has become the preferred first-line therapy for ES-SCLC; however, the mOS remains only 9–10 months, and the 2-year OS rate is <5% (3).

With the development of immunotherapy, treatment outcomes for SCLC have improved. Immunochemotherapy has replaced simple chemotherapy as the new first-line treatment standard for ES-SCLC (4). Compared with non-SCLC, which responds relatively well to immunotherapy, the overall effectiveness of immune checkpoint inhibitors in ES-SCLC is still not optimistic, which may be related to the immunosuppressive features of SCLC, such as lower CD8+ T-cell infiltration, expression level of major histocompatibility complex class I molecules, and expression level of programmed death-ligand 1 (PD-L1) (5, 6). Although most patients with ES-SCLC respond well to initial first-line immunochemotherapy, they rapidly relapse and require further treatment; however, backline therapeutic choices are scarce and frequently less effective. Therefore, effective combination therapy options are urgently required to delay SCLC progression. Radiotherapy, in combination with immunotherapy, is a promising treatment modality. Studies have shown that radiotherapy not only directly attacks cancer cells but also induces their immunogenic death, remodels the tumor immune microenvironment by upregulating tumor antigens, promoting their release and presentation, and improves the ratio of tumor-infiltrating lymphocytes to immunosuppressive cells, thus synergistically combating tumors with immune checkpoint inhibitors (7, 8). Whether combined radiotherapy can reverse the immune desert state of SCLC and further enhance its curative effect has become a research hotspot.

Here, we report that adding local radiotherapy during the immunotherapy maintenance period in a patient with ES-SCLC effectively boosted the clinical benefit of first-line immunotherapy and is expected to substantially prolong the overall survival with a favorable safety profile.

## 2 Case presentation

This study involved a human participant and was approved by the Ethics Committee and the Institutional Review Board of Changde Hospital, Xiangya School of Medicine, Central South University (Approval no: 2024-244-01; date: [September 6, 2024]). The study was conducted in accordance with local legislation and institutional requirements. The patient provided written informed consent to participate in the study and for the publication of any potentially identifiable images or data included in this article. This study complies with the standard reporting guidelines (CARE).

On December 26, 2022, a 54-year-old Chinese male presented to our hospital with complaints of cough, blood-stained sputum, a hoarse voice, shortness of breath after activity, and pain in the posterior lumbar back and right epigastrium lasting >1 month. The patient's general condition was poor; however, there were no symptoms of discomfort such as chills, fever, dizziness, headache, nausea, or vomiting. He was previously healthy but often stayed up late. The patient did not have any infectious or genetic diseases, nor did they have a family history of cancer. However, he did have a history of excessive drinking (>40 years) and smoking (>30 years). Liver palpation indicated that the lower edge of the tumescent liver was located two transverse fingers below the ribs, and no other evident physical findings were observed. Contrast-enhanced computed tomography (CT) of the chest and abdomen revealed a central-type tumor in the lower lobe of the left lung with obstructive pneumonia, invasion of the left pulmonary artery and vein, multiple mediastinal regional lymph nodes, and liver metastases (Figures 1A, B). A whole-body bone scan revealed numerous bone metastases throughout the body, including in the left scapula, sternum, left ribs, cervical vertebrae, thoracic vertebrae, lumbar vertebrae, and pelvis. No brain metastases were detected on magnetic resonance imaging

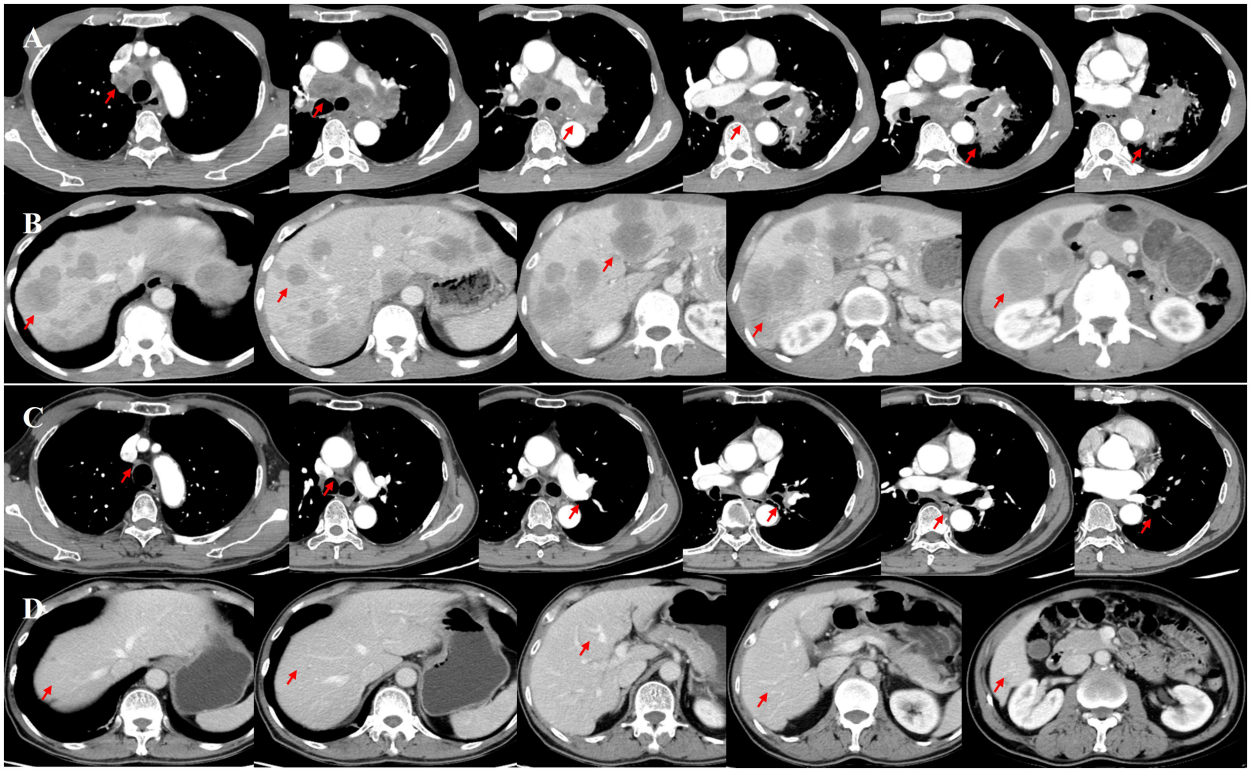


FIGURE 1
Enhanced computed tomography (CT) of the chest and abdomen was initially performed on December 27, 2022 **(A, B)**. After four cycles of immunochemotherapy, a follow-up enhanced CT was performed on April 22, 2023, showing the disappearance of the primary lesion in the left lung, notable shrinkage of the left lung hilar and mediastinal lymph nodes (short axis <10 mm), and absence of obvious metastatic lesions in the liver **(C, D)**.

(MRI). Bronchoscopic biopsy revealed SCLC with negative PD-L1 expression (Supplementary Figure 1). A diagnosis of ES-SCLC, cT4N3M1c2, stage IVB (International Association for the Study of Lung Cancer 9th Edition) was confirmed.

Immunochemotherapy is the preferred first-line treatment regimen. From January 05 to March 28, 2023, the patient was administered four cycles of serplulimab (4.5 mg/kg, day 1), carboplatin (AUC 5, day 1), and etoposide (100 mg/m$^2$, day 1–3); he experienced moderate gastrointestinal reactions and no other adverse events. The therapeutic effect evaluated using CT was a complete response (CR) (Figures 1C, D). He was started on serplulimab 200 mg (4.5 mg/kg) administered every 21 days as a maintenance regimen on April 23, 2023. A whole-body positron emission tomography (PET)-CT review was performed on October 27, 2023 (Figure 2), which showed the following: increased glycometabolism in the left lung hilar and mediastinal (group 2R/4R/8) lymph nodes (maximum standardized uptake volume [SUVmax]: 6.9), suggesting that local tumor cells were still active (partial response, PR); no glucometabolic concentrations in the liver and whole-body bone, suggesting that local tumor cells were inactive (CR); and two new annular nodules in the right frontal lobe, not accompanied by increased glucose metabolism, which were considered as brain metastases. Further brain MRI confirmed the diagnosis of brain metastases (oligo-progression) (Figure 3A).The patient underwent palliative radiotherapy (simultaneous integrated boost intensity-modulated radiation therapy [SIB-IMRT]) for the head lesions on October 31, 2023, with a planning gross target volume dose of 45 Gy in 10 fractions (fx) and planning target volume dose of 30 Gy/10 fx (once a day). He also underwent consolidative thoracic radiotherapy (cTRT) on December 18, 2023, with a planning target volume dose of 39 Gy/26 fx (twice a day). No significant adverse effects were observed. On February 23, 2024, a follow-up MRI showed CR of the metastatic head lesions (Figure 3B). The patient underwent strontium (Sr-89) chloride radionuclide therapy on June 30, 2023, January 29, 2024, and August 20, 2024, respectively, and bisphosphonate therapy once a month to reduce the risk of pathological fracture. The patient continued to receive maintenance therapy with serplulimab, and first-line immunotherapy was administered for up to 20 months. Patient compliance and tolerability were good. At the most recent follow-up examination, the patient exhibited no signs of deterioration, no immunotherapy-related adverse effects, and a high quality of life. We plan to maintain immunotherapy until disease progression or the occurrence of intolerable toxicity. The patient's treatment course is depicted in Figure 4.

## 3 Discussion

The era of immunotherapy for SCLC has dawned with the success of the IMPOWER133 and CASPIAN phase 3 clinical trials for ES-SCLC. However, in the immunotherapy groups in these two

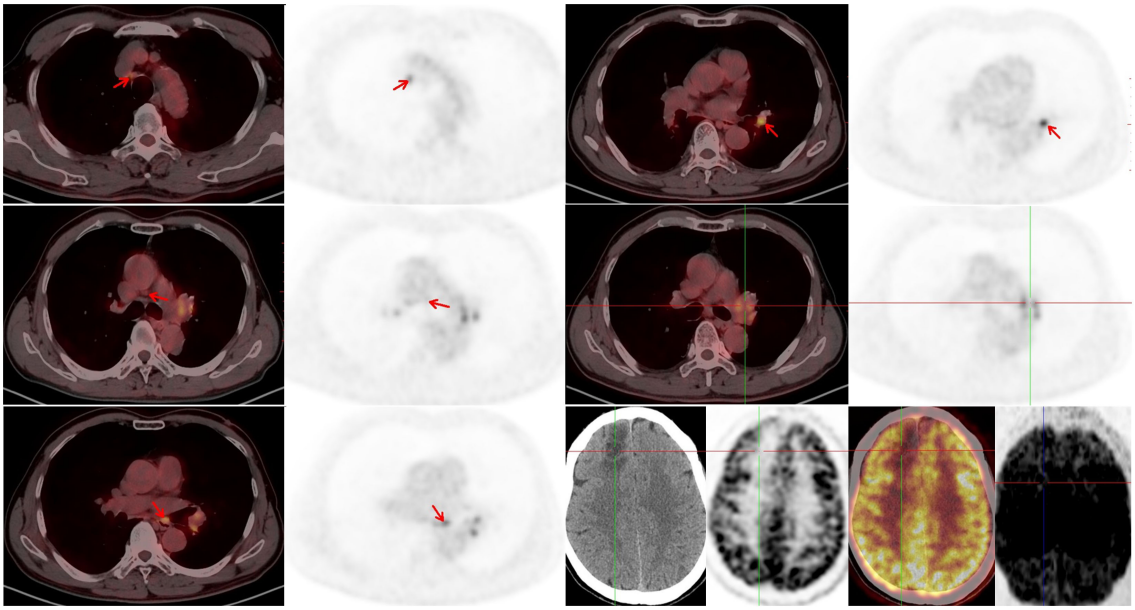

FIGURE 2
Positron emission tomography (PET)-computed tomography (CT) was performed on October 27, 2023. A residual tumor in the left lung hilar and mediastinal lymph nodes and new metastatic lesions in the brain can be observed.

studies, the median progression-free survival (mPFS) was approximately 5 months, whereas the mOS was only 12–13 months (9, 10). The ASTRUM-005, EXTENTORCH, and RATIONALE-312 trials with PD-1 inhibitors followed suit with positive results, showing that the mOS significantly exceeded that with atezolizumab and durvalumab (11–13). However, the overall survival benefits for patients have reached a bottleneck. Thus, there is a need to screen potential beneficiaries of immunotherapy, seek new therapeutic modalities and tactics to further increase the efficacy of first-line immunotherapy, and ultimately enhance the long-term survival outcomes in patients with ES-SCLC.

Radiotherapy and immunotherapy are mechanistically well synergized; however, cTRT was not authorized in any of these studies, and the advantages and safety of combining immunotherapy with thoracic radiotherapy (TRT) remain controversial. In an era of chemotherapy, TRT coupled with prophylactic cranial irradiation has reduced the incidence of intrathoracic recurrence by 50% and increased the 2-year overall survival rate (13% vs. 3%, P = 0.004) in patients with ES-SCLC who achieved PR or CR following chemotherapy (14). An analysis of the progression patterns in the IMpower133 study revealed that the predominant progression in both arms occurred in existing lesions, especially in the lung and lymph nodes (15). This indicates that TRT after first-line immunotherapy may be beneficial and that consolidation radiotherapy for primary and metastatic lesions may further improve the first-line mPFS and overall prognosis of patients with ES-SCLC after immunochemotherapy. PD-1 inhibitors are associated with a greater risk of pneumonia during first-line ES-SCLC treatment than PD-L1 inhibitors (16). However, in the present case, the patient did not develop pneumonia of any grade after TRT and PD-1 immunomaintenance therapy. We did not specifically analyze the SCLC molecular subtype; we only confirmed that the PD-L1 expression level was negative. Although the patient's condition was well-controlled using immunochemotherapy, brain radiotherapy (SIB-IMRT), and cTRT, we are still pondering whether the patient's brain metastasis could have been avoided, thus further improving the patient's prognosis by advancing the treatment time with cTRT and prophylactic cranial irradiation. In addition, we appreciate the importance of PET-CT examination before local radiotherapy, which can facilitate effective evaluation of the patient's tumor control status and provide guidance for accurately delineating the subsequent radiotherapy target volume. Owing to the high cost, the patient did not undergo PET-CT for metabolic assessment of the mediastinal lymph nodes following TRT. Phase I/II prospective and retrospective studies have provided preliminary evidence for the safety and effectiveness of radiotherapy and immunotherapy in patients with ES-SCLC (17–24). However, further investigations of the optimal dose, segmentation pattern, and intervention time of TRT for ES-SCLC in the context of immunotherapy are required. Currently, phase III clinical studies on first-line immunotherapy combined with TRT for patients with ES-SCLC are underway (NCT04028050; NCT05223647; NCT04402788), and the results of this research are expected to be of great clinical significance.

Anti-angiogenic drugs have been used as the first-line treatment for various tumors; however, their application in the first-line therapy of ES-SCLC is still being explored. Anti-angiogenic drugs modulate the tumor immune microenvironment and synergize with immunotherapy. The ETER701 study showed that a four-drug combination regimen (benmelstobart, anlotinib, and chemotherapy)

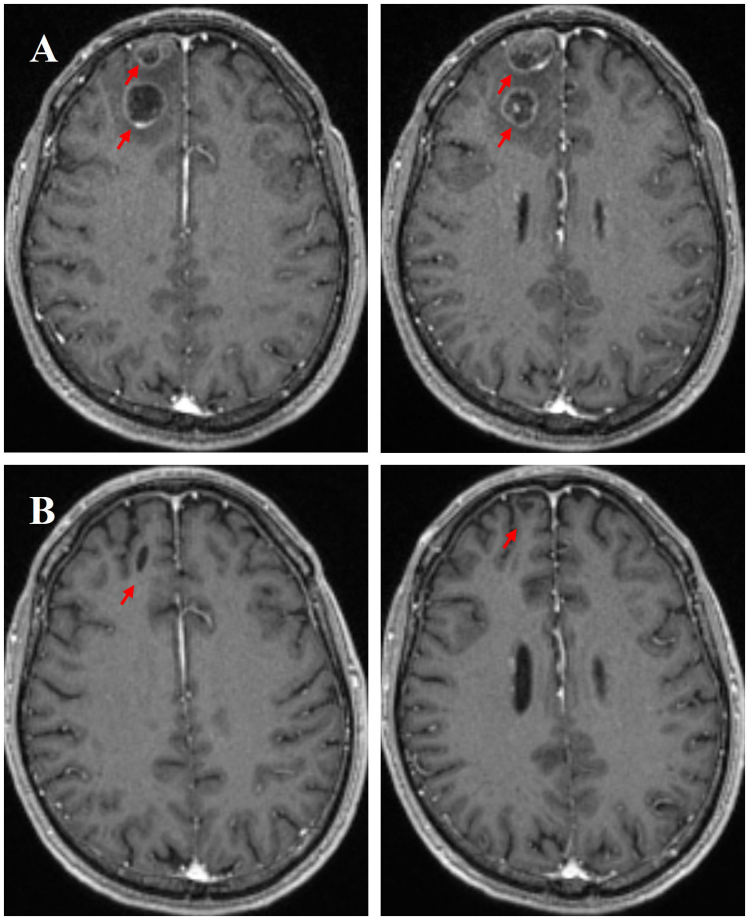


FIGURE 3
Enhanced magnetic resonance imaging (MRI) scans of the brain on October 28, 2023, further confirmed new brain metastases **(A)**. Following palliative radiotherapy, an MRI evaluation of the brain metastatic lesions was performed on February 23, 2024, showing a complete response (CR) **(B)**.

in ES-SCLC resulted in an mPFS of up to 6.9 months and the longest mOS (19.3 months) ever documented in the registry trials to date (25). At the 2024 American Society of Clinical Oncology annual meeting, a preliminary analysis of the BEAT-SC study showed that it reached its primary endpoint of investigator-assessed progression-free survival (PFS). Bevacizumab combined with atezolizumab + carboplatin or cisplatin + etoposide significantly prolonged the median investigator-assessed PFS when compared with the standard immunotherapy (5.7 months vs. 4.4 months, $P$ = 0.0060); mOS data are currently unavailable (26). The phase Ib/II trial of surufatinib, toripalimab, and the etoposide and cisplatin (EP) combination for the first-line therapy of ES-SCLC demonstrated its therapeutic potential (overall response rate: 97.1%; disease control rate: 100%), and subsequent survival results are highly anticipated (27). Preliminary results from a phase Ib clinical

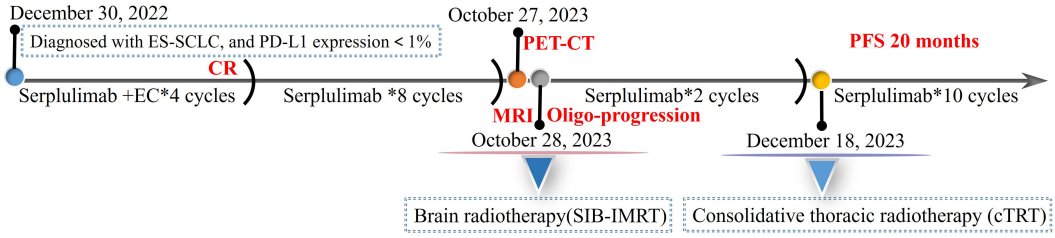


FIGURE 4
The course of disease and treatment. ES-SCLC, extensive-stage small-cell lung cancer; PD-L1, programmed death-ligand 1; EC, etoposide and carboplatin; CR, complete response; PET-CT, positron emission tomography-computed tomography; MRI, magnetic resonance imaging; SIB-IMRT, simultaneous integrated boost intensity-modulated radiation therapy; PFS, progression-free survival.

trial of the novel PD-1/VEGF biospecific antibody AK112 in conjunction with the EP regimen for the first-line treatment option of ES-SCLC showed promising antitumor efficacy and survival benefits with a manageable safety profile (28). Immunochemotherapy coupled with anti-angiogenic drugs is another potential strategy for improving the first-line treatment efficacy in patients with ES-SCLC. However, we also need to further explore the beneficiaries and safety of anti-angiogenic drugs combined with immunochemotherapy, optimize the drug maintenance dose and duration, and make the treatment more precise and individualized. Follow-up studies are expected to provide more evidence to maximize the clinical benefits for patients with ES-SCLC.

Based on immunochemotherapy, novel first-line treatment modes for ES-SCLC are being actively explored. Clinical studies of immunochemotherapy combined with tarlatamab (AMG757), a DLL3-targeting half-life extended bispecific T-cell engager (HLE BiTE®), tifcemalimab (JS004), a humanized IgG4 monoclonal antibody against B and T lymphocyte attenuator, and autologous natural killer cells for the first-line treatment of ES-SCLC are currently ongoing (29–31). Clinical studies are also underway for the addition of the novel chemotherapy drug lurbinectedin, a poly-adenosine diphosphate ribose polymerase inhibitor, or bomedemstat, a lysine-specific demethylase 1 inhibitor (NCT05191797), to first-line immunomaintenance therapy in ES-SCLC (32–34). The results of these studies are promising and provide valuable medical evidence and treatment options in clinical settings.

In summary, our case revealed the notable efficacy and safety of a PD-1 inhibitor (serplulimab) combined with chemotherapy and local radiotherapy for ES-SCLC with negative PD-L1 expression. In the first-line treatment of ES-SCLC, combining radiotherapy with immunotherapy is another major issue that requires further investigation. These questions and debates will guide the way forward for clinical trials, which will ultimately lead to breakthroughs in the quality of life and survival benefits of patients with ES-SCLC.

## 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 humans were approved by the Ethics Committee and the Institutional Review Board of Changde Hospital, Xiangya School of Medicine, Central South University. The studies were conducted in accordance with the local legislation and institutional requirements. The 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

HW: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. NW: Methodology, Writing – review & editing. SL: Methodology, Writing – review & editing. YD: Methodology, Writing – review & editing. TW: Methodology, Writing – review & editing. WT: Supervision, Writing – review & editing. WD: Supervision, Writing – review & editing. XL: Supervision, Writing – review & editing. YZ: Supervision, Writing – review & editing. JZ: Supervision, Writing – review & editing. ZX: Formal analysis, Methodology, Supervision, Writing – review & editing. ZW: Formal analysis, Methodology, Supervision, Writing – review & editing.

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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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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1493740/full#supplementary-material

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# Discovery and preclinical evaluation of BPB-101: a novel triple functional bispecific antibody targeting GARP-TGF-β complex/SLC, free TGF-β and PD-L1

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**Background:** In the tumor microenvironment (TME), the transforming growth factor-β (TGF-β) and programmed cell death receptor 1 (PD-1)/programmed death ligand 1 (PD-L1) signaling axes are complementary, nonredundant immunosuppressive signaling pathways. Studies have revealed that active TGF-β is mainly released from the glycoprotein A repetitions predominant (GARP)-TGF-β complex on the surface of activated regulatory T cells (Tregs), B cells, natural killer (NK) cells, and tumor cells. The currently available antibodies or fusion proteins that target TGF-β are limited in their abilities to simultaneously block TGF-β release and neutralize active TGF-β in the TME, thus limiting their antitumor effects.

**Methods:** We designed and constructed a bispecific, trifunctional antibody, namely, BPB-101, that specifically targets the GARP-TGF-β complex and/or small latent complex (SLC), active TGF-β, and PD-L1. The binding ability of BPB-101 to the different antigens was determined by ELISA, FACS, and biolayer interferometry (BLI). The blocking ability of BPB-101 to the TGF-β and PD-1/PD-L1 signaling axes was determined by reporter gene assay (RGA). The antitumor effect and biosafety of BPB-101 were determined in a transgenic mouse tumor model and cynomolgus monkeys, respectively. Stability assessments, including stability in serum, after exposure to light, after repeated freeze-thaw cycles, and after high-temperature stress tests had been completed to evaluate the stability of BPB-101.

**Results:** BPB-101 bound efficiently to different antigenic proteins: the GARP-TGF-β complex and/or SLC, active TGF-β, and PD-L1. Data showed that BPB-101 not only effectively inhibited the release of TGF-β from human Tregs, but also blocked both the TGF-β and PD-1/PD-L1 signaling pathways. In an MC38-hPD-L1 tumor-bearing C57BL/6-hGARP mouse model, BPB-101 at a dose of 5 mg/kg significantly inhibited tumor growth, with a complete elimination rate of 50%. Stability assessments confirmed the robustness of BPB-101. Furthermore, BPB-101 showed a favorable safety profile in nonhuman primate (NHP) toxicity studies.

**Conclusion:** BPB-101 is a potentially promising therapeutic candidate that may address unmet clinical needs in cancer immunotherapy, thus, BPB-101 warrants further clinical investigation.

KEYWORDS

**PD-1/PD-L1, TGF-β, GARP, GARP-TGF-β complex, small latent complex (SLC), Tregs, tumor microenvironment (TME), BPB-101**

## Introduction

Immunotherapy is radically altering the consistently poor prognosis of cancer patients by triggering long-term durable remission in certain patients (1, 2). Moreover, immunotherapy is playing a revolutionary role in cancer treatment and has reached a critical point, especially with the clinical application of immune checkpoint inhibitors (ICIs) and CAR-T cell therapies. Despite the great promise of ICIs, which include anti-programmed cell death receptor 1 (PD-1) antibodies, anti-programmed death ligand 1 (PD-L1) antibodies, and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) antibodies, only approximately 20~30% of patients benefit from ICIs (3, 4). Scientists have aimed to explore the factors that may limit ICIs' efficacy. One of the most important of these factors is the transforming growth factor-β (TGF-β). TGF-β has been suspected to play a key role in regulating the efficacy of cancer immunotherapy and has gradually become a focus of this research field (5, 6). Many studies have revealed that the TGF-β signaling axis is one of the main factors that lead to the development of tumor resistance to immunotherapy. Eduard Batlle reported that TGF-β in the tumor microenvironment (TME) determines T-cell exclusion and poor tumor response to ICIs (6). In addition, TGF-β in the TME also directly or indirectly suppresses the activity of innate immune cells, such as natural killer cells (NKs) and dendritic cells (DCs), by inhibiting the expression of the NKG2D ligand or interfering with antigen presentation (7, 8). Therefore, reducing the level of TGF-β in the TME has become a key goal (9, 10).

The production of TGF-β is a precisely regulated process. TGF-β is initially produced by immune cells, primary regulatory T cells (Tregs) and tumor cells in an inactive form (11). In the endoplasmic reticulum, following cleavage by the endoprotease Furin, dimerized latent TGF-β molecules form a hairpin small latent complex (SLC), which conceals the sites by which TGF-β binds to its receptor (12, 13). To date, the SLC has been shown to release active TGF-β through three pathways. The glycoprotein A repetitions predominant (GARP)-TGF-β pathway is one of the main pathways by which active TGF-β is released. GARP, which is a type I transmembrane cell surface docking receptor for the SLC, cooperates with αV integrins (GARP/αV) to release active TGF-β from the surface of Tregs, B cells, NK cells, platelets, cancer-associated fibroblasts (CAFs) and tumor cells, thereby promoting immunosuppressive functions of the TME (14).

Several GARP-targeting strategies have already shown some promising results, further inspiring research enthusiasm for this topic (15, 16). However, studies show that the GARP/αV integrin is the main pathway but not the only pathway involved in the production of active TGF-β. Active TGF-β can also be generated by the direct cleavage of the SLC by extracellular proteases or through the latent TGF-β binding protein (LTBP)/extracellular matrix (ECM)/αVβ6 axis (17, 18). Interference with the binding of the GARP-TGF-β complex has no apparent effect on the production of active TGF-β via other pathways or on active TGF-β that is already present in the TME. Thus, to effectively decrease the TGF-β level in the TME, it is necessary to neutralize the active TGF-β that are already present while also preventing the production of more active TGF-β molecules via a two-pronged approach.

Numerous studies have revealed the close connection between TGF-β and PD-L1, both of which are important components related to immune evasion (19, 20). Surprisingly, the inhibition of TGF-β is likely to induce the overexpression of PD-1 by tumor infiltrating lymphocytes (TILs), contributing to tumor cell survival (21). Active TGF-β promotes the production of exosomes with high levels of PD-L1 by cancer cells, and these exosomes mediate T-cell dysfunction via the early phosphorylation of T cell receptor (TCR) signaling domain. Moreover, TGF-β has been shown to be a drive of tumor tolerance to anti-PD-L1 immunotherapy (22, 23). In the TME, the PD-1/PD-L1 and TGF-β signaling pathways are overlapping but nonredundant pathways promote tumor survival. Thus, the suppression of TGF-β or PD-L1 alone has limited therapeutic efficacy (24, 25). Preclinical studies have shown that

**Abbreviations:** BLI, biolayer interferometry; CAFs, cancer-associated fibroblasts; CDR, complementarity-determining region; CMC, Chemistry, Manufacturing and Control; CRS, cytokine release syndrome; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DCs, dendritic cells; ECM, extracellular matrix; GARP, glycoprotein A repetitions predominant; ICIs, immune checkpoint inhibitors; LTBP, latent TGF-β binding protein; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; nAb, nanoantibody; NHP, nonhuman primate; NK, natural killer; PD-1, programmed cell death receptor 1; PD-L1, programmed death ligand 1; RGA, reporter gene assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean; SLC, small latent complex; TCR, T cell receptor; TGF-β, transforming growth factor-β; TILs, tumor infiltrating lymphocytes; TME, tumor microenvironment; Tregs, regulatory T cells.

TGF-β inhibition combined with PD-L1 blockade has a significant synergistic effect that far exceeds that of either single treatment alone (26, 27). This combination strategy is the focus of current clinical investigations.

To mitigate the negative impact of TGF-β in the TME and thereby enhance the therapeutic effects of immunotherapy, our group designed and developed a trifunctional bispecific antibody (BPB-101) that targets both the GARP-TGF-β and the PD-1/PD-L1 signaling axes. Unlike existing PD-(L)1/TGF-β(R) therapeutic molecules or anti-GARP monoclonal antibodies, BPB-101 was designed to simultaneously target GARP-TGF-β complex or the SLC, active TGF-β, and PD-L1 on the basis that simultaneous inhibition of these pathways may be more effective for enhancing antitumor activity than inhibition of any one pathway alone.

Herein, we describe the discovery and preclinical assessment of BPB-101, including *in vitro* cytological experiments, *in vivo* antitumor studies and the safety profile analyses in nonhuman primates (NHPs).

# Materials and methods

## Materials

The list of materials used in this study is available in the Supplementary Materials and Supplementary Methods in Supplementary Data Sheet 1.

## Cell lines and animals

The information of cells lines and animals in this study is available in the Supplementary Materials and Supplementary Methods in Supplementary Data Sheet 1.

## Purify analysis of BPB-101

BPB-101, BPB-GARP and BPB-PD-L1 were expressed in Expi293F cells grown in shake flasks. The cell culture supernatants were harvested by centrifugation and then passed over protein A agarose (MabSelect SuReTM from Cytiva). Bound antibodies were washed with buffer at pH 3.4 (Supplementary Methods in Supplementary Data Sheet 1). To further purify the antibodies and remove aggregates and fragments, cation exchange chromatography (CEX) was employed. The protein solution was adjusted to pH 5.0, and the CEX resin was equilibrated with 50 mM acetate (Sigma). Elution was performed using a linear gradient, and the peak fractions were collected. The samples were then analyzed for purity using size-exclusion chromatography (SEC) (Supplementary Methods in Supplementary Data Sheet 1).

## Binding of BPB-101 to GARP-TGF-β complex, active TGF-β or PD-L1

Briefly, 293F-GARP-TGF-β (4E9) or 293T-hPD-L1 cells were plated in 96-well plates and incubated with antibodies, followed by FACS analysis. In addition, human PD-L1 protein, human GARP-TGF-β complex, or human TGF-β protein was coated onto 96-well plates and incubated overnight. Antibodies were then added to the plates, followed by the addition of goat anti-human IgG-Fc-HRP. The biotin signal value was measured at 450 nm (Supplementary Methods in Supplementary Data Sheet 1). For detailed information on the antigens and antibodies used, please refer to the Supplementary Materials.

## Biolayer interferometry

The binding kinetics were determined using an Octet RED96E system with anti-human Fc AHC sensors (Sartorius, 18-5064) or AHC2 sensors (Sartorius, 18-5142) to capture antibodies (28, 29). After establishing a 120-second baseline step in SD buffer, a 1:1 serial dilution of the human antigen in SD buffer was performed. Subsequently, the binding and dissociation processes of the antibody and antigen were detected (Supplementary Methods in Supplementary Data Sheet 1). For detailed information about the antigens and antibodies used, please refer to the Supplementary Materials.

## Competition binding assays of BPB-101 with PD-L1 and CD80

293T-hPD-1 and 293T-hPD-L1 cells were plated into 96-well plates and cultured with a mixture containing antibodies, biotin-conjugated PD-L1 protein, or CD80-mFc protein. One hour later, the cells were centrifuged using an Eppendorf 5810R centrifuge and washed twice with FACS buffer. Following the addition of SA-PE or R-PE-goat anti-mouse IgG Fc, the cells were analyzed with flow cytometry.

## Luciferase reporter gene assay

To verify the blocking effect of BPB-101 on the downstream signaling of TGF-β and PD-L1, two reporter systems were utilized. The 293T-hPD-L1 and 293-TGF-β/GARP-αvβ6 (4D11) cells were used as upstream cells to provide hPD-L1 or TGF-β protein, respectively. Jurkat-PD-1-CD3zeta-NFAT-Luc2 and 293-SBE-res (1E9) cells were used as effector cells to generate a fluorescent signal in response to the upstream signaling. This signal was detected in conjunction with the One-Glo reagent (Supplementary Methods in Supplementary Data Sheet 1).

## Allogeneic mixed lymphocyte reaction experiment

PBMCs were resuspended in RPMI 1640 culture medium and adjusted to a concentration of 1E6 cells/mL, as measured using a BECKMAN VI-CELL XR. Mature DCs from another donor were resuspended at a concentration of 2E5 cells/mL. Then, 100 μL of PBMCs, 50 μL of DCs and 50 μL of antibodies were mixed well in 96-well plates. The mixture was cultured at 37°C for 5 days and then

analyzed using an ELISA kit (Supplementary Methods in Supplementary Data Sheet 1).

## Cytokine secretion of Tregs

The anti-human CD3 and anti-human CD28 (1 μg/mL) were coated onto 96-well plates and cultured at 4°C overnight. The plates were washed three times with DPBS. Tregs, cultured in RPMI 1640 containing 500 IU/mL recombinant human IL-2 (rhIL-2), were added to the plates at a concentration of 4E5 cells/well (Supplementary Methods in Supplementary Data Sheet 1). After five days, the supernatants were collected, and the TGF-β levels were measured using an ELISA kit. For detailed information about the antibodies used, please refer to the Supplementary Materials.

## Binding of BPB-101 to different immune cells in the blood

Red blood cells (RBCs) were removed from human blood samples using an RBC lysis buffer (BioLegend, 420302). Then, the FCR blocking reagent (Miltenyi, 130-059-901) was applied to block nonspecific binding sites on the immune cells. BPB-101 and hIgG1 were serially diluted in FACS buffer, and their binding to $CD4^+$ T cells, $CD8^+$ T cells, Tregs, pDCs, classical monocyte cells, nonclassical monocyte cells, B cells ($CD19^+$), NK cells and NKT cells was determined by FACS (30–32). For detailed information about of the antibodies used, please refer to the Supplementary Materials.

## Mouse studies

All *in vivo* work was approved by the Jiangsu Provincial Department of Science and Technology Animal Control Committee (AP-MIJ220068). The main experiments focused on investigating the biodistribution and antitumor activity of BPB-101 (Supplementary Methods in Supplementary Data Sheet 1).

## Statistics

Data are presented as the mean ± standard error of the mean (SEM) unless otherwise indicated. Statistical significance was analyzed by the unpaired two-tailed Student's t test. P values below 0.05 were considered to indicate statistical significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns: not significant).

# Results

## Screening, construction, and purification of BPB-101

An anti-GARP monoclonal antibody (mAb) was discovered using a hybridoma platform and further selected for its binding to the GARP-TGF-β complex, the SLC and active TGF-β. An anti-PD-L1 nanoantibody (nAb) was screened from a VHH library and then optimized in tumor-bearing mice. Subsequently, BPB-101was successfully generated by combining the intact heavy and light chains of the humanized anti-GARP antibody with the humanized anti-PD-L1 nAb (VHH). The VHH domain is located at the C-terminus of BPB-101 (Figure 1A). The sequence of BPB-101, which includes VH, VL and VHH, differs from that of other mAbs associated with GARP, TGF-β and PD-L1. The Fab arm of the anti-GARP component differs from the VH and VL regions of reference antibodies, with the greatest variability in the complementarity-determining regions (CDR)-H3 and CDR-L3. Furthermore, the CDRs (PD-L1-binding domains) of M7824 (a PD-L1/TGF-βRII inhibitor) and atezolizumab (a PD-L1 inhibitor) were compared with the C-terminus (a PD-L1 inhibitor) of BPB-101. The VHH sequence of BPB-101 significantly differs from those of atezolizumab and M7824, with sequence similarities of 72.88% and 72.50%, respectively. All these results indicate that BPB-101 has a unique amino acid sequence (Supplementary Table S1, Supplementary Figure S1). For subsequent studies, BPB-101 was further purified using protein A agarose resin. After affinity chromatography, BPB-101 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and no bands indicating impurities were observed (Figure 1B).

## BPB-101 is a unique trifunctional antibody that binds efficiently to the GARP-TGF-β complex, active TGF-β and PD-L1

There are three TGF-β isoforms: TGF-β1, TGF-β2, and TGF-β3. The binding of active TGF-β to the corresponding receptor leads to the phosphorylation and activation of the canonical signaling molecules SMAD2 and SMAD3. We examined the binding of BPB-101 to these three TGF-β isoforms and found that the binding of BPB-101 to human TGF-β2 and TGF-β3 was relatively weak compared to its binding to human TGF-β1 (Figure 2A, Supplementary Figures S2A, B). Therefore, TGF-β1 was selected for follow-up studies to test BPB-101's ability to block TGF-β and terminate TGF-β-dependent signaling. The ELISA results in Figure 2A ($1^{st}$ panel) suggested that BPB-101 has a strong avidity for active human TGF-β1, with an $EC_{50}$ of 0.031 nM; which was superior to that of M7824 ($EC_{50}$ = 0.19 nM). Moreover, the DS-1055a (a GARP inhibitor) and ABBV-151 (a GARP-TGF-β1 complex inhibitor) both failed to bind to human active TGF-β1 (Supplementary Figure S2C). The binding of BPB-101 to human PD-L1 is another important precondition of its biological activity. BPB-101, BPB-PD-L1 (the parental antibody of BPB-101), M7824, and atezolizumab bound to PD-L1 with similar $EC_{50}$ values ranging from 0.02 to 0.04 nM (Figure 2A, $2^{nd}$ panel). BPB-101's binding ability to the human GARP-TGF-β1 complex, with an $EC_{50}$ of 0.029 nM, was stronger than that of the positive controls (Figure 2A, $3^{rd}$ panel). Interestingly, in this study, we did not detect binding activity of ABBV-151, which could theoretically bind to the GARP-TGF-β complex. However, M7824, which theoretically does not bind to the GARP-TGF-β complex,

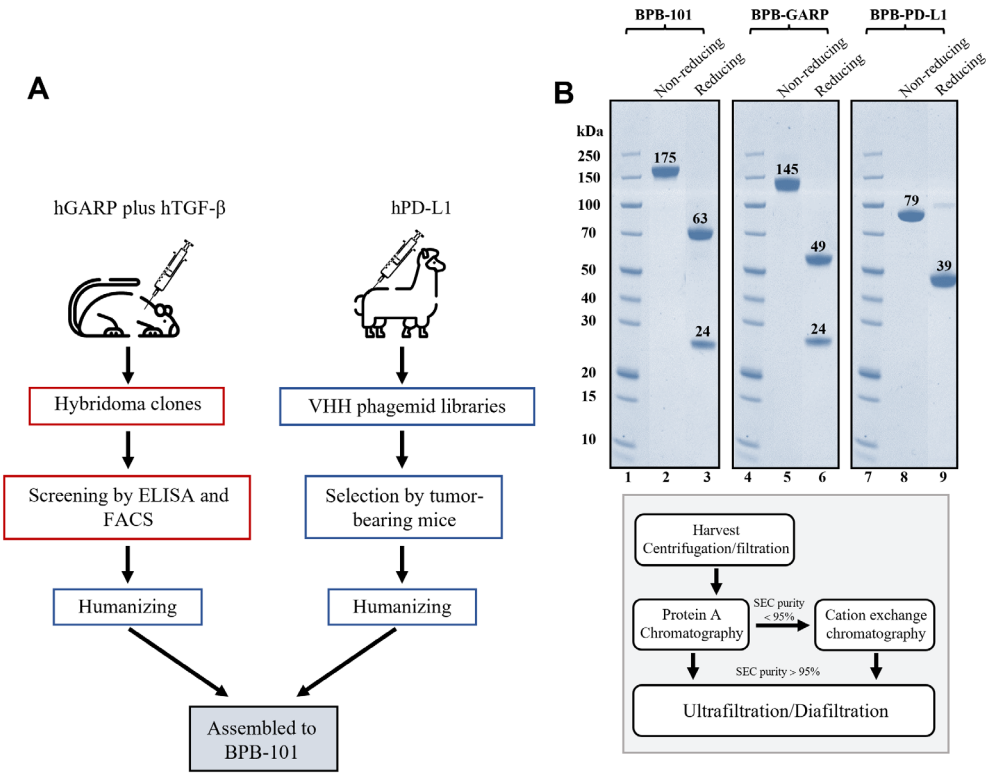


FIGURE 1
Screening, construction and purification of BPB-101. **(A)**, A simple schematic flow chart illustrates the construction and screening process of BPB-101. **(B)**, SDS-PAGE analysis of purified BPB-101, BPB-GARP (a parental mAb targeting GARP) and BPB-PD-L1 (a parental mAb targeting PD-L1) (upper panel). Schematic showing the purification of BPB-101, BPB-GARP and BPB-PD-L1 (lower panel). Lanes 1,4, and 7: the same marker, 2: BPB-101 in the nonreducing form, 3: BPB-101 in the reducing form, 5: BPB-GARP in the nonreducing form, 6: BPB-GARP in the reducing form, 8: BPB-PD-L1 in the nonreducing form, 9: BPB-PD-L1 in the reducing form.

produced a binding curve with an $EC_{50}$ of 0.18 nM. This result likely occurred due to a change in the conformation of the GARP-TGF-β complex antigen, as this phenomenon did not occur in subsequent cell-based binding experiments. The FACS data showed that the $EC_{50}$ values of BPB-101 in 293T-GARP-TGF-β1 complex cells (4E9) and 293T-hPD-L1 cells were 3.74 nM and 1.96 nM, respectively; these values were higher than those of M7824, which could not bind to 4E9 cells at all (Figure 2B). In conclusion, we confirmed that BPB-101 binds specifically to human TGF-β1, PD-L1, and the GARP-TGF-β1 complex in a dose-dependent manner.

We evaluated the avidity of antibodies for the GARP-TGF-β1 complex, the SLC and PD-L1 using biolayer interferometry (BLI) (Figure 2C, Supplementary Table S2). The dissociation constant ($K_D$) of BPB-101 for the GARP-TGF-β1 complex was less than 1 x $10^{-12}$ M due to its slow dissociation rate, and the avidity of the ABBV-151 control antibody for the GARP-TGF-β1 complex was nearly 3 orders of magnitude lower than that of BPB-101 (Figures 2Ci, ii, Supplementary Table S2). For the SLC, BPB-101 yielded a $K_D$ of <1 x $10^{-12}$ M, indicating a high binding efficiency. The remaining control antibodies-namely, ABBV-15, M7824 and GC1008 (a TGF-β1/2/3 inhibitor)-did not bind to the SLC (Figures 2Ciii, iv, Supplementary Table S2). The significantly lower $k_{off}$ value of BPB-101 indicated that it stably bound to the GARP-TGF-β1 complex or the SLC and was unlikely to dissociate after binding to these antigens. As expected, BPB-101 exhibited high avidity for PD-L1, with a $K_D$ of 4.96 x $10^{-10}$ M; this value was approximately 3-fold lower than those of M7824 and atezolizumab (Figures 2Cv–vii, Supplementary Table S2). Next, we evaluated BPB-101's ability to simultaneously bind to human PD-L1-expressing cells and human GARP-TGF-β1 complex-expressing cells. The percentage of double-positive cells in these populations tended to increase as the BPB-101 concentration increased from 0.00128 nM to 0.8 nM. At BPB-101 concentrations higher than 0.8 nM (4 nM, 20 nM and 100 nM), the dual binding of BPB-101 to the two cell lines became saturated (Figure 2D, Supplementary Figure S2D); these results suggested that BPB-101 has robust dual-target specificity.

## BPB-101 can simultaneously block the TGF-β and PD-L1 pathways and their downstream signals

It has been reported that, in addition to binding to PD-1, PD-L1 also binds to CD80 in its receptor form. When the PD-1/PD-L1 and CD80/PD-L1 pathways are activated, the ability of T cells to kill tumor cells is impaired (33, 34). Here, we assessed the ability of BPB-101 to target and block PD-L1, using atezolizumab and M7824 as controls. As expected, BPB-101 blocked the binding of PD-1/PD-L1 (Figure 3A) and CD80/PD-L1 (Figure 3B) interactions in a dose-dependent manner, with $IC_{50}$ values of 3.16 nM and 1.01 nM,

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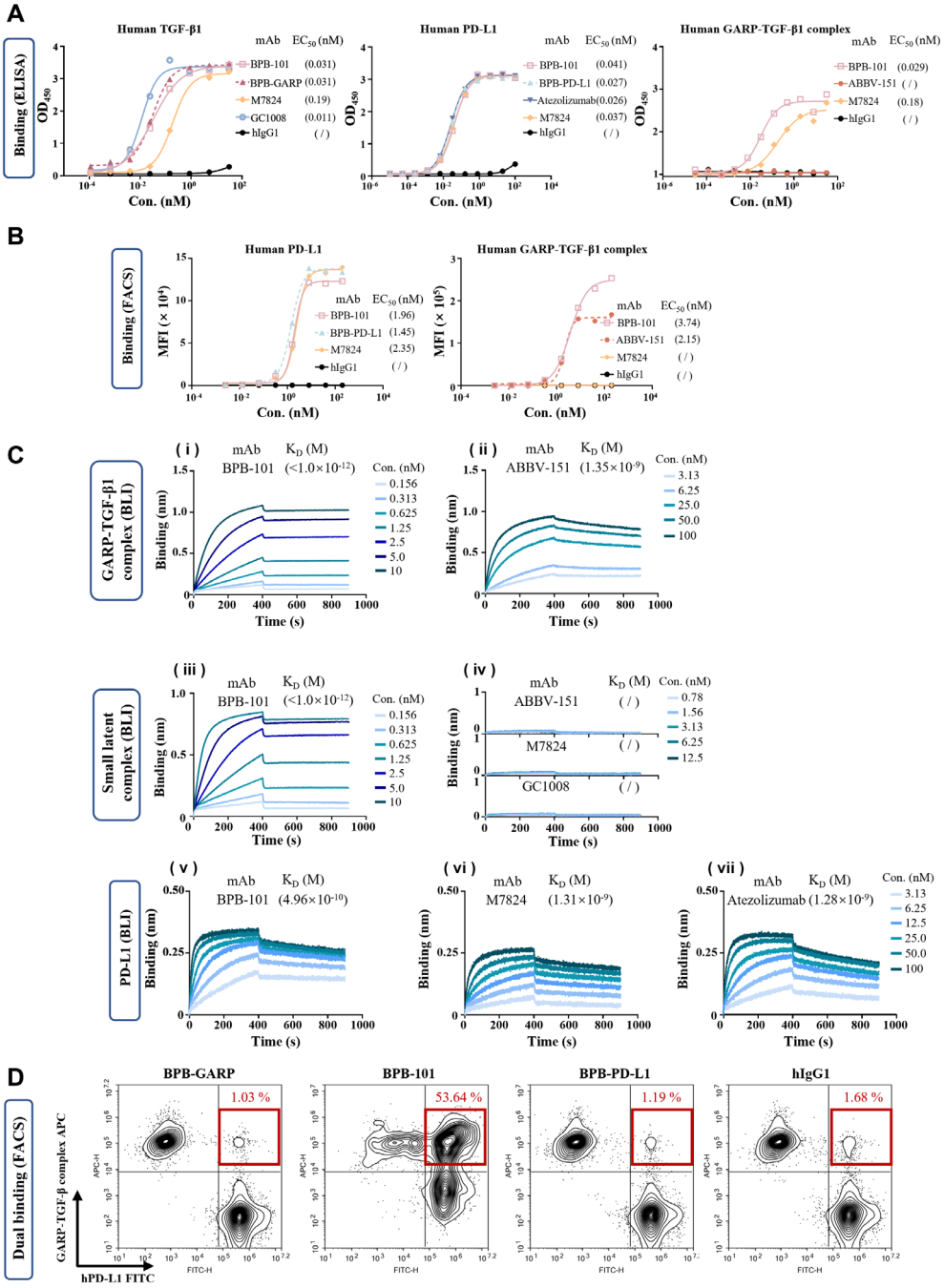


FIGURE 2
BPB-101 bound efficiently to human TGF-β, human PD-L1 and the human GARP-TGF-β1 complex. **(A)** Binding of different antibodies to human TGF-β, human PD-L1 or the human GARP-TGF-β1 complex as determined by ELISA. **(B)** Binding of different antibodies to 293T-GARP-TGF-β1 complex cells (4E9) or 293T-hPD-L1 cells as detected by FACS. **(C)** (i and ii) Biolayer interferometry (BLI) results showing the interaction of the GARP-TGF-β1 complex with immobilized BPB-101 and ABBV-151 (a GARP-TGF-β1 complex inhibitor) at 10 μg/mL. The concentrations of the GARP-TGF-β1 complex ranged from 0.156 to 10 nM for BPB-101 and from 3.13 to 100 nM for the other assays. The dissociation rate of BPB-101 was less than 1 x $10^{-7}$ 1/s, and it took more than 14 hours to achieve 5% dissociation; thus, an accurate $K_D$ could not be calculated (see Supplementary Table S1 for details). (iii and iv) BLI analysis of the SLC with immobilized BPB-101, ABBV-15, M7824 (PD-L1/TGF-βRII inhibitor) and GC1008 (TGF-β1/2/3 inhibitor) at 10 μg/mL. The concentrations of the SLC ranged from 0.156 to 10 nM for BPB-101 and ranged from 0.78 to 12.5 nM for the other assays. The binding of ABBV-151, M7824 and GC1008 to SLC was not detectable. (v - vii) The binding of human PD-L1 with immobilized M7824 and atezolizumab (PD-L1 inhibitor) at 10 μg/mL as detected by BLI. The concentrations of PD-L1 ranged from 3.13 to 100 nM for each assay. The dissociation constant expressed by $K_D$ was determined from the detailed binding traces of different mAbs. **(D)** Dual binding ability of BPB-GARP, BPB-101, BPB-PD-L1 and hIgG1 at 0.8 nM with CSFE-labeled 293T-hPD-L1 cells and FarRed-labeled 293F-GARP-TGF-β (4E9) cells.

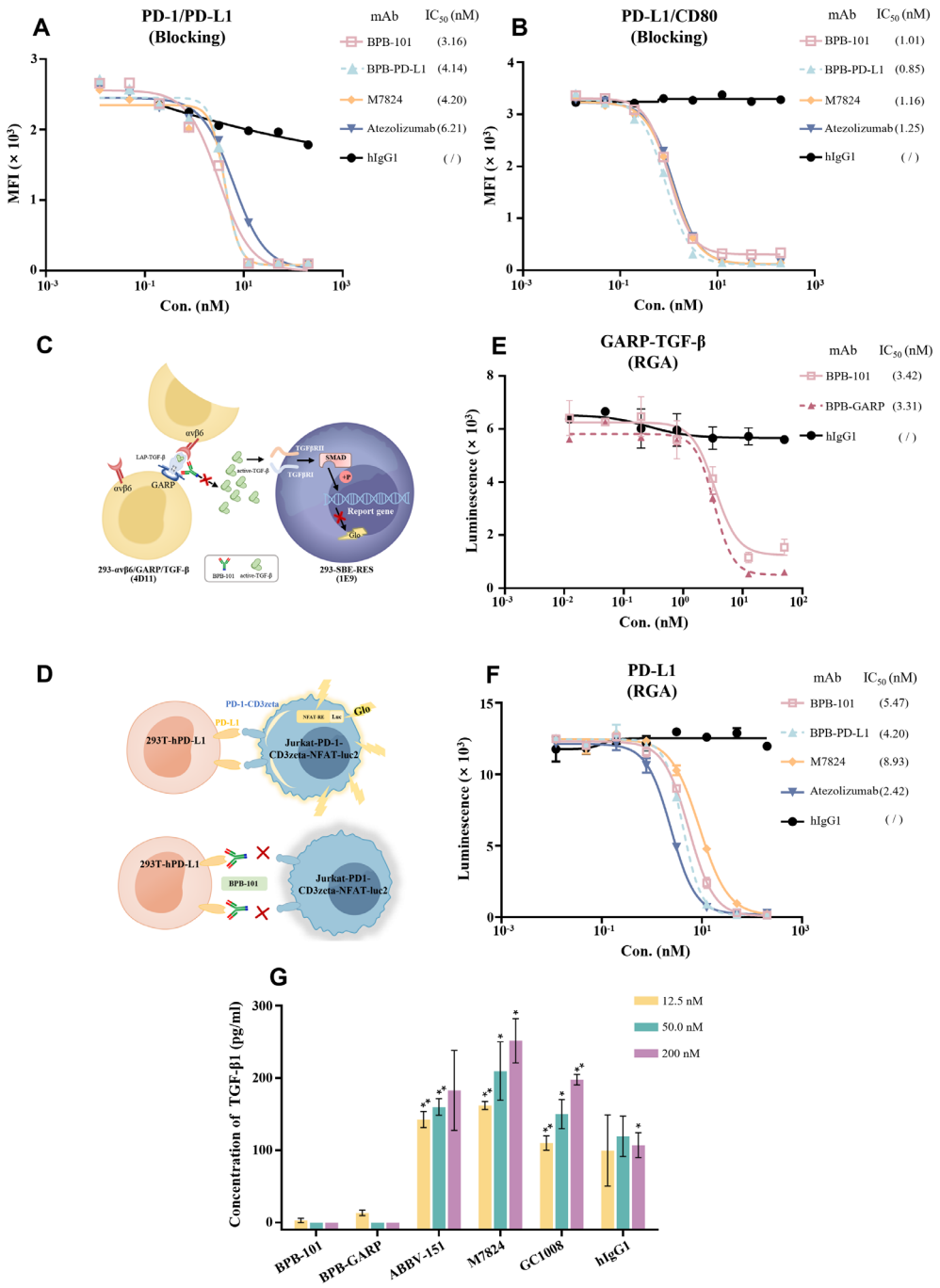


FIGURE 3
BPB-101 blocked the binding of PD-L1 to PD-1 or CD80 and the transduction of TGF-β and PD-L1 signals. **(A, B)** Abilities of BPB-101, BPB-PD-L1, M7824, atezolizumab and hIgG1 to block PD-1/PD-L1 and PD-L1/CD80 as detected by ELISA. **(C)** Schematic diagram of RGA of BPB-101 in blocking GARP-TGF-β signaling. **(D)** Schematic diagram of RGA of BPB-101 in blocking PD-1/PD-L1 signaling. **(E)** Luminescence results of 293-SBE-RES cells in RGA. **(F)** Luminescence results of Jurkat-PD1-CD3zeta-NFAT-luc2 cells in RGA. **(G)** The concentrations of TGF-β1 in the supernatants of Tregs cocultured with BPB-101, BPB-GARP, ABBV-151, M7824, GC1008 or hIgG1 (12.5 nM, 50 nM or 200 nM) for 5 days were detected by ELISA. The data are expressed as mean ± SEM. P values were calculated (different mAbs vs. BPB-101 at the same concentration). The statistical analyses were performed using unpaired two-tailed Student's t tests. (*$P < 0.05$, **$P < 0.01$, ns, not significant, not shown).

respectively; these values were significantly lower than those of M7824 and atezolizumab. Luciferase reporter gene assays were then used to further evaluate the inhibitory effect of BPB-101 on TGF-β and PD-L1 signal transduction. Two cell line systems were established for this assay: 1) HEK-293T cells expressing TGF-β-GARP-αvβ;6 and 293-SBE-RES cells (Figure 3C) and 2) HEK-293T cells expressing PD-L1 and a luciferase reporter driven by a native response element, namely PD-L1 effector cells (Jurkat-PD-1-CD3zeta-NFAT-luc2) (Figure 3D). These two systems were co-cultured in the presence of different mAbs, and the degree to which

the downstream effector cells were activated was determined by measuring the luciferase activity. BPB-101 and BPB-GARP obviously and effectively reduced the fluorescence signal intensity of the 293-SBE-RES cells (Figure 3E, Supplementary Figure S3), and the fluorescence signal intensity of the PD-L1 effector cells was decreased by BPB-101 and BPB-PD-L1. Moreover, in this system, the blocking ability of BPB-101 was weaker than that of atezolizumab and BPB-PD-L1, but stronger than that of M7824 (Figure 3F). The decreased in the downstream fluorescence signal demonstrated the strong ability of our antibodies to inhibit these two immunosuppressive signals. In addition, we evaluated the impact of our mAbs on TGF-β production. The abundant expression of GARP on the surface of Tregs is the main cause of the release of active TGF-β (35). When BPB-101 bound to the GARP-TGF-β complex on Tregs, it effectively prevented the secretion of TGF-β. Tregs were incubated with antibodies for 5 days, after which the TGF-β levels in the supernatants were measured by ELISA. We confirmed that BPB-GARP and BPB-101, but not ABBV-151, GC1008, M7824 or BPB-PD-L1, inhibited TGF-β release by Tregs (Figure 3G, Supplementary Figure S4).

## BPB-101 effectively enhances IFN-γ secretion by PBMCs

The allogeneic mixed lymphocyte reaction (MLR) is an experimental approach in which a mixture of lymphocytes from two unrelated individuals is cultured together, stimulating each other due to the different histocompatibility antigens on their membranes. This leads to cell division and proliferation, as well as the transformation of each lymphocyte. DCs, a class of APCs, can stimulate the activation and proliferation of T cells and induce the secretion of human IFN-γ (hIFN-γ). DCs that express PD-L1, which binds to PD-1 on T cells, can deliver negative immune regulatory signals and inhibit the secretion of hIFN-γ. In addition, Tregs and NK cells derived from PBMCs secrete TGF-β, which in turn inhibits the secretion of hIFN-γ (Figure 4A) (36, 37). We previously showed that BPB-101 could specifically bind to PD-L1 and the GARP-TGF-β1 complex, thereby blocking the PD-1/PD-L1 signaling pathway and TGF-β secretion (Figures 2A, 3). To verify whether BPB-101 relieves the associated immunosuppressive effects and promotes the secretion of hIFN-γ, we measured the auxiliary activating effects of BPB-101 on PBMCs in an MLR system. As expected, BPB-101, BPB-PD-L1 (a humanized monoclonal parental antibody) or atezolizumab treatment enhanced the activation of PBMCs in the presence of DCs, as indicated by an increased in the level of IFN-γ. At a concentration of 20 nM, the effects of BPB-101 and atezolizumab were similar, and both were slightly stronger than that of BPB-PD-L1. However, at a concentration of 80 nM, the effect of BPB-101 surpassed that of atezolizumab. The other humanized monoclonal parental antibody of BPB-101 (BPB-GARP) exerted almost no effect in this study system, even at high concentrations. Thus, the activity of the BPB-101 was superior to that of its constituent monoclonal antibodies (Figure 4B).

## Assessment of the potential *in vitro* side effects of BPB-101

To evaluate the potential side effects of BPB-101, whole blood samples from three healthy donors were incubated with different antibodies, and the binding of these antibodies to various immune cells was determined by FACS. The binding of BPB-101 to nine main types of immune cells was investigated. Compared with hIgG1, BPB-101 exhibited significantly greater binding to $CD4^+$ T cells, $CD8^+$ T cells and Tregs (Figure 5A), which was consistent with its outstanding ability to bind to the GARP-TGF-β complex and PD-L1 (Figure 2A). However, BPB-101 failed to bind to pDCs, classical monocyte cells, nonclassical monocyte cells, B cells, NK cells or NKT cells, all of which play key roles in immunoregulation (Figures 5B, C). On this basis, it is important to understand whether BPB-101 causes abnormal cytokine release, as antibody-induced cytokine release may induce fatal adverse effects, known as cytokine release syndrome (CRS), in the clinic (38, 39). Different mAbs were added to PBMCs, and the levels of IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ were secreted by PBMCs were monitored to assess the potential side effects of these mAbs. As shown in Figures 5D–I, the effect of BPB-101 was quite similar to that of the negative control (atezolizumab, an anti-human PD-L1 antibody). In contrast, the reference antibody (TGN1412, an anti-human CD28 antibody) significantly stimulated the secretion of these cytokines by PBMCs. These findings suggested that BPB-101 could bind specifically to $CD4^+$ T cells, $CD8^+$ T cells and Tregs without eliciting CRS.

## BPB-101 has satisfactory tumor-targeting properties and antitumor effects

According to the previous results of species cross-reaction experiment, BPB-101 can only bind to PD-L1 and GARP-TGF-β complex proteins of human and cynomolgus monkeys, but not to PD-L1 and GARP-TGF-β complex proteins of mice. Therefore, in the subsequent *in vivo* experiments, transgenic mice and cynomolgus monkeys were used as relevant species, respectively. The tumor-targeting ability of BPB-101 was explored in C57BL/6-hGARP mice bearing MC38-hPD-L1 tumors, with healthy C57BL/6-hGARP mice serving as controls. BPB-101 was first labeled with $^{89}$Zr according to a previously reported method (40, 41). $^{89}$Zr-BPB-101 was then intravenously administered, and the mice were scanned via PET/CT at preset times (Figure 6A). The radioactivity per unit volume of tumors, livers, spleens, kidneys, lungs and lymph nodes was analyzed by PMOD software. The percentage injection dose per gram of tissue (%ID/g) was calculated based on the administration dose. Once injected, the radiation of $^{89}$Zr-BPB-101 was immediately distributed in the tumors of tumor-bearing female or male mice, and the amount of mAbs in the tumor gradually increased over time. Twenty-four hours later, the radiation signal of the drug in tumors exceeded that in livers, and the accumulation of the drug in the tumors was consistently greater than that in any other tissue. A high concentration of $^{89}$Zr-BPB-101 remained in the tumor until 336 h,

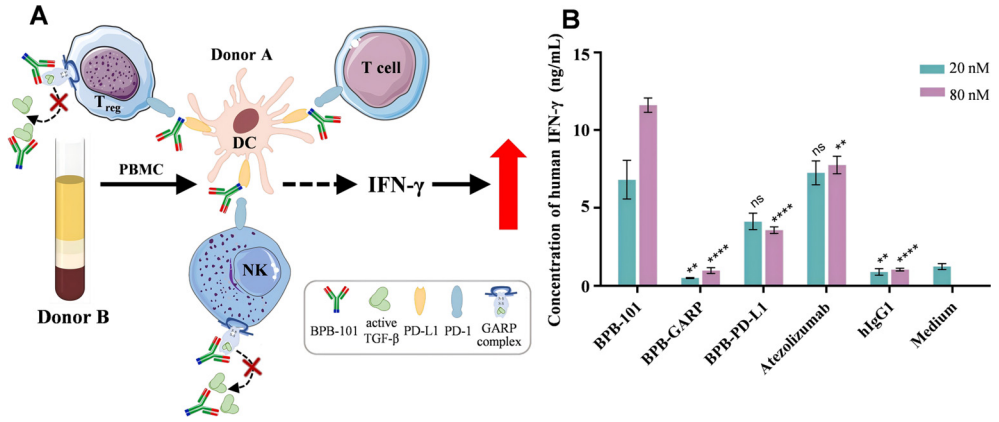


FIGURE 4
BPB-101 increased IFN-γ secretion by PBMCs. **(A)** Schematic diagram of the allogeneic mixed lymphocyte reaction assay. The anti-PD-L1 component is illustrated in blue at the C-terminus of BPB-101, while the Fab region, depicted in red, represents the anti-GARP complex. **(B)** PBMCs and DCs were cocultured with 20 nM or 80 nM BPB-GARP, BPB-PD-L1, BPB-101, atezolizumab or hIgG1 at for 5 days, and the concentrations of human IFN-γ in the supernatants were determined by ELISA. P values were calculated (different mAbs vs. BPB-101 at the same concentration). The data are expressed as the mean ± SEM. The statistical analyses were performed using unpaired two-tailed Student's t tests. (**$P < 0.01$, ****$P < 0.0001$, ns, not significant, not shown).

suggesting that BPB-101 gradually accumulated in the tumor (Figures 6B, C). However, in both healthy female and male mice, $^{89}$Zr-BPB-101 was concentrated in livers (Supplementary Figures S5A, B). We compared the distribution of BPB-101 in a series of tissues collected from tumor-bearing mice and tumor-free healthy mice. Figure 6D shows that there was a statistically significant difference in drug accumulation in the spleen between normal mice and tumor-bearing mice at 72 h. No statistically significant differences in drug distribution were noted at other time points or in other tissues. These data indicated that BPB-101 had satisfactory tumor-targeting ability. Moreover, the tumor volume of mice treated with $^{89}$Zr-BPB-101 tended to decrease over time, indicating that BPB-101 has a potential inhibitory effect on tumor growth (Supplementary Figure S5C).

The antitumor effect of BPB-101 was more pronounced in subsequent trials. C57BL/6-hGARP mice bearing MC38-hPD-L1 tumors were randomly divided into four groups and intraperitoneally injected with PBS, BPB-GARP (4.2 mg/kg), BPB-GARP + BPB-PD-L1 (4.2 + 2.3 mg/kg), or BPB-101 (5 mg/kg) twice a week for a total of four injections (Figure 6E). The changes in tumor volume and body weight of the mice were recorded every other day. Throughout the experiment, the tumor volume and tumor weight of the mice in the BPB-101 group decreased, and at the end of the experiment, the tumors in half of the mice (4/8) had completely disappeared (Figures 6F–H, Supplementary Figure S6). The average tumor volume of the mice in the BPB-101 group remained below 50 mm$^3$ at the end of the experiment. In contrast, the tumor volume of the mice in the PBS group continued to increase, eventually reaching approximately 700 mm$^3$ (Figure 6F). There were significant differences in tumor volume and tumor weight between the BPB-101 group and the PBS group on D18 (Figures 6F, G**).** Although the tumor volume in the BPB-GARP + BPB-PD-L1 group was significantly lower than that in the PBS group, and the tumor elimination rate reached 37.5% (3/8), there was no significant difference in tumor weight (Figure 6G). Additionally, the tumor volume in the BPB-GARP group was significantly lower than that in the PBS group. Compared to those in the BPB-GARP group, the tumor volume and tumor weight of the mice in the BPB-101 group decreased significantly (Figures 6F, G). The observed antitumor activity of BPB-101 was encouraging, and there was no evidence of tumor recurrence in our study, suggesting the potential of BPB-101 to sustain the immune response. The body weight of the mice increased slowly in all groups, and no visible toxic side effects were observed (Figure 6I).

## Evaluation of BPB-101 safety *in vivo*

To further evaluate the safety of BPB-101, a preclinical NHP toxicity study was conducted in cynomolgus monkeys. The cynomolgus monkeys were intravenously injected with 30 mg/kg or 100 mg/kg BPB-101 in a volume of 15 mL/kg, and control monkeys were injected with the same amount of solvent (15 mL/kg). The levels of IL-2, IL-6, TNF-α and IFN-γ in the serum were measured at preset times. Data in Table 1 show that the serum IL-2 levels of cynomolgus monkeys treated with BPB-101 (30 mg/kg or 100 mg/kg) was approximately 1.5 pg/mL one day after the fifth administration, a value similar to the maximum value in the control group (one day after the third administration). Serum IL-6 levels in the treatment groups peaked 6 hours after the end of the first administration and then decreased 24 hours after the first dose. Although serum IL-6 levels subsequently increased again with increasing dose, they remained below the initial peak observed 6 hours after the first administration. The levels of TNF-α and IFN-γ in the serum of cynomolgus monkeys in all the groups were below the lower limit of detection of the assay kit at each time point. Typically, CRS is characterized by a rapid, significant increase in cytokine levels over a short timeframe, with levels that continue to

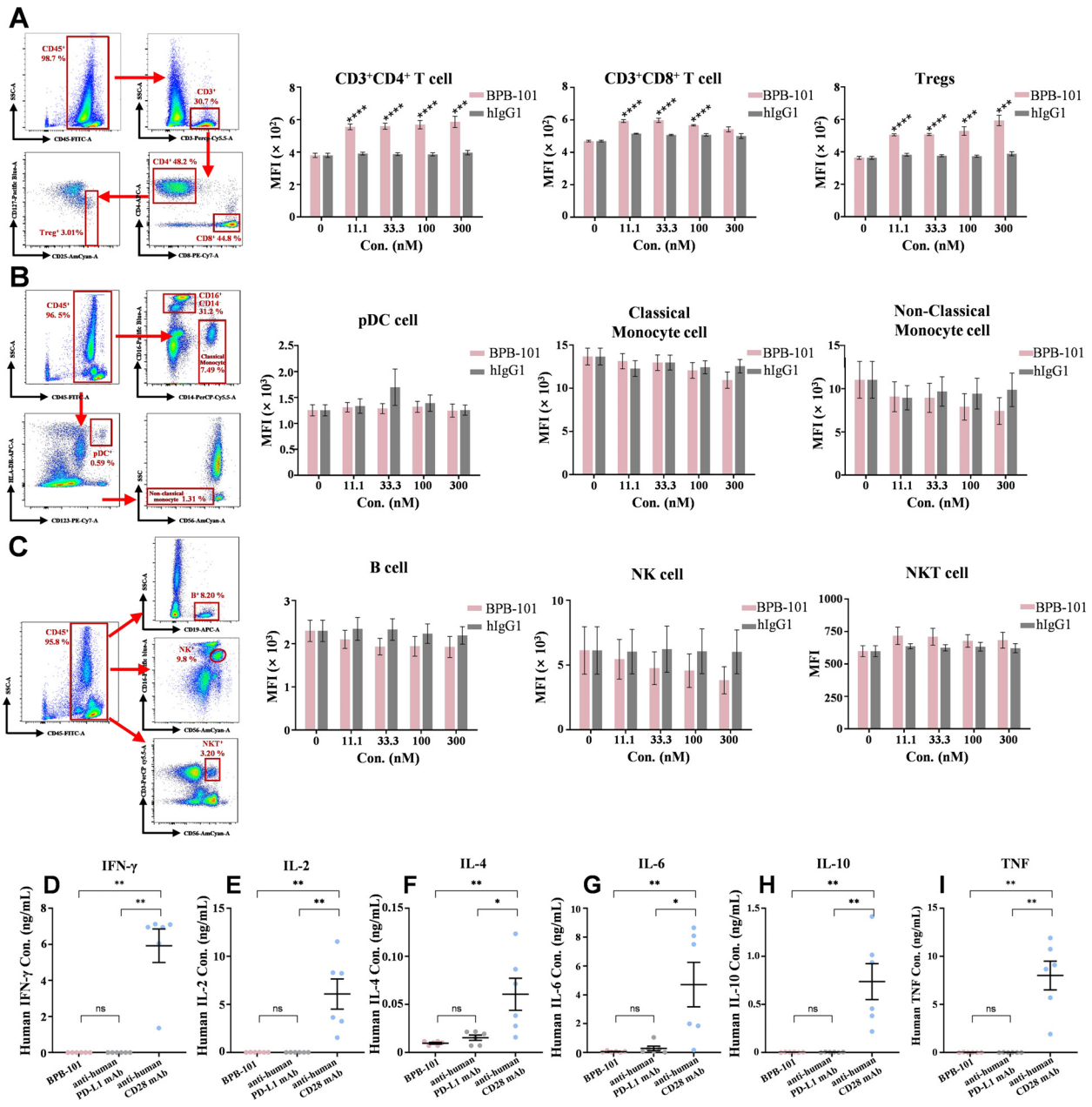


FIGURE 5
*In vitro* risk assessment of BPB-101. **(A)** FACS analysis of $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$) and Tregs ($CD3^+CD4^+CD25^+CD127^{low}$) in the blood. A representative sample is shown to illustrate the gating strategy. P values were calculated (BPB-101 vs. hIgG1 at the same concentration). **(B)** FACS analysis of pDCs ($HLA\text{-}DR^+CD123^+$), classical monocyte cells ($CD14^+CD16^-$) and nonclassical monocyte cells ($CD16^+CD56^-CD14^-$) in the blood. A representative sample is shown to illustrate the gating strategy. **(C)** FACS analysis of B cells ($CD19^+$), NK ($CD56^+CD16^+$) and NKT cells ($CD3^+CD56^+$) in the blood. A representative sample is shown to illustrate the gating strategy. All the values are presented as the mean values of three donors, and each experiment was repeated three times. An anti-human CD28 antibody (TGN1412) was used as a positive control, and an anti-human PD-L1 antibody (atezolizumab) was used as a negative control for the assessment of cytokine secretion by PBMCs. After incubation with three mAbs (10 μg/mL), the supernatants were collected, and the levels of IFN-γ **(D)**, IL-2 **(E)**, IL-4 **(F)**, IL-6 **(G)**, IL-10 **(H)** and TNF **(I)** in the supernatants were determined by FACS according to the experimental methods of the Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II. PBMCs were obtained from six healthy donors. All the data are expressed as the mean ± SEM. The statistical analyses were performed using unpaired two-tailed Student's t tests. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns, not significant).

increase and do not easily return to baseline (the cytokine level of cynomolgus monkey before drug injection). However, BPB-101 treatment did not enhance cytokine release. During the administration and recovery periods, all monkeys in the BPB-101 group maintained a good general condition with normal autonomic activity. Thus, BPB-101 displayed a favorable safety profile *in vivo*.

## BPB-101 exhibits an outstanding stability

Stability is one of the important factors that determines the biological function of antibodies. Here, we investigated the stability profile of BPB-101 in human serum. After incubating BPB-101 for one week in a solution containing a high concentration of serum

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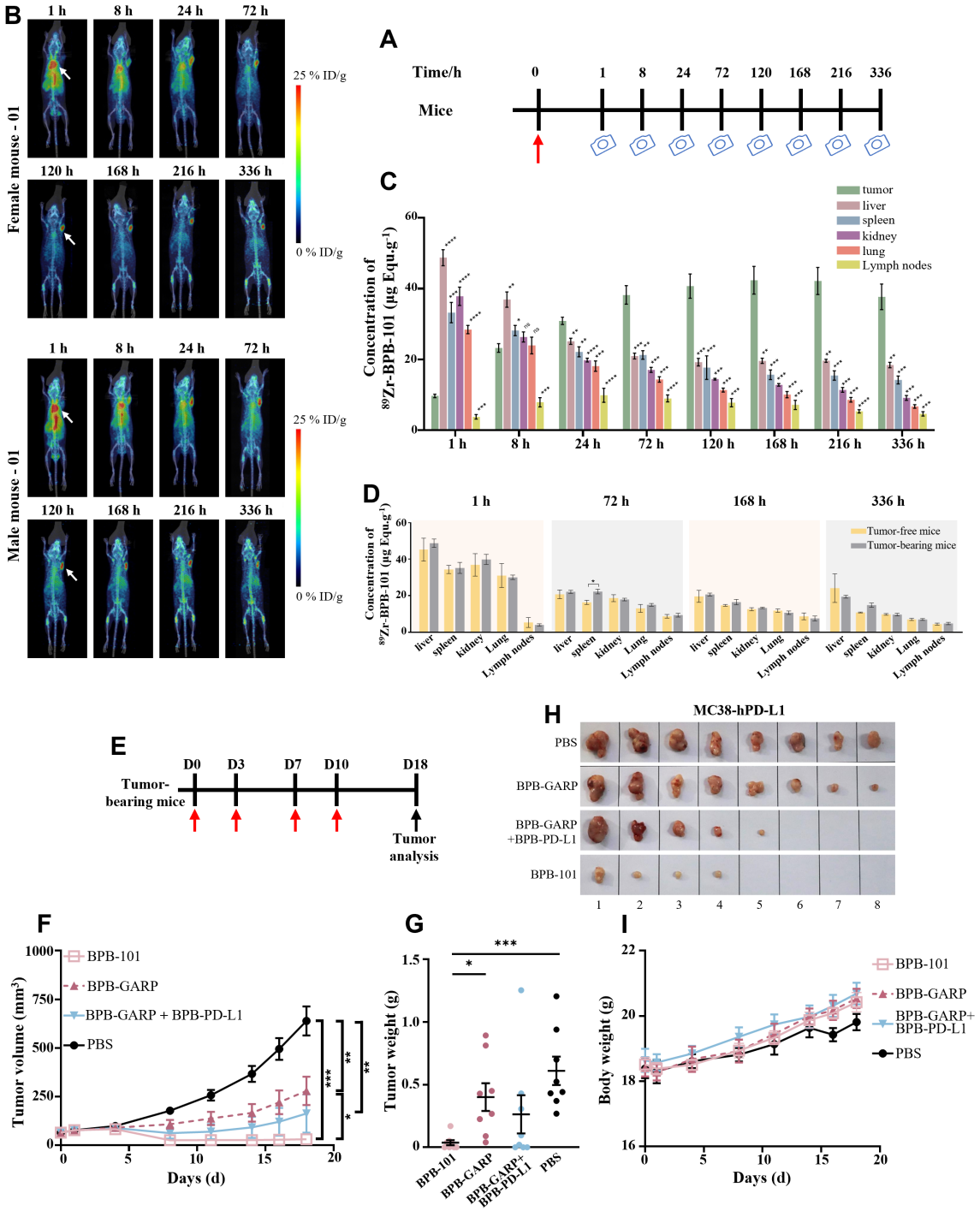


FIGURE 6
BPB-101 exhibited obvious tumor targeting ability and antitumor effects. **(A)** Schematic outline of the assay of BPB-101 biodistribution, red arrow: drug injection, camera: immune PET imaging. **(B)** Maximum intensity projection (MIP) slices obtained 1, 8, 24, 72, 120, 168, 216 and 336 hours after the administration of $^{89}$Zr-BPB-101 to tumor-bearing mice. (5 mg/kg, i.v., one dose). The white arrow indicates the tumor site. **(C)** Concentrations of $^{89}$Zr-BPB-101 in the tumors, livers, spleens, kidneys, lungs and lymph nodes of tumor-bearing mice at different time points (n = 4). **(D)** Biodistribution data of $^{89}$Zr-BPB-101 in the livers, spleens, kidneys, lungs and lymph nodes of tumor-bearing mice (n = 4) and tumor-free mice (n = 2) at 1 h, 72 h, 168 h and 336 h, respectively. P values were calculated (tumor-bearing mice vs tumor-free mice at the same time). **(E)** Schematic outline of the antitumor experiment; red arrow: drug injection, black arrow: tumor analysis. **(F)** Tumor-bearing mice were treated with BPB-101, BPB-GARP, BPB-101 + BPB-PD-L1 or PBS, and the tumor volume was recorded (5 mg/kg, i.p., four doses, n = 8). The P value shown on the top of the column is the comparison of tumor volume on D18. The results are presented as the mean ± SEM. **(G)** Average tumor weight of different mAb-treated mice on D18. **(H)** Images of tumors in different groups on D18 (the source data are shown in Supplementary Figure S6). **(I)** Changes in body weight of mice in all the groups. All the data are expressed as the mean ± SEM. The statistical analyses were performed using unpaired two-tailed Student's t tests. (*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.01, ****$P$ < 0.0001, ns, not significant, not shown).

TABLE 1 Cytokine analysis at various time points following intravenous administration of different doses of BPB-101 in cynomolgus monkey.

| IL-2 (pg/mL) | Control group | | 30 mg/kg group | | 100 mg/kg group | |
|---|---|---|---|---|---|---|
| | N | Mean ± SD | N | Mean ± SD | N | Mean ± SD |
| Before the first administration | 1 | 1.097 | / | / | / | / |
| 2 hours after the end of the first administration | 1 | 0.851 | / | / | / | / |
| 6 hours after the end of the first administration | 1 | 0.844 | / | / | / | / |
| 24 hours after the end of the first administration | 1 | 0.983 | / | / | / | / |
| 1 day after the third administration | 1 | 1.305 | 2 | 0.846 ± 0.0813 | / | / |
| 1 day after the fifth administration | / | / | 2 | 1.620 ± 0.6548 | 1 | 1.572 |
| **IL-6 (pg/mL)** | **Control group** | | **30 mg/kg group** | | **100 mg/kg group** | |
| | N | Mean ± SD | N | Mean ± SD | N | Mean ± SD |
| Before the first administration | 3 | 1.073 ± 0.6285 | 3 | 0.751 ± 0.4538 | 3 | 2.355 ± 1.5642 |
| 2 hours after the end of the first administration | 5 | 3.191 ± 1.0260 | 5 | 5.220 ± 4.1426 | 5 | 3.524 ± 1.0796 |
| 6 hours after the end of the first administration | 5 | 2.327 ± 1.2779 | 5 | 7.164 ± 7.4416 | 5 | 5.110 ± 2.9338 |
| 24 hours after the end of the first administration | 4 | 1.373 ± 1.1042 | 5 | 1.060 ± 0.5945 | 4 | 1.795 ± 1.3313 |
| 1 day after the third administration | 4 | 1.558 ± 1.6487 | 5 | 3.610 ± 3.3586 | 5 | 4.784 ± 3.8497 |
| 1 day after the fifth administration | 2 | 0.818 ± 0.1732 | 5 | 5.886 ± 5.5312 | 5 | 4.693 ± 4.1139 |
| **TNF-α (pg/mL)** | **Control group** | | **30 mg/kg group** | | **100 mg/kg group** | |
| | N | Mean ± SD | N | Mean ± SD | N | Mean ± SD |
| Before the first administration | / | / | / | / | / | / |
| 2 hours after the end of the first administration | / | / | / | / | / | / |
| 6 hours after the end of the first administration | / | / | / | / | / | / |
| 24 hours after the end of the first administration | / | / | / | / | / | / |
| 1 day after the third administration | / | / | / | / | / | / |
| 1 day after the fifth administration | / | / | / | / | / | / |
| **IFN-γ (pg/mL)** | **Control group** | | **30 mg/kg group** | | **100 mg/kg group** | |
| | N | Mean ± SD | N | Mean ± SD | N | Mean ± SD |
| Before the first administration | / | / | / | / | / | / |
| 2 hours after the end of the first administration | / | / | / | / | / | / |
| 6 hours after the end of the first administration | / | / | / | / | / | / |
| 24 hours after the end of the first administration | / | / | / | / | / | / |
| 1 day after the third administration | / | / | / | / | / | / |
| 1 day after the fifth administration | / | / | / | / | / | / |

Cytokine indicators that fall below the lower limit of quantitation (BLQ) are not included in the statistical analysis, hence the number of samples included in the statistics is reduced. A '/' indicates that all data in the group were below the detection limit of the assay kit.

(> 90%) at 37°C, it was still able to bind efficiently to GARP-TGF-β complex-expressing cells (Figures 7A, C), and PD-L1-expressing cells (Figures 7B, D), with little changes in the $EC_{50}$ values as determined by FACS at different time points. Moreover, we conducted other stability experiments, including assessments after exposure to illumination for 5 days (Figure 7E), after repeated freezing and thawing cycles for 3 cycles (Figure 7F), and after high-temperature acceleration tests (40°C, 4 weeks) (Figure 7G), were completed. The $EC_{50}$ values, as determined by ELISA, changed only slightly. All these results confirmed the excellent stability of BPB-101.

## Discussion

The evolution of tumor immunity has indeed unveiled treatment options for cancer patients, effectively prolonging survival and

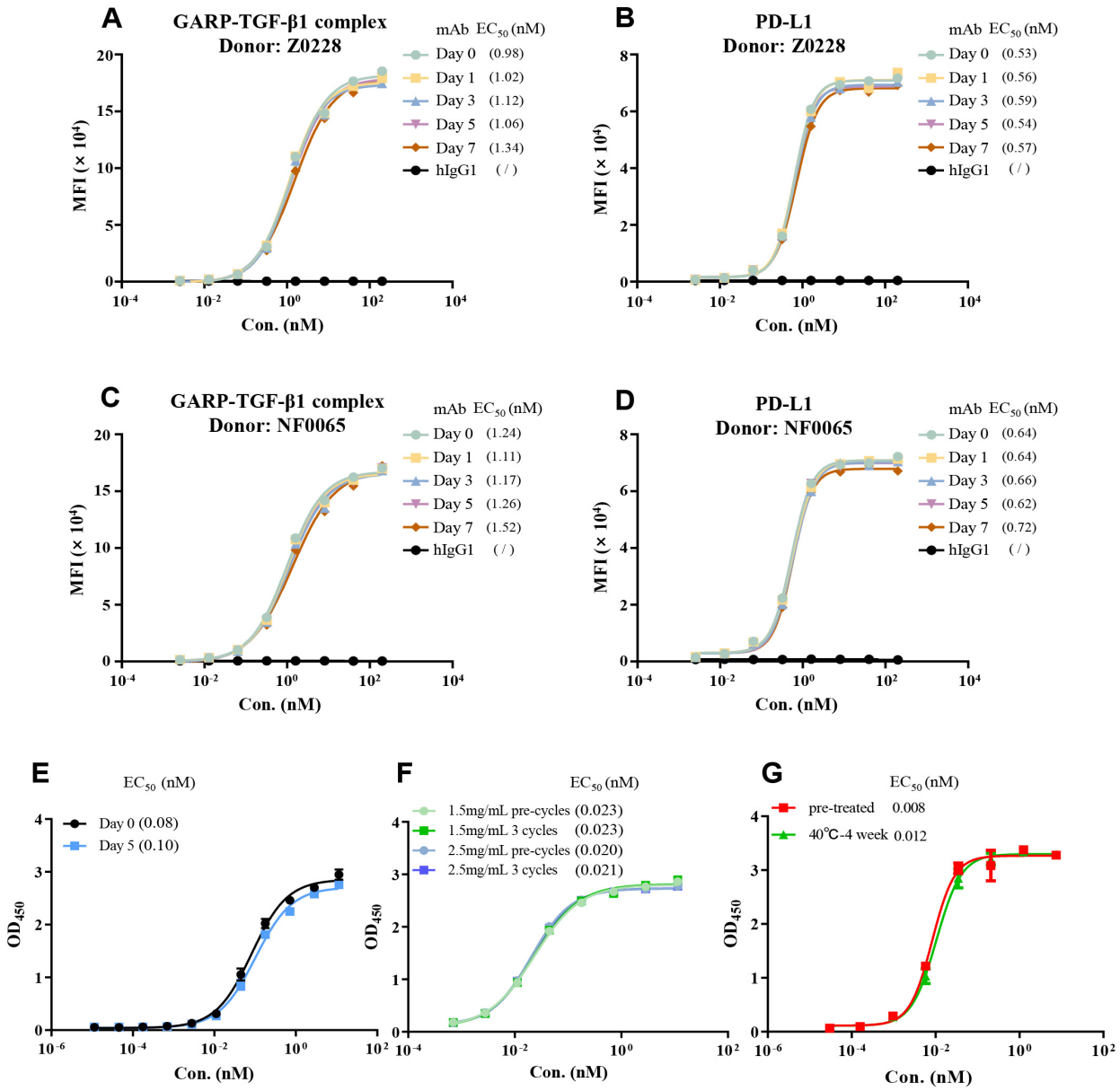


FIGURE 7
Evaluation of BPB-101 stability *in vitro*. **(A–D)** First, BPB-101 was incubated in a solution with a high concentration of serum (> 90%) at 37°C for 0, 1, 3, 5 or 7 days. Then, the ability of BPB-101 to bind to the GARP-TGF-β complex and PD-L1 was determined via FACS [**(A, B)** Donor: Z0228; **(C, D)** Donor: NF0065]. **(E)** The binding of BPB-101 to the GARP-TGF-β complex and PD-L1 after exposure to 5000 lux and 85 μW/cm$^2$ light conditions for 0 and 5 days was determined by ELISA. BPB-101 was placed horizontally during the exposure period. **(F)** Binding of BPB-101 to human TGF-β1 and PD-L1 before and after freeze-thaw cycles, as determined by ELISA. **(G)** Binding of BPB-101 to the GARP-TGF-β complex and PD-L1 after incubation at 40°C for four weeks, as determined by ELISA.

improving the quality of life. However, the fact that only a minority of patients respond to currently available therapies underscores the need for developing novel immune drugs. Within the TME, tumor cells orchestrate an environment conductive to their own growth and survival by directly or indirectly modulating various factors. TGF-β and the PD-1/PD-L1 pathways are two critical and well-studied mechanisms that suppress antitumor immunity in the TME. The development of antitumor drugs targeting the PD-1/PD-L1 axis has been relative success (42), with the launch of numerous products globally, including monoclonal antibodies against PD-1 or PD-L1, and bispecific antibodies targeting PD-1/CTLA-4 (43). Despite these advances, the development of therapeutics targeting TGF-β continue to encounter significant challenges (5).

TGF-β plays a dual role, not only regulating normal cell growth but also promoting tumor growth and metastasis in the advanced TME. It orchestrates tumor angiogenesis, facilitates tumor metastasis, and stimulates tumor fibroblast growth while simultaneously inhibiting the activities of various immune cells. Consequently, transforming the TME into an immune-supportive microenvironment that bolsters the antitumor functions of immune

effector cells is crucial. Tumor cells, in addition to immune cells, also produce TGF-β. Thus, merely inhibiting or eliminating the active TGF-β present in the TME is insufficient, as tumor cells can continue to thrive due to the continuous secretion of TGF-β by themselves or by immune cells.

In light of these insights, we developed a tri-functional bispecific antibody, BPB-101. The Fab arm of BPB-101 specifically targets the GARP-TGF-β complex and/or the SLC, as well as active TGF-β. The C-terminus of BPB-101 incorporates an anti-PD-L1 nAb (VHH), which can effectively disrupt PD-1/PD-L1 signaling. This bispecific antibody is designed to reduce TGF-β levels in the TME and curb the immune evasion tactics of tumor cells. Therefore, BPB-101 has the potential to significantly reverse the immunosuppressive microenvironment and reactivate systemic antitumor immunity.

Upstream of the TGF-β axis, four monoclonal antibodies against GARP have been studied in clinical trials: ABBV-151 (AbbVie), SRK-181 [Scholar Rock Inc. (5)], DS-1055a [Daiichi Sankyo (44)], and HLX-60 (Henlius, NCT05606380). These drugs target hGARP, LAP-TGF-β or the hGARP-TGF-β complex, blocking only one of the TGF-β production pathways, and they cannot neutralize active TGF-β that is already present in the TME. Additionally, drugs developed to target TGF-β or TGF-βR, such as GC1008 (Genzyme, phase III), SAR439459 (Sanofi, phase III), NIS793 (Novartis, phase III), M7824 (Merck, phase II/III), and SHR-1701 (Hengrui, phase III), can only neutralize active TGF-β and cannot block TGF-β production.

Our BPB-101 fully addresses the shortcomings of the aforementioned drugs. The data showed that BPB-101 efficiently bound to its targets-GARP-TGF-β complex, active TGF-β and PD-L1-at both the cell and protein levels. However, DS-1055a, which targets GARP, and ABBV-151, which targets the GARP-TGF-β1 complex, both failed to bind to active human TGF-β1.

Interestingly, we did not detect the binding activity of ABBV-151 to the GARP-TGF-β1 complex by ELISA. However, M7824, which theoretically does not bind to the GARP-TGF-β complex, produced a binding curve to the GARP-TGF-β complex. This result probably occurred due to conformational changes that occurred when the GARP-TGF-β complex antigen was coated, as this phenomenon was not observed in the FACS analyses. These data also suggest that cellular-level detection is necessary to analyze antibody function.

To verify whether BPB-101 also associates with the SLC, we developed a BLI-based assay. The results showed that BPB-101 efficiently bound to the SLC, although the exact binding site remains unclear. In contrast, ABBV-151, M7824 and GC-1008 did not exhibit binding to the SLC. These findings suggest that BPB-101 has the potential to block the three active TGF-β release pathways: cleavage of the LAP domain, the SLC-LTBP complex, and the GARP-TGF-β complex (10). Therefore, this molecule is distinct from currently available GARP/TGF-β-targeting antibodies or fusion proteins. Consequently, BPB-101 may have the potential to address TGF-β immunosuppression in the TME, a challenge that has plagued the field for decades.

The anti-PD-L1 VHH (BPB-PD-L1), derived from an alpaca antibody library, is characterized by small size, high affinity and stable performance (45), which may contribute to improved CMC (Chemistry, Manufacturing and Controls) production and drug stability.

Studies have shown that Tregs inhibit immunity in response to the environment, particularly by promoting tumor growth through the release of TGF-β (46). Furthermore, we demonstrated the ability of our antibody to inhibit TGF-β1 secretion by human Tregs, suggesting its potential for clinical application.

Safety, effectiveness, and drug availability are important criteria for evaluating therapeutic drugs. We confirmed that BPB-101 did not cause significant cytokine release *in vitro*. Furthermore, an *in vivo* safety evaluation of BPB-101 was conducted in cynomolgus monkeys with single-dose and multiple-dose administrations. This evaluation focused on the levels of IL-2, IL-6, TNF-α and IFN-γ at different time points after administration. Since there is no defined threshold for each cytokine, CRS is usually assessed based on trends in cytokine expression. CRS is characterized by a rapid and significant increase in cytokine levels over a short period, with difficulty in reverting to normal levels. However, in our study, the cytokine levels were very low (TNF-α and IFN-γ), similar to those in the control group (IL-2), or initially increased and then decreased (IL-6); these results were inconsistent with the characteristics of CRS. Therefore, we concluded that BPB-101 treatment did not enhance cytokine release and displayed a favorable safety profile.

Given that there is no species crossover within the GARP and PD-L1 genes between mice and humans, we utilized MC38-hPD-L1 tumor cells and human GARP transgenic mice for further studies. First, we demonstrated that BPB-101 effectively distributed to the tumor within an hour and continued to accumulate at the tumor site over time. Then, we further investigated the antitumor efficacy of BPB-101 *in vivo*. The antitumor activity of BPB-101 was notably better than that of monoclonal antibodies alone or in combination. The tumor volume of the mice in the BPB-101 group gradually decreased ($P < 0.001$ vs. PBS) during treatment. Moreover, tumors were completely eliminated in 50% (4/8) of mice, indicating the antitumor potency of BPB-101.

It has been reported that the use of TGF-β inhibitors increases the risk of bleeding (47). Specifically, Mayur S. Mitra et al. revealed that treatment with anti-TGF-β neutralizing monoclonal antibodies, which block all three isoforms, was associated with an increased risk of bleeding and cardiac toxicity in mice and monkeys (48). In a phase I clinical study of M7824, the incidence of bleeding-related AEs was 39.3%, and the incidence of grade 3 and higher bleeding-related AEs was 10.2% (49, 50). However, bleeding was not observed in our *in vivo* preclinical study with cynomolgus monkeys, indicating that the safety profile of BPB-101 may differ from that of TGF-β inhibitors.

Moreover, stability assessments, including evaluation of stability in serum, after exposure to light, after repeated freeze-thaw cycles, and after high-temperature stress tests, confirmed the stability of BPB-101.

In summary, BPB-101 effectively blocks the immunosuppression of TGF-β by simultaneously targeting the source of TGF-β production and neutralizing active TGF-β. Through this effect, coupled with the inhibition of the PD-L1 axis, BPB-101 elicits potent antitumor activity in preclinical models. Given these very encouraging preclinical observations, a good safety profile, and druggability, BPB-101 has entered clinical development to examine

its safety and preliminary efficacy in advanced-stage cancer patients. During the dose-escalation phase of the clinical trial, BPB-101 has shown good safety and has not induced CRS.

Research has shown that in patients with acute myeloid leukemia (AML), the expression of GARP on $CD4^+$ T cells is elevated, leading to increased levels of TGF-β1. This increase inhibits the antitumor activity of NK cells, resulting in their dysfunction. Further studies indicate that blocking TGF-β1 signaling can enhance the anti-tumor activity of NK cells in leukemia xenograft mouse models (51). Therefore, we believe that BPB-101 not only has potential for the treatment of solid tumors but also holds promise for applications in hematological malignancies. We anticipate that BPB-101 will benefit more cancer patients and fill an important gap in currently available treatment options.

However, there are still limitations and areas for further exploration. The intricate relationships and synergies among the three functions of BPB-101, its interaction with immune cells, and its precise mechanisms of action remain to be fully elucidated. Future studies should focus on analyzing the characteristics of immune cells at different tumor stages, as well as exploring more indications and disease models. The selection of appropriate biomarkers will also be critical for advancing the clinical development of BPB-101.

Moreover, the exploration of combination therapies and the ability to address unmet clinical needs remain paramount. Despite the promising preclinical performance and safety profile of BPB-101, understanding its place within the broader landscape of cancer therapy, particularly in combination with existing treatments, will be essential for maximizing patient benefit.

## 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

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Jiangsu Provincial Department of Science and Technology Animal Control Committee (AP-MIJ220068). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

WX: Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Data curation. JX: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. PL: Data curation, Investigation, Methodology, Writing – review & editing. DX: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. HC: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. HZ: Investigation, Methodology, Writing – review & editing. LZ: Formal analysis, Validation, Writing – review & editing. ML: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. SY: Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. MJ: Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. WY: Formal analysis, Investigation, Methodology, Writing – review & editing. JW: Supervision, Writing – review & editing. LD: Funding acquisition, Resources, Supervision, Writing – review & editing.

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## Conflict of interest

The authors are employees of Betta Pharmaceuticals Co., Ltd., which also provided funding for this study, thereby presenting a potential conflict of interest.

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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.2024.1479399/full#supplementary-material

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# Global trends and research hotspots in perioperative management of lung cancer: a bibliometric analysis from 2004 to 2024

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**Objective:** This article aims to analyze the current status and research hotspots of literature related to perioperative management of patients with Lung Cancer and provide reference for future research directions.

**Methods:** This study conducted a bibliometric analysis of research literature related to perioperative management of Lung Cancer published between 2004 and 2024, retrieved from the Web of Science database. R software and VOSviewer were used for analyzing keyword clusters and research themes, revealing trends and frontiers in this field.

**Results:** A total of 4,942 studies on perioperative management of lung cancer were included. In recent years, research in this area has shown a global upward trend, with particular focus on surgical risk assessment, complication prevention, and postoperative management. Perioperative biomarkers before and after surgery have emerged as a central focus due to their impact on diagnosis and treatment. The application of novel therapies, such as targeted drugs and immunotherapy, in perioperative management is also becoming a significant research hotspot. Additionally, China has been a leading contributor to research output in this field, demonstrating strong performance in international collaborations.

**Conclusion:** Perioperative management is a critical factor influencing the prognosis of Resectable lung cancer patients. Through a systematic analysis of the current status and research hotspots in perioperative management of lung cancer, this study provides valuable references for future clinical practice and research, particularly regarding the integration of novel therapies to optimize patient outcomes.

KEYWORDS

**lung cancer, perioperative management, immunotherapy, targeted drugs, bibliometric**

# 1 Introduction

Lung cancer is a malignancy that arises from cells in the lung tissue, typically originating from the cells lining the airways (1). Lung cancer is mainly divided into small cell lung cancer and non-small cell lung cancer (NSCLC), of which NSCLC accounts for 80%-85% (2, 3). Lung cancer is currently one of the most dangerous cancers globally, consistently ranking among the top three in incidence rates over the years (4). In 2022, there were approximately 19.97 million new cancer cases worldwide, with lung cancer accounting for 2.481 million cases, representing about 12.4% of all new cancer cases, once again making it the most common cancer (5). In China, lung cancer is both the most prevalent and the most deadly malignancy. According to epidemiological data from 2022, the incidence and mortality rates of lung cancer remain high, posing a serious threat to public health and presenting significant challenges to individuals and families (6).

Current treatments for lung cancer include surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy. For early-stage NSCLC, surgery is usually the best treatment option (7). In addition, although surgery is the first choice for early-stage lung cancer, 30%-55% of patients still experience recurrence and death, so perioperative treatment is very important (8). In the past few decades, adjuvant platinum-based doublet chemotherapy after surgery has been the standard treatment for early-stage NSCLC patients. Although adjuvant chemotherapy has an advantage in disease-free survival (DFS) compared to surgery alone, the overall survival improvement is not significant, only increasing by 5% (9). Therefore, more effective treatments are urgently needed. In cases of advanced or inoperable lung cancer, chemotherapy are commonly used. With the rapid development of targeted and immunotherapy, these treatments have emerged as new approaches for lung cancer, particularly for patients with specific genetic mutations, such as EGFR or ALK mutations. Due to the high mortality rate of lung cancer (10), and the challenges posed by Locally advanced lung cancer, tumor biological heterogeneity, and patient individual differences, treatment remains highly challenging (11). In this context, perioperative management of lung cancer has become a critical component for successful treatment, highlighting its importance (12).

Perioperative management encompasses the entire treatment process, from preoperative assessment and preparation to intraoperative procedures and postoperative recovery (13). It is not just a time-specific concept but a comprehensive set of management measures aimed at maximizing surgical success and minimizing postoperative complications through multidisciplinary collaboration and integrated treatments, ultimately improving patient survival rates and quality of life (14–16).

Preoperative assessment involves several key aspects, such as determining whether the patient's respiratory system can tolerate the surgical burden through tests like FEV1 (forced expiratory volume in one second) and DLCO (diffusing capacity of the lungs for carbon monoxide) (17). Preoperative interventions, including managing malnutrition, anemia, and chronic diseases, are also crucial, as improving these conditions can significantly enhance surgical safety and success rates (18). During the surgery, real-time monitoring of vital signs and oxygenation status is essential for ensuring surgical success. New surgical techniques, such as video-assisted thoracoscopic surgery (VATS), can greatly reduce the incidence of complications and accelerate postoperative recovery (19). Postoperative management is also vital for the long-term prognosis of lung cancer patients. Common postoperative complications include pulmonary infections, persistent air leaks, atelectasis, and arrhythmias (20). For some high-risk patients, postoperative treatments such as chemotherapy, radiotherapy, or targeted therapy are particularly important, as they can reduce the risk of tumor recurrence and improve survival rates. Additionally, regular follow-up and imaging surveillance are crucial for early detection of recurrences and timely intervention (21, 22). These processes can significantly speed up patient recovery and enhance prognosis.

While preoperative and postoperative care are vital components of lung cancer management, analyzing the academic research surrounding this topic provides a broader perspective on the field's growth and progress. Bibliometrics is a widely used method for analyzing academic publications (23). With the advent of scientific databases such as Web of Science (WOS), obtaining research data has become more convenient, driving rapid developments in bibliometric research (24). As a comprehensive analytical method, bibliometrics combines quantitative and qualitative analysis, revealing various characteristics of publications, including identifying countries, journals, authors, and institutions that contribute significantly to the field, displaying frequently cited research and commonly used keywords, and uncovering collaboration relationships among countries, institutions, and authors within specific scientific domains (25). Furthermore, bibliometric methods provide an overview of the evolution and development frontiers of a research field for new researchers (26). However, there is currently a lack of bibliometric analysis on the perioperative literature in early-stage lung cancer. Although the number of relevant research papers has steadily increased, the knowledge system, research hotspots, and trend development in this field remain unclear. To fill this gap, this study employs R software, VOSviewer, and CiteSpace to conduct a systematic analysis of the literature on perioperative NSCLC from 2004 to 2024. We aim to explore the changes and development trends of research hotspots in this field and identify potential hotspots for future research. Looking ahead, a better understanding of the current state and potential of the perioperative management of lung cancer field is of great significance for its sustainable development.

# 2 Materials and methods

## 2.1 Data collection

The data used in this study were retrieved and downloaded from Web of Science database core data collection (WOSCC). (purchased version by Guangxi Medical University) on August 18, 2024. We used the following search formula:(((((TS=("Preoperative Optimization" OR "Postoperative Care" OR "Perioperative Period" OR "Intraoperative Period" OR

"Postoperative Period" OR "Neoadjuvant treatment" OR "Adjuvant treatment" OR "Neoadjuvant therapy" OR "Adjuvant therapy" OR Perioperative OR Neoadjuvant-adjuvant OR "Preoperative Period")) AND TS=("Pulmonary Neoplasm" OR "Lung Neoplasm*" OR "Lung Cancer*" OR "Cancer of Lung" OR "Pulmonary Cancer*" OR "Cancer of the Lung"))) AND DT=(Article OR Review)) AND LA=(English)) AND PY=(2004-2024).

After removing irrelevant literature, we reviewed 4,957 papers. After eliminating duplicates and retracted articles, a total of 4,942 papers were analyzed. The retrieved papers were saved in plain text format and exported as full records along with their cited references.

## 2.2 Data analysis

To analyze annual publications, Origin 2018 was used. Additionally, R software (version 3.6.3) along with the bibliometrix package (version 4.0) (27), VOSviewer (version 1.6.17) (28), and CiteSpace (version 6.1.4) (29)were employed for data visualization and to create scientific knowledge maps (30). To ensure data accuracy and reliability, data extraction and analysis management were performed by two different authors independently.

VOSviewer was used for visualizing co-authorship networks of countries/institutions, co-citation analysis of sources, and co-occurrence analysis of keywords. In the co-authorship network analysis, the following parameters were set: minimum number of publications for countries ≥25, and minimum number of publications for institutions ≥19. For the co-citation analysis of sources, the parameter set was: minimum number of citations for sources ≥120. Additionally, in the co-occurrence analysis of keywords, the parameters were set as follows: minimum number of occurrences for keywords ≥17. Journal impact factors (IFs) were retrieved from the 2023 Journal Citation Reports (JCR). The study introduced the Multiple Country Publication Ratio (MCP_Ratio) as an indicator to assess the degree of scientific collaboration among different countries. The calculation formula is as follows: MCP_Ratio = MCP/(SCP + MCP), where MCP represents publications involving multiple countries and SCP represents publications from a single country. Additionally, frequency (Freq) is used to indicate the proportion of articles from a specific country relative to the total number of articles, calculated using the formula: Freq = Articles/Total Articles. To further clarify the purposes and specific tasks of each analytical platform, we have summarized the applications of these tools in Annex 1.

# 3 Results

## 3.1 Overview of literature in the field of perioperative management of lung cancer

A total of 4,957 documents were collected from WoSCC. After removing duplicates and retracted articles, 4,942 documents remained. Figure 1A shows that the number of publications related to perioperative management of lung cancer has increased annually. There was a slow increase from 2004 to 2010, a moderate increase from 2010 to 2017, a rapid increase from 2017 to 2021, and an explosive growth from 2021 to 2023. In 2023, the number of published documents reached 555, and as of August 2024, 366 documents have been published in this field.

According to the statistics based on the country of the corresponding authors, China (n = 1,481) has the highest productivity, followed by the USA (n = 1,148), Japan (n = 493), Italy (n = 290), and Germany (n = 181). Notably, among the top five countries by publication volume, China and the USA have multinational publication (MCP) ratios of 9.30% and 14.40%, respectively, which are significantly lower than those of Canada and the United Kingdom, with MCP ratios of 32.30% and 38.50% (Figure 1B, Table 1). Furthermore, Figure 2A indicates that China has the most extensive collaboration with other countries in the field of perioperative management of lung cancer research. Additionally, the collaboration map shows that The University of Texas System (n = 371) and Duke University (n = 148) are prominent centers of collaboration (Figure 2B; Table 2).

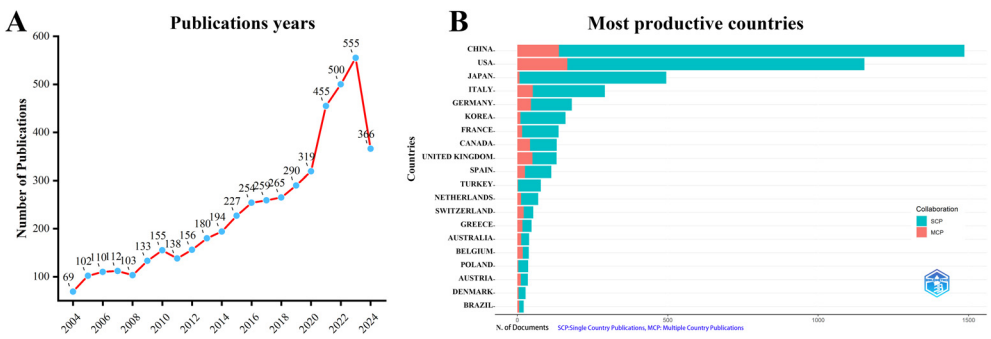


FIGURE 1
Annual publication trends in perioperative management of lung cancer research from 2004 to 2024 were analyzed.**(A)** Depicts the annual publication trends. **(B)** Illustrates the distribution of countries and the collaborative efforts among corresponding authors.

TABLE 1 Most relevant countries by corresponding authors.

| Country | Articles | SCP | MCP | Freq | MCP_Ratio |
|---|---|---|---|---|---|
| China | 1486 | 1348 | 138 | 0.300 | 0.093 |
| USA | 1154 | 988 | 166 | 0.233 | 0.144 |
| Japan | 495 | 488 | 7 | 0.100 | 0.014 |
| Italy | 291 | 240 | 51 | 0.059 | 0.175 |
| Germany | 181 | 136 | 45 | 0.037 | 0.249 |
| Korea | 160 | 150 | 10 | 0.032 | 0.063 |
| France | 137 | 121 | 16 | 0.028 | 0.117 |
| Canada | 131 | 89 | 42 | 0.026 | 0.321 |
| United Kingdom | 130 | 80 | 50 | 0.026 | 0.385 |
| Spain | 113 | 88 | 25 | 0.023 | 0.221 |
| Turkey | 78 | 76 | 2 | 0.016 | 0.026 |
| Netherlands | 69 | 57 | 12 | 0.014 | 0.174 |
| Switzerland | 53 | 32 | 21 | 0.011 | 0.396 |
| Greece | 47 | 30 | 17 | 0.009 | 0.362 |
| Australia | 39 | 26 | 13 | 0.008 | 0.333 |
| Belgium | 38 | 20 | 18 | 0.008 | 0.474 |
| Poland | 36 | 33 | 3 | 0.007 | 0.083 |
| Austria | 35 | 24 | 11 | 0.007 | 0.314 |
| Denmark | 27 | 23 | 4 | 0.005 | 0.148 |
| Brazil | 21 | 15 | 6 | 0.004 | 0.286 |

MCP, Multiple country publication; SCP, Single country publication.

## 3.2 Journals and co-cited journals

Using R software (version 3.6.3) with the Bibliometrix and ggplot2 packages, we analyzed the journals with the highest number of published articles and the most cited journals in this field. Additionally, VOSviewer (version 1.6.17) was utilized for co-citation analysis of journals. The results indicate that a total of 4,942 documents were published across 819 academic journals (Annex 2).

Table 3 and Figure 3A show that the journal with the highest number of published articles is the *Annals of Thoracic Surgery* (n = 268, IF = 3.6), followed by the *Journal of Thoracic Disease* (n = 247, IF = 2.1), the *European Journal of Cardio-Thoracic Surgery* (n = 186, IF = 3.1), *Lung Cancer* (n = 161, IF = 4.5), and the *Journal of Thoracic and Cardiovascular Surgery* (n = 139, IF = 4.9).

Additionally, Table 4 and Figure 3B reveal that the most cited journal is the *Journal of Clinical Oncology* (n = 12,683, IF = 42.1), followed by the *Annals of Thoracic Surgery* (n = 10,762, IF = 3.6), the *New England Journal of Medicine* (n = 6,942, IF = 96.2), the *Journal of Thoracic Oncology* (n = 6,715, IF = 21), and the *Journal of Thoracic and Cardiovascular Surgery* (n = 6,243, IF = 4.9).

The co-citation analysis of journals shows that the *Journal of Clinical Oncology* and *Annals of Thoracic Surgery* are prominent centers of collaboration (Figure 4). Furthermore, we observed that the *Annals of Thoracic Surgery* and *Journal of Thoracic Disease* are among the top journals in both publication volume and citation count. These findings suggest that the *Annals of Thoracic Surgery*, *Journal of Clinical Oncology*, and *Journal of Thoracic Disease* are likely the most representative journals in this field. Additionally, these results highlight a relative scarcity of publications on perioperative achievements in top-tier journals, underscoring the need to enhance the depth and quality of research in this area.

## 3.3 Most cited references and reference burst

We used the Bibliometrix package in R software to identify the top 20 most cited references in perioperative management of lung cancer research (Table 5). These references each have over 400 citations and span 15 different journals, indicating that significant breakthroughs in this field are still emerging. Interestingly, no single

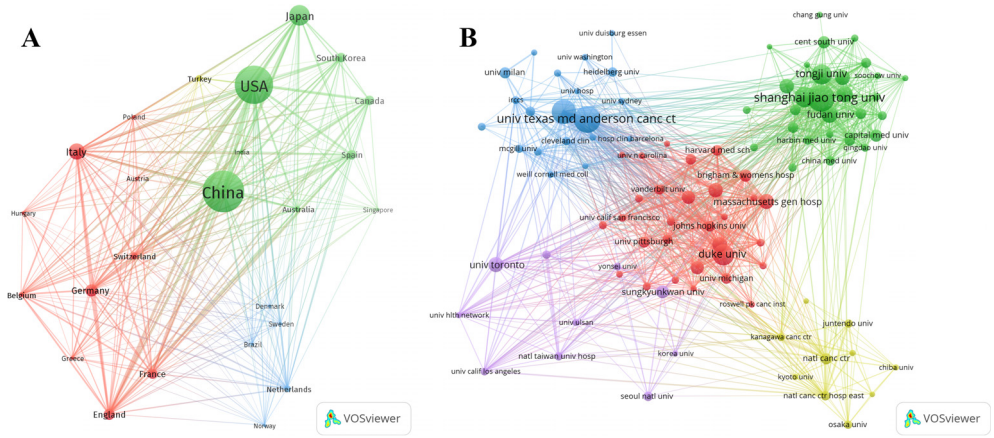


FIGURE 2
A map of countries involved in the field of perioperative management of lung cancer research from 2004 to 2024. **(A)** Map of cooperation between different countries. **(B)** Map of cooperation between different institutions.

TABLE 2 Most relevant affiliations in perioperative management of lung cancer.

| Affiliation | Articles(n) |
|---|---|
| The University of Texas System | 371 |
| Harvard University | 349 |
| The University of Texas MD Anderson Cancer Center | 309 |
| Shanghai Jiao Tong University | 234 |
| Memorial Sloan Kettering Cancer Center | 228 |
| University of Toronto | 199 |
| Sichuan University | 184 |
| University of California | 179 |
| Sun Yat-sen University | 171 |
| Central South University | 157 |
| Chinese Academy of Medical Sciences (Peking Union Medical College) | 150 |
| Duke University | 148 |
| Fudan University | 144 |
| Tongji University | 140 |
| UNICANCER | 139 |
| Zhejiang University | 136 |
| Yale University | 135 |
| Tianjin Medical University | 130 |
| Sungkyunkwan University | 129 |
| National Taiwan University | 123 |
| Peking Union Medical College | 118 |
| Mayo Clinic | 117 |
| Ruprecht-Karls-University Heidelberg | 116 |
| Université Paris Cité | 116 |
| Harvard Medical School | 114 |

journal dominates among the top 20 most cited references. The most cited references include "Integrated Genomic Characterization of Endometrial Carcinoma," "A View on Drug Resistance in Cancer," and "American Society of Clinical Oncology Treatment of Unresectable Non–Small-Cell Lung Cancer Guideline: Update 2003." However, upon closer examination, we found that these articles are predominantly original research and review articles within the perioperative management of lung cancer research field.

To identify influential citation bursts in perioperative management of lung cancer research, we performed an analysis using CiteSpace (selection criteria: top 25; number of states: 2; minimum duration: 2). The results revealed 98 references showing significant citation bursts, with 25 of them illustrated in Figure 5. The top three references with the most active citation bursts are "Cisplatin-Based Adjuvant Chemotherapy in Patients with Completely Resected Non–Small-Cell Lung Cancer" (intensity: 65.4), "The IASLC Lung Cancer Staging Project: Proposals for Revision of the TNM Stage Groupings in the Forthcoming (Eighth) Edition of the TNM Classification for Lung Cancer" (intensity: 62.78), and "Vinorelbine Plus Cisplatin vs. Observation in Resected Non–Small-Cell Lung Cancer" (intensity: 48.68).

Notably, the three most recent citation bursts are "Adjuvant Atezolizumab After Adjuvant Chemotherapy in Resected Stage IB–IIIA Non-Small-Cell Lung Cancer (IMpower010): A Randomised, Multicentre, Open-Label, Phase 3 Trial," "Neoadjuvant PD-1 Blockade in Resectable Lung Cancer," and "Gefitinib Versus Vinorelbine Plus Cisplatin as Adjuvant Treatment for Stage II–IIIA (N1–N2) EGFR-Mutant NSCLC (ADJUVANT/CTONG1104): A Randomised, Open-Label, Phase 3 Study." To gain deeper insights into the research frontiers and hotspots in perioperative management of lung cancer, we matched the DOIs of these 25 references with the titles in Annex 3.

In these referenced studies, randomized controlled trials (RCTs) constitute 27.3%, followed by phase studies (including Phase I, II, and III trials) at 22.7%, primarily focusing on the efficacy of various drugs during different stages of the perioperative period in lung cancer. These findings indicate that current research hotspots are centered on the combined use of neoadjuvant immunotherapy and chemotherapy, particularly in their application to NSCLC patients and their long-term prognostic outcomes.

TABLE 3 Top 10 journals with the most published.

| Sources | Documents | IF (2023) | Cites |
|---|---|---|---|
| Annals of Thoracic Surgery | 268 | 3.6 | 10762 |
| Journal of Thoracic Disease | 247 | 2.1 | 2180 |
| European Journal of Cardio-Thoracic Surgery | 186 | 3.1 | 5381 |
| Lung Cancer | 161 | 4.5 | 3495 |
| Journal of Thoracic and Cardiovascular Surgery | 139 | 4.9 | 6243 |
| Journal of Thoracic Oncology | 126 | 21 | 6718 |
| Frontiers in Oncology | 123 | 3.5 | 804 |
| Cancers | 104 | 4.5 | 638 |
| Clinical Lung Cancer | 87 | 3.3 | 966 |
| Thoracic Cancer | 87 | 2.3 | 537 |

## 3.4 Keyword clusters and evolution

Keyword clustering is an excellent way to understand the research hotspots and directions in a field. In this study, we extracted 6,467 keywords using VOSviewer. Table 6 shows that the top 20 keywords each appeared more than 210 times. Among them, the most frequently occurring keyword is "survival" (n = 850), followed by "surgery" (n = 743), "cell lung-cancer" (n = 676), "chemotherapy" (n = 655), "resection" (n = 620), "lobectomy" (n = 366), "therapy" (n = 329), and "open-label" (n = 324).

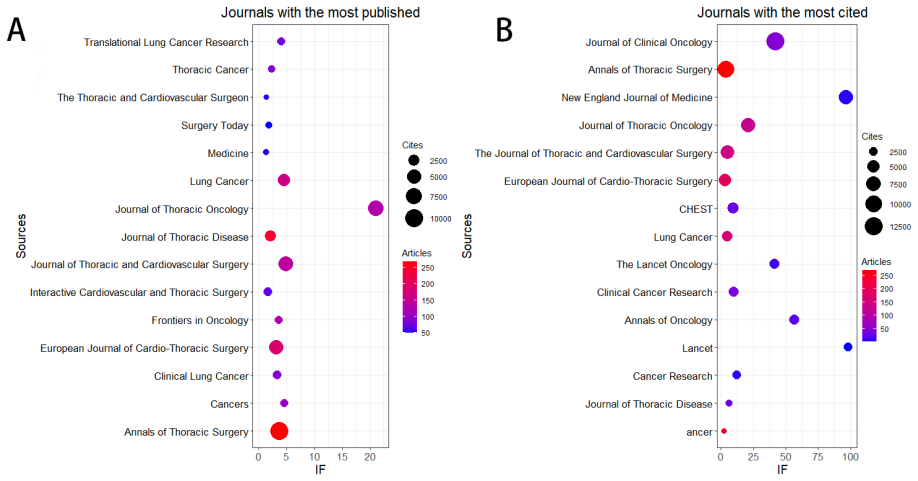


FIGURE 3
The journal with the highest volume of published articles and the journal with the most extensive citation count. **(A)** The journal with the highest quantity of published documents. **(B)** The journals with the most substantial citation counts.

Next, we selected 101 keywords with a minimum occurrence of 17 times to create a keyword clustering map (Figure 6). In the map, we observed five distinct clusters represented by different colors. The tumor types and related genes cluster (red points) includes 34 keywords such as breast cancer, colorectal cancer, esophageal cancer, gastric cancer, and non-small-cell lung cancer. The lung cancer treatment and perioperative management cluster (green points) contains 27 keywords, including meta-analysis, morbidity, mortality, outcomes, perioperative care, and perioperative period. The lung cancer surgical techniques and minimally invasive treatments cluster (blue points) has 15 keywords, including robotic surgery, segmentectomy, sleeve lobectomy, sublobar resection, and thoracotomy. The lung cancer treatment strategies and prognosis cluster (yellow points) comprises 14 keywords such as therapeutic approaches, prognostic outcomes, oncological management, treatment modalities, and survival metrics. The lung cancer immunotherapy and clinical research cluster (purple points) includes 11 keywords like immune checkpoint inhibitors, immunotherapy, neoadjuvant immunotherapy, chemoradiotherapy, and case report (Annex 4).

Additionally, we generated a trend topic map using the Bibliometrix package in R software (Figure 7). Trend topic maps are useful tools for identifying the chronological progression of research topics within a specific field, allowing us to examine the evolution of the field over time. By analyzing the trend topic map shown in Figure 7, we were able to identify the research focus and evolving trajectory of each stage in perioperative management of lung cancer research. Our findings indicate that the current research in this field mainly concentrates on pembrolizumab, open-label studies, and osimertinib.

Overall, our analysis reveals that recent lung cancer research has shifted focus toward novel targeted drugs and immunotherapy, with increased attention to postoperative management, survival rates, and new diagnostic technologies. In contrast, traditional randomized trials and some older keywords have seen a decline in focus.

TABLE 4 Top 10 journals with the most cited.

| Sources | Cites | IF (2023) | Documents |
|---|---|---|---|
| Journal of Clinical Oncology | 12683 | 42.1 | 48 |
| Annals of Thoracic Surgery | 10762 | 3.6 | 268 |
| New England Journal of Medicine | 6942 | 96.2 | 6 |
| Journal of Thoracic Oncology | 6715 | 21 | 127 |
| Journal of Thoracic and Cardiovascular Surgery | 6243 | 4.9 | 139 |
| European Journal of Cardio-Thoracic Surgery | 5381 | 3.1 | 186 |
| CHEST | 3906 | 9.5 | 32 |
| Lung Cancer | 3495 | 4.5 | 161 |
| Lancet Oncology | 3377 | 41.6 | 12 |
| Clinical Cancer Research | 3281 | 10 | 42 |

# 4 Discussion

## 4.1 General information

In this study, we collected a comprehensive corpus of 4,942 documents covering the period from 2004 to 2024. The analysis indicates a continuous upward trend in literature on perioperative management of lung cancer, with a gradual increase from 2004 to 2010, a moderate rise from 2010 to 2017, a rapid surge from 2017 to 2021, and an explosive growth from 2021 to 2023. This phenomenon can be attributed to the following three reasons: (1) Breakthroughs in Immunotherapy: Recent advancements in immunotherapy, particularly the development of immune checkpoint inhibitors (ICIs), have significantly impacted

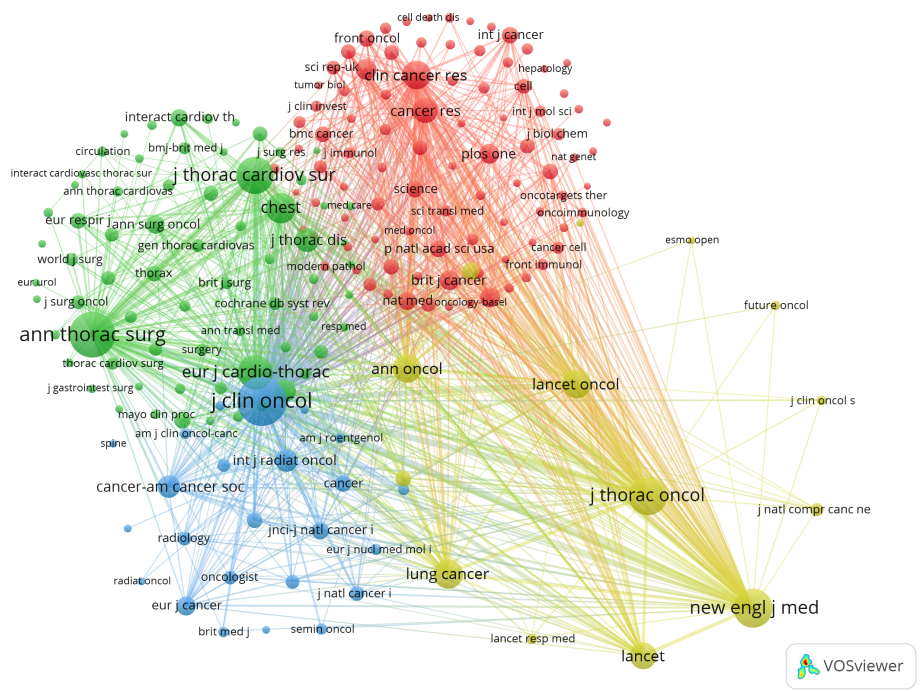


FIGURE 4
Co-cited journals related to perioperative management of lung cancer.

TABLE 5 Top 20 cited references related to perioperative management of lung cancer.

| Paper | DOI | Total Citations | TC per Year |
|---|---|---|---|
| GETZ G, 2013, NATURE | 10.1038/nature12113 | 3652 | 304.33 |
| VASAN N, 2019, NATURE | 10.1038/s41586-019-1730-1 | 1412 | 235.33 |
| PFISTER DG, 2004, J CLIN ONCOL | 10.1200/JCO.2004.09.053 | 1173 | 55.86 |
| WU YL, 2020, NEW ENGL J MED | 10.1056/NEJMoa2027071 | 1001 | 200.20 |
| HOWINGTON JA, 2013, CHEST | 10.1378/chest.12-2359 | 964 | 80.33 |
| HUNDAL R, 2014, CLIN EPIDEMIOL | 10.2147/CLEP.S37357 | 731 | 66.45 |
| CUZICK J, 2010, LANCET ONCOL | 10.1016/S1470-2045(10)70257-6 | 685 | 45.67 |
| CHAUDHURI AA, 2017, CANCER DISCOV | 10.1158/2159-8290.CD-17-0716 | 635 | 79.38 |
| YAMASHITA T, 2008, CANCER RES | 10.1158/0008-5472.CAN-07-6013 | 608 | 35.76 |
| BRUNELLI A, 2013, CHEST | 10.1378/chest.12-2395 | 568 | 47.33 |
| BURDETT S, 2014, LANCET | 10.1016/S0140-6736(13)62159-5 | 564 | 51.27 |
| WARTH A, 2012, J CLIN ONCOL | 10.1200/JCO.2011.37.2185 | 549 | 42.23 |
| VAN MEERBEECK JP, 2007, JNCI-J NATL CANCER I | 10.1093/jnci/djk093 | 539 | 29.94 |
| LARDINOIS D, 2006, EUR J CARDIO-THORAC | 10.1016/j.ejcts.2006.08.008 | 519 | 27.32 |
| PROVENCIO M, 2020, LANCET ONCOL | 10.1016/S1470-2045(20)30453-8 | 480 | 96.00 |
| KHORANA AA, 2005, CANCER-AM CANCER SOC | 10.1002/cncr.21496 | 434 | 21.70 |
| SCOTT WJ, 2007, CHEST | 10.1378/chest.07-1378 | 433 | 24.06 |
| CRINÒ L, 2010, ANN ONCOL | 10.1093/annonc/mdq207 | 430 | 28.67 |
| PILLAY B, 2016, CANCER TREAT REV | 10.1016/j.ctrv.2015.11.007 | 415 | 46.11 |
| ZHAO PF, 2019, J HEMATOL ONCOL | 10.1186/s13045-019-0738-1 | 405 | 67.50 |

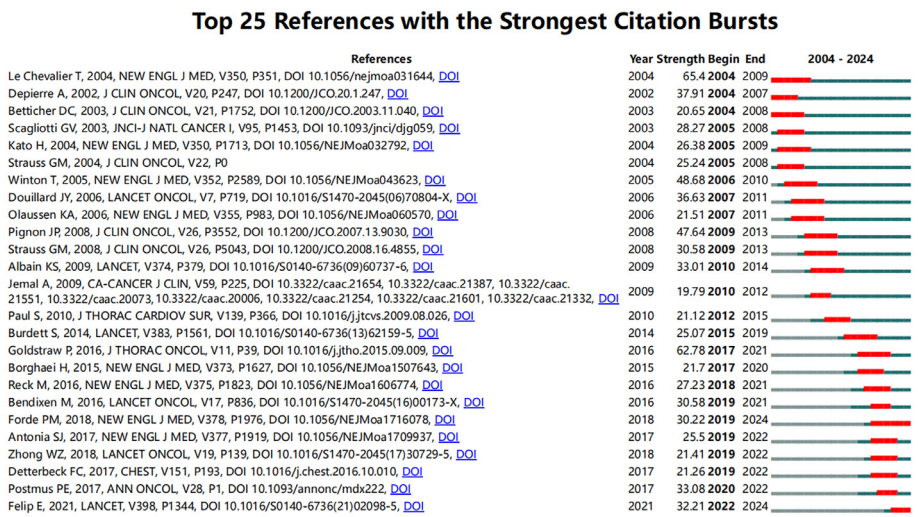


FIGURE 5
The top 25 most cited references on perioperative management of lung cancer.

perioperative management of lung cancer management. Clinical trials such as IMpower010 and CheckMate 816 have demonstrated the remarkable efficacy of immunotherapy in neoadjuvant and adjuvant settings. These studies not only improved patients' disease-free survival rates but also spurred researchers to explore optimal use of immunotherapy, leading to an increase in related literature (31). (2) Diversification of Perioperative Treatments: The strategies for perioperative treatment have become increasingly complex, encompassing various combinations of immunotherapy, targeted therapy, chemotherapy, and radiotherapy. This diversification has not only broadened treatment options but also expanded the scope of research, contributing to the surge in literature (32). (3) Advancements in Precision Medicine: With the development of genomic sequencing technologies, researchers have identified more biomarkers associated with lung cancer, enabling more personalized treatments. The application of precision medicine has further fueled research enthusiasm and driven growth in literature in the related field. In the field of perioperative management of lung cancer research, China has emerged as a leading country, producing the highest number of academic publications. This trend reflects the significant impact of lung cancer on China, which has attracted considerable attention from Chinese researchers. A total of 4,942 documents are distributed across 818 journals, with notable contributions from prestigious publications such as *Annals of Thoracic Surgery*, *Journal of Thoracic Disease*, and *European Journal of Cardio-Thoracic Surgery*. In particular, *Annals of Thoracic Surgery* stands out for its substantial number of published articles and significant citation count. This prominent performance underscores *Annals of Thoracic Surgery* as a key publication in the field of perioperative management of lung cancer research, affirming its central role in disseminating research findings in this domain.

TABLE 6 The top 20 keywords.

| Rank | Keywords | Count |
|---|---|---|
| 1 | survival | 850 |
| 2 | surgery | 743 |
| 3 | lung-cancer | 693 |
| 4 | cell lung-cancer | 678 |
| 5 | chemotherapy | 656 |
| 6 | resection | 622 |
| 7 | lobectomy | 366 |
| 8 | therapy | 329 |
| 9 | open-label | 324 |
| 10 | carcinoma | 299 |
| 11 | outcomes | 298 |
| 12 | management | 283 |
| 13 | impact | 267 |
| 14 | mortality | 260 |
| 15 | cancer | 256 |
| 16 | trial | 255 |
| 17 | complications | 250 |
| 18 | radiotherapy | 245 |
| 19 | risk | 233 |
| 20 | adjuvant chemotherapy | 229 |

## 4.2 Hotspots and development trends

Through a comprehensive analysis of literature clustering, keyword frequency, keyword clustering, and thematic evolution, we have identified the potential hotspots in perioperative

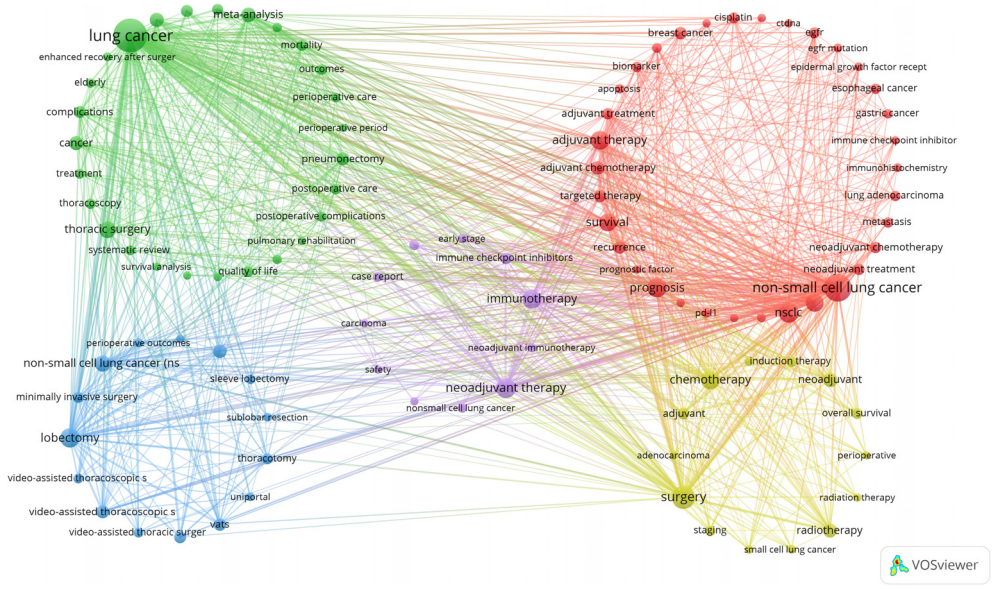


FIGURE 6
A co-occurrence map of keywords in the literature on perioperative management of lung cancer.

management of lung cancer research. The results indicate that the research frontiers and hotspots in this field are primarily focused on the following three aspects:

### 4.2.1 An Examination of the efficacy of immunotherapy and adjuvant treatments in contemporary lung cancer management

Over the past 20 years, advancements in targeted therapy and immunotherapy have significantly improved the survival rates and prognosis for patients with advanced NSCLC. Recent studies highlight their potential not only in advanced stages but also in early-stage, surgically resectable NSCLC. Comprehensive perioperative strategies, particularly neoadjuvant and adjuvant therapies, are becoming key methods to reduce recurrence rates and enhance treatment outcomes.

Immunotherapy, especially immune checkpoint inhibitors (such as PD-1/PD-L1 inhibitors), has been shown to have important clinical value in the perioperative treatment of patients with early-stage NSCLC (33, 34). Its main mechanism is to enhance the patient's immune system, identify and eliminate residual cancer cells, thereby reducing the risk of recurrence, including adjuvant immunotherapy and neoadjuvant immunotherapy (35).

Neoadjuvant immunotherapy (preoperative immunotherapy) is one of the focuses of current research. This model aims to reduce tumor size and improve resectability by using immunotherapy before surgery (36). Studies have shown that the combined use of

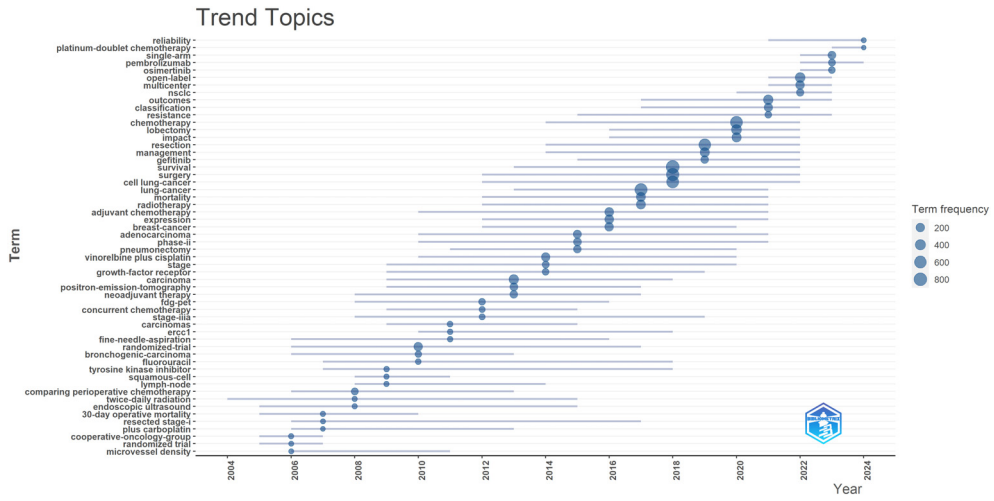


FIGURE 7
Trend topics in perioperative management of lung cancer research.

immune checkpoint inhibitors and chemotherapy not only significantly improves the R0 resection rate (complete resection rate), but also significantly improves the Pathological Complete Response (pCR), indicating that tumor cells have been fully eliminated before surgery. For example, the CheckMate 816 study showed that nivolumab combined with chemotherapy effectively improved progression-free survival (EFS) by an average of about 10.8 months (31.6 months vs. 20.8 months, hazard ratio (HR) =0.63, P=0.005), while achieving complete pathological response. The rate reached 24%, which was significantly better than the 2.2% of chemotherapy alone (P<0.001) (37). In addition, neoadjuvant immunotherapy can also activate the patient's immune system and help clear potential minimal residual disease after surgery, thereby reducing the risk of recurrence.

Postoperative adjuvant therapy is a strategy for patients who are not suitable for preoperative immunotherapy or have a small tumor burden. Postoperative adjuvant treatment (such as chemotherapy or immunotherapy) can effectively remove residual cancer cells and reduce the risk of recurrence (38). Especially for patients with low PD-L1 expression levels, postoperative immunotherapy can help enhance postoperative immune clearance and further prevent tumor recurrence. For example, the IMpower 010 study showed that atezolizumab as postoperative adjuvant therapy showed significant long-term benefit and prolonged DFS in patients with stage IB-IIIA NSCLC with high PD-L1 expression (39). In addition, pembrolizumab, as a PD-1 inhibitor, has also received good response in its application in metastatic NSCLC, supporting its potential in postoperative adjuvant therapy (40).

In order to maximize the therapeutic effect, when treating patients with NSCLC, the advantages of neoadjuvant and postoperative adjuvant therapy are usually combined, and immunotherapy and chemotherapy are used before and after surgery to minimize the possibility of recurrence, e.g. A clinical trial evaluating tolipalumab in combination with platinum-based chemotherapy in patients with resectable stage III NSCLC showed that compared with chemotherapy alone, tolipalumab significantly prolonged Event-free survival (EFS) and major pathological response rate(MPR), and the combined treatment regimen is controllable in terms of safety, providing a new adjuvant treatment option for this patient group (41, 42). This comprehensive treatment strategy is especially suitable for patients at high risk of recurrence and is one of the important directions for future lung cancer treatment.

Targeted therapy also provides notable benefits in the perioperative treatment of lung cancer, especially in NSCLC patients. Targeted therapy typically includes neoadjuvant therapy before surgery and adjuvant therapy after surgery, targeting specific genetic mutations (such as EGFR mutations) and demonstrating multifaceted advantages (43). The ADAURA study demonstrated that postoperative use of osimertinib significantly prolonged DFS and overall survival, with DFS benefits translating into statistically significant OS improvements, suggesting that adjuvant osimertinib can substantially reduce the risk of recurrence and improve long-term survival rates (44). However, the neoadjuvant use of osimertinib alone has shown suboptimal results (MPR 15%, pCR 0), and the benefits of combining osimertinib with chemotherapy in the neoadjuvant setting are still pending confirmation from the neoADAURA trial (45). As for the efficacy of neoadjuvant immunotherapy combined with chemotherapy in EGFR-mutant patients, there is currently no prospective evidence to suggest that perioperative immunotherapy benefits patients with resectable, driver mutation-positive lung cancer (46). While subgroup analyses from studies like Keynote671 indicated that EGFR-mutant patients had a better HR compared to wild-type patients (HR 0.09 vs. 0.48), smaller single-arm studies such as the NEOTIDE trial, which reported an MPR of 45% and pCR of 5%, are insufficient to establish the role of immunotherapy in EGFR-mutant patients during the perioperative period (47). Thus, while postoperative targeted therapy significantly improves survival, neoadjuvant targeted therapy has not shown the same benefits. For EGFR-positive patients, the optimal neoadjuvant treatment strategy, whether combining targeted therapy with chemotherapy or immunotherapy with chemotherapy, remains unclear and requires further research.

Similarly, targeted therapy has also demonstrated its efficacy in ALK-positive patients. The ALINA study further validated this by showing that, in patients with stage IB to IIIA ALK-positive NSCLC, adjuvant treatment with alectinib after complete resection reduced the risk of disease recurrence by 76% compared to chemotherapy (DFS HR=0.24, 95% CI 0.13–0.43, P<0.0001). Notably, the median DFS in the alectinib group has not yet been reached, while in the chemotherapy group, it was 41.3 months. Although overall survival data are still immature, the significant improvement in DFS highlights that alectinib is a safe and effective postoperative adjuvant therapy option for these patients (38).

However, it is important to note several limitations regarding the use of these therapies in perioperative management of lung cancer: (1) Immunotherapy may be ineffective in some patients due to Primary resistance, which can affect treatment efficacy (48, 49). (2) Due to serious adverse reactions of drugs, patients miss the opportunity for surgical treatment. (3) The impact of perioperative treatments is complex, and many mechanisms still require further investigation by researchers.

### 4.2.2 Novel biomarkers in perioperative assessment

Through the analysis of existing literature, circulating tumor DNA (ctDNA) and circulating tumor cells (CTC) have emerged as research hotspots in perioperative biomarkers for lung cancer in recent years. These biomarkers play a crucial role in the early diagnosis, treatment planning, monitoring of therapeutic response, and postoperative prognosis assessment of lung cancer.

The ctDNA, which consists of tumor DNA fragments present in the bloodstream, has shown great potential in tracking minimal residual disease and predicting tumor recurrence (43). The dynamic changes in ctDNA levels can reflect tumor burden, making monitoring during treatment highly significant. Studies have indicated that if ctDNA is not cleared in NSCLC patients following perioperative treatment, their risk of recurrence

significantly increases, suggesting the need for adjustment in the treatment plan (50, 51). As a non-invasive biomarker, ctDNA is particularly critical in the early detection of postoperative recurrence due to its rapid and sensitive characteristics (52, 53).

Minimal Residual Disease (MRD) refers to the small number of cancer cells that persist in a patient's body after treatment, which are often undetectable by standard diagnostic methods but can lead to disease relapse (54). The detection of MRD is crucial for evaluating the effectiveness of cancer therapy and guiding subsequent treatment decisions. Techniques such as flow cytometry, polymerase chain reaction, and next-generation sequencing are employed to identify these residual cells and assess their presence with high sensitivity (55). Monitoring MRD helps in predicting the risk of disease recurrence and tailoring treatment plans to prevent relapse, thus improving patient outcomes and advancing personalized medicine strategies (56).

Furthermore, the evaluation of postoperative inflammatory response plays an important role in cancer prognosis (57–59). Studies have shown that postoperative inflammatory status is closely related to the risk of cancer recurrence (60). Inflammatory markers such as C-reactive protein (CRP) and white blood cell count provide valuable information in postoperative monitoring (61). Moreover, some studies have found that elevated levels of postoperative inflammatory markers (such as IL-6) are associated with an increased risk of recurrence in NSCLC patients (62). Therefore, assessing postoperative inflammatory response not only helps in predicting the risk of recurrence but also may provide a basis for developing individualized treatment plans.

However, there are certain limitations: (1) Although ctDNA shows potential in tumor monitoring, the existing detection methods and standardized procedures for ctDNA still exhibit significant variability. Different detection methods and analytical platforms may lead to variations in results (63). While CTC detection is important for understanding cancer metastasis, its clinical application is limited by the sensitivity and specificity of current detection techniques (64).

### 4.2.3 Perioperative complications and prognosis in lung cancer

Lung cancer surgery-related perioperative complications and prognosis constitute a critical area of research within the field, as they profoundly influence both short-term recovery and long-term survival outcomes for patients. Perioperative complications, particularly air leaks, pleural effusions, pulmonary infections (50), and pulmonary thromboembolism (65), not only prolong hospitalization but may also lead to significant long-term health problems and a marked decline in quality of life.

In terms of prognosis, the occurrence of perioperative complications is closely related to patients' long-term survival and disease-free survival rates. Studies have shown that postoperative pulmonary complications can directly affect recovery and may indirectly influence tumor recurrence and overall prognosis through the induction of systemic inflammatory responses (66).

There are various strategies to prevent these complications, with patient nutrition being of paramount importance. Low protein levels and malnutrition have been found to be closely associated with a higher incidence of postoperative complications, particularly among elderly patients. Malnutrition can lead to poor tissue healing and weakened immune function, thereby increasing the risk of infections and other complications. For instance, research indicates that patients with a lower Prognostic Nutritional Index (PNI) are more prone to persistent air leaks, pneumonia, and other infectious complications (66–68).

The complexity and duration of surgery are also significant factors influencing complications. Longer surgical durations are typically associated with higher surgical invasiveness (69), such as thoracotomy, which causes greater damage to the chest wall and increases the risk of postoperative pulmonary complications (70).

Traditionally, patients were required to fast for at least six hours before surgery. However, recent studies have shown that moderate preoperative carbohydrate intake can significantly reduce postoperative endocrine responses. As a result, current guidelines allow the intake of clear liquids up to two hours before surgery (71). Additionally, regional anesthesia is an effective technique that can reduce endocrine metabolic responses and provide postoperative analgesia (72). Compared to general anesthesia, regional anesthesia significantly lowers the incidence of postoperative complications (73). Smoking markedly increases the risk of postoperative pulmonary complications (74); therefore, preoperative smoking cessation is an essential measure to improve lung health (75, 76). Overall, implementing these perioperative interventions can significantly enhance the outcomes of lung cancer surgery.

However, there are some limitations to these findings: (1) The occurrence of complications and prognosis may be influenced by various factors, such as underlying diseases and lifestyle habits, which many studies may not have adequately controlled for. (2) Many studies may focus only on short-term outcomes, neglecting long-term prognosis. For example, the long-term effects of perioperative complications may require longer follow-up to evaluate.

## 4.3 Future directions and limitations

### 4.3.1 Future directions

Pathological complete response of existing neoadjuvant immunotherapy combination chemotherapy regimens in NSCLC is approximately 20%, but there are significant differences across different molecular subtypes. Patients with KRAS and EGFR mutations respond particularly differently to neoadjuvant therapy. Future research should concentrate on optimizing treatment options based on the molecular characteristics of tumors. For instance, for patients with KRAS mutations, the emergence of KRAS G12C inhibitors provides new hope for enhancing perioperative outcomes. By combining KRAS inhibitors with immunotherapy or chemotherapy, it may be possible to significantly improve the pCR rate and reduce the risk of postoperative recurrence (77). For patients with EGFR mutations, although the results of current neoadjuvant treatments are suboptimal (for example, the pCR rate of osimertinib treatment is 0% and the MPR rate is only 15%), combining with new targeted drugs or signaling pathway modulators may yield better results in

the future (45). Strengthening molecular testing and clarifying different gene mutation types and their treatment responses will lay the foundation for further optimization of perioperative treatment strategies.

KRAS mutation is one of the most prevalent driver mutations in NSCLC, especially the KRAS G12C mutation, which is continuously activated via the RAS-MAPK signaling pathway and promotes the proliferation and survival of tumor cells. Persistent activation of this pathway not only increases tumor aggressiveness but also leads to resistance to traditional therapies such as chemotherapy (78). In recent years, the development of KRAS inhibitors (such as KRAS G12C inhibitors) has demonstrated initial success in treating patients with such mutations. Future research should focus on exploring the combined application of KRAS inhibitors and other therapies (such as immune checkpoint inhibitors and chemotherapy), particularly in the perioperative stage, using multiple strategies to inhibit the KRAS pathway and its related drug resistance mechanisms, thereby enhancing treatment efficacy before and after surgery (79). Additionally, further investigation into the cross-regulatory mechanisms of the RAS-MAPK pathway will help to target therapeutic interventions more effectively for patients with KRAS mutations (80).

EGFR mutations also play a crucial role in NSCLC, particularly in non-smoking patients. These mutations regulate the growth, proliferation, and survival of tumor cells through abnormal activation of the EGFR signaling pathway, which includes critical downstream pathways such as PI3K/AKT and JAK/STAT (81). Current EGFR-targeted therapies have achieved favorable clinical outcomes in postoperative adjuvant therapy, but their efficacy in neoadjuvant therapy is limited. This limitation may be linked to the complexity of the EGFR signaling pathway, variations in the tumor microenvironment, and resistance to immunotherapy (82). Therefore, future research should concentrate on developing new EGFR-targeting drugs or combination treatment regimens to improve perioperative treatment effects. Specifically, combining the inhibition of key nodes downstream of EGFR, such as the PI3K/AKT pathway, may yield better results during the neoadjuvant treatment phase (83).

For patients with KRAS and EGFR mutations, optimizing treatment models will be a key focus for future research. Comparative studies between neoadjuvant + surgery + adjuvant (sandwich model) and neoadjuvant treatment alone will help to determine the best perioperative treatment pathway. For patients with KRAS mutations, a strategy that combines KRAS inhibitors with immunotherapy or chemotherapy is anticipated to increase the surgical resection rate and reduce the risk of recurrence (84). For patients with EGFR mutations, in-depth research into the best targeted treatment options before and after surgery, especially through enhanced molecular testing and signaling pathway analysis, can help formulate more individualized treatment plans to further improve the pCR rate and overall survival rates (85).

### 4.3.2 Limitations

This study provides researchers with deeper insights into the perioperative field of lung cancer and explores new research directions. However, there are several limitations to this study.

First, despite our efforts to cover common terms related to perioperative management and lung cancer, different researchers may use expressions not included in our search, potentially leading to missed literature. In our literature search, we carefully selected terms associated with perioperative management and lung cancer. For perioperative management, we utilized terms such as "Preoperative Optimization," "Postoperative Care," and "Perioperative Period" to encompass the main phases: preoperative, intraoperative, and postoperative. Additionally, we included widely used terms like "Neoadjuvant Treatment" and "Adjuvant Treatment" to ensure we captured standard expressions for perioperative interventions. To further expand our search, we incorporated broader terms such as "Neoadjuvant-Adjuvant" and "Perioperative". Regarding lung cancer-related terms, we employed common keywords like "Lung Cancer*" and "Pulmonary Neoplasm*" and included synonyms (e.g., "Cancer of Lung") to ensure comprehensiveness. This diverse search strategy aims to reduce potential biases in terminology usage due to regional or disciplinary differences, ensuring that relevant literature is not overlooked. Second, we relied solely on the WoSCC database as our data source, which may have led to the exclusion of some publications from our analysis. Nevertheless, the WoS database is widely recognized as a high-quality digital literature repository and is considered one of the best options for bibliometric analysis (86–88). Therefore, our choice of data source is reliable. Additionally, our analysis was limited to English publications, which may introduce source bias. Lastly, while China faces a significant burden from lung cancer and has extensive research on this topic, we did not analyze Chinese literature, which may result in minor biases in our data analysis (89).

## 5 Conclusion

Our study clearly outlines the key research hotspots and frontiers in the field of perioperative management of lung cancer research. The following is a summary of the key points and research trends in this area:

a. Perioperative management of lung cancer research has garnered global attention, with China, the United States, Japan, and Italy being the most active countries. Extensive international collaboration has been observed among these nations.

b. *Annals of Thoracic Surgery* and *Journal of Thoracic Disease* are the most prominent journals for publishing perioperative management of lung cancer-related literature. Notably, *Annals of Thoracic Surgery* has the highest citation rate, indicating its significant influence in the field of perioperative management of lung cancer research.

c. In the perioperative management of lung cancer, the combination of adjuvant chemotherapy, targeted therapy, and immunotherapy represents a current hotspot and trend in surgical treatment.

d. The evaluation of biomarkers such as ctDNA and CTCs, as well as postoperative inflammation, is a rapidly emerging trend for facilitating personalized treatment plans in perioperative management of lung cancer management.

e. Research on complications and prognosis during the perioperative period, including air leaks, pleural effusion, pulmonary infections, and pulmonary embolism, represents a key focus and trend in the study of perioperative complications.

f. Future research should focus on the following key areas: enhancing the effectiveness of pCR and personalized treatment, investigating the impact of KRAS mutations and their associated signaling pathways on tumor biology, exploring the mechanisms of EGFR mutations and their signaling pathways, and optimizing perioperative treatment models.

Our research provides a comprehensive insight into the research trends and hotspots in the field of perioperative management of lung cancer. These findings not only enhance researchers' understanding of the field but also guide future exploration and innovation. By delineating current research patterns and potential focus areas, our study offers a valuable theoretical foundation and practical guidelines, supporting scholars in conducting more systematic and pioneering research in this critical area.

## Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: Available through the following URL: https://www.webofscience.com.

## Author contributions

QJ: Software, Writing – original draft, Writing – review & editing. ZW: Visualization, Writing – original draft. PL: Software, Writing – original draft. ZL: Methodology, Writing – original draft. HJ: Software, Writing – original draft. YC: Methodology, Writing – original draft. BZ: Software, Writing – original draft. YY: Writing – original draft, Writing – review & editing. YH: Writing – original draft, Writing – review & editing.

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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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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1500686/full#supplementary-material

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# B7-H3 in glioblastoma and beyond: significance and therapeutic strategies

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Cancer has emerged as the second most prevalent disease and the leading cause of death, claiming the lives of 10 million individuals each year. The predominant varieties of cancer encompass breast, lung, colon, rectal, and prostate cancers. Among the more aggressive malignancies is glioblastoma, categorized as WHO stage 4 brain cancer. Following diagnosis, the typical life expectancy ranges from 12 to 15 months, as current established treatments like surgical intervention, radiotherapy, and chemotherapy using temozolomide exhibit limited effectiveness. Beyond conventional approaches, the exploration of immunotherapy for glioblastoma treatment is underway. A methodology involves CAR-T cells, monoclonal antibodies, ADCC and nanobodies sourced from camelids. Immunotherapy's recent focal point is the cellular ligand B7-H3, notably abundant in tumor cells while either scarce or absent in normal ones. Its expression elevates with cancer progression and serves as a promising prognostic marker. In this article, we delve into the essence of B7-H3, elucidating its function and involvement in signaling pathways. We delineate the receptors it binds to and its significance in glioblastoma and other cancer types. Lastly, we examine its role in immunotherapy and the utilization of nanobodies in this domain.

KEYWORDS

**glioblastoma, B7-H3, CD276, signaling pathways, immune checkpoints, immunotherapy**

## 1 Introduction

Cancer is a significant health issue, being a disease of considerable concern. In 2020, it was the second leading cause of death in the United States, with 602,350 deaths, following heart disease, which claimed 696,962 lives. It is noteworthy that cancer stands as a primary cause of death among women aged 40-59 years and both genders within the 60-79 year age range. While there has been a reduction in mortality rates for certain cancers such as leukemia, melanoma, and kidney cancer, it is important to mention that other cancers like glioblastoma continue to sustain their high fatality rates (1).

Glioblastoma is the most common and malignant primary brain cancer. The World Health Organization (WHO) classifies glioblastoma as grade IV brain tumor. Currently

patients with glioblastoma first undergo surgical resection which is followed by radiotherapy and chemotherapy with temozolomide (2). Despite the combinatorial treatment, 5-year survival rate is less than 10% (3). Low survival rate of glioblastoma patients can be attributed to several factors. First, glioblastoma is a very heterogeneous cancer with different mutations (4). Second, it is very difficult to resect tumor completely due to the possibility of damaging nearby brain tissue (5). Third, tumor microenvironment (TME) suppresses the immune system (6). Fourth, blood-brain barrier (BBB) limits drug delivery to tumor (7). A recent publication by Lizhi Pang et al. describes a significant role of TME in glioblastoma. However, understanding the heterogeneity of glioblastoma remains challenging due to methodological limitations. Artificial intelligence (AI) could offer a step forward in uncovering brain tumor heterogeneity with introduction of different data analysis and predictive models. AI can significantly boost speed with analysis of large date such as single cell and protein interactions. Machine learning can predict different genetic drivers, tumor progression and patient survival. Such an approach could enable personalized and precise interventions, leading to reliable outcomes in scientific and medical advancements (8).

Recently, new drugs and methods of treatment have been developing including immunotherapy. Different approaches of immunotherapy have been developed including immune checkpoint inhibitors, oncolytic virus therapies, cancer vaccines, adaptive cell transfer and cytokine therapies. Immunotherapy has been to some extent successfully implemented in treatments of several cancers (9). In the case of glioblastoma, researchers have so far been able to reach phase 3 of clinical trials using immunotherapies where no significant improvements in terms of median overall survival have been shown (10–13).

New methods based on nanotechnology are developing including nanobodies. Nanobodies have several advantages over conventional antibodies. Some studies indicate that nanobodies might diffuse through BBB (14) and TME, giving hope for increased immunotherapeutic efficacy (15). One of the potential targets is ligand B7-H3, due to its high expression on different cancers and correlation to poor patient prognosis (16, 17). Several different approaches targeting B7-H3 are being investigated, including chimeric antigen receptor (CAR) T-cells as the most studied approach. In this review we focus our research on B7-H3 and its immunotherapy considering glioblastomas. Several other cancers will also be described. At last, nanobodies and their therapeutic use in immunotherapy will be presented including latest research on nanobodies in glioblastoma and B7-H3.

## 2 B7 homolog 3 (B7-H3)

B7 homolog 3 (B7-H3) is an immunoregulatory checkpoint glycoprotein and a member of B7 family regulatory ligands, responsible for regulating T cell lymphocytes and tumorigenesis. Members of this family are cluster of differentiation 80 (CD80 or B7-1), CD86 (B7-2), CD274 (B7-H1 or PD-L1), PDCD1LG2 (PD-L2), ICOSLG (B7-H2), CD276 (B7-H3), VTCN1 (B7-H4), VSIR (B7-H5), NCR3LG1 (B7-H6), HHLA2 (B7-H7) and ILDR2 (18). Members of B7 family are a group of proteins important for immune homeostasis. They are primarily involved in costimulatory and coinhibitory signals that modulate T-cell activation, proliferation, and function. The interaction between B7 proteins and their receptors can either suppress or enhance immune response, making them critical in the context of cancer or autoimmune diseases. B7-1, B7-2 and B7-H2 are important for regulating immune tolerance and naïve T cell activation. Meanwhile PD-L1, PD-L2, HHLA2, B7-H3 and B7-H4 are responsible for regulating immune response in peripherial tissues (19). B7-H3 has called for attention due to its high expression on cancer cells and lower or none in normal cells. Additionally, its high expression in cancer cells is correlated with poor prognosis (20–23). The expression of B7-H3 is higher in glioblastoma, while it shows minimal presence in normal tissues, making it an interesting therapeutic target. Some FDA approved drugs targeting B7-H3 such as $^{131}$I Omburtamab for neuroblastoma, Enoblituzumab for prostate cancer, and GSK5764227 (HS-20093) for small-cell lung cancer, highlights the progress in this area. The development of B7-H3 targeting drugs is advancing, and there is great potential to investigate their therapeutic effects in glioblastoma, a field where the full therapeutic potential has not yet been elucidated (24).

B7-H3 is a type I transmembrane glycoprotein with an extracellular domain containing IgV-like and IgC-like domain, transmembrane domain and short intracellular domain containing short cytoplasmic tail. Two isoforms of B7-H3 are discovered in humans, 2IgB7-H3 (25) and 4IgB7-H3 (26, 27). B7-H3 is located at 15q24.1. The size of 4IgB7-H3 is 534 amino acids and encode a 110 kDa protein (26), while 2IgB7-H3 is 306 amino acids and encodes 45-66 kDa (25, 28). In non-cancerous brain tissues of patients only 2IgB7-H3 was present, while in glioblastoma tissues 2IgB7-H3 expression was decreased and 4IgB7-H3 expression was expressed. Interestingly 2IgB7-H3 expression was higher in glioblastoma recurrences compared to newly diagnosed glioblastoma (20). Soluble B7-H3 (sB7-H3) is 16.5 kDa and is detected in serum levels of healthy individuals. Monocyte, dendritic cells, activated T cells and some cancer cell lines (ovarian, breast, lung) release sB7-H3. Evidence indicate that sB7-H3 is released with a help of matrix metalloproteinase (MMP) (29).

## 3 B7-H3 receptors

B7-H3 is known for its dual role in physiology, possessing stimulatory and inhibitory function in cancer development (30). Many factors influence this type of action. It may depend on cell type on which it is expressed, cell receptor (31), the isoform being present (32), posttranscriptional regulation (33), epigenetic and posttranslational modifications (21, 34).

### 3.1 TLT-2

The first possible receptor identified for 2IgB7-H3 was TLT-2, which was found to bind fusion protein of murine B7-H3 and human IgG1 (B7-H3Ig) in murine T cells. The interaction with TLT-2 resulted in enhanced T-cell activation, especially CD8+ T cells

correspondingly enhancing production of IFN-γ and IL-2. However, it should be considered that fusion protein B7-H3-Ig and innate membrane B7-H3 are different, the same as TLT-2 expression in transfected cells and TLT-2 normally found on cells (31). Studies show contradictory results, while some claim that both 2IgB7-H3 and 4IgB7-H3 inhibit T cell activation (35), others show the contrary (31). Additionally, Leitner et al. (35) showed that TLT-2 on human T cells does not serve as a receptor for neither 2IgB7-H3 or 4IgB7-H3 even though there is some kind of receptor on T cells that interact with B7-H3. Even though Hashiguchi et al. (31) found interaction with TLT-2 and B7-H3Ig in murine system, Leitner et al. (35) and Yan et al. (36) were not successful in reproducing the results (35). The existence of TLT-2 remains controversial, and a need of better understanding is crucial to elucidate described contradictories. It should be considered that although the same methods were used, fusion protein construct, transduction efficiency and cellular background can result in different outcomes.

### 3.2 Interleukin 20 receptor subunit alpha (IL20RA)

Using new interactome technique, Husein et al. identified another possible receptor – the interleukin 20 receptor subunit alpha (IL20RA) (37). IL20RA is a subunit of the IL20 along with IL20RB. Upon binding of its ligand, it can form heterodimer with IL20RB. IL20RA has many ligands including IL-19, IL-20, and IL-24 (38, 39). IL20RA is expressed in skin, ovary, placenta, lungs and testes (38). IL20RA is expressed in human skin, higher in adults compared to children skin (40). It is involved in many cancers by regulating signaling pathways. Overexpression of IL20RA was found in colorectal cancer (CRC) and was also associated with greater tumor diameter and poor prognosis (41). Knockdown of IL20RA in CRC cell lines downregulates Janus kinase 1 (JAK1) and signal transducer and activator of transcription 3 (STAT3) and consistently suppress tumor growth (42). On the other hand, activation of IL20RA in ovarian cancer cells, when they disseminate into peritoneal cavity, results in polarization of macrophages into anti-tumor M1 subtype (43). They also showed expression of IL20RA and IL20RB in human brain micro vessels (44). There are currently no findings connecting IL20RA and glioblastoma.

Using Conditioned Media AlphaScreen technology, IL20RA was confirmed as an interacting partner of recombinant B7-H3 protein. B7-H3 was expressed as biotinylated Avi-tagged extracellular domain using *E. coli*. Additionally, binding of recombinant B7-H3 on cells transiently expressing IL20RA was analyzed with immunofluorescence. Results confirm binding of B7-H3 only on cells expressing IL20RA. This is a promising finding of another B7-H3 receptor but should be confirmed with other studies (37).

### 3.3 Angio-associated migratory cell protein (AAMP)

Recently a new potential B7-H3 receptor has been identified adding another piece to a puzzle of dual B7-H3 functioning. Angio-associated migratory cell protein (AAMP) plays a role in cell migration and regulates angiogenesis (45). AAMP has also role in different cancers including being overexpressed in breast cancer (46) and gastrointestinal stromal tumors (47). Furthermore AAMP is associated with poor clinical outcomes in CRC patients promoting invasion and migration both *in vitro* and *in vivo* (48). Using yeast two-hybrid screening (Y2H) and mass spectrometry phosphorylation screen, 17 potential interacting partners on natural killer (NK) cells were identified. After bimolecular fluorescent complementation (BiFC) assay, 4 candidate genes were revealed: cluster of differentiation 164 (CD164), AAMP, receptor-type tyrosine-protein phosphatase alpha (PTPRA) and SLAM Family Member 7 (SLAMF7). After that endogenously binding partners were further accessed using co-immunoprecipitation (co-IP), results confirmed AAMP as the only interaction partner. Additionally, B7-H3 interaction with AAMP knockdown and control Jurkat cell lines was analyzed. The highest concentration of B7-H3 used equally inhibited the proliferation of both cell lines, while the lower concentration of B7-H3 inhibited the knockdown of Jurkat cells more, indicating that AAMP has at least a partial interaction with B7-H3 and reduces the antiproliferative effect of B7-H3 to a limited extent. To further validate the finding, they used multivariate expression of these two genes in glioblastoma patients and showed a positive correlation in expression. Also, poor prognosis is only observed for patients with high AAMP and B7-H3 expression. Tumors can glycosylate proteins and change their interaction with receptors additionally adding another level of complexity. CD164, PTPRA and SLAMF7 could not be verified further due to shorter interaction time, poorly evaluated antibodies or Y2H false positives (49).

## 4 B7-H3 posttranscriptional regulation

miRNAs represent a very unique group of small RNAs responsible for regulating gene expression. Deregulation of miRNAs can lead to the development of various cancers, as they may either silence genes that prevent tumor growth or activate oncogenic pathways. Their ability to influence multiple cellular processes makes them key players in cancer progression and potential targets for therapeutic intervention (50).

The regulatory effect of miRNA on the *B7-H3* gene is evident in several cancers, including osteosarcoma, clear cell renal carcinoma, multiple myeloma, medulloblastoma, mantle cell lymphoma, neuroblastoma, ovarian, colorectal and cervical cancer. Several miRNAs, such as miR-199a, miR-128 and miR-187, bind to the 3'UTR region of B7-H3 and regulate its expression (51–53). In neuroblastoma, it was found that depletion of miR-29 (miR-29a, miR-29b, and miR-29c) was associated with poorer patient survival. Experiments showed that these miRNAs are responsible for degrading B7-H3 mRNA and activating NK cells (54). Using TCGA data analysis, researchers found that decreased levels of miRNA 29c were linked to poorer survival in quadruple negative breast cancer (55). In head and neck squamous cell carcinoma, downregulation of miR-214-3p inhibited $CD8^+$ T cell activity and facilitated disease progression (56). miR-34a has been shown to

drive immune evasion in CRC cells by inhibiting SIRT1 and inducing B7-H3 and TNF-α in the tumor microenvironment (57). Similarly, miR187 was found to inhibit the growth and invasion of CRC cells by targeting B7-H3. This miRNA was downregulated in CRC compared to controls and was associated with shorter overall survival (53). In CRC, upregulation of miR-155 inhibits miR-143, leading to higher B7-H3 expression and the activation of T cells to release TGFβ, which promotes tumor growth (58). miR-128 was also found to downregulate B7-H3, which is typically highly expressed in CRC cells. This miRNA represses CRC migration and proliferation (59). Upregulation of miR-29c in CRC cells reduced B7-H3 expression, inhibiting cancer progression, invasion and migration (60). Similar findings were observed in ovarian cancer, where higher miR-29c expression downregulated B7-H3 and activated NK cells (61). In cervical cancer, higher expression of miR-199a was associated with inhibition of tumor migration, proliferation and invasion by targeting B7-H3. This effect was mediated through the AKT/mTor signaling pathway (62). In lung cancer, lower miR-145 expression was correlated with higher B7-H3 levels and increased lymph node metastasis (63). In medulloblastoma, reduced expression of miR-1253 resulted in increased B7-H3 expression, which promoted tumor cell proliferation and aggressiveness. miR-1253 was found to reduce tumor progression by arresting cells at $G_0/G_1$ phase of the cell cycle (64). In mantle cell lymphoma, increased miR-506 expression decreased B7-H3 levels, inhibiting cancer cell proliferation and metastasis (65). Nygren et al. comprehensively analyzed miRNAs regulating B7-H3 in breast cancer. They identified thirteen miRNAs that target B7-H3 directly by binding to the 3′-UTR region: miR-214, miR-363*, miR-326, miR-940, miR-29c, miR-665, miR-34b*, miR-708, miR-601, miR-124a, miR-380-5p, miR-885-3p, and miR-593. Moreover, miR-29c was related to significantly reduced risk of developing metastasis and had lower expression in higher grade tumors (33). Overall, the higher expression of these miRNAs in various cancers reduces B7-H3 levels, thereby inhibiting tumor growth. These findings provide a solid reason for developing more effective combination therapies that utilize miRNAs to target B7-H3 across different cancers.

# 5 Mechanism of action and role in cancer

Although B7-H3 is not an established cancer biomarker, lately it started to gain importance because of its overexpression in cancer compared to healthy tissues (66). B7-H3 is correlated to several cancer-related processes such as metabolism, angiogenesis, invasion and therapy resistance (67), including via JAK/STAT, NK-κb and PI3K pathways as summarized and illustrated in Figures 1, 2. B7-H3 is expressed in different cells in the tumor microenvironment such as cancer cells, cancer stem cells, dendritic cells, pro-tumor type 2 macrophages, myeloid-derived suppressor cells, monocytes, endothelial cells (ECs), NK cells and cancer-associated fibroblasts which indicates the extensive engagement of the B7-H3 ligand/receptor interaction within the tumor region (67).

## 5.1 B7-H3 expression in cancer

B7-H3 is highly expressed in several cancers (Figure 2). Zhang et al. reported B7-H3 expression in 93% of analyzed ovarian tumor samples

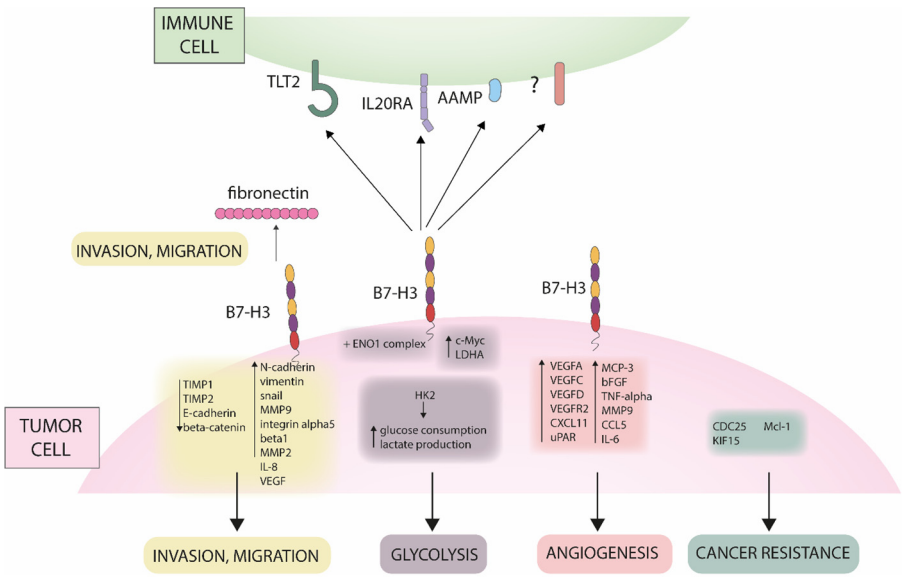


FIGURE 1
Different role of B7-H3 in cancer. The figure shows mechanisms of B7-H3 in promoting invasion, migration, glycolysis, angiogenesis and cancer resistance.

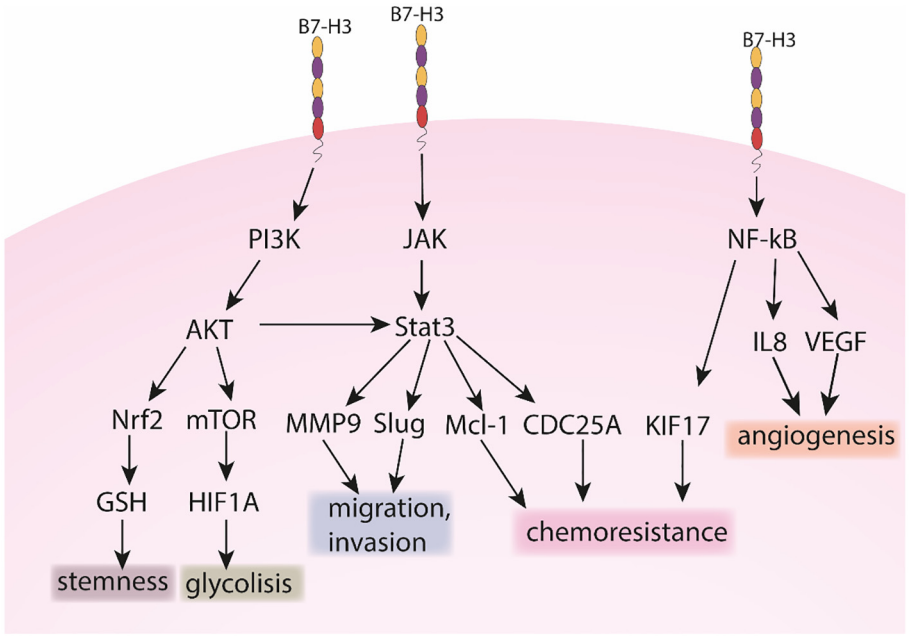


FIGURE 2
The role of B7-H3 in PI3K, JAK and NK-kB pathways.

(96/103), where the protein presented with membrane and cytoplasmic localization (68). In contrary, B7-H3 expression was not detected in non-neoplastic ovarian specimens. They also reported that high B7-H3 expression was positively correlated to increased recurrence and mortality. The results of their study also show association between B7-H3 expression in tumor vasculature and histological type, stage, recurrence incidence, and poor clinical outcome. MacGregor et al. used B7-H3 as a novel target to be used in combination with existing therapies to overcome immunosuppression in the TME of ovarian cancer cells (69). With immunohistochemistry (IHC), the authors showed that B7-H3 is expressed by both tumor and stromal cells in the epithelial ovarian TME. In flow cytometry, both tumor and stromal cells were positive for surface B7-H3 expression which was lower in tumor cells compared to stromal cells. Analysis of TCGA dataset showed that B7-H3 expression is positively correlated to stromal markers (fibroblast activation protein alpha (FAP) and platelet-derived growth factor receptor beta (PDGFRβ)) and negatively correlated to epithelial markers (EpCAM and E-Cadherin). Since B7-H3 was found to be broadly expressed on tumor cells, stromal cells, and antigen-presenting cells (APCs), the authors suggest that B7-H3 therapies can target multiple cell populations. However, because its expression on non-tumor cells can be induced under specific conditions, possible off-target effects and toxicities should be considered beforehand. Yonesaka et al. reported that 74% of the examined non-small cell lung cancer (NSCLC) tested positive for B7-H3 in IHC while normal lung cancer was not stained (70). They also examined responsiveness to anti-programmed cell death protein 1 (PD-1) therapy and B7-H3 expression levels and reported that patients whose tissues were not stained for B7-H3 benefited more from anti-PD-1 therapy than patients whose tissues stained positive for B7-H3. The authors suggest that besides correlated to poor survival, high B7-H3 expression may be correlated to refractoriness to anti-PD-1/programmed death-ligand 1 (PD-L1) immunotherapy by impairing CD8+ T-cell mediated tumor immunity. Figure 3A (72) shows mRNA expression of B7-H3 across different tumors *versus* normal tissue. In all tumors, except for three, the expression is higher in tumor *versus* normal tissue. Those three exceptions are cervical squamous cell carcinoma, acute myeloid leukemia, and pheochromocytoma and paraganglioma. The protein expression (Figures 3B–D) show that B7-H3 is highly expressed in glioblastoma also on protein level.

## 5.2 The dual role of B7-H3 in immune system regulation

B7-H3 was initially thought to be a T-cell stimulating protein, but more recent studies show that it acts as a T-cell inhibitor (73). One of the first studies to investigate the role of B7-H3 showed that it increases the proliferation of CD4+ and CD8+ T cells and is crucial for interferon-gamma (IFN-γ) formation during T cell activation. In mouse models, it was also proved to activate tumor-specific cytotoxic T cells and enhances antitumor immunity. However, another study showed that B7-H3 plays a co-inhibitory role, inhibiting the proliferation of CD4+ and CD8+ T cells and reducing IL-2 and IFN-γ levels. It has also been confirmed to inhibit NK cell activity and promote osteoblast differentiation (74). Oh et al. investigated the role of B7-H3 in osteoclast differentiation. B7-H3 is highly expressed in osteoclasts and its inhibition leads to inhibition of osteoclastogenesis as well as increased IFN signaling, signal transducer and activator of transcription 1 (STAT1) activation and indoleamine 2,3-dioxygenase (IDO) induction as a downstream mechanism (75). Zhang et al. analyzed the role of B7-

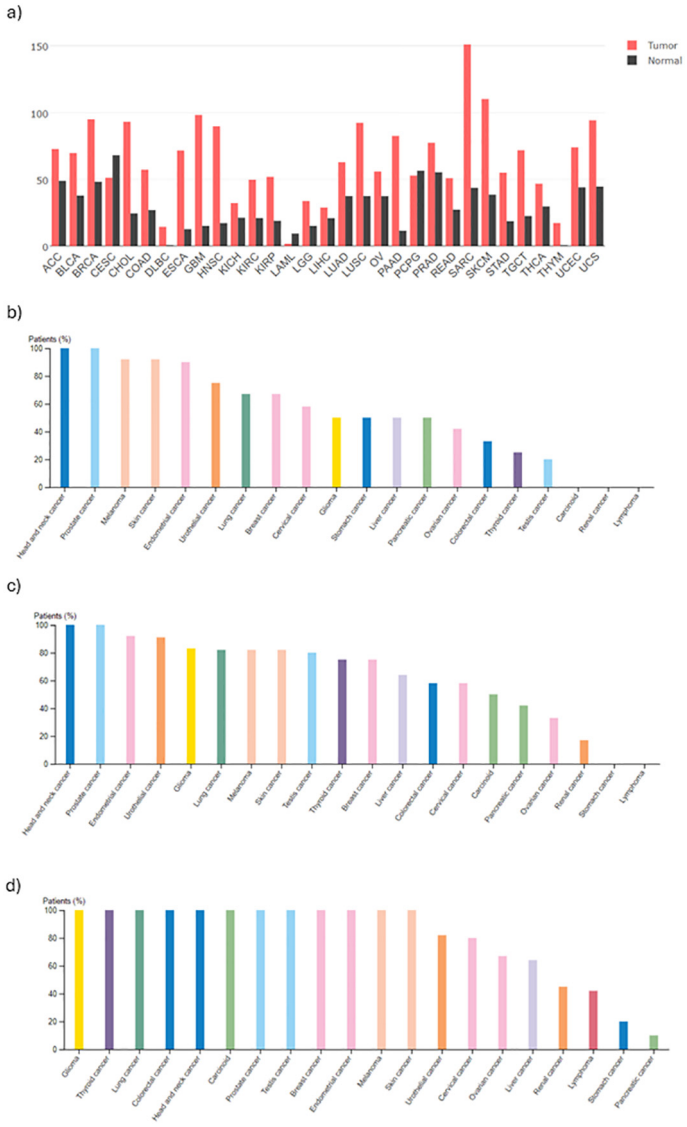


FIGURE 3
Summary of B7-H3 expression levels in different cancers. **(A)** mRNA expression in different cancers, obtained from GEPIA (71). Images of IHC staining obtained from the Protein Atlas (https://www.proteinatlas.org/) using three different antibodies **(B)** HPA009285, **(C)** HPA017139, **(D)** CAB017826.

H3 in children with allergic asthma. They found that children with asthma had higher levels of B7-H3 compared to control subjects. They also showed that B7-H3 is a target of miR-29c and is regulated by Th2/Th17 cell differentiation (76).

In cancer, Sun et al. have shown that overexpression of B7-H3 leads to a complete regression of 50% of lymphomas and significantly reduces tumor growth in mice. In addition, the mice acquired systemic immunity against tumor cells and antitumor immunity was mediated by CD8+ T cells and NK cells (77). Similarly, Muo et al. investigated the effect of overexpression of B7-H3 in mouse mastrocytomas. Transfection with the gene resulted in enhanced immunogenicity, which led to tumor regression. It also induced a clonal expansion of CD8+ cytotoxic T lymphocytes (CTL) (78). In human studies, Loos et al. observed that pancreatic cancer patients with high levels of B7-H3 had a better prognosis than patients with low levels. Furthermore, the expression correlated with the number of CD8+ T cells (79). Similarly, Wu et al. analyzed the expression of B7-H3 in patients with gastric cancer. Higher expression of B7-H3 in the tissues was associated with better survival (80). In contrary, several other studies have shown that higher expression of B7-H3 in cancer is associated with poorer survival and disease progression, such as in prostate cancer and NSCLC (23, 79, 81–85). As Suh et al. have shown, B7-H3 inhibits the proliferation of T cells, preferentially downregulating type 1 cells rather than type 2 cells (86). It has also been observed that B7-H3 is associated with a reduction of IFN-γ and TNF-α. These are known to be produced by activated T cells and are toxic to tumor cells (87). B7-H3 mediates the immunosuppression via CCL2-CCR2 axis, which leads to M2 macrophage migration and differentiation as observed in ovarian cancer (88). B7-H3 has also been investigated in inflammatory diseases, such as arthritis. Yang et al. discovered that there is

positive correlation between symptoms severity and B7-H3 expression on macrophages. The activity of B7-H3 is via NF-kB pathway. When mice with arthritis were treated with anti-B7-H3 antibody, the mice had weaker symptoms, and the inflammation reduced (89).

## 5.3 Role of B7-H3 in metastasis

Beside the immunological role, B7-H3 has an important role in tumor metastasis, as observed in several studies. Chen et al. investigated the role of B7-H3 in invasion. Downregulation of the gene led to reduced cell adhesion to fibronectin and reduced migration and invasion in melanoma and breast cancer cells. On the other hand, it played no role in proliferation (90). Similarly, in prostate cancer cells, Yuan et al. observed that downregulation of B7-H3 had no effect on cell proliferation. Also, B7-H3 blocks the interaction of cells with fibronectin and is related to increased cell migration and invasion (85). On the other hand, Yu et al. observed that B7-H3 promotes proliferation of lung cancer cell lines and similarly promotes invasion and migration. They also analyzed the expression of important epithelial and mesenchymal markers. After siRNA transfection, the levels of N-cadherin, vimentin and Snail were reduced, while the expression of E-cadherin was increased (91). Xie et al. investigated the role of B7-H3 in clear cell renal cell carcinoma. Knockdown of the gene led to a decrease in N-cadherin, MMP9, integrin alpha5 and beta1 genes. They showed that fibronectin promotes migration and invasion, but this effect is diminished in cells that have the B7-H3 gene knocked down. Their results also suggest that B7-H3 forms a complex with exogenous fibronectin (92). Furthermore, Xie analyzed the mechanism behind the B7-H3 related invasion in pancreatic cancer cells. sB7-H3 induced metastasis and invasion of cancer cells. It first upregulated Toll-like receptor 4 (TLR4) expression and then activated nuclear factor kappa B (NF-κB) pathway. This in turn increased the expression of IL-8 and vascular endothelial growth factor (VEGF) (93). Tekle et al. published a comprehensive study on the role of B7-H3 in melanoma cells. Similar as in previous studies, silencing of B7-H3 reduced migration and invasion *in vitro*. The effect was also translated *in vivo*, as silencing reduced metastatic potential and symptoms-free survival in mice and rats. The possible mechanism behind the metastatic potential of B7-H3 was also analyzed. In cells with knockdown, metastasis-associated proteins such as MMP2, Stat3 and IL-8 were decreased, while TIMP metallopeptidase inhibitor 1 (TIMP1) and TIMP metallopeptidase inhibitor 2 (TIMP2) were increased (94). In hepatocellular carcinoma (HCC) B7-H3 promotes cell invasion by targeting epithelial-mesenchymal transition (EMT) via partially activating JAK2/STAT3/Slug signaling pathway (95). Kang et al. first examined B7-H3 expression in HCC and found positive correlation between high B7-H3 expression and HCC metastasis. Moreover, increased B7-H3 intensity level was detected in metastatic HCC compared to other non-metastatic primary HCCs. Therefore, high B7-H3 expression was correlated to aggressive and metastatic HCC consequently also with cell migration. The authors reported that B7-H3 downregulation significantly reduces cell migration and matrigel invasion and has no effect on cell proliferation or apoptosis. In their study EMT-related protein E-cadherin was upregulated, while N-cadherin and vimentin were downregulated in B7-H3 silenced cells compared to negative controls. They propose B7-H3 to be used as a marker for tumor recurrence and/or metastasis. In a different study, Li et al. showed that silencing B7-H3 significantly suppressed migration and inhibited proliferation of lung cancer cells A549 and H460 compared to negative controls (96). Moreover, they reported that knock down of B7-H3 downregulated the expression of many integrin-associated proteins. In CRC, B7-H3 also may promote EMT by decreasing expression levels of E-cadherin and β-catenin and increasing expression levels of N-cadherin and vimentin (97). Liu et al. investigated the role of B7-H3 in the migration and invasion of CRC cells (98). With *in vitro* wound healing and transwell assays they observed that enhanced expression of B7-H3 promotes cell migration and invasion, respectively. The related pathway identified was Jak2/Stat3 which is known to be important in cell migration, invasion and metastasis. The authors reported MMP-9 to be a downstream target of B7-H3. Overexpression of B7-H3 increased phosphorylation of Jak2 and Stat3, which resulted in increased expression of MMP-9. B7-H3 is upregulated in bladder cancer where it promotes cell migration and invasion through the phosphoinositide 3-kinases (PI3K)/protein kinase B (AKT)/STAT3 signaling pathway (99).

## 5.4 Role of B7-H3 in glycolysis

As observed in several studies, B7-H3 has an important role in metabolism, particularly glycolysis. Nunes-Xavier analyzed the effect of B7-H3 levels in triple-negative breast cancer cell lines on sensitivity to 22 different anticancer drugs. In cells with B7-H3 knocked out, apoptosis inhibitor gene (API-2) and everolimus, which target the PI3K/AKT/mammalian target of rapamycin (mTOR) signaling pathway, showed greater inhibition of cell viability. In cells with B7-H3 knockdown, glycolytic capacity was reduced, while cells with B7-H3 overexpression exhibited higher glycolytic activity. Similar to previously observed, overexpression of B7-H3 had a weak effect on cell proliferation (100). Shi et al. investigated the role of B7-H3 in the metabolism in CRC cells. B7-H3 promotes glucose consumption and lactate production through the expression of hexokinase 2. In addition, B7-H3 also increased chemoresistance in cancer cells via hexokinase 2 (70). Li et al. similarly analyzed the effect of B7-H3 in oral squamous cell carcinoma. Elimination of B7-H3 resulted in decreased proliferation, colony formation, migration and invasion of the cells. B7-H3 also promoted glycolysis by regulating HIF1A hypoxia inducible factor 1 subunit alpha (HIFα) *via* the PI3K/AKT/mTOR pathway (101). Another mechanism was proposed by Zuo et al., who analyzed the role of B7-H3 in cervical cancer. Knockdown of B7-H3 resulted in decreased cell proliferation in the HeLa cell line. They also observed that B7-H3 forms a complex with the enolase 1 (ENO1) protein, confirmed by liquid chromatography–mass spectrometry (LC-MS) and IP. ENO1 is an important enzyme in glycolysis, and when the level of B7-H3 was reduced, adenosine triphosphate (ATP) and lactate production were also reduced. In addition, cellular Myc (c-Myc) and lactate dehydrogenase A (LDHA) were also reduced (102).

## 5.5 Role of B7-H3 in cancer resistance

B7-H3 has a significant role in cancer resistance to both, chemo- and radiotherapy. Ma et al. revealed that patients with low expression levels of B7-H3 presented with better overall survival compared to patients with high B7-H3 expression levels. It is reported that B7-H3 enhances chemoresistance of CRC cells by regulating the expression of cell division cycle 25A (CDC25A) through STAT3 signaling pathway in CRC cells (103). Moreover, overexpression of B7-H3 enhanced chemoresistance by reducing the G2/M phase arrest in a CDC25A-dependent manner. While the overexpression of B7-H3 promoted CRC cell viability and colony formation, chemotherapy-induced apoptosis was significantly decreased in B7-H3-overexpressing CRC cells *in vitro* and *in vivo*. Chemotherapy sensitivity was increased in cells with a stable knockdown of B7-H3. Ma et al. conclude that CRC cells can acquire chemoresistance through the B7-H3/CDC25A axis. In breast cancer, high expression levels of B7-H3 are correlated to poor outcome and resistance to commonly used chemotherapeutics such as paclitaxel (104). To study the role of B7-H3 in the sensitivity of metastatic breast cancer cells to paclitaxel, Liu et al. silenced B7-H3 in three breast cancer cell lines (MDA-MB-231, MDAMB-435, and MDA-MB-436). They reported that silencing B7-H3 enhanced the effect of paclitaxel chemotherapy compared to parental and control cells. Silencing B7-H3 abrogated the phosphorylation of Stat3 *via* inactivation of Jak2 and downregulated the direct target genes and anti-apoptotic factors induced myeloid leukemia cell differentiation protein (Mcl-1) and, to a lesser extent, survivin. On the contrary, its overexpression increased the phosphorylation of Jak2 and Stat3. *In vitro* results were also confirmed *in vivo* where the growth of B7-H3 knockdown xenografts was significantly inhibited upon paclitaxel treatment, while control tumors were only marginally affected. They concluded that Jak2/Stat3 pathway contributes to B7-H3–mediated paclitaxel resistance. Improving the sensitivity to paclitaxel by silencing B7-H3 is an important step into the clinical management of (metastatic) breast cancer. In CRC, B7-H3 enhanced the resistance to irradiation through upregulation of Kinesin family member 15 (KIF15) expression via NF-κβ which activated the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (105). Moreover, in CRC B7-H3 has an inducible effect on the polarization of macrophages from anti-tumor M1 into pro-tumor M2 (106).

## 5.6 Role of B7-H3 in angiogenesis

Several studies show that B7-H3 promotes angiogenesis. Wang et al. investigated the role of B7-H3 in CRC. As observed *in vitro* and *in vivo*, knocking down B7-H3 inhibits tube formation in human umbilical vein endothelial cells and leads to decreased expression of VEGFA, VEGFC, confirmed at mRNA and protein levels. The expression of VEGFA was induced via NF-κB, indicating the mechanism behind the angiogenesis (107). Purvis et al. investigated the role of B7-H3 in medulloblastoma, the most common embryonal neuroepithelial tumor. B7-H3 promoted angiogenesis by stimulating the secretion of VEGF. Afterwards, the conditioned medium of cells with B7-H3 overexpression was analyzed. Several pro-angiogenic factors were increased, including IL-6, IL-1, VEGF-D, VEGFR2, C-X-C motif chemokine ligand 11 (CXCL11), urokinase plasminogen activator surface receptor (uPAR), monocyte-chemotactic protein 3 (MCP-3), basic fibroblast growth factor (bFGF), tumor necrosis factor-alpha (TNF-α) and MMP-9, and chemokine (C-C motif) ligand 5 (CCL5 or RANTES) (108). In NSCLC Fan et al. investigated the role of B7-H3 in angiogenesis and alternative microvascular circulation, which is independent of angiogenesis, vascular mimicry. Cancer cell with B7-H3 knockdown resulted in decreased expression of E-cadherin and MMP-14, while there was no change in VEGF secretion. In the 3D model with B7-H3 knockdown, tumor growth was significantly reduced and the formation of capillary-like tubular structures was decreased. In the *in vivo* xenograft, B7-H3 knockdown resulted in significantly reduced tumor growth and decreased formation of vascular mimicry, while there were no changes in CD31+ endothelial vessels. Analysis of the supernatant of the cultured cells revealed no change in VEGF production between knockdown and mock cells. The proposed signaling pathway regulating vascular mimicry is via PI3K/AKT (109). Son et al. investigated the role of B7-H3 in angiogenesis. Suppression of B7-H3 resulted in decreased proliferation, increased apoptosis, and decreased migration of late endothelial progenitor cells, which are important for recovery from vascular dysfunction. However, this also led to increased tube formation and angiogenesis. This suggests that B7-H3 is necessary to maintain the cell population while blocking the promoted angiogenic differentiation (110). Lai et al. also showed that B7-H3 promotes VEGF secretion, that leads to angiogenesis (111). Seaman et al. conducted a comprehensive study on B7-H3's role in promoting angiogenesis. Their findings revealed high expression levels of B7-H3 on both cancer cells and tumor-associated blood vessels. Leveraging this expression pattern, they developed an anti-CD276 drug conjugate, effectively targeting both tumor cells and infiltrating blood vessels. This dual-targeting approach highlights B7-H3 as a significant therapeutic target for inhibiting tumor growth and vascular support within the tumor microenvironment (66).

## 5.7 B7-H3 in cancer stem cells

Several studies show that B7-H3 is associated with cancer stem cells. For example, Liu et al. have shown that B7-H3 is upregulated in breast cancer stem cells and its inhibition leads to inhibition of cancer stem cells, both *in vitro* and *in vivo*. Namely, B7-H3 binds to major vault protein (MVP) and activates MAPK/ERK kinase (MEK) and the MAPK kinase signaling pathway (112). A similar mechanism was observed in prostate cancer, where B7-H3 is overexpressed in cancer stem cells (113). B7-H3 is also overexpressed in cancer stem cells in head and neck squamous cell carcinomas and is associated with the escape of anti-tumor immunity. Anti-B7-H3 antibodies have been observed to eliminate cancer stem cells, inhibit tumor growth and lymph node metastasis *in vivo*. The blockade also reduces EMT (114). Xia et al. conducted

an in-depth analysis of B7-H3's impact on stemness in gastric cancer stem cells, revealing that B7-H3 enhances this stemness through the AKT/Nrf2 pathway (115). This effect is mediated by B7-H3's regulation of glutathione (GSH) synthesis, a critical factor in cellular redox balance that promotes cancer stem cell characteristics. Importantly, they demonstrated that inhibiting B7-H3 expression significantly reduces the stemness of these cancer cells, thereby suppressing tumorigenicity.

# 6 Role of B7-H3 in glioblastoma

There are reports that the expression level of B7-H3 in glioblastoma positively correlates to the malignancy grade and poor survival (116). Analyzing Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) data, Zhang et el. reported high B7-H3 mRNA expression levels in isocitrate dehydrogenase (IDH)-wild type glioblastoma, which was correlated with the malignancy grade i.e. predicted significantly worse patient survival (21). The authors also analyzed the methylation pattern of B7-H3 and discovered that the B7-H3 gene promoter was significantly hypomethylated in CGGA and TCGA glioblastoma samples. In addition to this Wang et al. performed genetic and clinical characterization of B7-H3 expression using RNAseq data from CGGA and TCGA and confirmed high B7-H3 expression in high-grade gliomas (117). They also identified methylation of B7-H3 promoter and miRNA-29 as potential regulators of B7-H3 expression. Their gene set enrichment analysis (GSEA) network analysis revealed B7-H3 is highly correlated to mitotic cell cycle, cell proliferation, angiogenesis and upregulated immune response i.e. higher malignancy. Takashima et al. analyzed the expression of 67 immunotherapy-related genes involved in T-cell status as well as stimulatory and inhibitory checkpoint molecules in 571 non-treated primary glioblastoma patients. Their results suggested that the expression of a single gene *B7-H3* and its combination with *GATA3* and *LGALS3* are effective for glioblastoma prognosis (118). High expression levels of B7-H3 in glioblastoma were also confirmed by Nehama et al. (119). The authors analyzed B7-H3 mRNA expression levels in TCGA dataset and reported 77% of primary and recurrent glioblastoma samples presented with high expression. They also evaluated protein expression levels with IHC and observed strong staining in 76% (35/46) of analyzed cases. The authors noted that glioblastoma cells recruited around blood vessels were intensely positive for B7-H3. Furthermore, Nehama et al. generated B7-H3-redirected chimeric antigen receptor (CAR) (B7-H3.CAR) T cells encoding CD28 or 4-1BB endodomains and CD19-redirected CAR (CD19.CD28) T cells as control and tested them with U87MG and U138MG glioblastoma cells. In 5 days after seeding, the authors observed complete or near-complete elimination of U87MG and U138MG cells. Controls remained viable. At last, the antitumor activity of B7-H3.CAR-T cells was tested in a xenograft murine model. Tumor regression was observed in samples treated either with B7-H3.CD28 CAR-T cells or B7-H3.41BB CAR-T cells. Results were additionally confirmed using glioblastoma neurospheres in coculture and engrafted in mice. *In vivo*, B7-H3.CAR-T cells encoding either CD28 or 4-1BB equally controlled tumor growth and prolonged survival in 50% of treated mice as compared to controls.

In glioma, B7-H3 promotes EMT through activation of the JAK2/STAT3/Slug pathway. Zhong et al. examined B7-H3 protein expression levels in glioblastoma and lower grade glioma (LGG) tissues with IHC and found out that high B7-H3 expression levels are common in glioblastomas compared to LGG. They established B7-H3-overexpressing and knockout glioma cells to study the effect of B7-H3 on cell proliferation and invasive potential. Their *in vitro* and *in vivo* results showed that overexpression of B7-H3 promotes cell proliferation and invasion. In addition, they reported that B7-H3 induces EMT processes through downregulation of E-cadherin and upregulation of MMP-2 and MMP-9 expression via a JAK2/STAT3/Slug-dependent signaling pathway (120). In accordance with previously published data, knockdown of B7-H3 decreased while its overexpression increased the migration and invasion of glioblastoma cells as reported by Zhang et al. (121). In the same study, with RNA sequencing the authors examined transcriptome changes after B7-H3 knockdown and showed decreased expression of MYC proto-oncogene, BHLH transcription factor (MYC) and increased expression of SMAD family member 6 (SMAD6). Both genes are correlated to the TGF-β signaling pathways which was reported to be significantly enriched.

In glioblastoma both splicing variants, 2IgB7-H3 and 4IgB7-H3, of B7-H3 are present. In particular, 4IgB7-H3 is restricted to glioblastoma cells and may serve as a target for therapy, while 2IgB7-H3 presents with higher expression in recurrent glioblastoma and increases resistance to temozolomide (20). Therefore, 4IgB7-H3 can be further explored for therapeutic purposes whereas 2IgB7-H3 can be used to track tumor recurrence (122).

## 6.1 Distribution of B7-H3 and PD-L1 expression in brain cancer

The Human Protein Atlas (123) is a combination of transcriptomics and antibody-based proteomics aiming to map human proteins at a single cell and spatial resolution. It provides information about gene and protein expression levels in different reference and cancer samples. To contextualize the relevance of B7-H3 in brain cancers, we analyzed its expression pattern in brain cancer and compared it to the expression pattern of the well-known immune checkpoint PD-L1). Findings are presented in Figures 4, 5.

In Figure 4 RNA expression overview with data from TCGA is given. Expression levels of B7-H3 and PD-L1 were analyzed in 153 glioma samples and showed a median expression of 25.2 FPKM for B7-H3 and 1.2 FPKM for PD-L1. Both B7-H3 and PD-L1 have low cancer specificity.

Analyzing single cell types (Figure 5, top), in particular glial cells showed low expression of B7-H3 (6.2 astrocytes, 5.2 oligodendrocyte precursor cells, 1.5 oligodendrocytes, 7.2 microglial, 7.2 Muller glia, 1.9 Schwann cells) and PD-L1 (0.6 astrocytes, 0.9 oligodendrocyte precursor cells, 0.8 oligodendrocytes, 8.1 microglial, 0.7 Muller glia, 0.5 Schwann cells). When it comes to cell line categories, both B7-H3 and PD-L1 have low cancer specificity. Expression of B7-H3 and

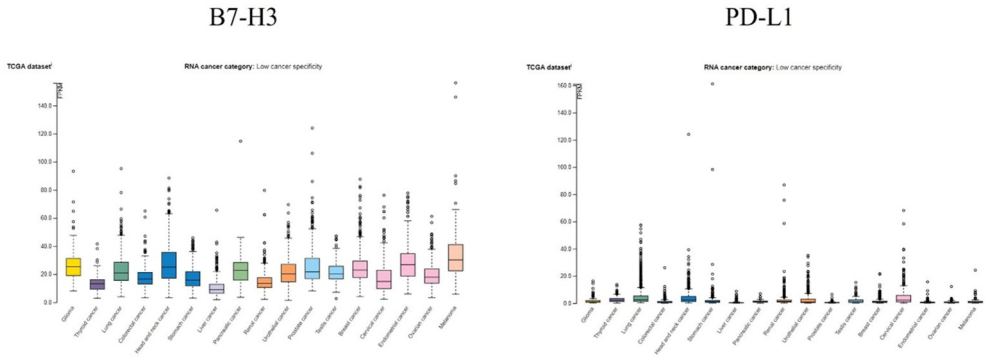


FIGURE 4
RNA expression overview of B7-H3 and PD-L1 in cancers analyzed in TCGA. FPKM, fragments per kilobase million. Data for B7-H3 and PD-L1 is from The Human Protein Atlas, access date 17 October 2024.

PD-L1 was analyzed in 80 brain cancer cell lines (Figure 5, middle) and showed a maximum average of 76.9 nTPM for B7-H3 and 8.5 nTPM for PD-L1. The more detailed analysis of expression of separate brain cancer cell lines (Figure 5, bottom) showed a maximum average expression of 181.7 nTPM for B7-H3 (cell line DBTRG-05MG) and a maximum average expression of 70 nTPM for PD-L1 (cell line IOMM-Lee).

## 6.2 Mechanisms of immune suppression of B7-H3 and PD-L1

Immuno surveillance refers to the processes by which host immune cells look for and recognize pathogens and altered cells such as cancer cells. Cancer cells however have developed various mechanisms to escape immune surveillance. The immunosuppression mechanisms of B7-H3 and PD-L1 are listed in Table 1.

# 7 B7-H3 in cancer immunotherapy

Immunotherapy is a developing field in cancer treatment using human own immune system to recognize and destroy tumor cells. Different types of immunotherapies were developed. In the next section, various immunotherapeutic approaches based on B7-H3 which are illustrated in Figure 6 will be explained with addition on using nanobodies as an approach to target tumors (30).

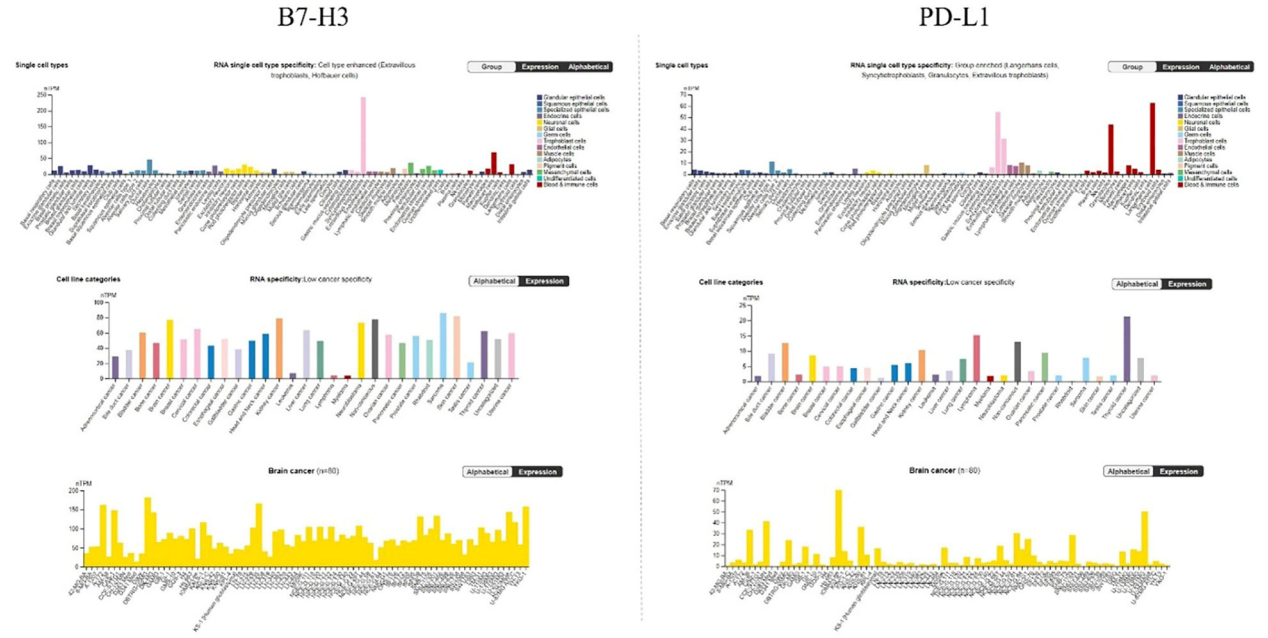


FIGURE 5
Expression levels of B7-H3 and PD-L1 in (top), RNA single cell type, (middle) different cancer cell lines, and (bottom) brain cancer cell lines. nTPM, normalized transcripts per million. Data for B7-H3 and PD-L1 is from The Human Protein Atlas, access date 17 October 2024.

TABLE 1 Mechanisms of immunosuppression of B7-H3 and PD-L1.

| Immune checkpoint | Mechanism of suppression | Reference |
|---|---|---|
| B7-H3 | B7-H3 expressed on APC or tumor cells recognizes a receptor on activated CD4+ and CD8+ cells | (26, 124) |
| | 2Ig and 4Ig isoforms of B7-H3 inhibit the proliferation of T cells and downregulate cytokine production | (125) |
| | Promotes production of IL-10 and TGFβ to favor immunosuppressive environment | (126) |
| | Inhibits activity of CD4+ T cells, CD8+ T cells, γδT cells, CAR-T cells, Vδ2 T cells, Th17 cells, CD3+ T cells, NK cells, macrophages, neutrophils, dendritic cells, and secretion of IFN-γ, IL-2, perforin, granzyme B to favor immunosuppressive environment | (127–130) |
| | Promotes polarization of type 2 macrophages | (106) |
| | Contributes to CCL2–CCR2–M2 macrophage axis-mediated immunosuppression | (88) |
| PD-L1 | Binds to PD-1 and exerts immunosuppressive regulatory effect via SHP2 by reducing the immune response of T cells | (131) |
| | PD-1/PD-L1 interaction:<br>- suppresses T cells activation and proliferation, promoting T cell dysfunction and apoptosis<br>- enhances function of $T_{reg}$ and induces immune tolerance<br>-promotes polarization of TAM and other immune cells into tumor-promoting phenotypes, facilitating immune escape and cancer progression | (132) |
| | Upregulation of PD-L1 and IL-10 expression in TAMs suppresses T cell proliferation and promotes tumor growth through the TLR4-MyD88-p38-STAT3 signaling pathway | (133) |
| | PD-1/PD-L1 axis inhibits NK cell-induced antitumor immunity *in vivo* | (134) |

APC, antigen presenting cells; PD-1, programmed cell death protein 1; SHP2, Src homology region 2 domain-containing phosphatase-2; $T_{reg}$, regulatory T cells; TAM, tumor associated macrophages; NK, natural killer cells

## 7.1 Monoclonal antibodies

Monoclonal antibodies are antibodies having only monovalent affinity for a single epitope. With changing their variable region, they can be designed to target any antigen or epitope (135). Therapeutic antibodies target cell surface antigens in cancers. The first developed monoclonal antibody for cancer treatment was against CD20 protein, which is highly expressed on cancerous B cells in non-Hodgkin lymphoma but not on healthy B cells (136). Until now, the WHO International Nonproprietary Names (INN) Program has assigned INN names to about 1,000 monoclonal antibodies, 530 of which are in the field of oncology (137). Success of monoclonal antibody in cancer treatment is now evident in multiple cancers including bladder cancer, sarcoma, colorectal cancer and breast cancer to name a few, targeting different proteins including those of B7-family PD-1 and PD-L1 (138).

Therapeutic monoclonal antibodies have different mechanisms of action: antibody-dependent-cellular-phagocytosis (ADCP), antibody-dependent-cellular-cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), radionucleotide delivery, cytotoxic drug delivery, neutralization, or signal blockade (139). The effect of the therapeutic monoclonal antibody is related to the antigen profile of the cancer, as well as the ability of the antibody to be internalized, activate Fcγ-receptors on innate cells, trigger activation of complement or block receptor-mediated oncogenic signaling (139). This depends on the isotope and nature of the monoclonal antibody (i.e., specific binding site, avidity of target binding, and particular conformation) and the target protein. Immune checkpoints enhance anti-tumor immune responses by restoring exhausted T cells. From the six major immune checkpoints for cancer immunotherapy, namely cytotoxic T-lymphocyte associated protein 4 (CTLA-4), programmed cell death 1 (PDCD1), cluster of differentiation 274 (CD274), inducible T-cell costimulator (ICOS), lymphocyte-activation gene 3 (LAG3), and CD40, ipilimumab targeted against CTLA-4 was the first monoclonal antibody approved by the Food and Drug Administration (FDA) in 2011. Ipilimumab activates the immune system by targeting CTLA-4, a protein receptor that downregulates the immune system, and is used in the treatment of metastatic melanoma. Ipilimumab mechanism of action is by blocking the binding of CTLA-4 to its ligands, inhibiting CTLA-4-mediated downregulation of T cells and promoting the interaction of CD80/CD86 with CD28 (137). This activates the immune system by increasing T cell expansion and enhancing the CTL-mediated anti-tumor immune response. Moreover, the IgG1-Fc region of ipilimumab binds to FcγRIIIa, induces ADCC and complement-dependent cytotoxicity (CDC) for enhanced anti-tumor efficacy by reducing $T_{reg}$ cells.

Monoclonal antibodies have also been tested against B7-H3. In case of ovarian cancer cell lines treating with monoclonal antibodies alone significantly inhibited cell growth. In combination with tyrosine inhibitor even better outcome appeared (140). Shi et al. demonstrated monoclonal antibodies to inhibit 2IgB7-H3 and 4IgB7-H3 known to be highly expressed in variety of tumors (141). This success led to testing monoclonal antibodies including monoclonal antibodies against B7-H3 in clinical trials (Table 2). However, there are no clinical trials in phase 3 yet.

For NCT02475213, patients with non-small cell lung cancer, head and neck squamous cell carcinoma, urothelial cancer and melanoma, were treated with enoblituzumab (investigational anti-B7H3 antibody), together with pembrolizumab, anti-PD1 inhibitor. 133 patients were enrolled in the study and 116 got treatment-related adverse effects, which were equal or treater than grade 3 in 28.6% of patients. There was also one treatment-related death due

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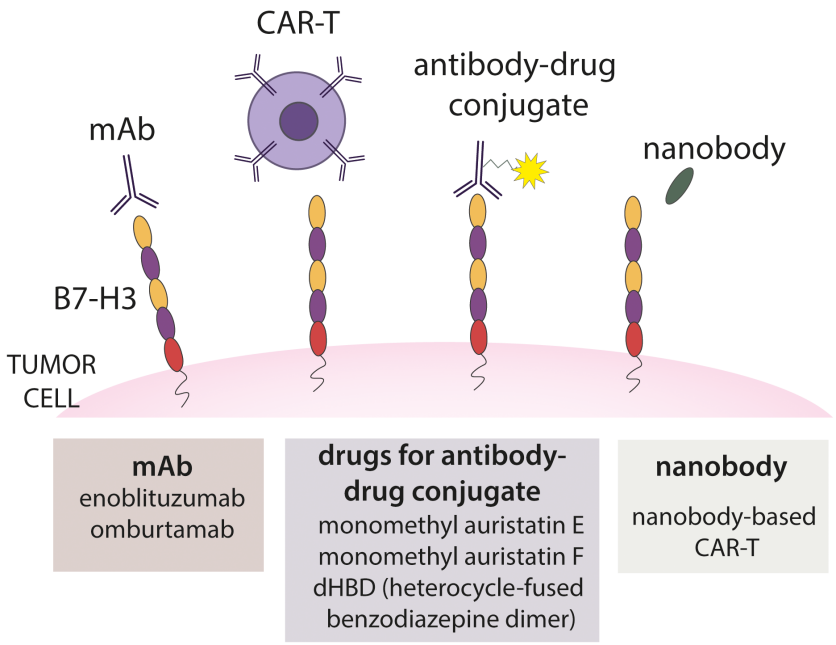


FIGURE 6
Different approaches of B7-H3 immunotherapy. Several approaches are presented, including targeting B7-H3 with monoclonal antibody, CAR-T, antibody-drug conjugate and nanobody.

TABLE 2 Clinical trials targeting B7-H3 with monoclonal antibodies (142–147).

| Trial number | Mechanism | Cancer | Stage | Goal | Result | Year |
|---|---|---|---|---|---|---|
| NCT02475213 | Dual target of Enoblituzumab with Pembrolizumab or Retifanlimab | NSCLC<br>Head and neck squamous cell carcinoma<br>Urothelial cancer<br>Melanoma | I/II | Outcomes and safety | Side effects in 87.2% of patients and grade ≥3 in 28.6% patients. One treatment-related death occurred (pneumonitis). Objective responses occurred in 33.3% patients with CPI-naïve HNSCC and in 35.7% patients with CPI-naïve NSCLC. | 2022 |
| NCT00089245 | $^{131}$I-Omburtamab | CNS malignancies | I | Efficacy and safety | In patients receiving cRIT for neuroblastoma, survival was markedly increased (median PFS 7.5 years)<br>Acute toxicities included < grade 4 self-limited headache, vomiting or fever, and biochemical abnormalities. Grade 3/4 thrombocytopenia was the most common hematologic toxicity. | 2022 |
| NCT01502917 | $^{124}$I-Omburtamab | DIPG | I | Safety | One (4%) of 28 patients had treatment-related transient grade 3 hemiparesis and one (4%) had grade 3 skin infection. No treatment-related grade 4 adverse events or deaths occurred. | 2018 |
| NCT02982941 | Enoblituzumab | Neuroblastoma<br>Rhabdomyosarcoma<br>Osteosarcoma Ewing sarcoma Wilms tumor<br>Desmoplastic small round cell tumor | I | Safety | No results posted | 2019 |
| NCT02381314 | Enoblituzumab plus ipilimumab | Melanoma<br>NSCLC and other cancers | I | Safety | No results posted | 2018 |
| NCT01391143 | Enoblituzumab | Refractory cancer expressing B7-H3 | I | Safety | No results posted | 2019 |

NSCLC, non-small cell lung cancer; HNSCC, CPI-naïve; CNS, central nervous system; cRIT -; DIGP, diffuse intrinsic pontine glioma; PFS, progression free survival.

to pneumonitis. The response in HNSCC was in 6 cases out of 18 and in NSCLC was 5 of 14. The treatment was discontinued in 13 patients (148). Both NCT00089245 and NCT01502917, include therapy based on anti-B7-H3 antibody labeled with radioactive iodine. At clinical trial NCT00089245, 37 patients received injections of radiolabelled omburtamab. 16 of patients had metastatic neuroblastoma and other had solid tumors with B7H3 expression, including medulloblastoma, ependymoma, melanoma and rhabdomyosarcoma, choroid plexus carcinoma, atypical thabdoid teratoma, chordoma, pineoblastoma and retinoblastoma. The drug was overall well-tolerated. The most common adverse effect was myelosuppression. Patients who had neuroblastoma had significant improvement in overall survival and progression-free survival. Seven survived without progressive disease for 13-17 years from treatment. Of the remaining 8, only 2 had CNA relapse and the 6 died of systemic relapse or chemotherapy-related toxicity (149). At clinical trial NCT01502917, 28 children with diffuse intrinsic pontine glioma were treated with omburtamab labeled with radioactive 124-iodine (150). There were no dose-limiting toxicities. One out of 28 patients had treatment-related transient grade 3 hemiparesis and one had grade 3 skin infection. There were no treatment-related grade 4 adverse effects or deaths. The systemic exposure was low. The radioisotope was retained in the brain for more than 8 days.

## 7.2 Chimeric antigen receptor T cells (CAR-T cells)

CAR-T cell therapy is one of the main approaches of effective adoptive T-cell therapy (ACT), which kills cancer cells by the therapeutic use of transferred T cells (151). CAR is a synthetic construct i.e. a bioengineered receptor that binds to target cell surface antigens (e.g. proteins, glycolipids, and carbohydrates) through a single-chain variable fragment (scFv) recognition domain. CAR-T cells technology is an *ex vivo* method, which genetically modifies patient derived cells for immunotherapeutic purposes. Either NK cells or T cells can be used for CAR-T. Currently, various generations of CAR-T cells have been developed, each varying in their capacity to eliminate cancer cells (152). CAR-T cells mediate major histocompatibility complex (MHC)-unrestricted tumor cell killing by allowing T cells to bind to their cell surface antigens through a scFv recognition domain (151). Then, CAR-T cells form a non-classical immune synapse (IS), which is required for their effector function. These cells mediate their anti-tumor effects through perforin and granzyme axis, the Fas and Fas ligand axis, and cytokine release for sensitizing tumor stroma. The outputs largely depend on the individual components of the receptor i.e. scFv, spacer and costimulatory domains. First generation of CAR-T cell lacked co-stimulatory molecules which made them less powerful and inconsistent (153). However, newer generations of CAR-T cells added co-stimulatory molecules and improved its efficacy. Unlike conventional T-cells, CAR-T cells can bind to an antigen irrespective of the MHC presentation. However, their limitation is binding to cell surface expressed antigens only. CAR-T cells are currently used to treat hematological malignancies; namely, 6 CAR-T cell products have been approved by the FDA (154). Research for solid tumors is limited due to immunosuppressive TME and lack of antigens (152). Additionally, research on solid tumors was halted due to two deaths caused during clinical phase I in men with prostate cancer (155).

In clinical perspective, Majzner et al. tested B7-H3 CAR T cells on models of solid pediatric tumors (osteosarcoma, medulloblastoma and Ewing sarcoma) in xenograft mice (156). The authors developed a B7-H3 CAR, based on MGA271 (Enoblituzumab), that preferentially binds tumor tissues and shows restricted recognition of normal human tissues. They showed that the B7-H3 CAR T cells mediate antitumor activity *in vivo* and cause regression of established solid tumors. In particular, the B7-H3 CAR-T cells eradicated the autochthonous DAOY and D425 medulloblastoma xenografts which was observed by bioluminescent imaging. The systemically administrated B7-H3 CAR-T cells also mediated regression and eradication of established osteosarcoma and Ewing sarcoma xenografts.

CAR-T technology was tested for B7-H3 *in vitro* and *in vivo* experiments. Although no clinical trials have been completed so far, many clinical trials are currently recruiting participants. CAR-T cells targeting B7-H3 shows high tumor specific killing ability *in vitro* and tumor suppression effect *in vivo* in PDX (patient derived xenograft) model in osteosarcoma (157). CAR-T cells targeting B7-H3 are also effective in inhibiting growth *in vitro* and *in vivo* of NSCLC (158), prostate cancer (159), glioblastoma (116), ovarian and triple negative breast cancer (160).

## 7.3 Antibody drug conjugates (ADC)

Antibody drug conjugates (ADC) are a class of drug typically composed of monoclonal antibodies covalently attached to a cytotoxic drug i.e. payload and a linker in between. ADC combine the advantages of specific targeting ability and potent killing affect to accurately and efficiently eliminate cancer cells (161). Because of the antibody specificity, ADC target only antigen-expressing cells and therefore do not cause systemic toxicity which is common in conventional chemotherapy (148). When the antibody binds to the cell surface antigen on the target cell, the ADC is internalized to form an early endosome, followed by a maturation into late endosomes and finally fused with lysosomes. Linker breakdown promotes ADC release of the cytotoxic drug by chemical- or enzyme-mediated release into lysosomes which eventually leads to cell death or apoptosis via targeting DNA or microtubules (148, 161–163). Their mechanism of action is eliciting immunogenic cell death, ADCC, ADCP and CDC effects, as well as dendritic cell activation upon interaction with cancer and immune cells (161, 162). The “bystander effect” i.e. diffusion of the payload from antigen-expressing cancer cells to adjacent cells and therefore killing these cells contribute to the cytotoxicity of ADC in heterogenic tumors (148). Binding of the ADC to its target may also interrupt its downstream function by preventing the antigen interaction with its binding partners (163). ADC induce tumor-specific adaptive immunity by increasing the infiltration of T cells into the TME. Typically, they remain stable in blood, are target-specific and release cytotoxic component in the vicinity of cells. All components of ADC need to be carefully selected, including the

right molecular target since they have an impact on safety, efficiency, and delivery of drug (161). Mostly used cytotoxic molecules inhibit tubulin, are immunomodulators or they damage DNA (164). Currently there are many ADC drugs that are under development for cancer treatment (161). First ADC drug granted approval by FDA was Mylotarg® (gemtuzumab ozogamicin) for the treatment of adults with acute myeloid leukemia (AML) in 2000 (161, 165). The first ADC for treatment of solid tumors was Ado-Trastuzumab Emtansine (Kadcyla®) or T-DM1 for treatment of HER2+ metastatic or locally advanced breast cancer and was approved in 2013 (162). Recently two studies demonstrated efficacy of ADC targeting B7-H3 in glioblastoma (166), lung, and breast cancer cell lines and PDX models (167). Study using ADC for killing glioblastoma showed greater killing potency for glioblastoma cells with greater expression of B7-H3. They used monomethyl auristatin E which is known microtubule-disrupting agent. When coupled with fluorescent conjugate, they showed specific accumulation in tumor cells *in vivo* (166). ADC used for targeting B7-H3 in lung and breast cancer cell showed the same affinity as unconjugated B7-H3 antibody. This ADC cause cell cycle arrest in S phase, DNA damage and apoptosis. In this case, they used monomethyl auristatin F, which is also microtubule-disrupting agent (167). When it comes to ADC treatment, many of the patients develop resistance and experience disease progression. Therefore, the mechanisms of resistance (eg. altered target cell surface expression, gene mutations, changes in trafficking and internalization, payload resistance) need to be studied for optimal results. In addition, combining ADC with other chemo- or immune-therapeutic approaches may increase their utility in cancer treatment (163).

## 7.4 Antibody dependent cell mediated cytotoxicity (ADCC)

The immune mechanism of antibody-dependent cell-mediated cytotoxicity (ADCC) involves three components: effector cells with NK cells being the major type *in vivo*, antibodies and target cells opsonized by the antibodies (168). ADCC is a method of combating tumors by introducing specific antibodies that attach to target tumor cells. This attachment triggers or recruits effector immune cells to recognize these foreign antibodies and initiate cell death through non-phagocytic mechanisms. Antigens bind to the target cells through their antigen binding fragment (Fab), while the interaction with the effector cells occurs between the fragment crystallizable region (Fc) portion of the antibody (30, 168). There are three different mechanisms that occur after the activation of effector cells: cytotoxic granule release, Fas signaling, and elaboration of reactive oxygen species (ROS). From these, the main mechanism of action in ADCC is release of perforins and granzymes from effector cell granules. Activated effector cells can also upregulate the expression of Fas ligand in order to cause apoptosis in the target *via* Fas signaling. Although IgG, IgA, and IgE can mediate ADCC, IgG is the most used immunoglobulin subclass for cancer therapeutic antibodies. They developed humanized mouse antibody against B7-H3, which was afucosylated afterwards. Flow cytometry showed binding on IgC1 and IgC2 domain of human B7-H3. ADCC was activated against medium and high expressing breast (MDA-MB-231) and lung cancer (NCI-H322) cell lines. Afucosylated protein induced ADCC even on low expressing B7-H3 cancer cells, while non-afucosylated did not. Additionally, the afucosylated protein showed dose-dependent antitumor efficacy in severe combined immunodeficient (SCID) mouse and small effect in new generation of severely immunodeficient (NOG) mouse (169). Xu et al. developed bispecific modified protein targeting B7-H3/PD-L1 and with modified Fc region enhanced ADCC. Fusion protein blocked PD/PD-L1 pathway, activated CD8+ cells and enhanced ADCC in tumor cells with higher expression of B7-H3 (170). Another study found that B7-H3 human mouse chimera antibody induced ADCC in primary leukemia cells but not in hematopoetic cells. Furthermore, treating PDX models in combination with antibody and human NK cells significantly prolonged survival (171).

## 7.5 Small molecular inhibitors

Another important way of fighting cancer is using small molecular inhibitors. These molecules target different important molecules in signaling pathways of cancers including VEGFR, human epidermal growth factor receptor (HER), RAS, JAK, mTOR and so on. Because of their small size, small molecular inhibitors can bind to a wider range of extracellular and intracellular targets when compared to antibodies. Based on target specificity, small molecule inhibitors are divided into selective and multikinase. Multikinase small molecular inhibitors repress multiple kinases in the tumor, they do not require precise detection and rely on histology. Selective small molecule inhibitors bind to a single target and inhibit its cell signaling. They can either inhibit its unusual function or reverse its regular action. Selective small molecule inhibitors are then further divided into two groups: selective small molecule kinase inhibitors and selective small molecule non-kinase inhibitors (172). Selection of a small molecule whether it is a multikinase or selective small molecule kinase is based on the number of kinases whose inhibitory activity $IC_{50}$ values are below 10 nM (173). Selective small molecule non-kinase inhibitors bind to targets beyond the kinome (i.e. the complete set of protein kinases encoded by the genome), thereby blocking subsequent functions to control tumors.

Protein kinase inhibitors are the main category of small molecule inhibitors. Protein kinase enzyme has important role in cell growth, proliferation and differentiation. Protein kinase catalyzes the transfer of γ-phosphate group from ATP to protein residues containing hydroxyl groups. Dysregulations of protein kinases are linked to various diseases including cancer. Because of this, protein kinases are studied as cancer therapeutic targets. The selective small molecule kinase inhibitors contain receptor-related kinase inhibitors, kinase inhibitors targeted intracellular signaling pathways, and inhibitors targeting other cytoplasmic kinases (172). As described in (174), protein kinase inhibitors are classified into six types: Type-I inhibitors bind to the active conformation of the kinase (DFG-Asp in, αC-helix in); type-I½ inhibitors bind to a

DFG-Asp in inactive kinase conformation with αC-helix out; type-II inhibitors bind to a DFG-Asp out inactive conformation; type III inhibitors restrain kinase activity by binding to an allosteric site; type IV inhibitors bind outside of the cleft; type V inhibitors are bivalent molecules that span two distinct regions of the kinase domain; and type VI inhibitors that bind covalently with the kinase active site. While type I-V inhibitors are all reversible, type VI are irreversible kinase inhibitors. The tyrosine kinase inhibitor imatinib was the first small molecule targeted drug that was approved by the FDA in 2001 (174).

Small molecular inhibitors have some important advantages that can be readily used like pharmacokinetic properties, low manufacturing cost, drug storage and transportation, small size, short half-lives, patient compliance and good distribution in tissues (174, 175). Moreover, small molecule inhibitors can be taken orally, and some of them can penetrate the BBB to control intracranial lesions (172). However, they also present with disadvantages such as low response rate and drug resistance. Understanding that the receptor(s) on activated T cells interact with the FG loop of the IgV domain of B7-H3, there is potential to create a small molecule inhibitor aimed at disrupting this precise binding region. However, it is important to acknowledge that unforeseen off-target effects may occur, underscoring the need for comprehensive assessment to minimize any potential negative outcomes. To the best of our knowledge, there are currently no clinical trials targeting B7-H3 protein and none *in vitro* or *in vivo* studies regarding B7-H3.

## 7.6 Nanobodies

Nanobodies are ~15kDa proteins derived from heavy-chain only antibodies produced by just few animals, such as camelids and sharks. They were first discovered by the group of Raymond Hammers in Belgium at Vrije Universiteit Brussel in 1993 in camelids (176). Camelids have two types of IgG antibodies, the conventional and heavy chain antibodies that are lacking light chains. Nanobody is a variable domain of this heavy chain antibody. These nanobodies have several advantages compared to conventional antibodies. Due to their smaller size, they exhibit better tissue penetration and faster clearance from the body. Additionally, they are more stable due to several amino acids substitution that make them more hydrophilic. They contain 3 binding domains i.e. complementarity determining regions (CDRs - CDR1, CDR2, CDR3) compared to 6 in classical antibodies, but still bind with affinities in nano/pico molar concentrations (177). The longer CDR3 of nanobodies forms a finger-like extension that binds to small hidden epitopes in the concave surface of the antigen or in the antigen gap with high affinity. Nanobodies can selectively bind multifunctional epitopes on the receptor, block disease-related signaling pathways or responses (178). Zarantonello et al. described the selection of the C1q specific nanobody C1qNb75 that is able to inhibit activation of the classical pathway (CP) of the complement system (179). The proposed mechanism of action is by occluding the IgG binding site on C1q.

Nanobodies as the single agents have not been yet developed and tested as therapeutics. However, their unique and potentially buried binding sites, small size, as well as simple expression and production, make nanobodies suitable for use in CAR-T therapy development. Li et al. developed nanobody-based CAR-T cells targeting B7-H3 for treating several different solid tumors. Nanobody against B7-H3 was retrieved from dromedary camel phage library and specific CAR-T cells were obtained first by cloning nanobody into CAR construct. Human peripheral blood mononuclear cells (PBMCs) were then isolated from healthy donors and stimulated with anti-CD3/anti-CD28 antibody-coated beads in presence of IL-2. The therapy efficiently lysed four neuroblastoma (NB) cell lines, two pancreatic cancer cell lines, triple-negative breast cancer cell lines and lung adenocarcinoma cell line. CAR-T cells were tested also in mice model of pancreatic cancer, where they had high antitumor activity (180). One of the most important components in CAR-T cell technology is the antigen-recognition domain (180). B7-H3 has two distinct epitope domains IgC and IgV; one of the clinically tested antitumor antibodies, 8H9, binds to the IgV domain. In their study, Li et al. showed that the B7-H3 CAR-T cells targeting IgC are more potent than those targeting IgV in pancreatic ductal adenocarcinoma (PDAC) and NB mouse preclinical models. They conclude that the antigen-binding epitope is crucial for the biological function of antibody-based CAR-T cells.

# 8 Therapeutic approaches for glioblastoma and other brain tumors

Table 3 below presents clinical trials targeting B7-H3 in glioblastoma patients. Currently there are five different clinical trials that are in phase of recruiting patients and all of them include CAR-T therapy. The majority of trials are Phase l.

All clinical trials are designed for glioblastoma patients with recurrent and refractory glioblastoma. For three of the clinical trials, the inclusion criteria are the B7H3 expression in tumor, determined by immunohistochemistry. For NCT05241392, the expression of B7-H3 should be more or equal to 30%. For NCT04077866 and NCT04385173 the inclusion criteria positive B7-H3 positive tumor expression by immunohistochemistry at the initial tumor or recurrent disease with H-score more or equal to 50. For NCT05474378 and NCT05366179, there is no inclusion criteria for B7-H3 expression. There is no outcome for the trial listed.

A case study reported evaluation of the therapeutic potential of B7-H3 targeted CAR T-cell therapy in treating recurrent glioblastoma (187). A 56-year old woman presented with recurrent glioblastoma after two craniotomies and standard treatment with chemotherapy during the last 2 years. High B7-H3 expression levels were confirmed in primary patient cells using flow cytometry. B7-H3 targeted CAR-T cells induced specific anti-tumor effect in the primary cells. ELISA results also indicated an activation effect of the CAR-T cells when cocultured with tumor primary cells. After detecting tumor recurrence, the patient received weekly intracavitary infusions of B7-H3 targeted CAR-T cells with a dose-escalating principle. Tumor reduction was detected using magnetic resonance imaging (MRI) and the clinical response was maintained for approximately 50 days after the administration of B7-H3 targeting CAR-T cells. However, in

TABLE 3 Clinical trials targeting B7-H3 in glioblastoma patients (181–186).

| ClinicalTrials ID | Disease | Mechanism | Stage | Status |
|---|---|---|---|---|
| NCT04077866 | Recurrent or refractory glioblastoma | Drug: Temozolomide<br>Biological: B7-H3 CAR-T | Phase l/ll | Recruiting |
| NCT04385173 | Recurrent or refractory glioblastoma | Drug: Temozolomide<br>Biological: B7-H3 CAR-T | Phase l | Recruiting |
| NCT05241392 | Recurrent glioblastoma | Biological: B7-H3-targeting CAR-T cells | Phase l | Recruiting |
| NCT05474378 | Recurrent glioblastoma | Biological: B7-H3-targeting CAR-T cells | Phase l | Recruiting |
| NCT05366179 | Recurrent or refractory glioblastoma | Biological: B7-H3-targeting CAR-T cells | Phase l | Recruiting |

cycles 6 and 7 the patient had another recurrence and dropped out of the clinical study. Headaches were reported as a side effect of the therapy starting from 3 h after infusion and increasing in follow up infusions. Headaches were probably as a result of inflammation response. The authors also detected a significant expansion of T cells in cerebrospinal fluid (CSF) samples obtained from the infusion device. In addition, they evaluated 16 inflammatory cytokines to assess immunologic changes in CSF and periphery blood before and after each cycle infusion. In particular, IL-2 and IL-6 levels increased significantly (factor >5) in periphery blood. The tumor became resistant to the treatment which may be a result of target antigen heterogeneity i.e. CAR-T cells were not able to completely eliminate the tumor cells especially those with low B7-H3 expression levels. Expansion of these cells may have led to tumor relapse.

Similarly, Tang et al. assessed bioactivity and safety levels of B7-H3 CAR-T cells against anaplastic meningioma (188). A 49-year old woman presented with multiple recurrent anaplastic meningiomas. IHC showed high and homogeneous expression levels of B7-H3, which was also confirmed with immunofluorescence staining of B7-H3 in primary cells from the patient tumor. Patient received 3 cycles of CAR-T cell treatment. Here, the patient also reported moderate headache as a side effect starting from 3 h after CAR-T cell infusion in 2$^{nd}$ and 3$^{rd}$ round. CAR-T cells were detected in CSF but were absent in peripheral blood. From the 6 inflammatory cytokines measured, only IL6 appeared with increased levels in the serum. This case study indicated that B7-H3-targeted CAR-T cells exhibited local antitumor responses against anaplastic meningioma. B7-H3 targeted CAR-T cells locally suppressed tumor progression without serious side effects which indicated the tolerability, safety and efficacy of the therapy.

Despite the promising early results, no B7-H3-targeting CAR-T cell therapies have been approved for clinical use.

## 9 Gaps in the current understanding of B7-H3 and future research directions

B7-H3 has gained attention in the fields of oncology and immunotherapy because of its expression pattern on tumor cells and safety profile (175). This also encouraged its potential use in therapeutic development, based on the knowledge and directions obtained from existing immunotherapeutic strategies involving other immune checkpoints such as PD-L1 and CTLA-4. Still, even with the progress in understanding the role of B7-H3 in cancer, there are several gaps in knowledge that remain to be filled. The molecular mechanisms by which B7-H3 contributes to cancer metastasis, angiogenesis and immune evasion can be elucidated by clarifying its exact intracellular signaling pathways and interactions with other immune checkpoints (e.g. PD-L1). In this regard, identifying the receptor of B7-H3 is crucial not only for elucidating its role in cancer, but also for understanding of its exact function and design of B7-H3 antagonists. The heterogeneity of B7-H3 expression within tumors and how it affects treatment as well as development of drug resistance should be explored in more detail. To evaluate its diagnostic and therapeutic potential, the expression profiles of B7-H3 in pre-malignant lesions, tumor-associated vasculature, metastases, recurrence as well as bodily fluids have to be determined. At last, development of suitable models for cancer modeling such as spheroids and organoids that will provide more information about the therapeutic potential of targeting B7-H3 alone and in combination with existing therapies (e.g. radio-, chemo- and immuno-therapy) will accelerate its translation into real clinical settings.

Two of the most extensively explored approaches are monoclonal antibodies and CAR-T cell therapy. The value of monoclonal antibodies in cancer treatment lies in their mechanism of action i.e. promoting cancer cell death by recognizing tumor associated antigens (TAA) on cancer cells, and stimulation of long-lasting antitumoral activities while leaving healthy cells untouched (189). Still, more than 20 years passed from the approval of the first monoclonal antibody for the treatment of autoimmune diseases, non-Hodgkin lymphoma and chronic lymphocytic leukemia (Rituximab, 1997) until the approval of the first antibody for treatment of solid tumors (Sacituzumab Govitecan, 2020). This can be a result of poor antibody penetration in tumor tissue and impaired homogeneous distribution, heterogeneous antigen expression on cancer cells and presence of immunosuppressive TME (190). Perhaps the combined use of multiple antibodies targeting various TAA will yield better results and improved patient outcomes. Bispecific antibodies that can

simultaneously target TAA and activate T cells or other effector immune cells may also be beneficial (190). Moreover, novel antibodies and antibody classes targeting different antigens or different epitopes on existing and to-be-determined therapeutic targets should be explored (191). With such an approach more cancer cells will be destroyed which will leave little space for development of escape mechanisms. Changes in gene/protein expression levels during the course of treatment which can influence the therapeutic response should not be overlooked. Development of a new generation of antibodies with improved specificity, penetration and distribution, reduced off target effects and increased potency should follow. Similar to ADC the limitation of the monoclonal antibodies is development of therapy resistance which emphasizes the need to deepen the knowledge about their mechanisms of action. Because of their expensive production and complex nature strategies for cost reduction should also be proposed.

To date, 6 CAR-T cell therapies against hematological cancers are already approved by the FDA (180). However, although promising, the application of CAR-T therapy to solid tumors is not that remarkable and is limited by various factors such as target antigen heterogeneity, trafficking and a hostile TME. To address the problem of antigen loss, bispecific CAR-T cells may be used to avoid antigen escape relapse. Moreover, trafficking of the CAR-T cells is another problem as locally delivered CAR-T cells may not be able to reach distant tumors and target them effectively (188). In addition, low or heterogeneous antigen expression is suboptimal for efficient CAR-T cell therapy as it can result in development of treatment resistance and tumor relapse. On the other hand, low antigen levels i.e. below the threshold for effective CAR-T on non-malignant tissues may be tolerable (156). Related to this, the antibody on which CAR-T is based, MGA271, shows minimal binding to normal tissues (156). In early phase clinical trials MGA271 was proven safe, without major toxicities and resulted in meaningful responses (192).

## 10 Conclusion

The extensive development of immunotherapeutic approaches and their success in the clinic have prompted scientists to further improve current therapies and search for immunotherapeutic targets that have clinical potential. One of these is the transmembrane protein B7-H3 that is highly expressed in various types of cancer. Because of its multifaceted role in several key cancer processes like promotion of cell migration, invasion and proliferation, B7-H3 is an attractive target for cancer treatment. In regard to immunotherapy, several approaches have been developed to date, including CAR-T, ADC, ADCC and nanobodies, particularly their use in CAR-T. The novel immunotherapeutic approaches are highly sought after for problematic cancers for which there are no effective therapies. One of these is glioblastoma, a particularly deadly cancer with a low survival rate that has remained unchanged for years. Since higher B7-H3 expression levels are correlated to the malignancy grade and poor survival of glioblastoma patients it is worth exploring it as a biomarker for disease progression. The existence of both splicing variants in glioblastoma opens up new avenues for research and clinical translation. Namely, the specific expression of 4IgB7-H3 in glioblastoma cells and higher expression of 2IgB7-H3 in recurrent glioblastoma and its correlation to temozolomide resistance indicate that B7-H3 can be explored as a therapeutic target, but also as a biomarker of tumor recurrence. However, for better designing of targeted diagnostic and therapeutic approaches its receptor should be determined.

## Author contributions

DB: Writing – original draft, Writing – review & editing. IJ: Writing – original draft, Writing – review & editing. AZ: Writing – original draft, Writing – review & editing.

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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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# Expanding the horizon of CAR T cell therapy: from cancer treatment to autoimmune diseases and beyond

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Chimeric antigen receptor (CAR)-T-cell therapy has garnered significant attention for its transformative impact on the treatment of hematologic malignancies such as leukemia and lymphoma. Despite its remarkable success, challenges such as resistance, limited efficacy in solid tumors, and adverse side effects remain prominent. This review consolidates recent advancements in CAR-T-cell therapy and explores innovative engineering techniques and strategies to overcome the immunosuppressive tumor microenvironment (TME). We also discuss emerging applications beyond cancer, including autoimmune diseases and chronic infections. Future perspectives highlight the development of more potent CAR-T cells with increased specificity and persistence and reduced toxicity, providing a roadmap for next-generation immunotherapies.

KEYWORDS

**CAR-T cells, adoptive immunotherapy, tumor microenvironment, solid tumors, autoimmune diseases**

## 1 Introduction

Adoptive cell therapy (ACT) is a novel strategy for cancer treatment. Human T cells are modified *in vitro* with genetic engineering technology and then reinfused into patients for therapeutic purposes. In particular, T cells engineered to express chimeric antigen receptor (CAR) have been shown to have significant clinical success, and the prognoses of many patients with hematological diseases have improved (1–3). Anti-CD19 CAR-T cells have shown unprecedented clinical effects in treating various diseases, including acute

lymphoblastic leukemia (ALL) in children and adults, non-Hodgkin lymphoma (NHL), diffuse large B-cell lymphoma (DLBCL), and chronic lymphocytic leukemia (CLL) (4–8).

At present, CAR-T cells are cultivated from T cells derived from peripheral blood *in vitro* and then genetically modified in the laboratory to introduce specific genes encoding CAR proteins so that these specific antigen receptors can be expressed on the T-cell surface. These engineered T cells are subsequently amplified in the laboratory and infused into the patient. Once in the body, they can identify and eliminate tumor cells bearing target antigens (Figure 1).

CAR is the key component of CAR-T cells responsible for binding to the corresponding antigens on the tumor cell surface for targeted therapy. Different tumor-associated antigens (TAAs) can be used as target antigens because of their specific high expression in tumor tissue. CAR has four major structures: an extracellular antigen recognition domain (usually a single chain variable fragment (scFv)), a hinge domain, a transmembrane domain, and an intracellular signal transduction domain (usually CD3ζ) (9, 10) (Figure 2). CAR is a synthetic receptor that enables T cells to recognize TAAs without the major histocompatibility complex (MHC). To improve the curative effects, five generations of CARs have been developed (Figure 2) (11). The first generation of CARs consisted of an extracellular antigen recognition domain fused with a transmembrane domain and the CD3ζ intracellular signaling domain (12). However, the results of clinical trials did not meet expectations because of the slow amplification and poor durability of the T cells. The second and third generations included one or two costimulating domains (usually CD28/4-1BB) to increase T-cell proliferation, cytotoxicity, and survival rates (13). Inducible second-generation transgenic proteins, such as the cytokine interleukin-12 (IL-12), were added to the fourth generation of CAR-T cells, also called T cells redirected for universal cytokine-mediated killing (TRUCKs), to improve antitumor activity (14). The design of the fifth generation of CAR is ongoing. Compared with the previous generations, fifth-generation CARs have been proven to have the ability to reactivate the immune system and maintain durability (15). They include the backbone chain of second-generation CARs and an added IL-2 receptor domain between the CD3 and CD28 signaling regions. Fifth-generation CARs are designed to simultaneously activate TCR, the costimulatory domain CD28, and cytokines.

CAR-T-cell therapy has achieved great success in treating hematological malignant tumors. To date, six CAR-T-cell therapies have been approved by the US Food and Drug Administration (16), and five have been approved by the Chinese National Medical Products Administration (NMPA) (Table 1).

CD19-targeted CAR-T cells are the first type of cell therapy with genetic engineering components approved in the US (17). CAR-T-cell therapy also has unique advantages in treating noncancerous diseases because of its excellent clinical effects (18–20). Experimental results suggested that the next major developments could be in areas beyond cancer, including autoimmune diseases, infectious diseases, and senescence-associated diseases, in which CAR-T cells will become widely applied. However, many problems with CAR-T-cell therapy exist in clinical practice (3, 8, 21). In many clinical trials, T lymphocytes are activated and amplified rapidly after CAR-T-cell infusion, causing excessive cytokine cascade release and finally leading to cytokine release syndrome (CRS) (22). Moreover, resistance triggered by loss of target is a key issue in CAR-T-cell therapy (23, 24).

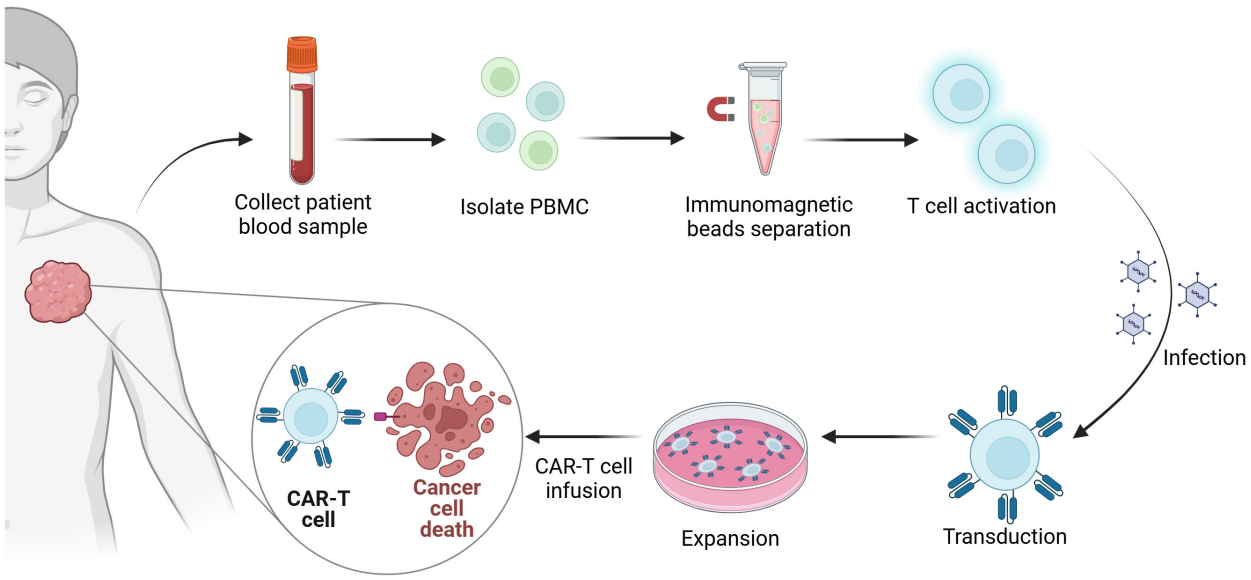


FIGURE 1
Manufacturing process of CAR-T-cell therapy. The first step in the generation of CAR-T cells is the collection of blood samples from the patient. Subsequently, peripheral blood mononuclear cells (PBMCs) are isolated from the collected blood samples. Immunomagnetic beads are utilized to isolate T cells, which are activated simultaneously. Next, the gene encoding the chimeric antigen receptor (CAR) is introduced into the T cells via viral infection. Finally, the CAR-T cells are expanded *in vitro* and then reinfused into the patient's body to eradicate tumors.

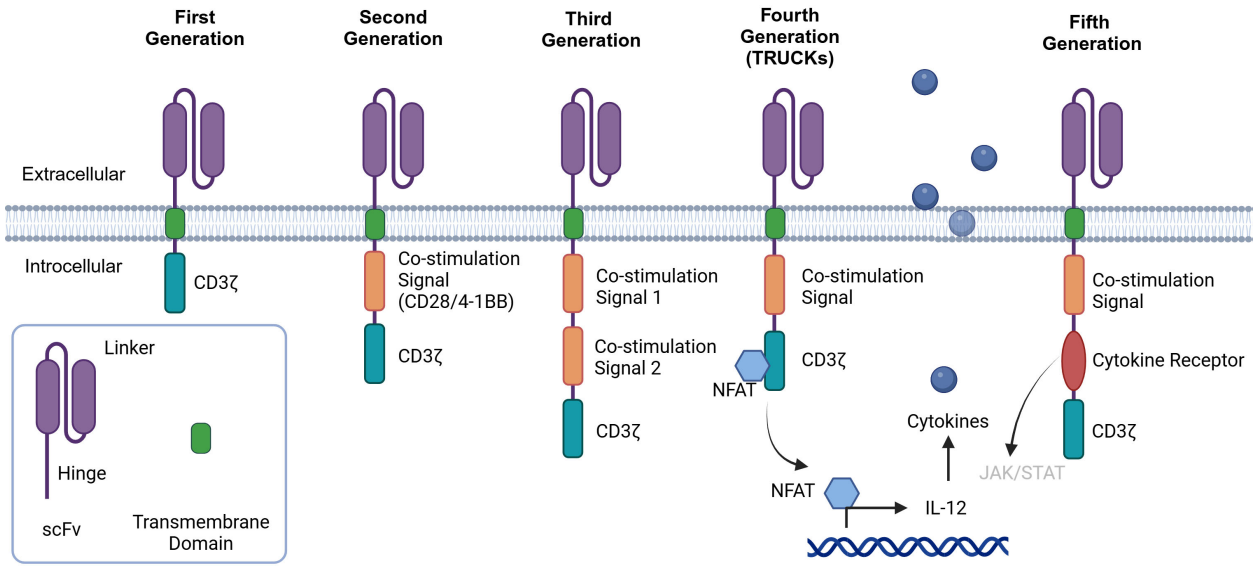


FIGURE 2
History of CAR-T-cell development. First-generation CAR-T cells are built upon the CD3-ζ chain. While they possess the ability to activate T cells, their antitumor efficacy is relatively limited. Second-generation CARs include a costimulatory molecule, such as CD28 or 4-1BB, which enhances the activation and functionality of CAR-T cells. Third-generation CARs further enhance the intracellular signaling domain by including a second costimulatory molecule. When a single-chain variable fragment (scFv) binds to tumor-associated antigens (TAAs), it activates the first signal through CD3ε and the second signal via two costimulatory signals, thereby strengthening the T-cell response. Fourth-generation CAR-T cells, also referred to as "cytokine-mediated killers at the cosmic level," are engineered to release modified genes into tumor tissue upon the binding of the CAR to targeted antigens. This unique design aims to increase antitumor activity in a more targeted and potent manner. Fifth-generation CAR-T cells are designed by adding a cytoplasmic IL-2R β-chain domain and a STAT3/5 binding site to the second-generation design. This modification is expected to further optimize the activation, proliferation, and antitumor capabilities of CAR-T cells.

There is still no CAR-T-cell therapy approved for solid tumor treatment in clinical practice because of the challenging characteristics of these tumors, such as loss of target antigen, an inhibitory tumor microenvironment (TME), and failed CAR-T-cell therapy caused by poor infiltration, a lack of tumor-killing ability and low durability (16, 25–28). In addition, the time-consuming and expensive nature of the treatment also presents challenges. A variety of strategies and methods have been used to overcome these hurdles, including arming CAR-T cells by knocking out PD-1 expression or secreting cytokines/chemokines and combining CAR-T-cell therapy with other therapeutic methods (29, 30). To date, over 700 clinical trials for CAR-T-cell therapy are underway (31), many of which focus on solid tumors.

Previous reviews have comprehensively discussed the problems faced by CAR-T-cell therapy, such as those in solid tumor treatment, resistance, and toxicity (2, 8, 24, 32). On this basis, here, we first summarize the mechanism of CAR-T-cell therapy in cancer treatment. Next, we analyze the challenges that CAR-T-cell therapy is facing, progress, and existing complications in the treatment of solid tumors and noncancerous diseases. Finally, we look ahead to the future development of CAR-T-cell therapy, proposing some viable and promising solutions to address various challenges. We also provide conclusions and recommendations, hoping for valuable insights for CAR-T-cell therapy.

## 2 Killing mechanisms of CAR-T-cell therapy

Normal T cells bind to target cells before killing them, forming immune synapses in the binding region. Similarly, CAR-T cells target tumor cells and form similar immune synapses, but their structure is slightly different from that of T cells, making the signaling pathway and triggering time slightly different (9). After CAR-T cells bind to tumor cells through immune synapses, they kill tumor cells through three mechanisms.

Initially, CAR-T cells secrete perforin and granzymes. Perforin can "dig holes" on the tumor cell surface, after which granzymes are transported into the tumor cells, damaging them directly or inducing cell apoptosis, which is considered the major killing mechanism of CAR-T cells (33). To ensure the precise killing of target cells, granzymes are anchored to the microtubes. After the formation of immune synapses, granzymes migrate to the interface, fuse into the plasmalemma of the center supramolecular activation cluster (cSMAC) (34), and are then released into the synaptic cleft by vesicles. In the synaptic cleft, perforin induces the formation of pores on the target cell membrane to promote the entry of proapoptotic granzymes. Upon entering the cytoplasm of target cells, granzymes can induce cell apoptosis by cleaving key substrates. The effects of perforin and granzymes are dependent on $Ca^{+}$ (35).

TABLE 1 CAR-T-cell therapies marketed in China and the United States.

| Listing country | Product name | Corporation | Approval time | Indication | CR | Target |
|---|---|---|---|---|---|---|
| USA | Kymriah (Tisagenlecleucel) | Novartis Pharma AG | 2017.8.30 | Precellular acute lymphocytic leukemia: recurrent or refractory diffuse large B-cell lymphoma | Complete response (CR):>90% | CD19 |
| USA | Yescarta (Axicabtageneciloleucel) | Kite Pharma | 2017.10.18 | Recurrent or refractory diffuse large B-cell lymphoma: Recurrent or refractory follicular cell lymphoma | non-Hodgkin lymphoma Complete response (CR):51% | CD19 |
| USA | Tecartus (Brexucabtagene autoleucel) | Kite Pharma | 2020.7.24 | Recurrent or refractory mantle cell lymphoma | mantle cell lymphoma Complete response:67% | CD19 |
| USA | Breyanzi (Lisocabtagene maraleucel) | Juno Therapeutics | 2021.2.5 | Recurrent or refractory diffuse large B-cell lymphoma | Complete response (CR):54% | CD19 |
| USA | Abecma (Idecabtagene vicleucel) | Bristol-Myers Squibb and bluebird bio, Inc | 2021.3.26 | Recurrent or refractory multiple myeloma | Complete response (CR):28% | BCMA |
| USA | Carvykti (ciltacabtagene autoleucel) | Legend Biotech Corporation | 2022.2.28 | Recurrent or refractory multiple myeloma | Complete response (CR):78% | BCMA |
| China | Axicabtagene Ciloleucel | Fosun Kite Biotechnology Co., Ltd | 2021.6.22 | Recurrent or refractory diffuse large B-cell lymphoma | | CD19 |
| China | Relmacabtagene autoleucel | JW Therapeutics (Shanghai) Co., Ltd. | 2021.9.7 | Recurrent or refractory diffuse large B-cell lymphoma | | CD19 |
| China | Equecabtagene Autoleucel | Nanjing IASO Biotherapeutics Co., Ltd. | 2023.6.30 | Recurrent or refractory multiple myeloma | | BCMA |
| China | Inaticabtagene Autoleucel | Juventas Cell Therapy Ltd. | 2023.11.7 | Recurrent type B acute lymphoblastic leukemia | | CD19 |
| China | Zevorcabtagene Autoleucel | CARsgen Therapeutics Co. Ltd. | 2024.2.23 | Recurrent or refractory multiple myeloma | | BCMA |

CAR-T cells can also induce apoptosis through the Fas/FasL (CD95L) pathway (23). FasL is a cytokine that can bind to the death receptor TNFRSF6/FAS. It induces apoptosis triggered by cytotoxicity during T-cell development. The Fas/FasL pathway participates in immune cell homeostasis in nonpathogenic cases. The Fas/FasL pathway is calcium independent. Tschumi et al. reported that CAR-T cells can utilize this pathway to mediate tumor killing (36, 37). After CTL cells recognize target cells, the FasL expressed at high levels on the cell surface recognizes Fas on the target cell surface, triggering the apoptosis program inside the target cell through Fas and leading to programmed cell death of the target.

FasL is a homotrimer. Once it binds to the trimeric ligand, Fas recruits the Fas-associated protein with a novel death domain (FADD) in the cytoplasm through the death domain of its intracellular segment. The amino terminus of FADD contains a death effector domain (DED), which interacts with the DED domain of Caspase-8 to recruit Caspase-8 to the Fas region, forming a death-induced signaling complex (DISC) (38). The Caspase-8 proenzyme is activated and self-cleaves to form active Caspase-8. Activated Caspase-8 can activate downstream Caspase-3 to form mature Caspase, which then mediates cell death by cleaving over 500 cell substrates, effectively executing the apoptotic program (39). CD30 is a membrane-protein receptor on activated lymphocytes and is a member of the tumor necrosis factor receptor superfamily (40). CD30 CAR-T cells not only target $CD30^+$ embryonal carcinoma (EC) cells through CAR-T cells but also eliminate surrounding CD30-ECs through Fas/FasL interactions. The Fas/FasL interaction between tumor cells and CAR-T cells can be used to reduce tumor escape caused by heterogeneous antigen expression or to increase the antitumor activity of CAR-T cells.

In addition to these two mechanisms, CAR-T cells can also secrete specific cytokines that promote CAR-T-cell competence, alter the TME, and further enhance antitumor activity. In a prostate cancer model, blocking TGF-β in T cells increased the ability of the cells to infiltrate, proliferate, and mediate antitumor responses. The efficacy of CAR-T cells can be increased through dominant negative TGF-β RII (dnTGF-β RII). CAR-T cells can specifically eliminate advanced tumors expressing prostate-specific membrane antigen

(PSMA) (41). Boulch et al. reported that the main mechanism by which anti-CD19 $CD4^+$ CAR-T cells eliminate tumors is the production of interferon (IFN)-γ rather than perforin, which mediates cytotoxicity. CAR4 T ($CD4^+$ CAR-T) cells form high concentrations of IFN-γ in the tumor microenvironment to eliminate IFN-γ-sensitive tumor cells at the distal end. The intrinsic sensitivity of tumor cells to the proapoptotic effects of IFN-γ is the main determinant of CAR4-T-cell therapy (42).

Abnormalities in certain signaling pathways make solid tumors more resistant to CAR-T cells than hematologic cancers are. Larson et al. reported that glioblastoma and other solid tumors are more resistant to CAR-T cells owing to the loss of genes in the IFN-γ receptor (IFNγR) signaling pathway (IFNGR1, JAK1, or JAK2). However, the absence of this pathway does not make lymphoma cell lines insensitive to CAR-T cells (43). Tregs in the TME downregulate the type I interferon (IFN1) receptor IFNAR1 on $CD8^+$ CTL through ADP–ribose polymerase family member 11 (PARP11). PARP11 is induced and overactivated in response to adenosine, thereby promoting the accelerated ubiquitination and degradation of IFNAR1. Therefore, partial INFAR1 deficiency disrupts CTL cytotoxicity and reduces their survival ability (44).

# 3 CAR-T cells beyond cancer

The success of CAR-T-cell therapy in the treatment of hematological cancers has sparked attempts to expand CAR-T-cell therapy to other therapeutic fields. To achieve significant therapeutic effects in cancer, almost all tumor cells must be eliminated. Moreover, in most other diseases, only a portion of pathological cells need to be cleared to achieve the therapeutic goal. Similarly, the TME is an important obstacle to CAR-T-cell therapy for cancer, but in most other diseases, target cells do not exist in environments similar to the TME and are more accessible (18, 45). CAR-T cells have been applied to autoimmune diseases (46–48), infectious diseases (49–51), senescence-associated diseases (52), and other fields (Figure 3, Table 2).

## 3.1 Autoimmune diseases

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the body recognizes its own antigens as foreign antigens, leading to the activation of its own effector B and T cells. This type of attack on normal cells may lead to fatigue, inflammation, and, in severe cases, even death. In clinical trials, some therapeutic effects have been achieved by targeting B cells, but their efficacy is very limited because severe SLE is not easy to treat and still requires the use of drugs for maintenance after treatment (45). CAR-T cells targeting CD19 have shown the ability to eliminate pathological B cells in cancer; thus, researchers propose that CAR-T-cell therapy may have applications in the treatment of SLE. Mackensen et al. reported the use of CAR-T-cell therapy to treat 5 patients diagnosed with SLE (53). CD19 CAR-T cells were used to target B cells in SLE

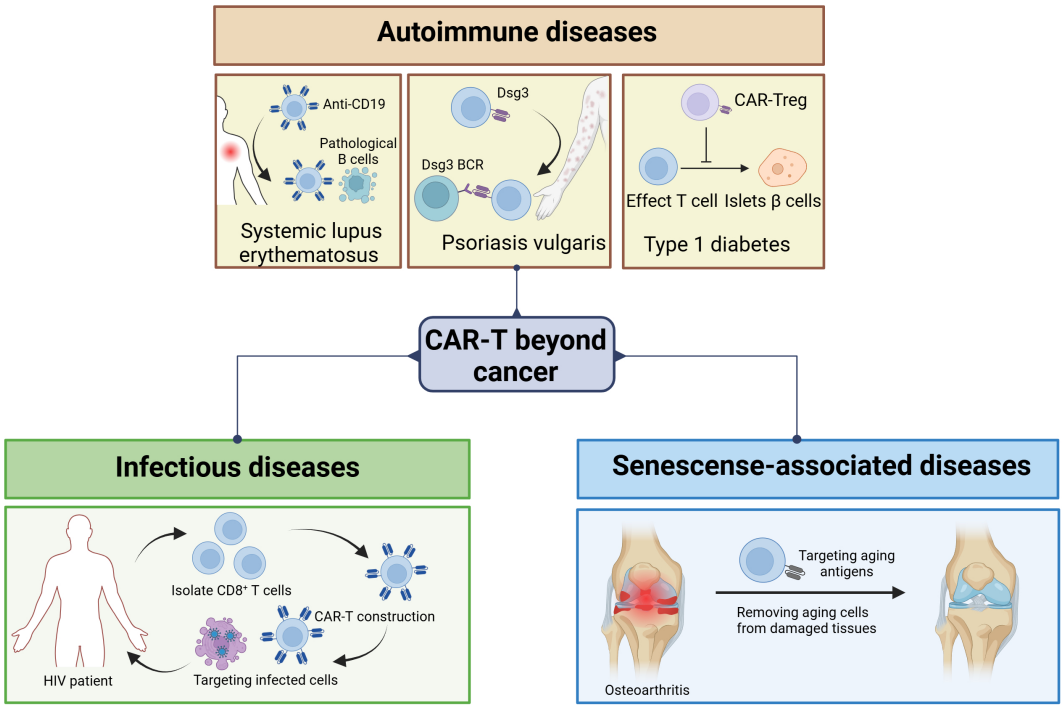


FIGURE 3
CAR-T cells for the treatment of noncancerous diseases. CAR-T cells are emerging in the field of noncancer disease treatment. CAR-T-cell therapy is not limited to cancer treatment. It has demonstrated significant potential in multiple areas, including autoimmune diseases, chronic infectious diseases, and diseases associated with aging. In autoimmune diseases, targeting CD19 can be used to treat SLE, and targeting Dsg3 can be used to treat PV, while CAR-Tregs can be used to treat type 1 diabetes. In infectious diseases such as AIDS, CAR-T-cell therapy is a potential way to clear host cells. With respect to senescence-associated diseases, CAR-T cells promote longevity by removing aging cells.

TABLE 2 CAR-T therapy for other diseases.

| Type | Disease | CAR cell | Target | Report time |
|---|---|---|---|---|
| Autoimmune disease | Systemic lupus erythematosus | CAR T | CD19 | 2022.10 |
| Autoimmune disease | Psoriasis vulgaris | CAAR T | Dsg-3 | 2016.6 |
| Autoimmune disease | Type 1 diabetes | CAR Treg | InsB-I-Ag7 | 2019.9 |
| Infectious diseases | AIDS | CAR T | HIV Host cell | 2024.5 |
| Senescence-associated disease | Senescence | CAR T | uPAR | 2020.6 |

patients, and in all five patients, CAR-T-cell infusion improved their condition after the discontinuation of immunosuppressive drugs.

Psoriasis vulgaris (PV) is a life-threatening autoimmune disease caused by autoantibodies against Desmoglein3 (Dsg3). In PV, pathogenic memory B cells express anti-Dsg3 B-cell receptors (BCRs). Thus, targeting the elimination of anti-Dsg3 memory B cells should be able to cure PV without the risk of universal immune suppression. Ellebrecht et al. created a chimeric autoantibody receptor (CAAR) (46), using their own Dsg3 as the extracellular domain of CAAR to enable T cells to kill autoimmune B cells in the PV. CAAR T cells are a targeted therapy for antibody-mediated autoimmune diseases, which may generate long-term memory CAAR-T cells and potentially cure the disease.

Type 1 diabetes is an autoimmune disease characterized by the destruction of islet β cells by autoeffector T cells, leading to reduced insulin secretion and dysregulation of blood sugar. Inhibiting this autoimmune response is the main goal of the prevention and treatment of type 1 diabetes. Therefore, targeting regulatory T cells (Tregs) for therapy is a major area of focus (48). Spanier et al. developed a "TCR-like" CAR derived from an antibody that recognizes the insulin B peptide (InsB) present in I-Ag7 (54, 55). After *in vivo* administration, InsB-I-Ag7 CAR Tregs markedly reduced the development of spontaneous diabetes in NOD mice. Designing more effective CARs that target appropriate antigens can enhance the functional and targeting properties of Tregs.

## 3.2 Infectious diseases

CAR-T-cell therapy can also be used to treat infectious diseases. AIDS is an epidemic caused by human immunodeficiency virus (HIV) infection and has caused millions of deaths in recent decades. Although combined antiretroviral therapy (cART) has made considerable progress in inhibiting HIV replication, it has not been able to eliminate cells that are latently infected with HIV, and infected individuals remain HIV-positive for life (51). Lifetime antiretroviral therapy is needed to maintain control over virus replication. Therefore, there is an urgent need for new treatment strategies to eliminate the virus in the host to treat AIDS.

Researchers generate and amplify CAR-T cells that target HIV-infected cells from the patient's blood and then reinject CAR-T cells into the patient's body for therapeutic purposes. $CD8^+$ T cells were collected from HIV patients, and CAR gene transduction was performed. After verifying the specificity and effectiveness of anti-HIV therapy *in vitro*, HIV-specific CAR-T cells were reinjected into the patient's body to kill cells infected with HIV. The cytotoxic T cell (CTL) response is a key component of host immunity against HIV infection (56). In addition to restraining HIV replication during acute infection, enhancing the HIV-specific CTL response before the virus is activated can lead to rapid and effective killing of infected cells (57). Owing to this strong selective pressure, HIV quickly acquires mutations to evade CTL recognition. Unless cART is begun in the early stages of HIV infection, the vast majority of latent viruses carry CTL escape mutations (58). Equipping $CD8^+$ T cells with CARs that can recognize various HIV antigens is crucial for the treatment of HIV. After binding to HIV envelope proteins, these CARs can trigger T-cell activation, proliferation, and cytokine production *in vitro*. CD4 receptors are used to construct anti-HIV CAR-T cells (59). CD4 interacts with gp120 during HIV infection and has a natural high affinity for HIV. CAR-T cells, which are based on CD4 receptors, have been shown to have the same level of cytotoxicity as natural CTLs. However, despite the marked advantages of CD4 receptors, CAR-T cells based on CD4 receptors are susceptible to HIV infection, and further research is needed to identify safer and more effective design targets.

## 3.3 Senescence-associated disease

The abnormal accumulation of aging cells produces an inflammatory environment, leading to chronic tissue damage and resulting in various senescence-associated diseases, such as atherosclerosis and osteoarthritis. Therefore, removing aging cells from damaged tissues can alleviate the symptoms of these diseases and even promote longevity (60). CAR-T cells targeting aging cells have the ability to act as senolytics. The characteristic molecules expressed on the aging cell membrane serve as unique antigen markers that can be selected for CTL-mediated senolytic activity and clearance. Amor et al. conducted a study on CAR-T-cell therapy targeting urokinase-type plasminogen activator receptor (uPAR) in mouse models (60). uPAR is a cell membrane protein that is widely expressed during the aging process and is associated with extracellular matrix remodeling. During replication, oncogene induction, and toxicity-induced aging, uPAR is upregulated on the surface of aging cells (61). uPAR-specific CAR-T cells can effectively eliminate aging cells *in vitro* and *in vivo*. CAR-T-cell therapy for senescence-associated diseases shows great promise. However, many key issues still need to be addressed. Aging cells exhibit hysteresis, as their phenotype depends on various stress factors to which they are subjected before symptoms appear. Moreover, the

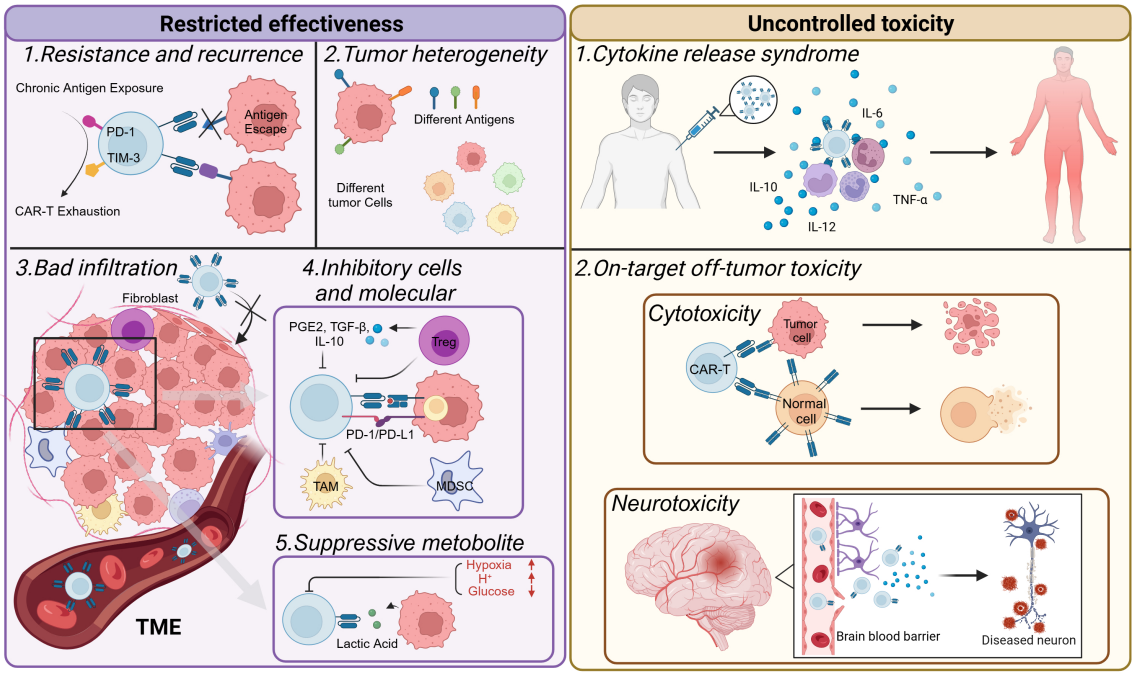


FIGURE 4
Challenges faced by CAR-T-cell therapy. CAR-T-cell therapy is constrained primarily by issues related to effectiveness and toxicity. CAR-T cells may become exhausted, resulting in short-lived resistance. This can lead to antigen escape in tumors, causing tumor recurrence. The heterogeneity of tumors also limits the efficacy of CAR-T-cell therapy. In the tumor microenvironment, various environmental changes occur. For example, it is often challenging for T cells to infiltrate solid tumors. In addition, immunosuppressive cells are recruited, inhibitory molecules are expressed, and metabolic reprogramming takes place. These factors collectively make it difficult to achieve a favorable prognosis when treating solid tumors. Moreover, CAR-T-cell therapy can sometimes produce excessive toxicity to the human body, inducing CRS or other on-target off-tumor toxicities and damaging normal cells in the body.

aging phenotype is strongly heterogeneous owing to factors such as the tissue from which it originates and the functions it performs, and every step from cell experiments to mouse experiments and even to clinical applications faces more complex transformations (52). Therefore, it is necessary to continuously optimize and test CAR-T cells to clarify the mechanisms.

# 4 Challenges facing CAR-T-cell therapy

The use of CAR-T-cell therapy for hematologic counters faces many challenges. These include side effects induced by CAR-T cells, as well as problems related to resistance and recurrence. Although the successful application of CAR-T-cell therapy in treating hematologic cancers has spurred research into its potential use in solid tumors (2, 6, 11, 25, 62), it faces even more daunting challenges (Figure 4).

The tumor microenvironment (TME), characterized by its distinctive immunosuppressive features, is not only an accomplice in the development of resistance but also the primary factor contributing to the challenges faced by CAR-T-cell therapy in treating solid tumors. The TME is a complex entity that encompasses immune cells, stromal cells, blood vessels, cytokines, and the extracellular matrix. These various components interact with one another, continuously fueling tumor progression (63). This complex interaction sets the stage for the multiple roles that the TME plays in different stages of tumor development. In the early stages of tumor development, the TME promotes cancer cell growth, supports tumor colonization and invasion, and facilitates angiogenesis to overcome hypoxia and the acidic environment (64). The immunosuppressive TME can prevent CAR-T cells from effectively entering solid tumors. Moreover, it suppresses their immune effects, causing insufficient activation and exhaustion of CAR-T cells.

## 4.1 Resistance and recurrence

Although the efficacy of CAR-T-cell therapy is remarkable, resistance remains a crucial issue. Cancer recurrence after CAR-T-cell therapy is a major clinical challenge. In BLL patients treated with anti-CD19 or anti-CD22 CAR-T cells, the recurrence rate can reach 50% 12 months after infusion (24, 65). CD19 gene deletion and mutation may lead to the loss of the CD19 antigen on tumor cells, resulting in CD19-negative recurrence (66). CAR-T-cell resistance can be divided into primary resistance and secondary resistance. Primary resistance is due to a lack of response to CAR-T cells, whereas secondary resistance is characterized by cancer relapse after the initial brief response. The causes of resistance can be attributed to the dysregulation of CAR-T cells, the escape of tumor cells, and the inhibition of the TME (32).

From the perspective of immune cells, CAR-T-cell dysfunction is an important cause of resistance. Dysfunction of T cells may be caused by various factors, including long-term antigen stimulation, immune deficiency, or other undetermined factors (67, 68). Overactivation and high target affinity can exhaust T cells, reducing their cytotoxicity and persistence, potentially causing cell death. Exhausted T cells have low proliferation and cytotoxicity and high inhibitory receptor expression (PD-1$^+$ TIM-3$^+$). Since exhaustion is a tumor escape mechanism (69), reducing exhaustion and aging can promote CAR-T-cell function. Compared with endogenous CTLs, CAR-T cells have genetically modified antigen receptors that target tumor cells. However, their artificial design affects their effectiveness. CARs with CD28 costimulatory domains have poorer persistence than those with 4-1BB (24, 70). The manufacture of CAR-T cells requires a large number of healthy T cells from patients. Low-quality T cells can reduce the treatment efficiency of CAR-T-cell therapy. Chemotherapy can reduce patient lymphocytes and degrade product quality. Additionally, owing to age-related immune decline, young donor-derived CAR-T cells perform better than do those from elderly donors. Patients with advanced cancer may also be unable to provide high-quality T cells to manufacture CAR-T cells (71).

Tumor cells have multiple ways to escape immunity and develop resistance, and one of the most common mechanisms is the loss or downregulation of target antigens on tumor cells. Antigen escape, mutation, downregulation, or loss can lead to tumor cells evading recognition and clearance by the immune system, reducing the therapeutic effect of CAR-T-cell therapy and causing tumor recurrence (24, 72). CD19 CAR-T-cell primary resistance occurs in 10–20% of ALL patients; in a Philadelphia Children's Hospital trial, 20 of 55 ALL patients experienced recurrence, 13 (24%) of whom were CD19 negative (73). Antigen loss or escape may become a major hurdle to cancer treatment, as there is greater heterogeneity in target antigen expression (66). Tumor cells can have genetic mutations that change their antigen structure, so they are sometimes unrecognizable by CAR-T cells. They can also evade by downregulating or losing antigens. There is also non-antigen-deficient resistance. In 2020, Singh et al. proposed that death receptor signaling dysfunction in cancer cells directly reduces CAR-T-cell killing (74). In ALL cells that resist CAR-T-cell attack, genes associated with activating cell death pathways (FADD, BID, CASP8, and TNFRSF10B) are missing, whereas genes required to resist cell death pathways (CFLAR, TRAF2, and BIRC2) are enriched. In the presence of antigens, prolonged survival of cancer cells can cause T-cell dysfunction. This effect is amplified when CAR-T-cell dysfunction is induced. This mechanism seems to rely on two stages: initial resistance to death receptor-driven killing, followed by antigen-driven CAR-T-cell dysfunction. The dysregulation of death receptor signaling in ALL leads to CAR-T-cell failure, further exacerbating the disease and promoting the development of resistance.

The tumor microenvironment plays a significant role in driving the development of resistance to CAR-T-cell therapy. Within the tumor microenvironment, inhibitory immune cells such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs) contribute to CAR-T-cell exhaustion (32). For example, Tregs usually express the inhibitory molecule PD-L1. PD=L1 can then directly inhibit the function of CAR-T cells, thereby reducing their ability to effectively target and eliminate tumor cells. Moreover, immunosuppressive cells and tumor cells secrete inhibitory cytokines, such as IL-10, IL-4, and TGF-β. These cytokines can reduce the effector function of CAR-T cells. This reduction in function further contributes to the development of resistance to CAR-T-cell therapy (75–77). It is crucial to further understand the mechanism of CAR-T-cell resistance and identify which patients are most likely to experience recurrence to optimize CAR-T-cell therapy.

## 4.2 Tumor heterogeneity

Tumor cells from different sources exhibit distinct morphological and phenotypic features, which is also one of the reasons solid tumors are difficult to treat. In cancer treatment, identifying ideal target antigens is crucial for the effectiveness of therapies. The first major difference between solid tumors and hematologic tumors is that it is more difficult to find ideal target antigens in solid tumors (16). The susceptibility of CAR-T cells to tumor-associated antigens (TAAs) determines the difficulty with which CAR-T cells can accurately recognize solid tumors. Moreover, owing to the loss of antigens and the absence of MHC presentation, solid tumors are difficult to target, resulting in extremely high heterogeneity of tumor cells. In addition, solid tumors exhibit TAA heterogeneity between tumor types (primary and metastatic) and patients with the same cancer (78). This high heterogeneity of solid tumors impacts immunotherapy efficacy, as the immune targets are limited to specific cells, hindering widespread killing.

Despite these challenges, researchers have explored potential target antigens to improve the treatment of solid tumors. EGFRvIII, a tumor-specific antigen (TSA) for CAR-T cells, is expressed only on human tumor cells and not on normal cells (79). CAR-T cells targeting EGFRvIII can accurately target tumor cells, which can improve therapeutic efficacy and reduce toxicity. However, the situation of target antigens in solid tumors is more complex. The shortage of TSA severely limits the use of CAR-T-cell therapy to treat solid tumors, as solid tumors rarely express TSA, which is different from hematological cancer, which commonly expresses TSA-CD19 (80). Therefore, targeting TAAs is an alternative method to overcome the shortage of TSAs. For most solid tumors, the most common antigens are TAAs, which are expressed at high levels on the tumor surface but also at low levels in normal tissues (81). TAAs are used in most ongoing clinical trials of CAR-T-cell therapy for solid tumors. Notably, CAR-T cells targeting TAAs are likely to cause damage to normal tissues, and potential risks should be fully evaluated before their use. With the continuous development of new technologies, high-throughput sequencing and other methods have enabled us to obtain more information about patients, including their specific mutation sites. This makes it possible to screen for new antigens and apply CAR-T-cell therapy to target new antigens in the future, potentially revolutionizing the treatment landscape of solid tumors.

## 4.3 Difficulties in CAR-T-cell infiltration

Even if solid tumor surface target antigens are present, CAR-T-cell trafficking into tumors is needed. In hematologic tumors, circulating CAR-T cells can directly damage tumor cells. However, in solid tumors, multiple obstacles must be overcome to enable infiltration (82). It is difficult for CAR-T cells to penetrate tumor tissue through the blood system of solid tumors. Solid tumors have unique histopathological features, such as concentrated blood vessels (83), tumor-associated fibroblasts, and myeloid cells that form the extracellular matrix (ECM). These features, in turn, contribute to the difficulty of T-cell infiltration into solid tumors. Although these features are beneficial for the growth of solid tumors, they increase the difficulty of T-cell infiltration at the tumor site, preventing continuous contact between T cells and tumor cells, which is necessary for T cells to exert their cytotoxic and antitumor effects. Ideally, the entry of T cells into the TME is a gradual process: tumor cells die and release antigens. Antigen-presenting cells (APCs) process and present tumor antigens. Interactions between APCs and T cells lead to T-cell activation, after which T cells reach the tumor site through the blood system, killing tumor cells by recognizing tumor antigens and releasing more antigens, resulting in a cascade amplification effect (84).

The most direct cause of T-cell infiltration disorders is insufficient T-cell recognition, which is often due to a lack of tumor antigens (85). After identifying tumor antigens, the antigens are processed, and the corresponding antigen peptide MHC-I class complexes on their surfaces are expressed. However, the absence of tumor antigens results in CAR-T cells being unable to effectively recognize tumor cells. In addition, changes in APC surface molecules, such as downregulation of MHC-I, limit the presentation of antigen peptide MHC-I complexes (84). Lysosomes are also associated with reduced infiltration of $CD8^+$ T lymphocytes. In pancreatic ductal adenocarcinoma (PDAC), the autophagy-related receptor NBR1 induces the degradation of MHC-I on the surface of tumor cells, which further affects the T-cell response (86). These findings indicate that defects in the tumor antigen processing and presentation pathways inhibit T-cell initiation and hinder the effectiveness of cancer immunotherapy.

Tumor blood vessels are crucial for T-cell infiltration, but in many solid tumors, these vessels are twisted and diseased, especially in the tumor core (87). Damage to tight junctions and increased permeability lead to hypoxia, acidosis, and necrosis, inhibiting T-cell function and antitumor immunity. In the TME, cells secrete tumor angiogenic factors. Vascular endothelial growth factor (VEGF) regulates tumor angiogenesis by binding to VEGFR1-3. Anti-angiogenic therapy targeting VEGFR can normalize the tumor vasculature and be combined with CAR-T-cell therapy to increase infiltration (88). As a hallmark of cancer, hypoxia is caused by increased oxygen demand triggered by tumor cell proliferation and insufficient blood supply by angiogenesis (89–91). Hypoxia inducible Factor 1 (HIF1) is a hypoxia-activated transcription factor. Hypoxia inhibits T-cell infiltration by recruiting immunosuppressive cells. CCL28 and VEGF, which are induced by CCL28 and VEGF, affect angiogenesis and T-cell trafficking. Moreover, under hypoxic conditions, in combination with TGF-β, the expression levels of CD39 and CD73 in tumor tissue are upregulated. CD39 and CD73 convert ATP to adenosine, which binds to A2AR, inhibiting the production of cytokines such as IL-2 and T-cell development and proliferation (92). Hypoxia and TGF-β can ultimately upregulate the expression of CD39 and CD73 in tumor tissue. The sequential conversion of ATP to extracellular adenosine is catalyzed by CD39 and CD73. Adenosine binds to the adenosine A2A receptor (A2AR) and inhibits the production of cytokines such as IL-2, restraining the development and proliferation of T cells (93, 94).

In addition, the lack of chemokine expression involved in T-cell infiltration in tumor tissue and the presence of dense ECM in solid tumors lead to a reduced ability of CAR-T cells to migrate and invade tumor cells (95). The transport of CTL cells to the tumor site can be affected by the interaction between some chemokine receptors on CTL cells and their corresponding chemokines. The lack of some chemokines, including CXCL9, CXCL10, CCL4, and CCL5, has been reported to lead to the inhibition of T-cell infiltration (84). However, some chemokines, such as CXCL12, are unfavorable for T-cell infiltration into tumors. Stromal cells, especially cancer-associated fibroblasts (CAFs), are the main producers of CXCL12. CXCL12 produced by CAFs can mislead CTLs into the extracellular matrix of the tumor and prevent them from entering the tumor (96). Moreover, high expression of CXCL8 is associated with a decrease in the number of T cells in tumors, an increase in neutrophil and monocyte infiltration, and a limited response to immune checkpoint inhibitors (ICIs) ( (97). These results reveal the regulatory role of chemokine receptor and ligand interactions in CTL homing to the TME.

## 4.4 Inhibitory immune cells and molecules

Solid tumors typically contain numerous immunosuppressive cells, such as regulatory T cells (Tregs), myeloid suppressive cells (MDSCs), and tumor-associated macrophages (TAMs). Tregs are a special subgroup of $CD4^+$ T cells that are responsible for suppressing immunity to prevent certain types of physiological damage caused by Th cell overactivation (98). In malignant tumors, Tregs may inhibit immune cell-mediated antitumor responses. In several cancers, including breast cancer, melanoma, and lung cancer, the increase in tumor-invasive Tregs is related to the low survival rate of patients (99). MDSCs are derived from myeloid progenitor cells, including immature macrophages, immature granulocytes, and immature dendritic cells (DCs). MDSCs have been shown to aggregate in tumors as T-cell inhibitors, secrete various inhibitory cytokines, and upregulate the expression of certain immunosuppressive molecules (100). In addition to being phagocytic and antigen-presenting cells, macrophages secrete various factors with immune-supportive or immunosuppressive functions. In the TME, these cells are called TAMs, and tumor malignancy is positively correlated with TAM enrichment in different solid tumors (101). These immunosuppressive cells help

to protect solid tumor cells from being killed by the host immune system.

Tumor-derived factors are a class of soluble factors that inhibit the efficacy of CAR-T-cell therapy for solid tumors. They are usually secreted by immunosuppressive cells, such as Tregs, which can secrete large amounts of PGE2, TGF-β, IL-10, and other substances that inhibit T-cell proliferation. TGF-β plays an important role in restraining antitumor responses. TGF-β can downregulate $CD8^+$ effector T-cell function and promote Treg maturation (83). Therefore, the function of $CD8^+$ T cells can be improved by binding to TGF-β, which means that in CAR-T-cell therapy, the population, infiltration, and persistence of T cells can be increased to boost the antitumor response.

The inhibitory function of immune responses is induced by immune checkpoint ligands, which are usually overexpressed in solid tumors. These ligands can induce the expression of immunosuppressive molecules or their receptors, including PD-L1/PD-1, T lymphocyte immunoglobulin mucin 3 (TIM-3), indoleamine 2,3-dioxygenase 1 (IDO-1), lymphocyte activation gene 3 (LAG-3), and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (62). Their inhibitory effects on T lymphocyte activation lead to tumor immune escape. For example, PD-L1 binds to PD-1, suppressing CAR-T-cell activation and increasing tumor immune tolerance. TIM-3 contributes to T-cell exhaustion in the TME. Recent immunotherapy research has focused on blocking these checkpoints to alleviate immune suppression and restore immune function. Combining immune checkpoint inhibitors such as CTLA-4 and PD-1 with CAR-T cells targets immunosuppressive cells in the TME (82). PD-L2, which is expressed on the surface of DCs, macrophages, mast cells, and some B cells, is the second ligand, after PD-L1, that can bind to PD-1. PD-L2 is also significantly expressed in the TME of renal cell carcinoma (RCC) and lung squamous cell carcinoma (LUSC). The coexpression of PD-L1 and PD-L2 in tumor cells significantly inhibits antitumor immune responses (102). In the future, the application of anti-PD-L2 monoclonal antibodies may overcome the limitations of anti-PD-L1 monoclonal antibody drugs.

## 4.5 Metabolism in the TME

The highly active metabolic pathways unique to tumor cells can cause significant changes in the composition of nutrients and other small molecules within the tumor microenvironment, which have critical impacts on immune responses. The high metabolic activity of tumor cells and the chaotic vascular system within the TME may lead to nutrient exhaustion and hypoxia, and this situation establishes metabolic competition between tumor cells and infiltrating immune cells (103).

Glucose restriction in the TME substantially affects T-cell responses. Owing to their highly active metabolic function, the ability of tumor cells to take up glucose is greatly increased, resulting in a low-glucose environment in the TME. Research has shown that low-glucose conditions (0.1 mM) inhibit the production of phosphoenolpyruvate (PEP), an intermediate product of glycolysis in T cells, disrupting calcium-dependent NFAT signaling and inhibiting T-cell proliferation, activation, and cytokine production, leading to a decrease in T-cell survival ability (104).

Hypoxia is caused by an imbalance in the oxygen consumption of rapidly proliferating tumor cells, coupled with an insufficient oxygen supply caused by abnormal tumor angiogenesis (105). Owing to their high density, tumor cells exceed the limit of oxygen diffusion from capillaries (106). Therefore, diffusion limitations, leakage, and deformities of the tumor vascular system lead to a hypoxic environment. At the molecular level, the adaptation of tumor cells to the hypoxic TME is largely mediated by the hypoxia inducible factor (HIF) family (107). Although tumor cells can adapt to the hypoxic environment, immune cells are inhibited under these conditions. *In vitro* experiments have shown that, compared with normoxia, low concentrations of oxygen significantly reduce the proliferation and function of T lymphocytes and promote their apoptosis (108). Hypoxia can delay the differentiation of effector T cells and reduce the generation of effector T cells and cytokines such as IFN-γ and IL-2. Accordingly, in the hypoxic environment of the TME, CAR-T cells are notably restrained and have difficulty functioning normally.

Many metabolites produced by cancer cell metabolism can also affect infiltrating T cells. *In vitro* studies of $CD8^+$ T cells in mice and humans have shown that elevated levels of extracellular lactate and $H^+$ in the TME can inhibit T-cell proliferation, survival, cytotoxicity, and cytokine production (103). Tumor cells tend to choose the metabolic mode of glycolysis, leading to the accumulation of lactate in the microenvironment and acidification of the TME. Changing cell markers and preventing cell differentiation can lead to carcinogenesis, increasing the survival and proliferation of tumor cells, stimulating angiogenesis, and inhibiting immune responses by altering several immune infiltrating cells. The immunoregulatory role of lactic acid has drawn much attention. It affects T-cell activity and CAR-T-cell efficacy by promoting the infiltration of immunosuppressive cells such as TAMs, MDSCs, and Tregs. Xiong et al. combined epigenetics with tumor lactate metabolism. Methyltransferase-like 3 (METTL3) regulates tumor metabolic reprogramming by controlling the expression of glucose transporters (GLUTs), lactate dehydrogenase (LDHA), and enolase 1 (ENO1) in tumor cells. High METTL3 expression in cancer (109) significantly increases glucose uptake and lactate production, enhances glycolysis, and promotes tumor growth (110). On this basis, lactic acid in the TME promotes the expression of METTL3 in TAMs through histone lysine lactylation (Kla) modification. On the other hand, lactic acid lactates the zinc finger domain of METTL3, promoting the methylation of JAK1 mRNA, which combines with YTHDF1 to improve translation efficiency and promote the activation of the JAK1-STAT signaling pathway, initiating the expression of downstream immunosuppressive molecules and resulting in immunosuppression (109). Immunosuppressive cells subsequently lead to poor therapeutic effects by inhibiting CAR-T cells.

## 4.6 Toxicity of CAR-T cells

Immunotherapy has unique toxicity that traditional cytotoxic chemotherapies or small-molecule inhibitor do not have (111–113).

Patients receiving CAR-T-cell therapy can experience many potentially life-threatening toxicities, and CRS is one of the most clinically importance and dangerous. CRS occurs because of the high-level immune activation of immune cells, followed by the release of many inflammatory cytokines, which are most commonly observed in therapies involving T cells (8, 114, 115).

Researchers define CRS as "a supraphysiologic response following any immune therapy that results in the activation or engagement of endogenous or infused T cells and/or other immune effector cells. Symptoms can be progressive, must include fever at onset, and may include hypotension, capillary leak (hypoxia), and end-organ dysfunction (111)." The clinical manifestations of CRS involve multiple systems and are accompanied by life-threatening complications, including cardiac dysfunction, respiratory failure, kidney and liver failure, etc. Fever is usually the first sign of CRS (116). Low-level CRS manifests as an influenza-like illness characterized by fatigue, muscle soreness, etc. According to the severity of the disease, CRS is divided into 5 grades (111), ranging from grade 1 (fever at 38°C) to grade 5, which may result in death. After treatment with CD19 CAR-T-cell therapy, 54% to 91% of patients develop CRS, and 8.3% to 43% of patients develop severe CRS (22). In a clinical trial involving 75 patients receiving tisagenlecleucel (CD19 CAR-T) treatment, there was a brief increase in the serum IL-6, IFN-γ, and ferritin levels during the CRS period after infusion. These increases were often more pronounced in Grade 4 CRS patients than in lower-grade patients. Similar trends were also observed for other cytokines, including IL-10, IL-12, IL-1β, IL-2, IL-4, IL-8, and TNF-α. A brief increase in C-reactive protein was observed in most patients, but the variability was high (117).

IL-6 is an important cytokine that plays a crucial role in host defense by regulating immune and inflammatory responses. The IL-6/IL-6R signaling pathway is the main mediator of CRS occurrence; therefore, targeting IL-6 for anticytokine therapy is an effective treatment method to mitigate CRS symptoms. In classical intracellular signaling, IL-6 binds to IL-6R on the membrane and interacts with membrane glycoprotein 130 (gp130), which induces dimerization and leads to intracellular signal transduction, thereby activating and releasing inflammatory cytokines (118). Tocilizumab is a humanized monoclonal antibody against IL-6R that can competitively inhibit the binding of IL-6 to receptors and has been widely used to treat rheumatism. Tocilizumab has been reported to have little effect on the efficacy of CAR-T-cell therapy and relatively few substantial side effects. In a clinical study of patients receiving tisagenlecleucel treatment, tocilizumab was administered to 13 patients with cardiovascular dysfunction, with fever and tachycardia rapidly subsiding within 4 hours after treatment (112).

A significant challenge faced by CAR-T-cell therapy for solid tumor patients is that most candidate target antigens are typically coexpressed in healthy tissues, which poses a considerable risk of "on-target, off-tumor" toxicity (25). Multiple clinical trials have reported varying degrees of "on-target, off-tumor" toxicity (79, 119, 120). In these trials, CAR-T cells targeted both tumor cells and normal cells, leading to significant damage and severe side effects such as neurotoxicity.

Neurotoxicity is a common and potentially life-threatening adverse reaction to CAR-T-cell therapy. Neurological damage is often associated with CRS that occurs during or after CD19 CAR-T-cell therapy and, in rare cases, may threaten a patient's life (121, 122).

The neurotoxicity caused by CAR-T cells is diverse, and patients may experience symptoms such as hallucinations, cognitive deficits, aphasia, speech disorders, seizures, and encephalomyelitis (123–128). Like CRS, neurotoxicity is classified into 5 grades according to severity, ranging from mild to severe. Neurotoxicity may occur simultaneously with certain symptoms of CRS (such as hypotension), but neurotoxicity may also occur in patients who do not exhibit typical CRS symptoms or after CRS subsides (129). According to reports, neurotoxicity typically first appears on the second day after CAR-T-cell infusion and can last until the third or fourth week (130), resulting in a highly variable course. Therefore, close monitoring of neurotoxicity is necessary during the use of CAR-T-cell therapy.

In a clinical trial, the manifestations of 25 adult patients with neurotoxicity syndrome who received CAR-T-cell therapy at Massachusetts General Hospital were described (131). This clinical trial involved 23 NHL patients and 1 ALL patient receiving CD19 CAR-T-cell therapy, along with 1 liver cancer patient receiving AFP-targeted CAR-T-cell therapy. Among the 25 patients, 12 (48%) experienced grade 1-2 neurotoxicity, and 13 (52%) experienced grade 3-4 neurotoxicity. Tocilizumab does not seem to reduce the severity of neurotoxicity. Instead, it may increase exacerbate it. Neurotoxicity is primarily treated with corticosteroids, and its symptoms are controlled (132). Although corticosteroids are widely used, the extent to which they affect the anticancer effects mediated by CAR-T cells is still unclear, and further clinical trials are needed to determine their usage standards.

The pathogenesis of neurotoxicity remains obscure (133). Severe neurotoxicity may be related to the levels of C-reactive protein, various serum cytokines, and other proteins, such as IL-2, IL-6, and IL-10, as well as TNF-α and Granzyme B (125, 133). When the blood–brain barrier is broken, CAR-T cells can enter the cerebrospinal fluid and may significantly increase cytokine levels in the cerebrospinal fluid (134), causing neurotoxicity. In 2020, Parker et al. proposed a possible neurotoxicity mechanism (135). CD19 has been reported to be expressed in brain cells that protect the blood –brain barrier. In mouse models, when CAR-T cells are infused into mice, even if the mice lack B cells, their brain blood–brain barrier permeability increases. In contrast, when human CD19 was used as a CAR-T-cell target, there was no significant change in blood–brain barrier permeability. Compared with other B-cell proteins, such as CD20, CD19 immunotherapy has a higher incidence rate of neurotoxicity. Therefore, they concluded that the expression of CD19 molecules on the surface of cells other than B cells (such as

blood–brain barrier cells) is the fundamental cause of CAR-T-cell-induced neurotoxicity.

## 4.7 High cost of CAR-T cells

CAR-T-cell therapy, an emerging immunotherapy, has achieved significant therapeutic success, but its high cost makes it difficult for ordinary people to afford. In China, five CAR-T-cell therapies have been approved for the market, including Zevorcabtagene Autoleucel (BMCA), Axicabtagene Ciloleucel (CD19), Relmacabtagene Autoleucel (CD19), Equecabtagene Autoleucel (BCMA), and Inaticabtagene Autoleucel (CD19), priced at 1.15 million yuan, 1.2 million yuan, 1.29 million yuan, 1.166 million yuan, and 0.99 million yuan, respectively. How to solve the problem of high costs has always been a topic of concern for R&D personnel. The high price of CAR-T cells is caused by various reasons. First, CAR-T cells are personalized products, and each batch of CAR-T cells corresponds to only one patient; thus, it is not possible to reduce costs by treating more patients. Second, CAR-T-cell therapy involves complex production processes and treatment procedures with high-quality control, production, and management costs (136). The clinical trial cost of CAR-T-cell therapy is also very high, and in addition to treatment, there are additional considerations for health care costs (137), which all contribute to the high cost of CAR-T-cell therapy.

# 5 Future perspectives

Although CAR-T-cell therapy has achieved many exciting results, in future research, we still need to study the mechanisms more deeply and find solutions to the problems, including cost, specificity, resistance, toxicity, and TME inhibition (Figure 5).

## 5.1 Decreasing the cost of CAR-T-cell therapy

### 5.1.1 Establishing cell lines

There are two main reasons CAR-T-cell therapy is expensive (1): The process is very cumbersome, involves multiple aspects, and requires a dedicated medical team (2). Currently, CAR-T-cell therapy is approved only for the treatment of certain tumors in the bloodstream, and its unsatisfactory effect on solid tumors greatly limits its large-scale application. Personalized therapy makes it difficult to reduce labor costs. At present, CAR-T-cells are produced mainly by autologous CAR-T-cell technology, in which T cells are collected from patients themselves and processed. If CAR-T cells are constructed into stable cell lines, it will become possible to prepare them in advance, ensure the timeliness of treatment, and achieve large-scale production, which will greatly reduce costs. By selecting appropriate host cells, constructing plasmid expression vectors containing CAR genes,

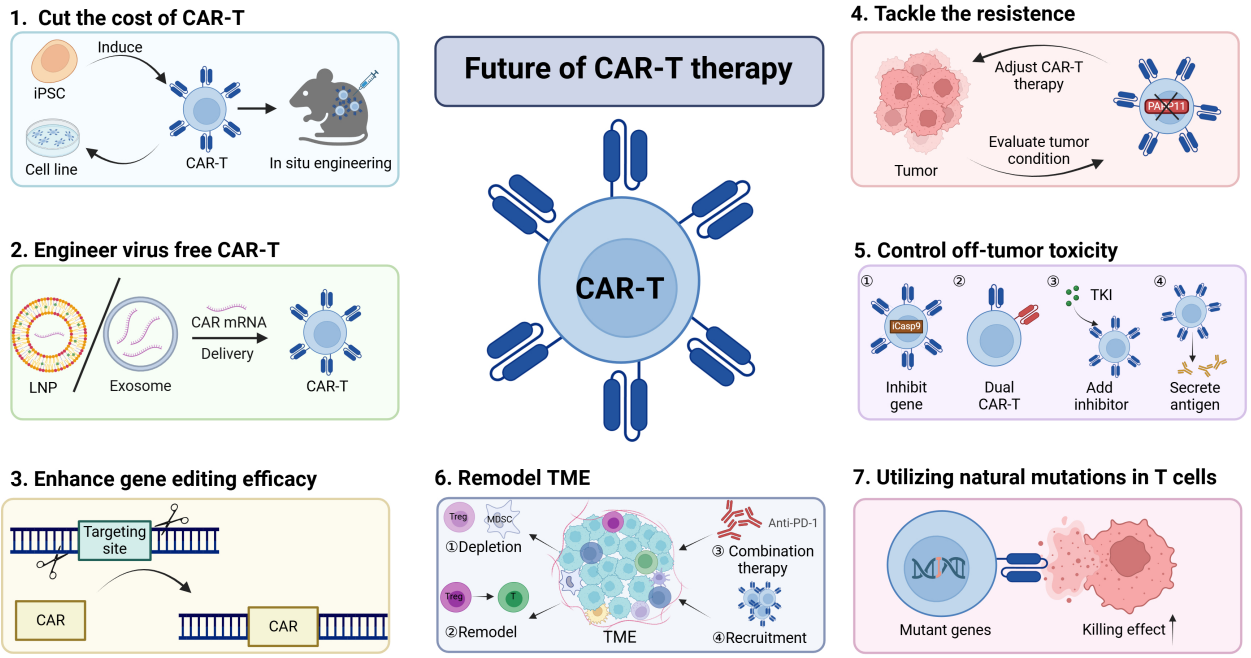


FIGURE 5
Harnessing multiple strategies to increase CAR-T-cell efficacy. The efficacy of CAR-T-cell therapy can be increased by employing various strategies.(1)The cost of CAR-T cells can be reduced by inducing CAR-T cells from iPSCs and cell lines or through *in situ* engineering approaches. (2) Virus-free engineering of CAR-T cells with LNPs or exosomes is an effective means to decrease genotoxicity.(3-4)The discovery of novel gene editing methods and the exploration of new targets have the potential to increase CAR-T-cell efficacy and overcome tumor resistance to CAR-T-cell therapy.(5)It is essential to control excessive toxicity by constructing new types of CAR-T cells.(6-7)Remodeling the TME or leveraging natural mutations can also contribute to the enhancement of CAR-T-cell efficacy. Collectively, these approaches can be utilized to improve CAR-T-cell therapies, offering promising avenues for the advancement of this field in the treatment of various malignancies.

and transfecting them into cells, we can screen stable expression cell populations using appropriate selection pressure and continuously culture them *in vitro*. Of course, there are some issues here, such as challenges in the process of building cell lines. Allogeneic CAR-T cells may lead to life-threatening GVHD. Moreover, these allogeneic CAR-T cells may be rapidly eliminated by the host immune system, reducing their antitumor activity and treatment outcomes.

### 5.1.2 iPSCs

Human induced pluripotent stem cells (hiPSCs) can provide an unlimited source of T cells for the development of CAR-T cells, with the potential to produce ready-made T-cell products. The combination of iPSCs and CARs provides an encouraging opportunity for cancer treatment, simplifying cell therapy methods for cancer patients and greatly reducing costs. In this case, gene editing of T-iPSCs (iPSC-derived T cells) using CAR would be a promising strategy to obtain T-iPSCs without restriction and generate functional T cells with a defined phenotype for therapeutic purposes. In a previous study, Themeli et al. isolated peripheral blood mononuclear cells (PBMCs) from healthy donors and transduced them with two retroviral vectors (138). Under laboratory conditions, the transduced T cells produce T-iPSCs, which are then transduced using a lentiviral vector encoding CD19-28z-CAR. The transduced iPSCs differentiate into T cells expressing CAR and endogenous TCR. A CD19-T-iPSC line targeting the CD19 antigen was successfully generated in a mouse model of human $CD19^{+}$ Burkitt lymphoma. *In vivo* cytotoxicity assays revealed strong antitumor activity and significant tumor regression.

However, the combination of T-iPSCs and CARs is still in its early stages of development and requires further experimental research (139). There are still challenges in the differentiation, maturation, and functionality of current iPSC-derived CAR-T cells, especially iPSC-derived T cells, which typically exhibit characteristics similar to those of gamma delta T cells and lack the powerful function of mature alpha beta T cells in the peripheral blood (140). iPSC-derived T cells are not considered readily available products. Owing to the presence of endogenous TCR or HLA mismatches, they cannot be used for third-party patients (138). The most common adverse reaction associated with TCR is GVHD, where donor T cells recognize host antigens as foreign antigens and subsequently destroy them.

## 5.2 *In situ* engineering

CAR-T cells produced *in vivo* provide an effective alternative immunotherapy for cancer treatment. This method requires a vector to deliver the CAR-encoding construct to T cells to express the CAR to eradicate the tumor. Therefore, an increasing number of studies have reported gene delivery systems for *in vivo* CAR-T-cell therapy based on viral vectors and polymer nanoparticles. Delivery methods based on biomaterials may change the manufacturing process of CAR-T cells, significantly reducing costs (141–143). Regulating the tumor microenvironment with biomaterials can also substantially increase the therapeutic effect of CAR-T cells against solid tumors. The *in situ* engineering of CAR-T cells can avoid the current need to extract autologous cells from patients, allows large-scale production, and facilitates storage and transport. Nanoparticles can be formulated and infused into patients when necessary. In terms of drug administration, *in situ* engineering can also save patients from the step of chemotherapy pretreatment (144). The modified CAR-T cells can also immediately recognize cancer cells *in vivo* and do not require additional ex vivo expansion steps. In one study, Smith et al. developed a polymer platform based on PBAE/PGA nanocarriers for the *in vivo* delivery of a CD19 CAR (145). The tumors were eradicated in approximately 70% of the mice treated, while the remaining mice showed notable regression, and an increase of 58 days in average survival time was observed. The therapeutic effect was comparable to that of CD19 CAR-T cells produced by infusion using standard methods *in vitro*. Fibrosis affects millions of heart disease patients. By delivering modified mRNAs into lipid nanoparticles (LNPs) that target T cells, transient antifibrotic CAR-T cells were generated *in vivo*, and CD5-targeted LNPs were injected into a heart failure mouse model to evaluate the efficacy of these reprogrammed CAR-T cells *in vivo*. The mRNA encoding CAR was effectively delivered to T lymphocytes, resulting in the *in vivo* generation of immediately effective CAR-T cells *in vivo*. Moreover, the accumulation of antifibrotic CAR-T cells in the spleen preserves target antigens, which can reduce fibrosis after injury and restore cardiac function (146).

## 5.3 Virus-free transduction

The majority of CAR-T cells are currently prepared through lentiviral transduction. Although lentiviruses can induce permanent expression of CARs, the uncertainty of the insertion site may lead to serious adverse reactions in patients receiving CAR-T-cell therapy (147). In addition to safety issues, there are various obstacles to the production and *in vivo* transfection of CAR-T cells by lentiviral transduction, including limited load, high cost, and *in vivo* immunogenicity. There is an urgent need for a safer and cheaper CAR-T-cell production strategy to replace lentiviral transduction.

mRNA transduction is a promising strategy for inducing transient CAR expression in T cells to avoid the adverse effects associated with viral vectors. Using mRNA to induce CAR expression has several advantages over viral transduction. First, mRNAs do not need to be integrated into the host cell genome, allowing CARs to be expressed in a short period of time, effectively avoiding the long-term risks related to CAR-T cells *in vivo*. Moreover, *in vitro* transcription is beneficial for optimizing the structure of CAR mRNA, making it easier for CAR proteins to be translated and expressed in T cells (148). mRNA-based CAR-T-cell therapy effectively kills tumor cells and achieves therapeutic results similar to those of lentivirus-mediated CAR-T-cell therapy. However, the most common requirement for mRNA delivery is electroporation, which may cause cytotoxicity (149). However, LNPs have been designed to deliver mRNA to T cells (150). This platform induces CAR expression at a level equivalent to electroporation with markedly reduced cytotoxicity. Furthermore,

exosomes are phospholipid bilayer vesicles secreted by living cells into the extracellular microenvironment. Exosomes contain many bioactive molecules and, together with their corresponding receptors, can mediate intercellular communication (151). Owing to their biocompatibility, low immunogenicity, and ability to penetrate the blood–brain barrier, exosomes are potential RNA drug carriers. By utilizing engineered exosomes, CAR-T cells can be directly generated from peripheral blood mononuclear cells (PBMCs), providing a methodological reference for the development of safer and more cost-effective CAR-T-cell therapies in the future (152).

Virus-like particles (VLPs) are multiprotein structures that are very similar to viruses, but owing to the lack of a viral genome, they can be safely used. These VLPs originate from both enveloped and nonenveloped viruses, self-assemble from one or more viral structural proteins, and can spontaneously form particles close to the size and shape of the original virus. Genetic materials such as DNA or RNA can be packaged into VLP nanocages and delivered to target cells, demonstrating the potential of VLPs as gene editing vectors (153, 154). Engineered DNA-free virus-like particles (eVLPs) have been developed for packaging and delivering base editors or Cas9 ribonucleoproteins, which mediate efficient base editing in several mouse and human cells (155). For example, regarding v3 and v3b PE-eVLPs, a single injection of v3 PE-eVLPs can restore the expression of treatment-related proteins in the retina and partially salvage visual function in two genetic blindness mouse models (156). The use of VLPs instead of traditional viral vectors for gene editing delivery can increase the safety of CAR-T cells.

## 5.4 Improving the site specificity of gene editing

The emergence of CRISPR/Cas9 technology, which is simple, reliable, and effective, has increased the efficiency and specificity of gene editing. It has been used for sequence knock-in or knockout in mammalian genome editing. Cas9 nuclease, guided by s small guide RNA (sgRNA), induces double-stranded DNA breakage (DSB), which is repaired through nonhomologous end joining (NHEJ), leading to gene function loss. Compared with NHEJ, after double-stranded DNA is cleaved by sgRNA, homologous directed repair can deliver larger gene sequences to precise sites in the genome (157–159). The HDR process can perform precisely targeted nucleotide substitution at designated sites (160).

The current research focus is on the precise editing of CAR-T cells using the CRISPR/Cas9 system, which can install the required genes in the genome with or without the introduction of DSBs. These tools and strategies can be directly applied to negative regulatory factors of T-cell function, target genes to specific loci, and produce repeatable, safe, and effective universal CAR-T-cell products for cancer immunotherapy (158). The latest progress in CRISPR technology has enabled endogenous genes to target and intervene in human CAR-T cells, unleashing the therapeutic potential of CAR-T-cell therapy. Strategies based on CRISPR have been used to develop next-generation CAR-T cells (161). Guiding the CD19-specific CAR to the T-cell receptor alpha constant (TRAC) locus not only leads to uniform expression of the CAR in human peripheral blood T cells but also increases the efficacy of T cells. In a mouse model of acute lymphocytic leukemia, the edited cells were significantly superior to traditional CAR-T cells. These findings emphasize the potential of the CRISPR/Cas9 system in advancing immunotherapy (162). CRISPR/Cas9 technology can also be used to knock down genes related to T-cell exhaustion and restore the loss of epigenetic targets, with the potential to prevent or even reverse CAR-T-cell dysfunction. Targeting inhibitory receptors, transcription factors, or other mediators of CAR-T-cell dysfunction through gene editing can reactivate injected cell products (158).

Although HDR can be used to insert specific DNA templates for the precise restoration of DNA sequences, this pathway is characterized by limited efficiency and high rates of unexpected insertion or deletion mutations, which render the repair ineffective. In addition, relying on homologous recombination limits the range of targeted diseases (163). The CRISPR/Cas-mediated single base pair editing system has been designed to overcome these limitations. DNA base editors (BEs) include the catalytic fusion of damaged Cas nucleases and base-modifying enzymes, which act on single-stranded DNA (ssDNA) rather than double-stranded DNA (dsDNA). When the guide RNA binds to the target DNA site, the base pairing between the guide RNA and the target DNA strand causes a small segment of single-stranded DNA in the "R-loop" to shift. The DNA base in this single-stranded DNA is modified by deaminase. To increase the efficiency of gene editing in eukaryotic cells, the catalytic inactivation of nucleases creates a gap in the unedited DNA strand, inducing the use of the edited strand as a template to repair the unedited strand (164, 165). A study combined nonviral CRISPR/Cas9 nuclease-assisted knock-in and Cas9-derived base editing techniques to perform DSB-free knockout within a single insertion range, inserting CAR into the TRAC gene, as well as two knockouts to silence MHC I and MHC II expression. This method increases the site specificity of gene editing and can effectively generate edited CAR-T cells with a translocation frequency comparable to that of unedited T cells, thereby enhancing the safety of CAR-T cells (166).

Another type of gene editing tool, prime editors (PEs), is a new method that can expand the scope of precise DNA editing without donors. It can be used for all transition and transposition mutations, as well as small insertion and deletion mutations. PEs do not require double-stranded DNA breakage and can perform almost any substitution, small insertion, or small deletion in the DNA of living cells. Prime editing requires at least one programmable nickel enzyme and polymerase fusion, as well as an extended guide RNA that can specify target sites and template the required genome editing (167, 168). One study designed a powerful knock-in (KI) strategy using PEs, called primed microhomology-assisted integration (PAINT), which utilizes reverse transcription of single-stranded microhomology sequences to promote targeted KIs in different types of cells (169). An improved version, PAINT 3.0, maximizes editing efficiency and reduces the possibility of off-target editing. By using PAINT 3.0, a reporter gene can be transformed into a housekeeping gene, with an editing efficiency

of up to 80%, which is more than 10 times greater than that of traditional homologous directed repair methods. PAINT 3.0 can efficiently target nonviral genomes in primary T cells and generate functional CAR-T cells with specific tumor-killing abilities. Overall, DNA base editing and prime editing tools can perform precise nucleotide substitutions in a programmable manner without the need for donor templates. Both DNA base editing and prime editing have significant potential as tools for the precise editing of CAR-T cells.

## 5.5 Counteracting resistance

Understanding the mechanisms of resistance can provide more appropriate treatment strategies to address this problem. B-cell maturation antigen (BCMA) is crucial for the normal function of plasma cells, so its absence is not common. Samur et al. determined through single-cell transcriptome analysis of bone marrow samples that the absence of BCMA is one of the mechanisms of resistance in CAR-T cells (170). The therapy achieved clinical effects during the initial infusion of CAR-T cells in the patient, but resistance developed later, manifested as recurrence and a lack of response to the second infusion of CAR-T cells. These findings emphasize that myeloma cells may still be able to survive without BCMA. Accordingly, CAR-T-cell therapy may result in the selection of BCMA-negative myeloma cells may be selected, leading to resistance. Therefore, it is possible to consider sequencing the BCMA locus before continuous BCMA-targeted therapy to fully evaluate the growth of rare BCMA-deficient myeloma cells and adjust treatment strategies in a timely manner.

The TME helps to evade antitumor immunity and generate resistance to treatment. Therefore, targeting certain key factors in the TME is a key approach for addressing CAR-T-cell resistance. Zhang et al. reported that TME factors such as regulatory T cells and adenosine downregulated the type I interferon (IFN1) receptor IFNAR1 on $CD8^+$ cytotoxic T lymphocytes (CTLs) (44). These events rely on poly-ADP ribose polymerase-11 (PARP11), which is induced in tumor CTLs and serves as a key regulatory factor for the immunosuppressive TME. Ablation of PARP11 can prevent the loss of IFNAR1, increase the antitumor activity of CTLs, and inhibit tumor growth in an IFNAR1-dependent manner. Therefore, the genetic or pharmacological inactivation of PARP11 increases the therapeutic efficacy of CAR-T cells. CAR-T-cell-based therapy designed to target PARP11 has shown superior efficacy against solid tumors.

## 5.6 Controlling off-target toxicity

To alleviate the "on-target, off-tumor" toxicity of CAR-T-cell therapy, it is necessary to carefully balance effective T-cell activation to ensure antitumor activity and decrease the potential for uncontrolled activation, which may generate immunopathology. The inducible cysteine aspartate protease 9 (iCasp9) "safety switch" provides a solution to remove improperly activated CAR-T cells (171). The induction of iCasp9 depends on the administration of the small-molecule dimerization drug AP1903, which rapidly induces apoptosis in transduced cells and prioritizes the killing of activated cells expressing high levels of transgenic genes. The iCasp9 gene has been incorporated into the vector used for preclinical studies and has demonstrated effective and reliable suicide gene activity in phase 1 clinical trials. By significantly improving the safety of CAR-T-cell therapy, iCasp9/AP1903 suicide gene technology can promote its wider clinical application.

In addition to ensuring the safety of CAR-T-cell therapy, increasing the targeting ability of CAR-T cells is crucial. One of the characteristics of tumor cells is the mixed expression of antigens. Designing dual-targeted CAR-T cells can maximize their ability to prevent tumors from escaping immune system detection and limit their off-target toxicity. Dual-targeted CAR-T-cell therapy can achieve dual effects, selectively targeting another B-cell malignant tumor target while targeting CD19. In this way, even if cancer cells develop an evasion mechanism, this therapy can switch targets in a timely manner to continuously attack cancer cells. Roybal et al. designed a combined and activated T-cell circuit in which a synthesized Notch receptor for one antigen induced the expression of a CAR for a second dual-target antigen. These dual receptors and gate T cells are armed and activated only in the presence of dual-antigen tumor cells. This precise dual receptor circuit opens the door to a wider range of immune recognition for tumors (172). Editing ready-made CD19/CD22 dual-targeted CAR-T cells through CRISPR can allow these edited cells to be used as a new treatment for ALL (173).

If the dose of CAR-T cells can be precisely controlled at different time points, toxic side effects can be reduced. Mestermann et al. used the tyrosine kinase inhibitor (TKI) dasatinib as a lead compound (174). Dasatinib eliminates CAR signaling by blocking the adenosine triphosphate binding site of LCK and immediately blocks the function of $CD8^+$ and $CD4^+$ CAR-T cells. This blocking effect does not affect the activity of CAR-T cells and is quickly and completely reversible after the removal of dasatinib, confirming that dasatinib can be used as a clinical on/off switch for CAR-T cells. Similarly, the clinically approved drug lenalidomide can also serve as an ON and OFF switch for CAR-T cells (175). Another study identified "super degron" tags that increase sensitivity to lenalidomide-induced degradation and used these degradable tags to generate off-switch-degradable CARs. A lenalidomide-induced dimer system was designed to obtain split CARs that require both lenalidomide and target antibody activation as ON switches. *In vivo*, ON-switch split CARs exhibit lenalidomide-dependent antitumor activity and OFF-switch degradation of inflammatory cytokines while maintaining antitumor efficacy. By utilizing synthesized Notch receptors and transcriptionally connecting multiple molecules to recognize events, different multireceptor cell recognition circuit libraries can be constructed (176). These synthetic circuits allow engineered T cells with integrated extracellular and intracellular antigen recognition, providing

robustness to heterogeneity, and enable achieve precise recognition by integrating up to three different antigens with positive or negative logic. Connecting multiple molecular recognition events in synthetic circuits provides a powerful method for designing cell-level recognition.

Abnormal secretion of cytokines is also one of the factors leading to off-target toxicity in CAR-T cells. By designing CAR-T cells that self-regulate the production of inflammatory cytokines, researchers can simultaneously address the toxicity of CRS and increase its ability to attack tumors, which is highly important for the treatment of cancer patients. One study designed CD19 CAR-T cells capable of secreting anti-IL-6Ra single-stranded variable fragment (scFv, known as Toci), which can reduce the severity of CRS (177). In a humanized NSG-SGM3 mouse model, CAR-T cells that secreted single-chain variable fragments (Toci) derived from tocilizumab produced lower CRS-related toxicity, resulting in greater safety than single-dose systemic administration of tocilizumab. Compared with traditional CD19 CAR-T cells, these Toci-secreting CAR-T cells show superior *in vivo* antitumor efficacy. This method of engineering T cells to self-regulate the production of inflammatory cytokines is a clinically compatible strategy that may simultaneously improve safety.

## 5.7 Remodeling the TME

### 5.7.1 Targeting immunosuppressive cells

In addition to targeting tumor cells directly, targeting other cells in the tumor microenvironment is a promising method. Tumor-associated macrophages (TAMs) are the most widely infiltrating immune cells in the TME. In clinical practice, a high number of TAMs is closely related to a poorer prognosis of various cancers. Because of the crucial role of TAMs in tumor development, the clearance of TAMs may be a method to alter the immunosuppressive environment of the TME and promote an antitumor immune response (178). In a previous study, Sánchez-Paulette et al. designed a CAR-T-cell that targets F4/80 (179), which can not only effectively clear TAMs and relieve the immunosuppression caused by TAMs but also promote the tumor antigen-specific T-cell immune response, thus inhibiting the growth of various tumors. These findings provide support for the subsequent development of CAR-T-cell therapy for clearing TAMs.

### 5.7.2 Remolding of immunosuppressive cells

Owing to the high plasticity and tumor infiltration ability of macrophages, researchers are attempting to turn them into tumor-killing weapons through gene editing. The “Don’t eat me” signaling pathway involving CD47/SIRPα is a key mechanism by which tumor cells evade macrophage phagocytosis. CRISPR/Cas9 gene editing technology can be used to knock down SIRPα in macrophages, increasing tumor killing and phagocytic ability (180). In 2018, CAR was first used to modify macrophages. Equipping macrophages with tumor antigen-specific CARs creates a potential weapon for targeted tumor killing, especially for solid tumors (181). Cheng et al. designed a series of CARs targeting CD19 or CD22, named CAR Ps. After transfecting these CAR Ps into macrophage cells, they observed strong specificity and phagocytic ability toward human Burkitt lymphoma cells both *in vitro* and *in vivo* (178). Similarly, a 2020 study demonstrated that CAR gene editing in human macrophages can guide their phagocytic activity against tumors (182). Adenovirus vectors can be used to modify HER2 CAR-M cells. Transfecting viral vectors into PBMCs from tumor patients and differentiating them into macrophages,can increase their likelihood of maintaining their tumor-inhibiting activity, which can help them overcome the transition to an immunosuppressive state. One of the main advantages of CAR M-cell therapy is its ability to create a proinflammatory environment within the tumor. The proinflammatory TME is also friendlier to other immune cells, such as T cells. Once T cells enter the tumor, they can recognize tumor antigens presented by macrophages and target cancer cells for destruction. In summary, CAR-M-cell therapy has shown significant advantages in the treatment of solid tumors (183).

The tumor immune response can also be improved by regulating the differentiation of suppressive immune cells. MDSCs are a mixture of immature myeloid cell populations with high heterogeneity and immunosuppressive activity. All-trans retinoic acid (ATRA) can promote the differentiation of MDSCs into granulocytes, macrophages, and DCs and increase the host antitumor immune response by neutralizing the production of ROS (184). The promotion of the development of MDSCs into normal monocytes and granulocytes not only reduces the number of MDSCs but also increases the number of mature myeloid cells, inhibiting tumor growth (185).

## 5.8 Increasing the effectiveness of CAR-T cells by combination therapy

With the emergence and development of various new immunotherapies, combination therapy has gradually become a popular way to treat tumors. The combination of CAR-T cells and immune checkpoint inhibitors may achieve remarkable therapeutic effects (186). PD-1 blockade increases the survival of CAR-T cells and promotes the killing of PD-L1-positive tumor cells. Research data suggest that PD-1-targeted combination therapy may help improve the therapeutic efficacy and persistence of CAR-T cells in patients. The current research further supports the combination of anti-PD-1 monoclonal antibodies and CAR-T-cell therapy and allows its use for treatment in clinical trials of GD2-specific CAR-T cells in patients with neuroblastoma.

When combined with a nanoparticle RNA vaccine (187), the CAR antigen can be delivered throughout the body to the lymphatic regions, stimulating CAR-T cells. The presentation of natural folding targets on resident antigen-presenting cells promotes the homologous and selective expansion of CAR-T cells. This leads to the successful infiltration of CAR-T cells and tumor regression in

mouse models. Using CAR-T cells with the help of DC vaccines is a reliable method for solid tumor treatment (188). One study constructed a combined method of CAR-T cells and DC vaccines. After the two types of cells were cocultured, CAR-T cells proliferated extensively and exhibited an increased CD45 $RO^+$ $CCR7^+$ phenotype. The injection of DC vaccines increased the immune-killing effect of CAR-T cells *in vivo*, the number of CAR-T cells in the peripheral blood of the mice was higher, and they persisted longer, indicating that DC vaccines could increase the persistence of CAR-T cells in mice.

## 5.9 CAR-T-cell recruitment and infiltration

The attraction of chemokines and the interaction between chemokines and receptors are the first steps in the process of T-cell trafficking to tumors. Therefore, modifying CAR-T cells in the tumor microenvironment to target enriched chemokines may be a feasible strategy for optimizing CAR-T-cell trafficking efficiency, especially in solid tumors. One study reported that several CXCR2 ligands are expressed at relatively high levels in human hepatocellular carcinoma tumor tissues and cell lines compared with other chemokines (189). However, both human peripheral T cells and hepatocellular carcinoma tumor-infiltrating T cells lack the expression of CXCR2. In xenograft tumor models, the expression of CXCR2 in CAR-T cells notably promotes tumor infiltration and enhances the antitumor effect of these cells. Another study introduced CXCR2 into CAR-T cells for the treatment of PDAC, and the results revealed that CXCR2 CAR-T cells not only reduced the volume of transplanted PDAC tumors but also completely eliminated the formation of metastatic tumors. CXCR2 plays an important and promising role in increasing the efficiency of CAR-T-cell therapy for primary and metastatic PDAC (190).

By delivering specific antigens to tumor cells in a specific way, we can also increase the specific killing ability of CAR-T cells and improve the infiltration effect. Certain types of bacteria selectively colonize tumor cores with immune privileges and can be engineered into antigen-independent therapeutic delivery platforms. To address the issue of CAR-T-cell infiltration, Vincent et al. developed a probiotic-guided CAR-T-cell (ProCAR) platform (191), in which tumor-colonizing probiotics (*E. coli* Nissle1917) release synthetic targets and label tumor tissue for CAR-mediated *in situ* lysis. This system demonstrated that CAR-T-cell therapy for solid tumors with unknown antigens is safe and effective in various models of human and mouse cancer and is a potential method for treating heterogeneous, cold tumors and poorly infiltrated solid tumors. However, humans are more sensitive than mice to endotoxins. Structural modification of LPS can continuously reduce TLR4 stimulation without disrupting bacterial activity or tumor colonization. This modification further limits bacterial growth and reduces immunogenicity, promoting safe systemic delivery and repeated administration (192).

## 5.10 Utilizing natural mutations in T cells

In human T-lymphomas, the evolution of the disease actively selects for mutations that increase T-cell fitness under challenges similar to those faced by therapeutic T cells. Therefore, it may be possible to use these mutations to improve T-cell therapies. T-cell tumors acquire mutations that increase their adaptive capacity. These mutations can undergo positive selection in the immunosuppressive microenvironment of solid tumors, enhancing the survival and development ability of tumor cells. In a previous study, Garcia et al. constructed a T-cell library containing 71 mutants and 45 wild-type controls (193). These mutations were introduced into human and mouse T cells, and their effects on the T-cell phenotype *in vitro* and *in vivo* were evaluated. This approach identified a novel gene fusion, CARD11-PIK3R3, that is capable of significant T-cell efficacy. CARD11-PIK3R3 promoted CAR-T-cell antitumor activity, reduced T-cell dosage requirements, and alleviated the need for T-cell exhaustion preconditioning. In addition, many researchers have used knockout T lymphoma antioncogenes to enhance T-cell therapy while triggering no malignant mutations (194). However, a patient was reported to develop CAR-T-cell lymphoma after being infused with BCMA-targeted CAR-T cells, which indicated that mutations had occurred before CAR-T-cell manufacturing (195). Therefore, additional safety methods, such as suicide switches, can be used to remodel T cells, increase control over CAR-T cells, and prevent T-cell exhaustion after sufficient treatment efficacy is achieved.

# 6 Conclusion

CAR-T-cell therapy, as one of the most promising methods for cancer treatment, has changed the treatment pattern of some hematologic cancers and may be used for the treatment of solid tumors and other diseases. The high cost of CAR-T-cell therapy is due mainly to its personalization and difficulty in mass production. By constructing cell lines, inducing relevant cells with iPSCs, and performing transient transduction *in vivo*, the production cost of CAR-T cells can be significantly reduced, and their universality can be enhanced. New gene editing technologies, such as the CRISPR/Cas9 system, can increase the specificity of CAR loci and thereby increase the safety of CAR-T-cell therapy. The effective treatment of solid tumors may depend on combination therapy. We can use immunosuppressants, tumor vaccines, and other methods in combination with CAR-T cells and improve the safety of therapy through modification strategies. In clinical trials, the incidence and severity of anti-CAR immune reactions should be reduced, and the immunogenicity should be monitored in patients receiving CAR-T-cell therapy to enable timely and effective responses to possible resistance. To increase the low infiltration rate, we can modify immunosuppressive cells that readily infiltrate the TME so that they express tumor-killing elements instead of inhibitory elements, directly disintegrating the tumor from inside. It is also possible to modify tumor microorganisms to deliver specific antigens to tumor cells and

express chemokines. This modification can enhance both antigen specificity and CAR-T-cell infiltration ability. Although many challenges remain in improving the efficacy of CAR-T-cell therapy, especially in solid tumors, and many methods have room for further optimization, we believe that CAR-T-cell therapy is highly promising. The development of CAR-T cells that can be used for the treatment of a wider range of patients can reduce costs and greatly increase the feasibility and popularity of immunotherapy.

## Author contributions

ZSY: Writing – original draft. BH: Writing – original draft. QW: Visualization, Writing – review & editing. FR: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. ZNY: Funding acquisition, Supervision, Writing – review & editing. HZ: Funding acquisition, Supervision, Writing – review & editing.

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# Immune microenvironment of tumor-draining lymph nodes: insights for immunotherapy

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Tumor-draining lymph nodes (TDLNs) play a crucial role in modulating tumor immune responses and influencing the efficacy of immunotherapy. However, our current understanding of the microenvironment within these lymph nodes remains limited. Tumors not only impair the anti-tumor activity of $CD8^+$ T cells by creating an immunosuppressive microenvironment, but they also facilitate immune evasion and promote metastasis by altering the structure and function of TDLNs. Research has shown that tumor-specific memory $CD8^+$ T cells ($T_{TSM}$) within TDLNs are essential for the efficacy of immune checkpoint inhibitors, such as PD-1/PD-L1 blockers. Moreover, the abnormal structure of TDLNs, along with the presence of immunosuppressive cells—such as regulatory T cells (Tregs), regulatory B cells (Bregs), and immunosuppressive dendritic cells (DCs)—contributes to tumor-mediated immune evasion. Therefore, gaining a deeper understanding of the immune microenvironment within TDLNs is essential for improving the effectiveness of immunotherapies and developing novel therapeutic strategies. This review explores various TDLN-based therapeutic strategies, addressing the controversies surrounding lymph node dissection, the use of TDLNs as a source of tumor-infiltrating lymphocytes (TILs) for therapy, targeting immunosuppressive cells within TDLNs, and methods to reverse the structural abnormalities of TDLNs. These strategies offer valuable insights and potential directions for advancing tumor immunotherapy.

KEYWORDS

**tumor-draining lymph nodes, immune microenvironment, immunosuppressive cells, immunotherapy, PD-L1/PD-1, $CD8^+$ T cells**

## 1 Introduction

In recent years, tumors have been recognized as highly complex systemic diseases that, in addition to their malignant proliferation, induce profound alterations across various bodily systems. These changes collectively create "sanctuaries" that shield the tumor from immune system surveillance. Specifically, tumors actively construct immunosuppressive microenvironments that significantly undermine the efficacy of $CD8^+$ T cell-mediated anti-

tumor responses, which are central to immune defense mechanisms (1). Currently, tumor immunotherapy strategies, particularly immune checkpoint blockade, aim to rejuvenate the anti-tumor potential of $CD8^+$ T cells within the tumor microenvironment (TME) (2). This approach has achieved significant efficacy in clinical practice and has emerged as one of the most promising therapeutic strategies in oncology. However, substantial challenges remain in clinical settings: only a small percentage of patients with solid tumors respond to immunotherapy, and the majority fail to achieve durable or substantial therapeutic benefits (3).

As research into tumor immunology advances, the pivotal roles of TDLNs in immunotherapy has garnered increasing attention. It has been discovered that the effectiveness of immune checkpoint inhibitors, including PD-L1 inhibitors, not only depends on the reactivation of pre-existing exhausted $CD8^+$ T cells within the TME, but also relies heavily on the continuous influx of newly activated and expanded effector T cells from the periphery, particularly within the TDLNs (4). The PD-1-PD-L1 interaction in the TDLN, rather than within the tumor itself, can serve as a predictive marker for the clinical efficacy of PD-L1 inhibitors, particularly in tumors like metastatic melanoma (5). Moreover, studies have shown that tumor antigen-specific memory $CD8^+$ T cells, which are crucial responders to PD-L1 inhibitors, are predominantly located within TDLNs (6). However, as tumors progress, they can significantly suppress anti-tumor immune responses by reprogramming the TDLN microenvironment. This process includes reshaping the structural architecture of TDLNs and regulating the differentiation and function of immunosuppressive cells, which ultimately limits the efficacy of immunotherapy. Therefore, a comprehensive understanding of the mechanisms driving TDLN immune microenvironment remodeling is essential for elucidating tumor immune evasion, enhancing the efficacy of immunotherapies, and developing novel therapeutic strategies. This review summarizes the current research on TDLN immune microenvironment remodeling and its implications for immunotherapy. Additionally, we explored relevant therapeutic strategies, aiming to provide new insights and references for future cancer immunotherapy.

# 2 The crucial role of tumor-draining lymph nodes in tumor immunotherapy

## 2.1 TDLN serves as the initiating site of the anti-tumor immunity cycle

During tumor progression, the TDLN is not only serves as the primary site where tumor cells arrive via the lymphatic system but also acts as the initiation point for anti-tumor immune responses. The tumor immunity cycle is a sequential process wherein the immune system first recognizes tumor cells, activates specific T cells, and then directs them to the tumor site for elimination. The most crucial steps of antigen presentation and T cell activation occur precisely within the TDLN (7). Recent studies have shown that naive T cells, upon activation in the TDLN in the presence of tumors, do not immediately differentiate into effector cells. Instead, they enter a "stem-like" state, from which they can develop into either exhausted T cell precursors/progenitors ($T_{pex}$ cells) or $T_{TSM}$ (6, 8). These cells express high levels of stem cell-related markers such as TCF-1 but lack effector molecules like granzyme B and perforin (9). It is only after migrating to the tumor microenvironment and receiving additional co-stimulatory signals and cytokine stimuli that these precursor cells differentiate into fully functional effector $CD8^+$ T cells, acquiring the capacity to kill tumor cells. However, numerous studies have indicated that within the TME, activated $CD8^+$ T lymphocytes often lose their ability to develop into memory cells due to persistent antigen stimulation and the inhibitory effects of the immune suppressive milieu. Initially, these activated T cells differentiate into $T_{pex}$ cells, which represent a less exhausted state, but eventually progress into terminally exhausted T cells (10–14). This process is marked by a progressive loss of effector function, proliferative capacity, and memory potential, accompanied by sustained high expression of immune checkpoint molecules, such as PD-1 and TIM-3 (15). Based on this understanding, immune checkpoint blockade (ICB) therapies, particularly targeting PD-1/PD-L1, have shown promise in partially reversing the exhaustion of $CD8^+$ T cells and, consequently, controlling tumor progression.

The prevailing viewpoint within the field is that $T_{pex}$ cells within the TME are the primary responders to PD-1/PD-L1 ICB treatment (16). However, it is important to recognize that $T_{pex}$ cells represent a relatively small proportion of the TME, constituting only about 5% (17). Moreover, recent research has revealed that after receiving PD-1/PD-L1 ICB therapy, patients with tumors develop novel TCR clones of tumor-localized, antigen-specific $CD8^+$ T cells that were previously absent (18). These findings suggest that the tumor-specific T cells responding to ICB treatment are largely dependent on the continuous replenishment of $CD8^+$ T cells from outside the tumor, particularly from the TDLNs (4, 19); however, this replenishment process diminishes as the tumor progression advances. For example, in a study on head and neck cancer, $T_{pex}$ cells in uninvolved lymph nodes (LNs) were found to undergo activation and differentiation following PD-L1 treatment, whereas these signals were impaired in lymph nodes affected by cancer metastasis (18). A study utilizing FTY720 to inhibit T cell recruitment from the TDLN to the tumor site showed a substantial effect on early tumor response (20); however, at later stages of tumor progression, this inhibition had minimal impact on tumor progression. This observation suggests that, as tumors progress, T cell activation within the TDLN may become impaired. In 2023, Professor Ye Liling's team conducted a series of tumor transplantation and lymph node excision experiments, which clarified that PD-1/PD-L1 ICB therapy primarily mobilizes TDLN-$T_{TSM}$ (6). These cells then expand and differentiate into $T_{pex}$ cells, which subsequently enter the TME to exert their effects, rather than attempting to reverse the exhaustion of $T_{pex}$ cells already infiltrated within the tumor. Furthermore, the TDLN-$T_{TSM}$ cells play a critical role in determining the therapeutic efficacy of PD-1/PD-L1 ICB treatment. The studies highlight the significant role of TDLNs in shaping the anti-tumor immune response and

influencing the response to ICB treatment. They underscore the importance of exploring the immune microenvironment of TDLNs to better understand the mechanisms underlying tumor immune evasion and resistance to immunotherapy.

## 2.2 The TDLN is a crucial structure within the lymphatic system for tumor metastasis

During tumor progression, tumors remotely regulate the immunosuppressive microenvironment within TDLNs through mechanisms such as the lymphatic transmission of inhibitory cytokines, tumor-derived extracellular vesicles (TDEVs), and other soluble factors (Figure 1). Additionally, tumors alter the adhesion properties within lymph nodes by upregulating metastasis-related genes and secreting lymphangiogenic factors like VEGF and extra cellular vessels (21, 22), which stimulate the formation of lyric vessels and facilitate tissue metastasis. Tumor cells can also exit lymph nodes by invading lymph node blood vessels after nodal metastasis and enter the bloodstream, thereby colonizing distant organs (23, 24). In mouse models, PD-L1 immunotherapy was found to impair the activation of $CD8^+$ T cells in metastatic lymph nodes (25). Similarly, a clinical study involving 68 non-small cell lung cancer patients demonstrated that patients with metastasis to lymph nodes often exhibited poorer responses to immunotherapy (26). Consequently, when cancer spreads to the lymph nodes, selective lymph node dissection and clearance are often performed to prevent further metastasis. However, the necessity of lymph node clearance remains controversial, as it does not always lead to significant improvements in survival rates (27–30). Research on colorectal cancer shows that lymph node and distant metastases originate from independent subclones in 65% of cases, while 35% share a common origin, indicating a complex and diverse evolutionary relationship in metastasis. This diversity may explain the limitations of lymph node dissection effectiveness (31).

The TDLNs represent both a critical site for anti-tumor immune responses and a key location for tumor metastasis, making their microenvironment exceptionally complex. Nevertheless, current research has predominantly focused on the immunosuppressive microenvironment of tumors, leaving a significant gap in our understanding of the immune landscape within TDLNs.

# 3 Factors of TDLN immune tolerance

As tumors progress, significant alterations occur in both the anatomical structure and cellular composition of TDLNs. These alterations directly or indirectly impair immune cell functions, enabling tumors to evade immune surveillance and continue their growth and dissemination. Gaining a deeper understanding of these structural modifications is crucial for enhancing the efficacy of tumor immunotherapy.

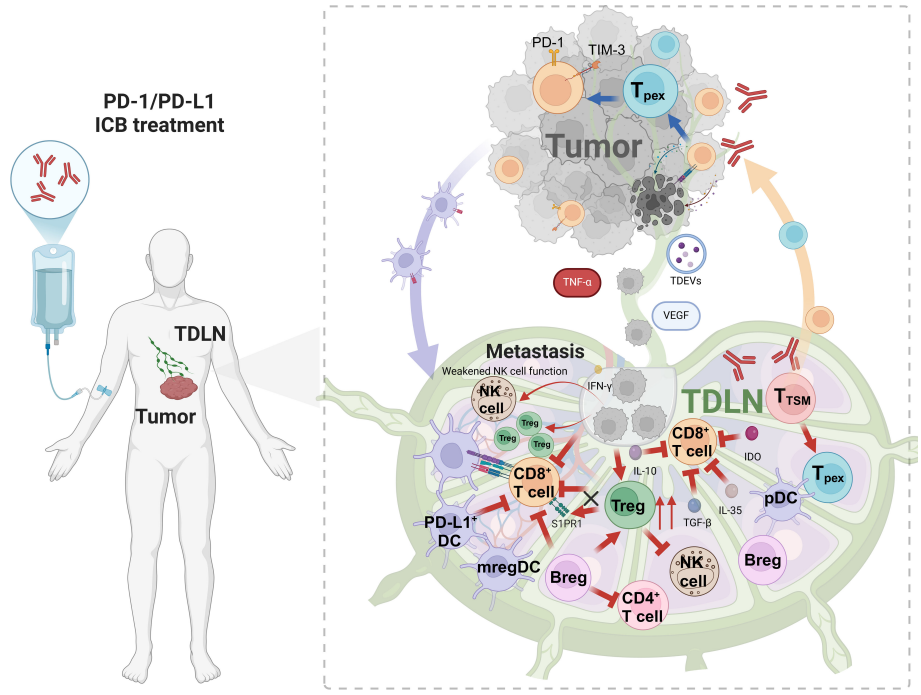


FIGURE 1
The immunosuppressive microenvironment within TDLNs. TDLN is the starting point of anti-tumor immunity and activates initial T cells. The antigen presented by DC activates CD8+ T cells (such as Tpex/$T_{TSM}$), which migrate to tumors and differentiate into effector cells. At the same time, TDLN promotes metastasis: VEGF induces lymphangiogenesis, and immune cells construct an immunosuppressive environment. Treg(IL-10/PD-1), mregDC(IDO), and Breg(IL-10/TGF-β/IL-35) cooperate to suppress immune response, drive tumor escape, and limit treatment response. Figure was created with Biorender.com.

## 3.1 Immunosuppression mediated by TDLN structural remodeling

In the early stages of tumor development, tumors disrupt or remodel the lymph node structure at distant sites, fostering the creation of an immunosuppressive microenvironment within TDLNs and laying the groundwork for tumor metastasis. It has been observed in animal models that tumor cells influence this process by regulating factors such as integrin αIIb and VEGF, which promote extensive lymphangiogenesis within the TDLNs which results in the dilation of lymphatic sinuses and the rapid proliferation of lymphatic endothelial cells (22, 32–34). Although recent research of this phenomenon is limited in human cancer models, it has undoubtably been observed in TDLNs of patients (35–37). Consequently, the increased flow of lymphatic fluid enhances the accumulation of tumor-derived factors in the TDLNs, further suppressing the local immune environment and accelerating tumor metastasis to these lymph nodes (38). Additionally, the expansion and dedifferentiation of high endothelial venules (HEVs) represent key features of tumor-induced lymph node remodeling. Over the course of tumor progression, the density of HEVs increases, followed by their gradual expansion and dedifferentiation, which significantly affect lymphocyte recruitment (39, 40). However, After tumor colonization in TDLNs, tumor cells cause HEV numbers to drop, which limits lymphocyte recruitment into the colonized node (41). Moreover, fibroblastic reticular cells (FRCs) in the TDLN contribute to the deposition of the extracellular matrix components, enhancing their proliferative capacity and driving fibrotic remodeling of the surrounding ductal structures. This remodeling alters the structural flexibility of the lymph nodes, which impacts immune cell trafficking and function (42).

## 3.2 The immunosuppressive cells in TDLNs

### 3.2.1 Tumor immunosuppression mediated by Treg cells in TDLN

A subset of $CD4^+$ T cells, known as regulatory T cells (Tregs) (43, 44), exhibits potent immunosuppressive properties, which contribute to tumor immune evasion. Within the TME, Tregs suppress the immune response against tumors through both direct intercellular interactions and the release of soluble cytokines, thereby playing a critical role in enabling tumors to evade immune surveillance. During tumor progression, tumor-derived signals and lymph node colonization by tumor cells direct the accumulation of Tregs in TDLNs (45–48). It has been observed that Tregs infiltrate TDLNs in significant numbers during cancer progression, and their accumulation often correlates with poorer clinical outcomes (49). Additionally, the Tregs in TDLNs exhibit enhanced immunosuppressive functions, such as upregulated expression of co-inhibitory molecules (e.g., CTLA-4 and PD-1) and increased secretion of immunosuppressive cytokines, including TGF-β and IL-10 (50) (Figure 1). Tregs in TDLNs also hinder the migration of activated $CD8^+$ T cells to the tumor site by downregulating sphingosine-1-phosphate receptor 1 (S1PR1) on these lymphocytes (Figure 1). This impairment in $CD8^+$ T cell trafficking limits their cytotoxic potential and further suppresses the anti-tumor immune response. Moreover, Tregs have been shown to inhibit the function of natural killer (NK) cells in TDLNs, thereby promoting tumor metastasis (51). In TDLNs, Tregs also uptake lactate through the high expression of monocarboxylate transporter 1 (MCT1) (52), which enhances PD-1 expression on Tregs. This mechanism contributes to resistance to PD-1 blockade therapy. Interestingly, while depleting Tregs could disrupt TDLN function and expand the T cell zone, it might also indirectly influence T cell recruitment and disrupt immune surveillance-stroma interactions, potentially accelerating tumor progression (53).

### 3.2.2 Tumor immunosuppression induced by DC subsets in TDLNs

Dendritic cells (DCs), as professional antigen-presenting cells, are central to orchestrating the anti-tumor immune response. DCs capture, process, and present tumor antigens, express the major histocompatibility complex molecules, and provide co-stimulatory signals essential for T cell activation. In TDLNs, DCs also activate naive T cells, initiating the immune response against tumors. However, during tumor progression, DCs undergo a functional shift from immune activation to immune suppression (54–56). The ability of DCs to activate immune responses or induce immune tolerance in TDLNs mainly depends on their activation status (57). In the tumor milieu, insufficient activation of DCs results in ineffective antigen presentation, preventing the proper activation of T cells against tumors. Moreover, tumor-derived signals, such as type II interferons, induce the expression of PD-L1 on DCs, which then migrate to TDLNs. These PD-L1-expressing DCs, predominantly located in the germinal centers and cortical regions of TDLNs, suppress the activation of $CD8^+$ T cells through the PD-1/PD-L1 interaction (20) (Figure 1). Studies have shown that the accumulation of $PD\text{-}L1^+$ conventional DC1 (cDC1) cells in TDLNs is associated with an increase in terminally exhausted $CD8^+$ T cells, as well as an elevated risk of metastasis or disease recurrence in patients with ovarian cancer, oral squamous cell carcinoma, and lung metastatic melanoma (20, 58). Abundant PD-1/PD-L1 interactions of $PD\text{-}1^+$ T cells and $PD\text{-}L1^+$ cDCs in TDLNs has also been linked to poor survival and distant disease recurrence in cancer patients (4). Single-cell analysis has identified a subset of mature DCs, termed regulatory DCs (mregDCs), which accumulate in TDLNs during tumor progression. These mregDCs express immune regulatory genes such as Cd274, Cd200,and Pdcd1lg2, along with mature genes such as Ccr7,Cd40, and Il12b (59). These cells enhance their migratory capacity and migrate to TDLNs, while simultaneously downregulating the expression of toll-like receptors (TLRs) and lectin-like receptors, which impairs their ability to capture and present antigens. Additionally, mregDCs secrete chemokines and express adhesion molecules that attract Treg to form a distinct Treg-mregDC-lymphatic niche. This network further

inhibits the initiation and maintenance of the anti-tumor immune response through the secretion of immunosuppressive cytokines and the expression of immune checkpoint molecules (60).

Despite representing a small proportion of the total DC population, mregDCs exert potent immunosuppressive effects through both direct cell-to-cell interactions and the secretion of immune regulatory factors. These cells suppress the antigen-presenting function of neighboring DCs, inhibit $CD8^+$ T cell responses, and promote the differentiation of Tregs and Th2 cells (61). Some studies have also identified plasmacytoid DCs expressing indoleamine 2,3-dioxygenase (IDO) in TDLNs (Figure 1), which directly activate Treg cells to enhance immunosuppressive functions (62–65). Furthermore, tumor cells can inhibit tumor immunity not only by affecting the function of DCs but also by altering their quantity (66). Thus, the role of DCs in TDLNs is complex and highly heterogeneous, with tumor-derived factors driving the functional plasticity of these cells. Identifying strategies to specifically eliminate or correct immunosuppressive DC populations in TDLNs remains a significant challenge in the field of cancer immunotherapy.

### 3.2.3 Tumor immunosuppression induced by Bregs within the TDLNs

In recent years, regulatory B cells (Bregs), a novel subset of B cell, have garnered increasing recognition for their role in immune regulation. Bregs primarily mediate immune tolerance by generating inhibitory cytokines such as TGF-β and IL-10, which suppress excessive inflammatory responses (67–69). Bregs are crucial in the outcome of chronic inflammatory diseases, and recent studies have highlighted their importance in the TME. In tumors such as gastric cancer, pancreatic cancer, and lung cancer, Breg cells are found to increase abnormally as the tumor progresses (70, 71). Moreover, Bregs exert their immunosuppressive effects primarily by inhibiting the functions of $CD4^+$ T and $CD8^+$ T cells by secreting IL-10, IL-35, and TGF-β (Figure 1). And promote the differentiation and proliferation of Treg cells, further contributing to immune evasion by the tumor (21, 72, 73). Notably, Bregs accumulate in TDLNs, where they play a significant role in promoting tumor growth (74–76). Bregs hinder the synthesis of IL-17 and IFN-γ by Th17 and Th1 cells, respectively, and prevent the differentiation of Th17 cells within the tumor microenvironment in an IL-10-dependent manner (77, 78). Furthermore, Bregs in TDLNs are more prone to differentiate and exert immunosuppressive effects, secreting cytokines such as TGF-β and IL-35 (72, 73). These findings underscore the critical role of Bregs in suppressing immune surveillance and promoting metastasis within the TDLN by modulating immune responses and enhancing immunosuppression in the tumor microenvironment.

## 3.3 Immune suppression mediated by tumor cells in TDLNs

Tumor colonization in lymph nodes induces tumor immune tolerance and further promotes distant metastasis (47). Numerous studies have shown that the primary tumor plays a preparatory role in establishing an immunosuppressive microenvironment within TDLNs to facilitate subsequent lymph node metastasis through the secretion of vesicles, cytokines, and other factors (79–82). One key mechanism involves the activation of lymphangiogenesis via VEGF, which alters the lymphatic matrix in the TDLN (83). Additionally, (Figure 1) TNF-α plays a crucial role in promoting inflammatory reactions within the TDLN by binding to TNF receptors (TNFR1 and TNFR2) on cell surfaces. This interaction activates downstream signaling pathways, leading to the production of inflammatory mediators such as IL-1, IL-6, and IL-8, which inhibit immune cell recruitment to the TDLN (84). Furthermore, tumor cells can remodel the TDLN into an immunosuppressive microenvironment that supports metastasis by secreting immunosuppressive molecules within exosomes. These exosomes, including melanoma-derived exosomes carrying PD-L1, shuttle tumor-derived signals to TDLNs, thereby promoting immune evasion. For example, melanoma-derived exosomes enhance PD-L1 expression on immature myeloid cells (IMCs) in mice, resulting in the inhibition of T cell activation. Interferon-gamma (IFN-γ) further augments PD-L1 expression on these exosomes, further impairing immune responses (81, 85). Once tumor cells metastasize to the lymph nodes, they directly influence the TDLN microenvironment. Tumor cells establish a hypoxic and acidic environment, accompanied by the release of large quantities of immunosuppressive cytokines such as IL-10 and TGF-β. This milieu not only promotes the expansion and activation of Tregs but also induces immune cells in the TDLN to adopt an immunosuppressive phenotype. As a result, the immunological function of the lymph nodes is suppressed, enabling the tumor to evade immune surveillance more effectively (86, 87).

# 4 Tumor treatment strategies based on TDLNs and prospects

## 4.1 Controversy surrounding lymph node dissection

Lymph node dissection is widely recognized as an essential procedure for reducing tumor burden and lowering the risk of distant metastasis (88–90) (Figure 2). Currently, lymph node dissection is classified into selective, systematic, or radical clearance. The prevailing view suggests that systematic or radical dissection provides a better prognosis compared to selective dissection. A 15-year follow-up study found that the gastric cancer-related mortality rate in the D1 group (local lymph node dissection) was significantly higher than in the D2 group (radical lymph node dissection) (91). Similarly, in thymic neuroendocrine tumors, radical lymph node dissection has been identified as the best treatment option (92). In non-small cell lung cancer (NSCLC), lymph node dissection is associated with a lower risk ratio compared to local biopsy or excision (29). However, with the growing understanding of the role of TDLNs, the practice of lymph node dissection has become increasingly controversial. For instance, a clinical study on esophageal cancer found that patients undergoing extensive lymph node dissection did not exhibit

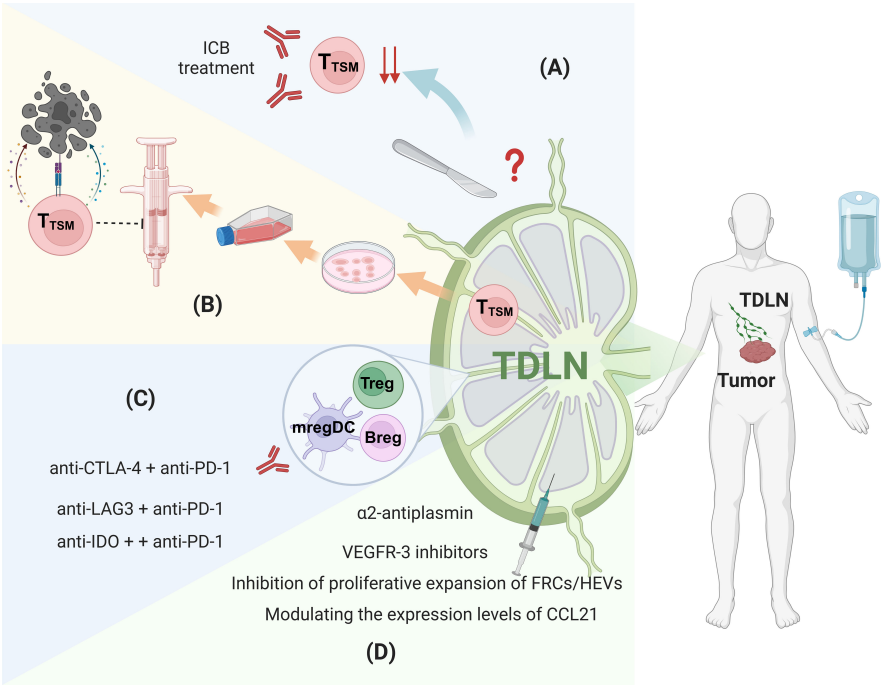


FIGURE 2
Therapeutic strategies targeting the immunosuppressive microenvironment of TDLNs. **(A)** Lymph Node dissection, **(B)** Seed cells for ACT therapy, **(C)** Targeting the immunosuppressive cells, **(D)** Reversing abnormal structure. Figure was created with Biorender.com.

significantly better five-year overall survival rates, suggesting that more aggressive lymph node clearance may not necessarily improve prognosis (93, 94). Similarly, a study on recurrent non-small cell lung cancer patients, who were grouped based on the number of dissected lymph nodes (using a cutoff of 16), revealed that patients with more than 16 lymph nodes dissected showed poorer responses to immunotherapy (95). In endometrial cancer, the prognosis was found to be unaffected by pelvic lymphadenectomy (96). Notably, a clinical study on stage I-II ovarian malignant germ cell neoplasms demonstrated that the 10-year disease-free survival rate was greater in the group that does not undergo lymph node dissection compared to the group that did (97). Likewise, in melanoma, immediate completion lymph-node dissection after sentinel-node metastasis did not increase melanoma-specific survival compared to observation (98). In breast cancer, sentinel lymph node dissection alone was non-inferior to axillary lymph node dissection for 10-year overall survival in certain patients (99). These findings suggest that the traditional "one-size-fits-all" approach to lymph node dissection may not be optimal. TDLNs not only serve as a pre-metastatic niche for tumors but also play a critical role in initiating the anti-tumor immune response. As such, lymph node dissection may inadvertently disrupt key immune events within the TDLNs and eliminate crucial cell populations essential for an effective immunotherapy response. Therefore, considering the functional diversity, cellular heterogeneity, and dynamic plasticity of TDLNs driven by tumor evolution, more comprehensive and systematic studies are required to guide clinical decisions regarding lymph node dissection.

## 4.2 TDLNs as potential sources of seed cells for adoptive cell therapy

Adoptive cell therapy(ACT), a form of adoptive cell therapy, has emerged as a promising cancer treatment in recent years (100). This therapy involves isolating T lymphocytes that specifically recognize tumor antigens from the patient, expanding them ex vivo, and reinfusing them to target and eliminate tumor cells. Currently, peripheral blood or tumor tissue serves as the primary source of ACTs. However, these T cells often exhibit exhaustion due to prolonged exposure to the immunosuppressive TME, resulting in suboptimal therapeutic outcomes and limited durability. The discovery of $T_{TSM}$ within the TDLN, which demonstrate enhanced proliferative capacity and reduced exhaustion, offers a promising alternative source of ACTs. $T_{TSM}$ cells derived from TDLN exhibit remarkable anti-tumor activity in animal models and are more effective in combination with PD-1/PD-L1 ICB therapy (6). This indicates that T cells from TDLNs could serve as superior seed cells for anti-tumor adoptive T cell therapy, providing new insights and considerations for future ACT treatment strategies (Figure 2). Chimeric Antigen Receptor T-cell (CAR-T) therapy is also a highly anticipated treatment approach. However, its current limitations include the limited selection of tumor targets, off-target toxicity, and relatively poor efficacy in solid tumors (101–104). Given that T cells in TDLNs are T cells with strong tumor specificity, utilizing them for CAR-T therapy may offer greater potential than conventional CAR-T treatments. However, research in this area is still in its early stages, and experimental evidence is yet to be established.

### 4.3 Targeting the immunosuppressive cells in TDLNs

Immunosuppressive immune cells, such as Tregs, Bregs, and mregDCs, accumulate in TDLNs and contribute to the establishment of an immunosuppressive microenvironment. Targeting these immunosuppressive cells in both the tumor site and TDLNs could lead to more favorable treatment outcomes.

Animal models have demonstrated that combining CTLA-4 and PD-L1blockade results in a favorable shift in the balance of effector T cells and Tregs within the local tumor microenvironment, showing greater efficacy than monotherapy with either blocker (105). Furthermore, studies have indicated that combined anti-CTLA-4 and anti-PD-1 therapy induces a durable anti-tumor response and enhances adoptive cell therapy in mice (106). Clinical trials also support these findings. For example, a phase III trial involving 945 patients with unresectable stage III and IV melanoma demonstrated that patients receiving the combination of ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) had longer median progression-free survival compared to those receiving either therapy alone (107). Additionally, the combination of nivolumab with relatlimab (anti-LAG3) was approved by the FDA as first-line treatment for stage III/IV melanoma due to its progression-free survival benefit (108). In the TDLN, mregDCs express immune checkpoints such as IDO and PD-L1, making IDO an attractive therapeutic target. In a clinical trial, the combination of nivolumab with a vaccine targeting immune cells expressing IDO and PD-L1 induced immune responses in more than 93% of patients (109). The vaccine-responsive T cells, including $CD4^+$ and $CD8^+$ T cells, were able to target both cancer cells and immune cells expressing these markers. Follow-up studies showed sustained and significant therapeutic effects in some patients (110) (Figure 2). These findings suggest that targeting the immunosuppressive microenvironment in TDLNs in combination with local tumor treatments, such as PD-1/PD-L1 blockade, may improve cancer treatment outcomes. In addition to combination ICB, there are other approaches to targeting immunosuppressive cells. Recent studies have shown that delivering antigens to the interfollicular regions (IFRs) of lymph nodes using specific immunization formulations can enhance Type 1 immune responses (Th1 responses), potentially providing a complementary strategy to reverse immunosuppression in TDLNs (111). Moreover, targeted delivery of adjuvant nanoparticles, vaccine antigens, and other molecules to TDLNs can also help improve the immunosuppressive microenvironment (112, 113).

### 4.4 Reversing abnormal structural changes in TDLNs

Several therapeutic strategies aim to reverse the structural changes in TDLNs caused by tumor derived factors, which contribute to immunosuppression. These strategies focus on inhibiting tumor-induced lymph node remodeling and angiogenesis. For example, the fibrinolytic inhibitor α2-antiplasmin has been shown to restrict lymph node remodeling and cancer metastasis by inhibiting fibrin degradation, thereby preventing lymphatic dilation and abnormal changes (114). In addition, VEGFR-3 inhibitors have been verified to reduce the proliferation of lymphatic endothelial cells (LECs), further impeding vascular remodeling and lymphangiogenesis (115). Targeted therapies that address lymph node structural remodeling can also restore immune cell distribution by blocking the signaling pathways involved in immune cell migration. Inhibiting of FRCs and HEVs can prevent the formation of the immunosuppressive microenvironment by regulating immune cell migration and positioning (116). Of these, treatment of FRCs and HEVs has focused on reducing stress and alleviating tumor-induced impaired lymphocyte recruitment. In mice, treatment with losartan, is an angiotensin receptor blocker that relieves solid stress, reduces collagen in LN metastatic lesions, Restores the presence of different HEV, and beautiful intrinsic lyric infiltration (41). Furthermore, modulating the expression levels of chemokines, such as CCL21, can improve immune cell distribution and function, thereby restoring normal TDLN structure. Interventions targeting physical changes, such as reducing FRC proliferation and abnormal extracellular matrix (ECM) deposition, can alleviate the immunosuppressive state in TDLNs, promoting the recovery of anti-tumor immune responses (Figure 2).

## 5 Conclusion and discussion

TDLNs serve as critical crossroads in cancer, orchestrating anti-tumor immune responses while simultaneously being exploited by tumors to foster immunosuppression and metastasis. These nodes are essential for initiating immunity, particularly through T cells that underpin the efficacy of therapies like PD-1/PD-L1 blockade. Yet, tumors disrupt this role by inducing structural remodeling—such as lymphangiogenesis, and recruiting immunosuppressive cells, including Tregs, Bregs, and immunosuppressive DC subtypes, creating a suppressive microenvironment that hampers immune function. Emerging therapeutic strategies offer promise, such as leveraging TDLN-derived T cells for adoptive cell therapy and targeting immunosuppressive pathways to enhance immunotherapy outcomes. However, the TDLN's complexity and dynamic nature present challenges, including difficulties in drug delivery and the need for reliable biomarkers to predict responses. Advancing these approaches will require a deeper understanding of TDLN plasticity and the exploration of combination therapies to synergistically improve cancer treatment efficacy.

## Author contributions

JW: Writing – original draft. DL: Writing – original draft. HL: Conceptualization, Writing – review & editing. MH: Conceptualization, Writing – review & editing.

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# Revisiting the role of cancer-associated fibroblasts in tumor microenvironment

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Cancer-associated fibroblasts (CAFs) are integral components of the tumor microenvironment playing key roles in tumor progression, metastasis, and therapeutic resistance. However, challenges persist in understanding their heterogeneity, origin, and functional diversity. One major obstacle is the lack of standardized naming conventions for CAF subpopulations, with current systems failing to capture their full complexity. Additionally, the identification of CAFs is hindered by the absence of specific biomarkers, limiting the precision of diagnostic and therapeutic strategies. *In vitro* culture conditions often fail to maintain the *in vivo* characteristics of CAFs, which complicates their study and the translation of findings to clinical practice. Although current detection methods, such as antibodies, mRNA probes, and single-cell transcriptomics, offer insights into CAF biology, they lack standardization and fail to provide reliable quantitative measures. Furthermore, the dynamic interactions between CAFs, tumor cells, and immune cells within the TME remain insufficiently understood, and the role of CAFs in immune evasion and therapy resistance is an area of ongoing research. Understanding how CAFs influence drug resistance and the immune response is essential for developing more effective cancer therapies. This review aims to provide an in-depth analysis of the challenges in CAF research, propose future research directions, and emphasize the need for improved CAF-targeted therapeutic strategies. By addressing these gaps, it seeks to highlight the potential of CAFs as targets for overcoming therapeutic resistance and enhancing the efficacy of cancer treatments.

KEYWORDS

**cancer-associated fibroblasts, tumor microenvironment, therapeutic resistance, biomarkers, immune modulation, targeted therapies**

## Introduction

Cancer-associated fibroblasts (CAFs) are a critical component of the tumor microenvironment (TME) and play pivotal roles in cancer progression, metastasis, and therapeutic resistance (1–3). As the most abundant stromal cells within tumors, CAFs are involved in various processes that facilitate tumor growth, including remodeling of the

extracellular matrix (ECM), promoting angiogenesis, and influencing immune responses. Additionally, CAFs contribute to the metabolic reprogramming of the tumor, establishing a pro-tumorigenic microenvironment (4). Recent research has highlighted the complex and often dualistic nature of CAFs, as they can exhibit both tumor-promoting and tumor-suppressing activities depending on their activation state, subpopulations, and interactions within the TME (2).

Despite their central role in cancer biology, significant challenges remain in understanding CAFs due to their heterogeneity, dynamic plasticity, and the lack of specific markers for accurate identification (5). The classification of CAF subpopulations is further complicated by the absence of standardized naming conventions, making it difficult to compare findings across studies (2). Moreover, CAFs exhibit a high degree of functional diversity that varies between different tumor types and stages, complicating efforts to develop universal therapeutic strategies targeting CAFs (6). Their ability to modulate immune responses, promote resistance to conventional therapies, and contribute to immune evasion has made them a significant target in cancer treatment (7). For instance, CAFs can inhibit the infiltration of immune cells into the tumor, enhance the immunosuppressive microenvironment, and reduce the efficacy of both chemotherapy and immunotherapy.

The potential for CAF-targeted therapies is considerable, but several obstacles remain, including the difficulty in isolating CAFs and maintaining their characteristics *in vivo* and *in vitro* (4). Furthermore, understanding the molecular mechanisms underlying CAF interactions with tumor cells, immune cells, and the ECM is essential for the development of effective CAF-targeted treatments. This review aims to provide a comprehensive analysis of the current challenges in CAF research, exploring their roles in tumor progression, therapeutic resistance, and immune modulation, while also discussing promising future directions for targeted therapies that aim to overcome the barriers CAFs present in cancer treatment.

## Fibroblasts and CAFs

Despite ongoing discussions about the precise definition of fibroblasts, they were originally identified about 150 years ago as spindle-shaped cells that produce collagen in connective tissues (8) (Figure 1). Current evidence suggests that fibroblasts in healthy tissues are dormant mesenchymal cells located within the extracellular matrix's interstitial fibers. These cells can become activated in response to specific conditions such as wound healing, tissue inflammation, and organ fibrosis. In the context of

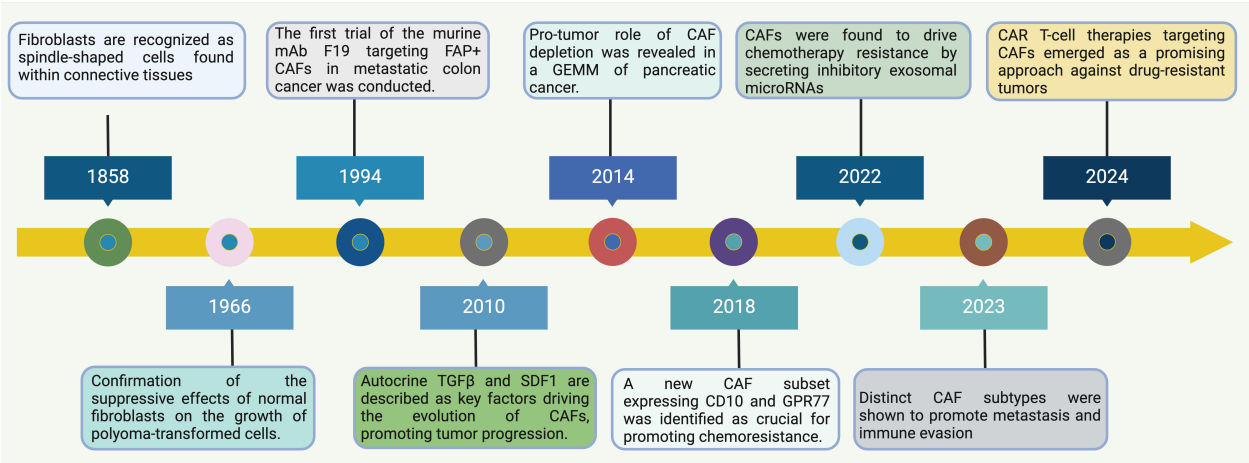


FIGURE 1
Timeline of Discoveries in Normal Fibroblasts and CAFs. This timeline illustrates significant milestones and the evolving focus of research on fibroblasts, especially in relation to their role in cancer. Starting from the historical identification of fibroblasts as spindle-shaped cells in connective tissues in 1858, the understanding of CAFs has evolved significantly (15). By 1966, research confirmed the suppressive effects of normal fibroblasts on the growth of polyoma-transformed cells, highlighting a functional aspect of fibroblasts in modulating cellular transformation (16). Progressing through the timeline, by 1994, the first trial targeting FAP+ CAFs using murine mAb F19 in metastatic colon cancer was conducted, illustrating a move towards targeted CAF therapies (17). In 2010, autocrine factors like TGFβ and SDF1 were identified as key drivers in the evolution of CAFs, promoting tumor progression, showcasing the increasing understanding of the molecular mechanisms by which CAFs influence cancer dynamics (18). Further advances in 2014 highlighted the role of CAFs in the depletion processes in a genetically engineered mouse model of pancreatic cancer, signifying the impact of CAFs beyond the tumor cells themselves (19). By 2018, the identification of a new CAF subset expressing CD10 and GPR77 was crucial for understanding chemoresistance, pointing towards the heterogeneity within CAF populations and their varied roles in cancer progression (20). Recent discoveries in 2022 and 2023 have established that CAFs drive chemotherapy resistance by secreting inhibitory exosomal microRNAs and promote metastasis and immune evasion through distinct CAF subtypes, respectively (21). These findings underscore the multifaceted roles of CAFs in facilitating tumor aggression and resisting therapeutic interventions. Finally, CAR T-cell therapies targeting CAFs have emerged as a promising approach against drug-resistant tumors, marking a pivotal shift towards leveraging immune system modifications to combat the complex interactions within the TME driven by CAFs (22). This shift not only represents a novel therapeutic strategy but also highlights the ongoing evolution of understanding in the field of tumor immunology and the critical role CAFs play in shaping cancer outcomes. Abbreviations: CAF, Cancer-Associated Fibroblast; FAP, Fibroblast Activation Protein; GEMM, Genetically Engineered Mouse Model; CAR, Chimeric Antigen Receptor; TGF, Transforming Growth Factor; SDF1, Stromal Cell-Derived Factor 1; GPR77, G Protein-Coupled Receptor 77

cancer, analogous processes include cancer development (referred to as the 'cancer wound'), inflammation that promotes tumor growth, and tumor-associated fibrosis (9–11). Activated fibroblasts in cancer settings are variously known as CAFs, tumor-associated fibroblasts, peritumoral fibroblasts, myofibroblasts, or reactive stromal fibroblasts (12, 13). These CAFs adapt alongside cancer cells, adopting a pro-tumor phenotype that allows them to thrive and expand within the complex tumor microenvironment, thereby facilitating tumor progression (14).

Fibroblasts are spindle-shaped, non-epithelial, non-immune cells embedded within the extracellular matrix (ECM) that are easily propagated in adherent cell culture (23, 24). They play a key role in the stroma of gastrointestinal organs, where they are well-organized, much like in other tissues. Throughout the gastrointestinal tract, a reticular network of stromal cells is closely associated with the epithelial basement membrane (25). The subepithelial plexus, consisting of reticular stromal cells, completely surrounds the glandular axis from the stomach to the rectum (26). This compartment is dynamic, with a radial axis of proliferation and differentiation similar to that of the epithelium, originating from gremlin 1-expressing intestinal reticular stem cells (27). These stem cells give rise to intestinal reticular cells, likely overlapping with FOXL1+ subepithelial telocytes and GLI1+ mesenchymal cells, which together form an essential mesenchymal niche supporting intestinal stem cells (26–28). Beneath this compartment is a loosely organized network of fibroblasts within the lamina propria that interact with one another as well as with deeper stromal elements, including smooth muscle, blood vessels, nerves, and inflammatory cells (25, 29, 30). Functionally, fibroblasts are crucial in regulating ECM synthesis and facilitating paracrine and juxtacrine signaling to adjacent epithelial cells, thereby influencing their growth and differentiation (23, 31). They are also poised to respond to tissue damage, whether due to injury or tumorigenesis.

Cancer-associated fibroblasts (CAFs) are broadly recognized as the fibroblasts located within and surrounding tumors (14). This group comprises native, normal fibroblasts, as well as activated, proliferating (Ki67+) or recruited fibroblasts in response to cancer-derived stimuli. These newly formed CAFs may arise through several mechanisms, which will be discussed later. Despite the advancements in immune cell immunophenotyping and subtyping, a definitive marker for CAFs remains elusive (14, 32, 33). This gap has led to the identification of overlapping, incomplete, or distinct CAF populations in different studies, with markers that label both CAFs and other cell types. These challenges have complicated the interpretation of many studies, which will be addressed further.

## The origin of CAFs

Due to the absence of specific biomarkers, identifying the origin of CAFs remains challenging. Current evidence primarily supports that fibroblasts originate from primitive mesenchymal cells, while CAFs arise from activated fibroblasts within local tissues (34, 35). Studies have shown that normal fibroblasts can proliferate, become activated, and express CAF markers by internalizing exosomes released from bladder cancer cells. Further research indicates that bladder cancer cells transform normal fibroblasts into CAFs through exosome-mediated transmission of transforming growth factor-beta (TGF-β) and SMAD signaling pathways (35, 36). Figure 2 illustrates the intricate and multifactorial processes involved in the transformation from normal fibroblasts (NFs) to CAFs. The recent research highlights the roles of key molecules and pathways, including transforming growth factor-beta 1 (TGF-β1), osteopontin, and interleukin-1β (IL-1β) (37–39). These elements initiate the transformation by engaging their respective receptors on NFs, subsequently activating downstream signaling cascades such as TGF-β/Smads and NF-κB, pivotal for modulating gene expression linked to the CAF phenotype (40). Exosomes from cancer cells, carrying miRNAs and lncRNAs, significantly contribute to the transformation of NFs into CAFs, mediated by pathways including TGF-β/Smads, JAK/STAT, and MAPK (41–43). These exosomes facilitate a feedback loop that enhances the conversion process. Additionally, the diagram delineates how alterations in glucose metabolism, driven by the hypoxia-inducible factor-1α (HIF-1α) pathway, are crucial for metabolic reprogramming essential for the survival and function of CAFs (44). HIF-1α are stabilized through both hypoxia-dependent mechanisms, where low oxygen levels inhibit their degradation, and hypoxia-independent pathways, such as activation by growth factors that affect the proteasome pathway (45). Key target genes involved in the transition from NFs to CAFs include VEGF for angiogenesis, GLUT1 for glucose metabolism, LOX for extracellular matrix remodeling, and CA9 for pH regulation (42, 46, 47). These genes contribute to the CAF phenotype, enhancing their ability to support tumor progression. Moreover, the transition to a CAF phenotype is driven by changes in cellular homeostasis, regulated by the activation of cytoskeletal proteins and the secreted phenotype, primarily through JAK/STAT and p53 signaling pathways. This comprehensive portrayal underscores the dynamic network of interactions that define CAF biology (Figure 2).

Additionally, a novel microfluidic model has been developed to regulate the three-dimensional tumor microenvironment (TME) *in vitro*, revealing that exosomes derived from melanoma can drive the differentiation of endothelial cells into CAFs through endothelial-mesenchymal transition (EndMT). Moreover, exosomes derived from mesenchymal stem cells (MSCs) have been shown to inhibit EndMT and induce CAFs to undergo reverse differentiation back into endothelial cells (48). This suggests that exosomes with the ability to reverse CAF differentiation could serve as effective carriers for anti-tumor drugs. Furthermore, epithelial cells in the TME can differentiate into CAFs via epithelial-mesenchymal transition (EMT) (49). Subsequently, CAFs secrete cytokines that promote EMT in tumor cells, ultimately facilitating tumor invasion and metastasis (50). Other studies have demonstrated that TGFβ1 can induce the differentiation of bone marrow-derived MSCs into CAFs by activating the JAK/STAT3 signaling pathway, promoting the migration and invasion of colorectal cancer cells (51). Various

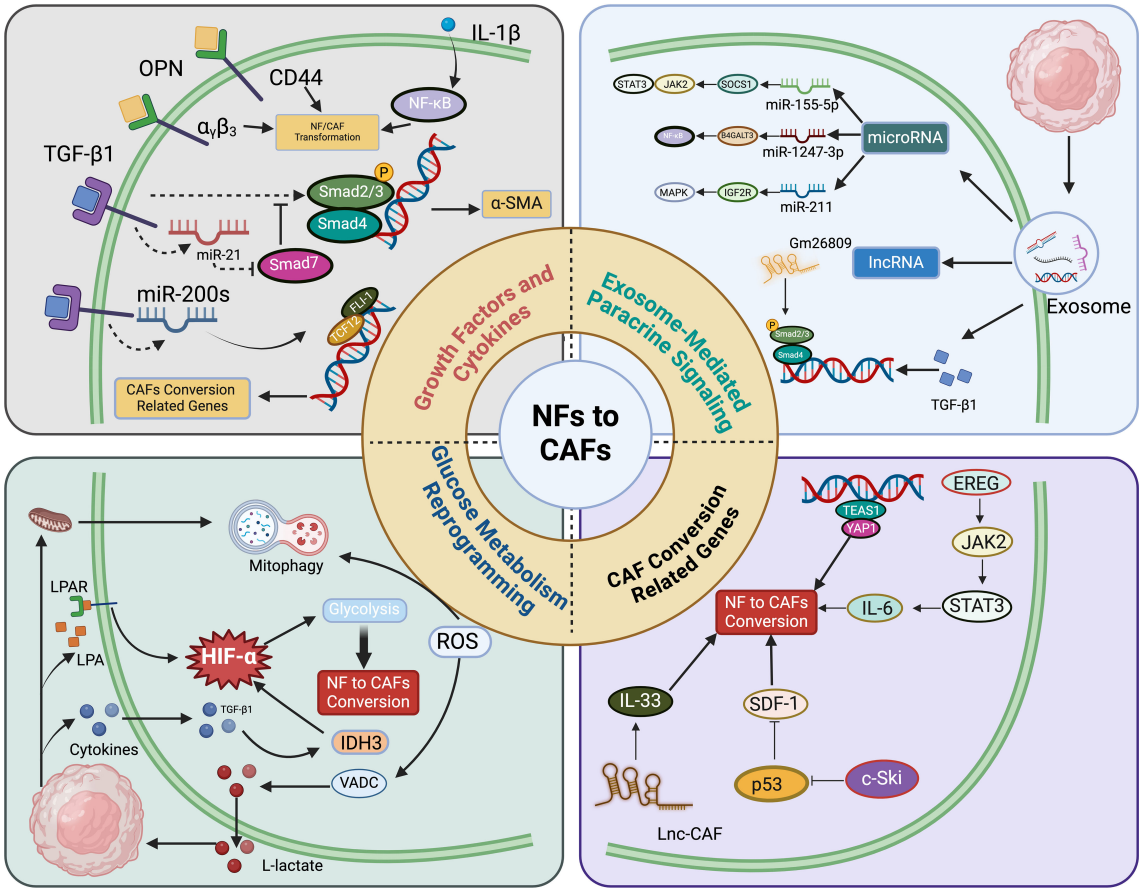


FIGURE 2
Conversion from normal fibroblasts (NFs) to cancer-associated fibroblasts (CAFs) involves multiple molecular mechanisms. a) Growth factors and cytokines like transforming growth factor-beta 1 (TGF-β1), osteopontin (OPN), and IL-1β interact with their respective receptors in NFs, subsequently activating downstream effectors such as miRNAs and CD44 (61–63). These molecules modulate the expression of genes associated with the CAF phenotype through the TGF-β/Smads and NF-κB signaling pathways (64, 65). b) Exosomes derived from cancer cells, carrying entities such as miRNAs and lncRNAs, induce the transformation of NFs into CAFs (65). This conversion is facilitated by signaling pathways including TGF-β/Smads, JAK/STAT, NF-κB, and MAPK cascades. c) The shift from NF to CAF is also driven by alterations in glucose metabolism, with the HIF-1α signaling pathway playing a crucial role in this metabolic reprogramming (66). d) Variations in cellular homeostasis prompt a self-driven transition to CAFs by regulating the activation of cytoskeletal proteins and the secreted phenotype, primarily through the JAK/STAT and p53 signaling pathways (67).

studies also support that PDGFα-CAF cells originate from MSCs (52). Additionally, several other sources of CAFs have been identified, including hematopoietic stem cells (HSCs), cancer stem cells (CSCs), adipocytes, pericytes, and stellate cells (47, 53–60). However, there is limited evidence supporting these origins, and their relevance to different tumor types remains uncertain.

In summary, CAFs originate from fibroblasts, epithelial cells, endothelial cells, bone marrow-derived MSCs, HSCs, CSCs, adipocytes, pericytes, and stellate cells (47, 53–60). This diversity in origin contributes to the heterogeneity of CAFs, with different origins being regulated by distinct signals or factors, resulting in varied differentiation pathways. Further research into CAF origins may provide insights into the discovery of biomarkers, therapeutic targets, signaling pathways, and activation mechanisms, all of which hold significant clinical potential. However, regardless of their cellular origin, the state, phenotype, and function of CAFs dynamically evolve throughout tumor progression, varying across different pathological stages (Figure 3).

CAFs originate from a variety of sources, including resident fibroblasts, epithelial cells, endothelial cells, bone marrow-derived mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), cancer stem cells (CSCs), adipocytes, pericytes, and stellate cells. Each origin is associated with distinct signaling pathways.Common CAF markers and gene signatures

In general, normal fibroblasts exhibit significant heterogeneity, which is reflected in differences in their morphology, behavior, and gene expression (as reviewed in (68)). In addition to the intrinsic heterogeneity within fibroblasts, other stromal cells share similarities with fibroblasts, particularly in the expression of so-called ‘specific’ markers. As a result, the complexity of the stromal compartment, along with the existence of multiple fibroblast subpopulations, makes it challenging to accurately isolate and define CAFs based on specific marker expression. Despite these challenges, numerous CAF markers are well-documented in the literature. Among the downregulated markers are caveolin-1 (69) and laminin (70), while frequently reported upregulated CAF

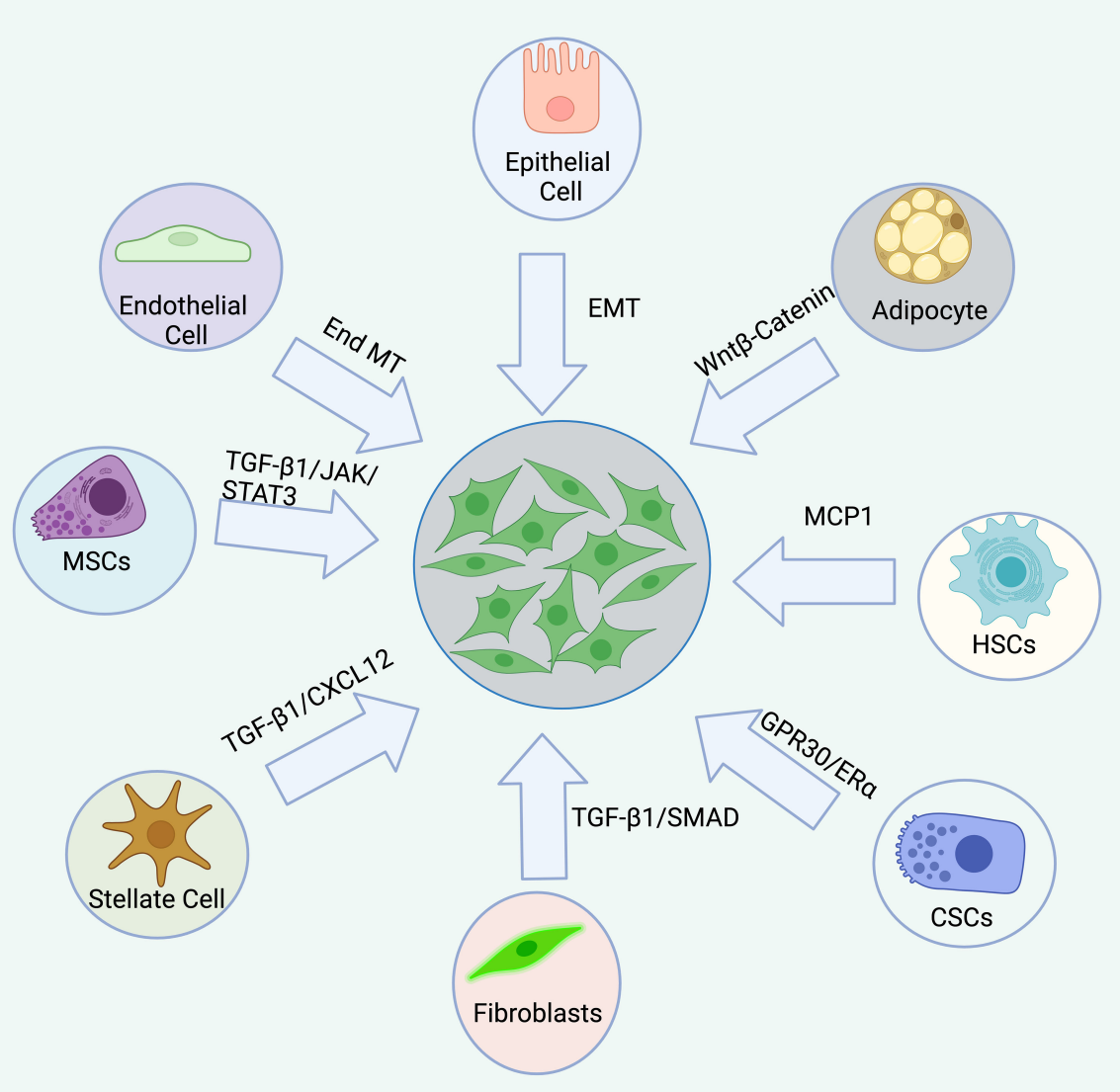


FIGURE 3
The origin of CAFs are complex, diverse, and heterogeneous. CAFs originate from a variety of sources, including resident fibroblasts, epithelial cells, endothelial cells, bone marrow-derived mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), cancer stem cells (CSCs), adipocytes, pericytes, and stellate cells. Each origin is associated with distinct signaling pathways.

markers include alpha-smooth muscle actin (aSMA) (71), vimentin (72), and fibroblast-specific protein 1 (FSP1) (32). However, Sugimoto et al., using a cancer mouse model, compared six markers from various mesenchymal cell groups residing in the stroma. They found that aSMA, platelet-derived growth factor receptor beta (PDGFRβ), and chondroitin sulfate proteoglycan 4 were expressed by fibroblasts, myofibroblasts, and vascular-associated cells, while vimentin and type I collagen also lacked specificity for fibroblasts (32). These findings raise questions about the specificity of these commonly accepted CAF markers. Moreover, aSMA was found to be downregulated, rather than upregulated, in the stromal compartment of prostate cancer (73). This highlights the heterogeneity within the stromal compartment and suggests that no definitive marker exists to clearly distinguish between mesenchymal subpopulations. Despite the fact that aSMA is often elevated in a cancer-type-specific manner and is considered a general marker for mesenchymal cells, it is incorrectly regarded as a universal marker for all CAFs (12, 74).

Several studies have identified a 'CAF gene expression profile' (75–77) and even 'CAF signatures' that can predict tumor outcomes (78, 79). A closer analysis of these studies reveals that, at the individual gene level, the differences between these profiles outweigh the similarities. However, when viewed from a broader perspective, examining gene families rather than individual genes, different cancer types and stages reveal similar groups of genes involved in processes such as cell adhesion, immune response, and extracellular matrix (ECM) modulation (75, 80, 81). This pattern aligns with expectations, as various cell types exposed to similar conditions tend to converge in performing the same functional tasks, albeit through different sets of genes.

## Function of CAFs

The functions of CAFs has employed a range of methodologies from basic cell culture experiments and animal models to correlational studies within extensive patient groups. These methodologies have disclosed a broad spectrum of CAF functions. The relatively straightforward process of culturing CAFs and corresponding normal fibroblasts from patient samples has significantly advanced the understanding of CAF mechanisms. Notably, CAFs are extremely efficient at depositing and remodeling the extracellular matrix (ECM) in the tumor microenvironment. This capability is regulated by RHO and RAB GTPase-mediated mechanisms controlling integrin-linked adhesions and the actomyosin cytoskeleton, which is associated with the suppression of the CD36 transmembrane receptor (82–85). Furthermore, CAFs synthesize enzymes that form matrix crosslinks and engage in force-driven ECM remodeling, contributing to the increased rigidity of tumor tissues. Although these chemical crosslinks are stable, the secretion of matrix proteases by CAFs facilitates dynamic remodeling of the tumor matrix, creating pathways that enable cancer cell migration. Eph–ephrin signaling mediated by direct cell contact also affects cancer cell motility (86). Beyond fostering local invasion, CAFs enhance metastasis in experimental settings, a process linked to their ECM remodeling activities (87–90). At secondary metastatic sites, newly activated fibroblasts support the development of extensive metastases through various mechanisms, including the production of matrix molecules like tenascin and periostin, which fortify cancer cell signaling via WNT pathways (91–93). Recent findings suggest that alterations in ECM structure can also impact the migration of immune cells, with significant implications for tumor immune surveillance (90, 94, 95).

Alterations in matrix production and tumor mechanics driven largely by CAF activities have multifaceted implications for cancer. Enhanced tissue stiffness activates signaling pathways in cancer cells that promote survival and proliferation (96). Additionally, increased mechanical stress can compress blood vessels, inducing hypoxia, which fosters more aggressive cancer phenotypes and hampers drug delivery (97–99). Changes in tissue mechanics might also play a role in the onset of cancer and pre-malignant conditions, as evidenced by the relationship between mammographic density and breast cancer risk (85). Strategies aimed at disrupting the interactions between CAFs and the mechanical properties of tumors for therapeutic benefit are currently under investigation.

CAFs are also significant producers of growth factors, cytokines, and exosomes that encourage tumor growth and influence responses to therapy (42, 100, 101). They secrete TGF-β, leukemia inhibitory factor (LIF), growth arrest-specific protein 6 (GAS6), fibroblast growth factor 5 (FGF5), growth differentiation factor 15 (GDF15), and hepatocyte growth factor (HGF), which drive the invasive and proliferative behaviors in cancer cells (102–107). HGF, in particular, is noted for promoting resistance to BRAF-targeted therapies by activating an alternative BRAF-independent pathway for ERK–MAPK signaling (108, 109).

The secretome of CAFs additionally affects other components of the tumor microenvironment (110–114). VEGF from stromal cells can initiate angiogenesis, while a variety of cytokines and chemokines from CAFs act on different leukocytes, including CD8+ T cells, regulatory T cells, and macrophages, yielding both suppressive and promotional immune responses (114). However, the overarching impact of CAFs is immunosuppressive, mediated by factors like IL-6, CXCL9, and TGFβ, which notably reduce T cell activity (115). Recent observations of antigen cross-presentation by CAFs have highlighted their role in modulating CD4+ and CD8+ T cell responses (116, 117). Clinical studies further corroborate a negative correlation between CAF presence and CD8+ T cell levels (118). Moreover, IL-6 may also foster systemic immunosuppression through metabolic effects (119). Disrupting CXCL12 from CAFs enhances T cell-mediated tumor control, and targeting focal adhesion kinase (FAK) in cancer cells simultaneously reduces stromal fibroblast activation and the formation of an immunosuppressive milieu (120). Nonetheless, the role of tumor necrosis factor (TNF) produced by CAFs is complex; while TNF can activate fibroblasts, its tumor-promoting, immunosuppressive effects are tied to the suppression of TNF signaling (121, 122).

Lastly, the metabolic exchange between cancer cells and CAFs provides another layer of interaction, where autophagy in stromal fibroblasts produces alanine used by pancreatic ductal adenocarcinoma cells to energize the tricarboxylic acid cycle (123–125). Additionally, metabolic irregularities in CAFs may link to altered immune regulation, potentially through IL-6 production or the depletion of immunomodulatory amino acids (126).

## CAFs in cancer

CAFs play a multifaceted role in cancer progression and metastasis by fostering a conducive tumor microenvironment through several mechanisms (103, 127, 128). Firstly, CAFs contribute to cancer cell stemness and metastatic capabilities by engaging in paracrine signaling with cancer stem cells, enhancing their self-renewal and propagation abilities which are crucial for tumor aggressiveness and metastasis (42, 129). Secondly, they promote tumor angiogenesis by secreting pro-angiogenic factors like VEGFA and PDGF and altering the extracellular matrix to enhance vascular formation, facilitating tumor growth and the dissemination of cancer cells (130–132). Thirdly, CAFs are instrumental in mediating immunosuppression within the tumor microenvironment by secreting factors such as TGF-β and IL-6, which modulate immune cell function and contribute to immune evasion by the tumor (133–135). Lastly, they are involved in metabolic reprogramming known as the "Reverse Warburg Effect," where they supply cancer cells with metabolic intermediates necessary for energy production, thus supporting the energetic demands of rapidly proliferating tumor cells (136, 137). Through these interactions, CAFs are key players in enabling cancer progression and the establishment of metastatic sites, making them significant targets for therapeutic intervention.

## CAFs contribute to cancer stemness

CAFs are integral to the tumor microenvironment (TME) and play a pivotal role in the maintenance and enhancement of cancer cell stemness, characterized by self-renewal and the ability to propagate, which are key traits of cancer stem cells (CSCs) (138–140). These cells are known to drive tumor aggression, contribute to resistance against therapies, and facilitate metastasis. CSCs are identified through various markers such as CD44, CD24, CD133, LGR5, SOX2, AQP5, ESA, PAF1, and CXCR4, though these markers lack high specificity (141). CAFs interact with CSCs predominantly through paracrine signaling, supporting a conducive niche for tumor growth (142). Research has highlighted that certain CAF subsets secrete molecules that directly enhance CSC properties. For example, a specific subset of CAFs expressing CD10 and GPR77, activated by NF-kB, has been shown to enrich CSCs in breast and lung cancers by releasing IL-6 and IL-8 (20). Similarly, in bladder cancer, CAFs stimulated by interferon from cancer cells can enhance stemness through the WNT5a/β-catenin signaling pathway (143). In the context of hepatocellular carcinoma, CAFs boost cancer cell stemness via the ERK1/2-FRA1-HEY1 pathway by secreting hepatocyte growth factor (144). Additionally, CAFs are known to produce exosomes that perpetuate stemness across various cancer types (145, 146). They also indirectly facilitate the recruitment and stemness of myeloid-derived suppressor cells through FAP-dependent mechanisms (146). Given the significant role of these paracrine interactions, targeting such pathways might offer new therapeutic avenues, particularly through manipulation of WNT signaling which is crucial in mediating interactions between CAFs and CSCs, affecting both the active and dormant CSCs (147).

## CAFs promote angiogenesis

Angiogenesis, the formation of new blood vessels, is essential for tumors to secure a greater supply of oxygen and nutrients. This process is predominantly driven by hypoxia within the tumor environment. Under these low-oxygen conditions, cancer cells release vascular endothelial growth factor A (VEGFA), which targets VEGF receptor 2 (VEGFR2) on adjacent endothelial cells (ECs) or on circulating endothelial progenitor cells derived from the bone marrow, thus initiating angiogenesis (148). This angiogenic cascade includes degradation of the basal lamina and extracellular matrix, EC proliferation, the development of vascular sprouts, and eventual vessel stabilization. Additionally, molecules like delta-like ligand 4 (DLL4) and angiopoietin 2 (ANGPT2) are vital for angiogenesis (149).

CAFs secrete a range of pro-angiogenic growth factors such as VEGFA, CXC-chemokine ligand 12 (CXCL12), fibroblast growth factor 2 (FGF2), and platelet-derived growth factor (PDGF) (150). CXCL12, also recognized as stromal cell-derived factor 1 (SDF-1), promotes tumor proliferation and angiogenesis via the CXCL12/CXCR4 pathway (151). This interaction triggers diverse signaling pathways, including the G-protein coupled/PI3K/AKT/NF-κB axis and the Ras-MEK1/2-Erk1/2 axis, leading to angiogenic responses (152). FGF2, part of the heparin-binding growth factor family, engages FGF receptors to stimulate multiple angiogenic activities and interacts with the VEGF pathway (153). Moreover, the PDGF/PDGFR signaling pathway is crucial in the development of connective tissue and wound healing, with studies indicating that PDGF-C upregulation in CAFs can promote angiogenesis even in the absence of VEGF activity (154, 155).

Beyond direct activation through paracrine signaling, CAFs also indirectly enhance angiogenesis via biomechanical properties of the tumor microenvironment, such as matrix stiffness (156). CAFs produce enzymes like lysyl oxidase (LOX) and lysyl hydroxylase 2 (LH2), which increase collagen and elastin cross-linking, thus raising matrix stiffness (157, 158). Research has shown that higher matrix stiffness correlates with enhanced VEGF binding by endothelial cells, which is influenced by β1 integrins (159). This relationship extends to complex pathways involving Ca2+ influx and HIF-1α ubiquitination in hepatocellular carcinoma angiogenesis, highlighting the multifaceted role of the ECM in angiogenic regulation (160). However, conflicting findings in studies like those by Bao et al., which suggest that increased stiffness may suppress VEGF secretion in certain cancer types, underscore the complexity of these interactions and the need for more nuanced research (161).

## CAFs dedicate in metabolic changes in cancer

Despite residing in a nutrient-limited tumor microenvironment (TME), cancer cells demonstrate a remarkable capability for continuous proliferation, aided by metabolic adaptations within the TME. Notably, Warburg et al., about a century ago, documented that cancer cells preferentially convert glucose to lactate to generate ATP—even in the presence of sufficient oxygen—a process now known as the "*Warburg Effect*" (162). Warburg attributed this metabolic peculiarity to mitochondrial dysfunction in cancer cells. However, subsequent studies in cancer metabolism have revealed that not all tumor cells are wholly reliant on this pathway; some retain mitochondrial functionality and can engage in oxidative phosphorylation (OXPHOS), illustrating the Warburg Effect's variability across different tumor environments (163).

This reverse *Warburg Effect* is primarily propelled by oxidative stress induced by cancer cells. The release of reactive oxygen species (ROS) by cancer cells heightens oxidative stress in stromal components, causing autophagosomes to merge with lysosomes, which leads to mitochondrial degradation in CAFs. This process also results in the breakdown of caveolin-1 (Cav-1) via the HIF-1α/NF-κB pathway (136). The subsequent reduction of Cav-1 in CAFs further increases ROS in cancer cells, fostering a feedback loop that amplifies oxidative stress and disrupts the NF-kB pathway (164). Additionally, TGF-β, a key regulator in cancer metabolism, influences ROS levels by modulating the expression of α-SMA and NOX4 in fibroblasts, thereby promoting oxidative stress (165–167).

Through the reverse *Warburg Effect*, CAFs under oxidative stress due to cancer-derived ROS undergo aerobic glycolysis, producing lactate and pyruvate. These metabolites are then utilized by neighboring oxidative cancer cells for further metabolic activities. While the transmission of ROS in this process has been documented, the detailed mechanisms by which cancer cells and CAFs initiate and adapt to these metabolic changes remain less explored. Nevertheless, targeting the Reverse Warburg Effect presents a theoretical possibility to disrupt cancer metabolism, potentially offering new avenues for therapeutic intervention aimed at curbing tumor growth by altering the metabolic interplay between cancer cells and their stromal environment.

## CAFs mediate immunosuppression

Chronic inflammation, immune cell infiltration, and the ability of cancer cells to evade immune surveillance are recognized as key hallmarks of cancer progression (168). Research has demonstrated the dual role of the immune system in both suppressing and promoting tumor development, a phenomenon termed "cancer immunoediting." This process involves three distinct stages: elimination, equilibrium, and escape (169).

During the elimination phase, innate and adaptive immune responses work in tandem to identify and destroy dysplastic cells before they progress to clinically detectable tumors. However, certain cancer cells may acquire immune-evasive or poorly immunogenic traits, allowing them to survive immune attacks and transition into the equilibrium phase. In this stage, the growth of neoplastic cells is restricted, and their immunogenicity is shaped under the selective pressure exerted by adaptive immunity, predominantly involving T cells and associated cytokines. Persistent immune selection pressure can lead these cancer cells to develop immunosuppressive or immune-evasive phenotypes, ultimately facilitating their progression to the immune escape phase. At this stage, cancer cells evade immune control entirely, resulting in unchecked growth, the formation of clinically evident tumors, and potentially metastasis (170). Despite significant progress, the complex mechanisms underlying cancer immunoediting remain incompletely understood, posing a barrier to developing effective immunotherapy strategies.

CAFs, as major components of the TME, play a pivotal role in promoting immune evasion. One of their key immunosuppressive mediators is TGF-β, which modulates the immune microenvironment by affecting T cell differentiation and proliferation through the inhibition of transcription factor activation triggered by Ca2+ influx (171). In ovarian tumors with T cell exclusion, elevated TGF-β expression and stromal activation are critical drivers of T cell exclusion. TGF-β reduces MHC-I expression on ovarian cancer cells *in vitro* and activates fibroblasts to promote extracellular matrix production, forming a physical barrier that impedes T cell infiltration (172). Additionally, TGF-β suppresses dendritic cell function, inhibits the development of cytotoxic natural killer (NK) cells and their secretion of IFN-γ, and polarizes macrophages towards an M2 phenotype with immunosuppressive, anti-inflammatory, and pro-angiogenic properties (173, 174). Beyond TGF-β, CAF-derived CXCL12 is a potent chemokine that modulates immune suppression. It restricts the migration of CD8+ T cells, sequestering them away from the tumor stroma, and inhibits NK cell proliferation, keeping them in a quiescent state (175, 176). Another significant CAF-secreted molecule in the immune microenvironment is IL-6, which is highly expressed in the inflammatory CAF (iCAF) subtype (176). IL-6 contributes to the accumulation of tumor-infiltrating lymphocytes and regulates neutrophil survival, activation, and function via the IL-6/STAT3/PD-L1 signaling axis (177, 178). Furthermore, CAFs secrete inhibitory immune checkpoints (iICPs) such as PD-1 and LAG3, further enhancing immune suppression within the TME (179).

These findings underscore the critical role of CAFs in facilitating tumor immune escape by creating an immunosuppressive microenvironment, thereby supporting cancer progression and resistance to immune-mediated therapies.

Non-follicular adaptive immune stimulating CAFs and Antigen-presenting CAF

In the context of cancer, non-follicular adaptive immune-stimulating CAFs and antigen-presenting CAFs (apCAFs) play distinct roles in modulating the immune response (180, 181). Non-follicular adaptive immune-stimulating CAFs, which predominantly reside outside organized lymphoid structures like cancer-associated tertiary lymphoid organs (CaTLOs), demonstrate a capacity to interact with the adaptive immune system beyond traditional immuno-suppressive roles (180). These CAFs are primarily characterized by their ability to activate and recruit adaptive immune cells rather than just innate immune interactions, marking a significant shift from the traditional view of CAFs as solely tumor-promoting entities (182).

Antigen-presenting CAFs (apCAFs), on the other hand, have a specialized role in direct immune modulation by presenting antigens to T cells via MHC class II molecules (116). CAFs expressing MHC class II, initially identified by Tuveson and colleagues in pancreatic cancer as apCAFs, have been subsequently confirmed by other research groups to be present in pancreatic, breast, and lung cancers (116, 183–187). This subset of CAFs is effective in initiating T cell responses, including the activation of effector CD4+ T cells (183–186). By presenting cancer antigens, apCAFs can influence T cell phenotypes and contribute to the immunological architecture of the tumor microenvironment (114). This function is critical for orchestrating localized immune responses against tumors and for supporting ongoing immune surveillance and anti-tumor activity.

Together, these roles underscore a complex interplay where CAFs can both suppress and stimulate immune responses, highlighting their dual potential as targets for cancer therapy (114, 188, 189). This emerging understanding challenges the traditional paradigm of CAFs and opens new avenues for therapeutic strategies aimed at modulating the tumor microenvironment to enhance anti-cancer immunity.

## CAFs facilitate cancer metastasis

Cancer metastasis is a multifaceted process encompassing various stages. It initiates with the migration and invasion of tumor cells into adjacent tissues, proceeds through intravasation into the bloodstream, follows by circulation and extravasation, and culminates in the colonization of new sites (190). CAFs significantly facilitate this process via paracrine signaling and direct physical interactions. The mobility of tumor cells, crucial for their migration and invasion, is often enhanced by the epithelial-mesenchymal transition (EMT), characterized by the loss of cell polarity and adhesion, which imparts a mesenchymal phenotype conducive to migration and invasion (191, 192). The necessity of EMT in all metastatic events remains debated.

CAFs are known to boost the migratory and invasive capabilities of cancer cells by secreting chemokines and exosomes. For instance, in gastric cancer, CAFs stimulated by TGF-β1/Smad2/3 signaling significantly upregulate hyaluronan and proteoglycan link protein 1 (HAPLN1), enhancing tumor migration and invasion (193, 194). In esophageal squamous cell carcinoma, CAF-like cells produce plasminogen activator inhibitor-1 (PAI-1), which augments migration and invasion through the PAI-1/low-density lipoprotein receptor-related protein 1 (LRP1) axis via Akt-Erk1/2 pathways. Furthermore, exosomes secreted by CAFs containing miR-18b and miR-382-5p have been documented to promote cancer cell migration and invasion through EMT induction (194). Additionally, CAFs facilitate EMT by modulating matrix stiffness and signaling pathways, such as the TWIST1/G3BP2 and EPHA2/LYN/TWIST1 pathways (195).

Apart from inducing EMT, CAFs also directly drive cancer cell migration through exerted physical forces. *Labernadie* and colleagues discovered a mechanism wherein CAFs apply physical force to cancer cells via heterophilic adhesion involving N-cadherin on CAFs and E-cadherin on cancer cells, mediated by β-catenin and α-catenin/vinculin interactions (196). *Erdogan* and team demonstrated that CAFs create and align a fibronectin-rich matrix, facilitating CAF-cancer cell association and directional migration through the nonmuscle myosin II/PDGFRα/α5β1-integrin/fibronectin pathway (197). Moreover, CAFs express membrane-anchored metalloproteinases (MT1-MMPs) that degrade collagen, easing tumor cell penetration and movement within the ECM (198).

Intravasation, a critical phase preceding circulation, involves tumor cells penetrating leaky, immature blood vessels formed during angiogenesis, often characterized by inadequate endothelial cell junctions and abnormal pericyte coverage (199). Various factors, including TGF-β, VEGF, and SOX2, have been identified as regulators of both intravasation and extravasation processes in metastasis (200, 201). CAFs enhance both hematogenous and lymphatic metastasis, with mechanisms involving signaling pathways such as periostin/integrin/FAK/Src/VE-cadherin, VEGFC/VEGFR3, and IL-6/IL-6R (202–204).

CAFs are dynamic participants in the metastatic process, not merely passive entities. For instance, circulating CAFs (cCAFs) found in the blood of patients with metastatic breast cancer correlate with clinical metastasis (205). These cCAFs, along with other cell types, form heterotypic clusters that influence survival and proliferation of circulating tumor cells (CTCs), potentially via soluble factors (206).

The target site microenvironment, often hostile to CTCs, is preemptively modified by the primary tumor, creating pre-metastatic niches (PMNs) (207). PMNs are shaped by cytokines and exosomes from the tumor and TME, with CAFs playing a dual role in their activation. CAF-derived factors, such as the non-coding RNA LncSNHG5 and extracellular vesicles, modify distant fibroblasts to enhance their tumor-supportive capabilities (208). Additionally, CAFs themselves undergo activation during PMN formation, further emphasizing their central role in cancer metastasis and providing potential therapeutic targets to hinder tumor growth and spread (209).

# CAF-targeting therapy

Recent years have witnessed significant advancements in therapies targeting cancer-associated fibroblasts (CAFs), focusing on three main objectives (1): directly or indirectly depleting CAFs, (2) mitigating or abolishing their tumor-promoting and immunosuppressive activities, or (3) reprogramming or normalizing CAFs towards a more dormant state. These strategies are outlined below.

## Chemotherapy targeting CAFs

Initially identified by Tuveson and colleagues, CAFs in various tumors express fibroblast activation protein (FAP), a unique membrane-bound serine postprolyl peptidase known for its additional endopeptidase activity (210). Val-boroPro (Talabostat), a competitive inhibitor of prolyl peptidase and an orally administered drug, demonstrated some control over tumor growth by degrading the extracellular matrix (ECM) in mouse models (211). However, it failed to show therapeutic efficacy in human clinical trials for metastatic colorectal cancers (212). Sibrotuzumab, a humanized anti-FAP monoclonal antibody, inhibited the dipeptidyl peptidase activity of FAP but did not show efficacy in suppressing pancreatic cancer growth in patients, despite the radiolabeled version of the antibody accumulating in tumors as visualized by SPECT (213, 214).

Utilizing FAP's enzymatic activity, anti-CAF prodrugs or protoxins that couple cytotoxic agents with a dipeptide containing a FAP cleavage site have been developed (210, 215). These prodrugs, which remain inactive until cleaved by FAP upon systemic delivery, have induced tumor lysis and growth inhibition when injected intratumorally in human breast and prostate cancer xenografts (215). Similarly, immunotoxins like Anti-FAP-PE39 have suppressed tumor growth and enhanced recruitment of tumor-infiltrating lymphocytes (216). Other novel approaches include monoclonal antibodies conjugated with cytotoxic agents or bispecific antibodies that target both FAP on CAFs and death receptor 5 on tumor cells, showing potent antitumor effects (216).

## Immunotherapy targeting CAFs

Numerous approaches have been developed to bolster immunity against FAP-expressing cells, notably CAFs, and to curb cancer proliferation (2, 21, 180, 189, 217). Immunization using dendritic cells transfected with FAP mRNA has effectively curtailed the growth of both implanted and intravenously introduced tumors (218). This effect was amplified when the vaccine targeted both FAP and a tumor-associated antigen simultaneously. These dendritic cell vaccines, when used in conjunction with an anti-fibrotic agent, have effectively activated both innate and adaptive immune responses, leading to enhanced NK cell function, boosted anti-tumor humoral responses, and potentiated cytotoxic CD8+ T cell activity across diverse tumor models (218). Additionally, adenoviral vaccines targeting FAP have selectively eliminated CAFs by activating a CD8+ T cell response, thereby reducing tumor growth and metastasis in various murine cancer models (114, 189, 219). A significant study employing a transgenic mouse model engineered to express the diphtheria toxin receptor under the FAP promoter demonstrated that depletion of FAP + CAFs via diphtheria toxin enhanced the efficacy of anti-cancer vaccines (220). Oral administration of an anti-FAP DNA vaccine markedly reduced new blood vessel formation, tumor growth, and metastasis in orthotopically injected breast carcinoma models (221), and the co-administration of doxorubicin significantly improved the drug's intratumoral absorption and extended the survival of the treated mice (221).

Adoptive transfer of chimeric antigen receptor (CAR)-T cells specifically designed to target FAP-expressing cells has shown promise in depleting FAP+ populations, including CAFs, thus limiting tumor stroma formation and enhancing the effectiveness of chemotherapeutic agents (222–224). However, this strategy has been linked with severe adverse effects such as profound bone marrow toxicity and cachexia, underscoring the need for more selective targeting in CAF-based therapies, an area that continues to be vigorously researched (225). Furthermore, near-infrared photoimmunotherapy (NIR-PIT) represents a novel technique for selectively depleting FAP-positive cells within the tumor microenvironment, showing efficacy in inhibiting tumor growth in a co-culture xenograft model of human esophageal squamous cell carcinoma without negative side effects (226, 227). Combining anti-FAP+ CAF therapy with 5-fluorouracil (5-FU) has proven to surpass the effectiveness of 5-FU alone in overcoming chemoresistance (228).

## Functional modification/reprogramming targeting CAFs

Reverting activated CAFs to a quiescent state involves the use of agents like all-trans-retinoic acid (ATRA), minnelide (which disrupts the TGF-β signaling pathway), and calcipotriol (228–232). The angiotensin receptor II antagonist losartan has reduced TGF-β-mediated CAF activation, enhancing drug delivery and the efficacy of immunotherapy, and is being studied in clinical trials for pancreatic cancer treatment (98, 233–235). Efforts to block immunosuppressive ligands of key CAF signaling pathways, including IL-6 (185, 186), LIF (187), and TGF-β (124, 126) aim to suppress or eliminate cancer cells (236–240).

The CXCL12/CXCR4 axis, crucial in cancer progression and immunosuppression, involves CXCL12 from CAFs recruiting CXCR4-expressing cells that support angiogenesis and tumor growth (241–243). Inhibiting this pathway using the CXCR4 antagonist plerixafor has significantly reduced fibrosis and improved immune cell infiltration and checkpoint inhibitor efficacy (244). Other strategies that inhibit CAF functions include TGF-β blockade, NFkB inhibitors to overcome chemotherapy resistance, and Smoothened hedgehog pathway inhibitors (IPI-926) (102, 245–247) (Figure 4).

## Targeting CAF control cancer cell dormancy

Initial research has established cancer cell dormancy as a critical factor contributing to drug resistance and recurrence, yet dormant cancer cells (DCC) continue to be challenging to detect clinically, highlighting a significant obstacle in overcoming drug resistance (248). The process of dormancy entry and escape involves complex interactions between tumor cells and the TME, with cytokines and chemokines secreted by CAFs playing a vital role (249). Consequently, a promising strategy to prevent cancer recurrence involves targeting these CAF-secreted factors. This approach focuses on drugs that specifically target these factors, potentially inhibiting dormancy-associated mechanisms.

### Inhibiting microenvironment interactions to prevent cancer cell reawakening

Maintaining tumor cells in a dormant state is critical to preventing metastasis and recurrence. It is imperative to develop therapeutic strategies that inhibit the communication between CAF-driven signaling and the supportive TME involved in dormancy escape (250). An effective target could be uPAR, which maintains the dormant state of cancer cells and limits metastasis (251, 252). For instance, ATN-292 reduces migration in human pancreatic cancer cells by blocking the uPA to uPAR binding (253), and a novel anti-uPAR monoclonal antibody has shown antitumor effects in gastric cancer by disrupting this interaction (254). The small molecule uPA inhibitor, WX-671, combined with gemcitabine, although well tolerated, did not improve survival outcomes compared to gemcitabine alone in a phase II trial (255).

High levels of TGF-β1 in the TME trigger dormancy escape, with CAFs being a primary source of TGF-β. Inhibitors targeting TGF-β1 interactions or receptor kinase activities are strategies to keep tumor cells dormant. Agents like SRK-181, which binds to the pro-segment of TGF-β1 preventing its activation, and LY2157299, a small molecule TGF-βRI kinase inhibitor known as Galunisertib, have shown promise in clinical trials (256–259). Another TGF-βRI inhibitor, Ki26894, has demonstrated efficacy in reducing invasiveness and bone metastasis in gastric cancer (260). Moreover, anti-inflammatory therapies targeting pro-inflammatory cytokines secreted by CAFs have been studied. NSAIDs such as sulindac and celecoxib, which inhibit COX-2

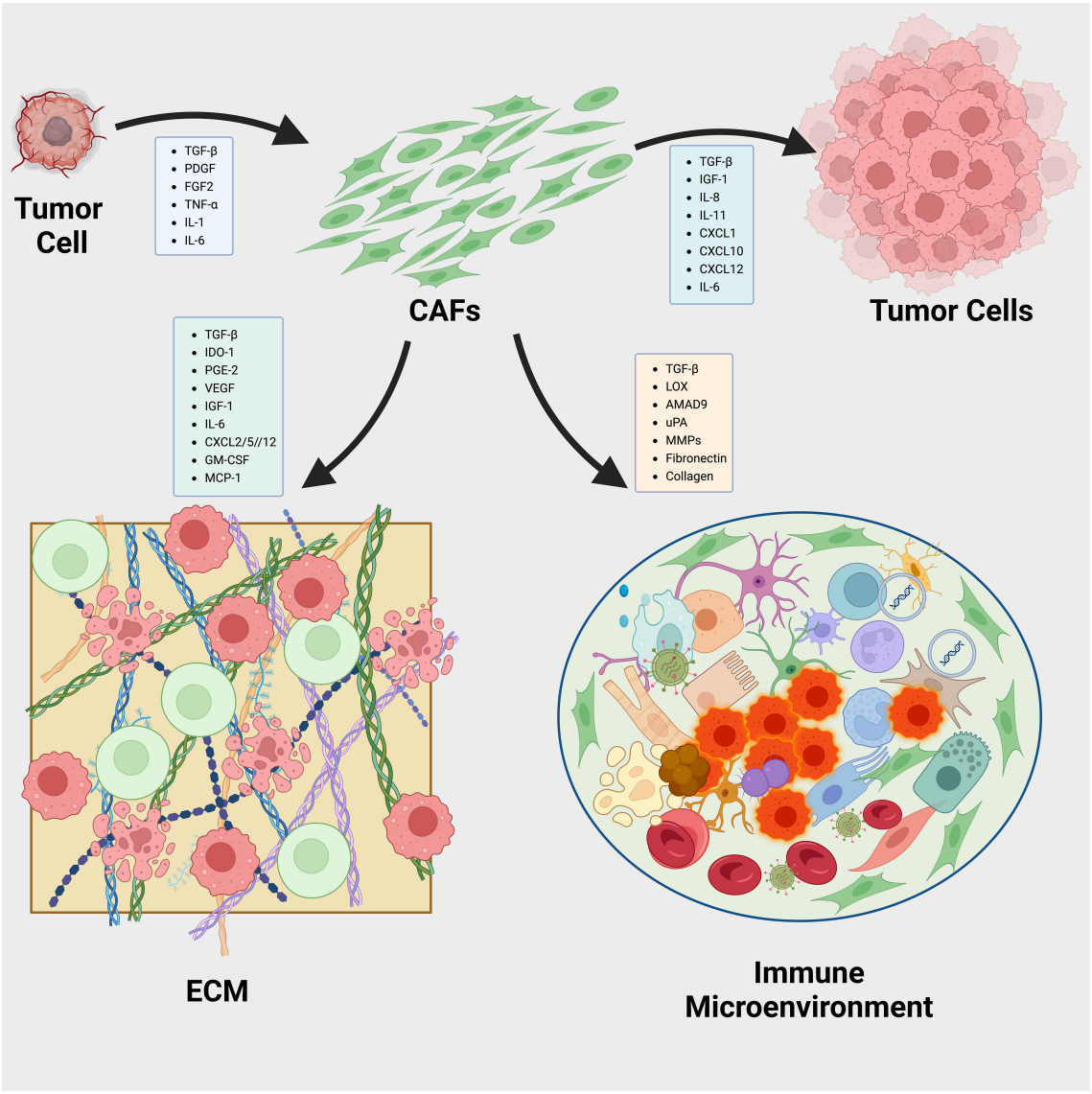


FIGURE 4
Dynamic Interactions Between CAFs and the Tumor Microenvironment in Cancer Progression. Reciprocal interactions between cancer-associated fibroblasts (CAFs) and the tumor microenvironment (TME) play pivotal roles in cancer progression via three primary mechanisms: a).Tumor-derived cytokines influence the behavior of CAFs, prompting them to release pro-tumorigenic factors that further drive the cancerous process. b).CAFs are instrumental in organizing and cross-linking the extracellular matrix (ECM) components, thus modifying the ECM's structure and functionality to facilitate tumor growth and metastasis. c).Factors secreted by CAFs also modulate immune cells within the TME, including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and dendritic cells (DCs). This modulation helps establish a tumor immune microenvironment (TIME) that promotes tumor development.

activity, have shown efficacy in gastrointestinal cancers in both preclinical and clinical settings. Sulindac is under investigation in a phase III trial for its potential to reduce adenomas and secondary cancers (261, 262), and celecoxib is being studied to enhance response rates in advanced colorectal cancer treatment (263, 264).

CAF-mediated ECM remodeling significantly contributes to dormancy escape. Targeting ECM molecules like collagen and FN, which regulate integrin roles in dormancy to proliferation transitions, is a potential strategy. Anti-integrin therapies like Volociximab have shown positive results in clinical trials (265, 266).

Lastly, inhibiting enzymes like LOX or LOXL2, which are implicated in chemoresistance and metastasis growth, could prevent dormant cancer cell awakening. Agents like Simtuzumab and EGCG have been evaluated for their efficacy in reducing LOXL2 activity and TGF-β1 signaling, showing potential in clinical trials (267, 268).

In conclusion, reinforcing the dormant state and inhibiting the pathways facilitating dormancy escape through targeted therapies offers a promising avenue for managing cancer progression and recurrence.

### Activate dormant cancer cells for improved treatment response

Understanding cancer dormancy has led to strategies aimed at preventing cells from becoming dormant or awakening dormant cancer cells (DCCs) to increase their sensitivity to treatment. One approach involves targeting dormancy-inducing factors influenced by CAFs. For instance, TGF-β2, which promotes dormancy via the TGF-βRIII pathway, is targeted by AP 12009 (Trabedersen), an antisense oligonucleotide (ASO) that has shown safety in phase I/II studies for pancreatic and colorectal cancer (269, 270).

Another CAF-secreted factor, DKK-1, helps maintain cancer cell dormancy (271). DKN-01, a humanized monoclonal antibody that inhibits DKK-1, is currently being evaluated in clinical trials for gastrointestinal (GI) cancers (272, 273). Notably, a phase II trial is investigating DKN-01 in combination with Tislelizumab and possibly chemotherapy for metastatic gastric cancer or gastroesophageal junction adenocarcinoma (274). Additionally, DKN-01 is being tested with pembrolizumab in advanced esophageal cancer in a phase Ib trial and in combination with bevacizumab and chemotherapy for advanced colorectal cancer in another ongoing phase II study (NCT05480306) (272, 275).

Other factors like GDF-10 and BMP4, also secreted by CAFs, have been implicated in promoting dormancy, although targeted therapies for these factors in GI cancers have yet to be explored (276–278). By inhibiting these CAF-derived factors, it is possible to either prevent entry into or trigger exit from dormancy. Implementing such strategies early in treatment may prevent tumor cells from developing robust malignancy. A summary of targeted factors by therapeutic agents in GI cancers emphasizes potential approaches to manage cancer dormancy at various stages (Figure 5).

## CAFs and therapeutic resistance

Resistance to cancer therapy often results in poor patient outcomes, underpinned by complex and dynamic mechanisms. Konieczkowski et al. introduced a convergence-based framework to understand cancer drug resistance, identifying major causes such as pathway reactivation, pathway bypass, and pathway indifference (279). Beyond genomic alterations in tumor cells, the role of CAFs in therapeutic resistance has been well-established, with their influence extending across multiple facets. CAFs affect the mechanical properties of the tumor microenvironment (TME), enhancing matrix stiffness which can impede the penetration of chemotherapeutic drugs. For example, gastric CAFs that express calponin 1 activate the ROCK1/MLC pathway, increasing matrix stiffness and contributing to resistance against 5-fluorouracil (5-FU) in cancer cells by activating the YAP signaling pathway (280). CAF-derived exosomes are also pivotal in mediating resistance within the TME (281). Annexin A6 in CAF-derived extracellular vesicles (EVs) activates the integrin β1-FAK-YAP signaling pathway, promoting the formation of a tubular network in the ECM that reinforces chemotherapeutic resistance (282). In breast cancer, CAF-derived circulating EVs containing the full mitochondrial genome enhance estrogen receptor (ER)-independent oxidative phosphorylation (OXPHOS), which induces therapy-resistant dormant cancer stem-like cells, leading to resistance to endocrine therapy (283). Targeting the YAP signaling pathway may be effective in overcoming the mechanical resistance encountered in targeted therapy. Regarding immunotherapy, CAFs activated by the IL-17/Act1/HIF1α pathway can lead to collagen deposition, enhancing PD-L1 resistance and reducing cytotoxic T cell infiltration (284).

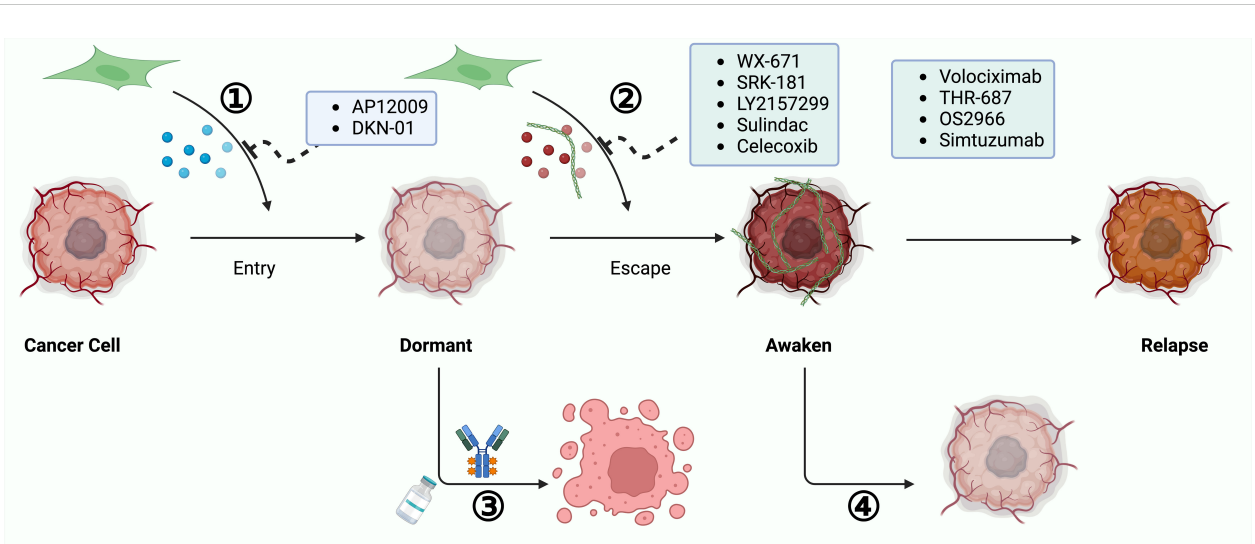


FIGURE 5
Approaches to Combat Cancer Recurrence by Targeting Dormant Cancer Cells. Targeting dormant cancer cells presents a viable strategy for preventing cancer recurrence. 1). Reactivating Dormant Cancer Cells for Therapeutic Sensitivity: This involves awakening dormant cancer cells to increase their susceptibility to treatments. Strategies include obstructing the secretion of dormancy-inducing factors by CAFs or reactivating cellular proliferation signals. 2).Sustaining Permanent Dormancy of Cancer Cells: This strategy aims to prevent cancer cell reactivation and subsequent growth by blocking pathways facilitated by CAFs that enable dormancy escape. By focusing on these dormant cancer cells, it is possible to avert cancer recurrence and enhance patient outcomes.

Another subtype of CAF, ecm-myCAF, has been shown to elevate PD-1 and CTLA4 protein levels in Tregs, boosting TGFβ-myCAF cellular content and mediating primary resistance to immunotherapy. Combining tumor-targeted therapy with CAF-targeted strategies, such as the FAP5-DM1 monoclonal antibody conjugated to maytansinoid, has demonstrated prolonged inhibition of tumor growth and complete regressions in xenograft models of multiple cancers (222). Additionally, CAFs contribute to radiotherapy resistance; upon irradiation, CAFs polarize towards the iCAF subtype via IL-1a, leading to oxidative DNA damage and p53-mediated therapy-induced senescence in iCAFs, which in turn facilitates chemoradiotherapy resistance and disease progression (285) (Figure 6).

## Challenges and directions

The challenges surrounding CAFs are multifaceted, ranging from classification issues to their dynamic roles within the TME. One major challenge lies in the lack of a uniform and comprehensive naming standard for CAF subgroups. An ideal naming convention should consider factors such as cell lineage, functional roles, biomarkers, clinical correlations, immune regulation, immune response, and metabolic status, integrating all these aspects to advance our understanding of CAFs. Another obstacle is the difficulty in identifying the origin of CAFs, which is compounded by the absence of specific biomarkers. A promising approach to this issue would involve the combined use of multiple biomarkers and the quantitative assessment of their variations to enhance specificity, particularly by focusing on distinct CAF subpopulations. Moreover, *in vitro* culture of CAFs presents significant challenges, as most of their *in vivo* characteristics tend to change due to alterations in culture conditions and passage (291, 292). I t is crucial to establish culture environments that closely mimic the TME and to track phenotypic changes during cultivation to preserve CAF traits. Despite the use of various methods for detecting CAFs, including antibodies, mRNA probes, and single-cell transcriptome analysis, there is still a lack of standardized, accurate, and universally applicable quantitative methods for their detection. While single-cell transcriptomics is already shedding light on CAF heterogeneity, further application of this technology is essential for deepening our understanding of CAF subpopulations (293). Another critical gap is the lack of longitudinal studies examining CAFs across different experimental stages, such as

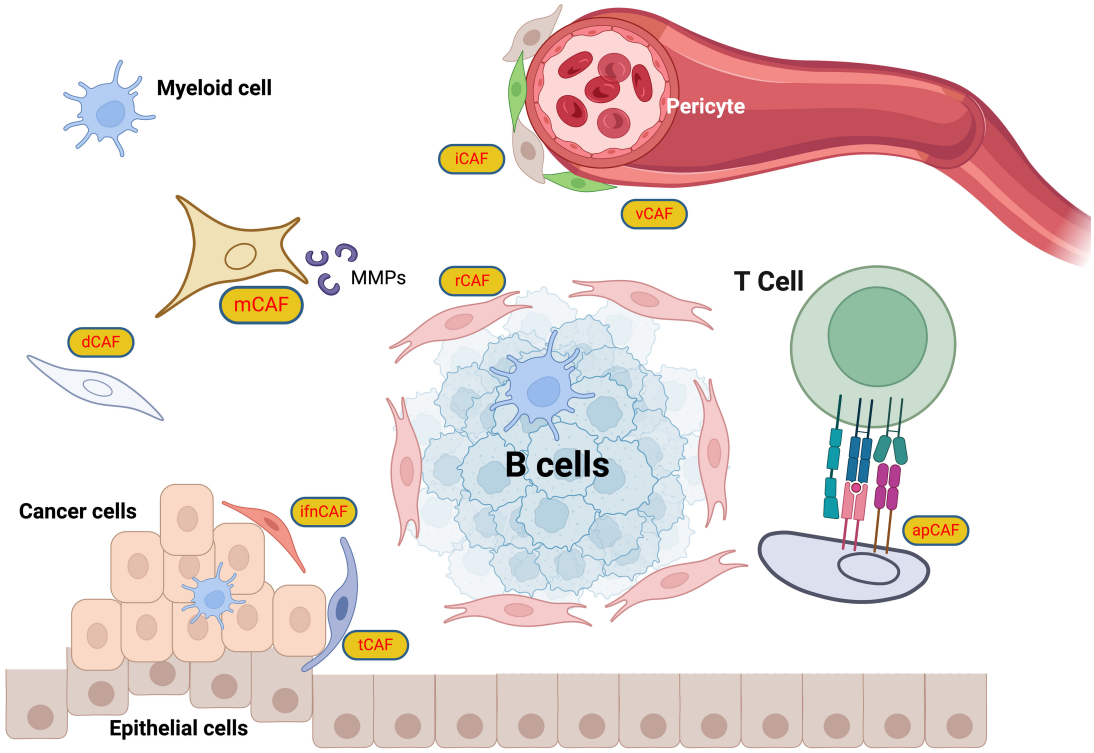


FIGURE 6
CAF classification scheme. The CAFs are categorized into specific roles: iCAFs (inflammatory CAFs) that modulate inflammation within the TME (286); rCAFs (regulatory CAFs), which might play roles in tumor regulation (2); dCAFs (developmental CAFs) associated with developmental processes (103); tCAFs (TGF-β producing CAFs), known for TGF-beta production influencing tumor growth and immune evasion (287); vCAFs (vascular CAFs) involved in vascular dynamics (288); ifnCAFs (interferon-producing CAFs), which might interact with immune pathways through interferon production (289); and apCAFs (antigen-presenting CAFs) that potentially present antigens to T cells, facilitating immune system interactions (290). This classification highlights the multifunctional nature of CAFs, underlining their importance in tumor progression, immune modulation, and the structural integrity of tumors, thus providing crucial insights for targeting these cells in cancer therapy strategies.

primary tumor growth, early isolation, and long-term passage, as well as across varying clinical stages (294). Such studies are necessary to improve our knowledge of CAF origins, subpopulations, heterogeneity, and plasticity in relation to tumor progression. Additionally, there is a need for horizontal studies that compare CAF subpopulations between different types of tumors and correlate these populations with clinical features to better understand the impact of CAFs on disease progression and treatment responses (2).

The regulation of CAFs within the TME is also not fully understood, and a deeper exploration of the dynamic interplay between CAFs, tumor cells, and other elements of the TME from biochemical, metabolic, immunological, and physical perspectives is necessary. Understanding how CAFs evolve in response to tumor progression and TME changes is equally crucial. Furthermore, while CAFs are known to influence immune responses within the TME, their crosstalk with immune cells remains poorly defined, highlighting the need for more research into how CAFs contribute to immune evasion and therapy resistance.

## Discussion

CAFs represent a crucial, yet complex, component of the TME, influencing various aspects of cancer progression, metastasis, and therapeutic resistance. This review has highlighted the multifaceted roles of CAFs in shaping the TME, including their involvement in ECM remodeling, immune modulation, angiogenesis, and metabolic reprogramming. The dynamic and heterogeneous nature of CAFs, however, complicates their classification and therapeutic targeting. Current research on CAFs has emphasized the need for a more standardized system to categorize the various CAF subpopulations based on their lineage, function, biomarkers, and interactions within the TME. Understanding the underlying mechanisms of CAF activation and their dualistic roles—either promoting or suppressing tumor growth depending on the context—remains a critical challenge for therapeutic strategies.

To effectively address the highlighted lack of specific CAF markers, concerted efforts are needed to identify and validate reliable markers that enhance specificity. This can be achieved by employing advanced genomic and proteomic technologies to analyze diverse cancer types, facilitating the discovery of unique CAF profiles. Additionally, the lack of specific biomarkers for CAF identification remains a significant hurdle, limiting their effective targeting in clinical practice. Although several biomarkers have been proposed, the lack of specificity for CAFs means that they cannot be universally applied in clinical settings. The use of advanced techniques, such as single-cell transcriptomics, holds promise for resolving the complexity of CAF subpopulations and identifying precise markers for their targeting. Another critical issue need to be discussed is the challenge of maintaining CAF phenotypes *in vitro*, as their characteristics often change when cultured outside the TME. Optimizing culture conditions to better preserve the *in vivo*-like properties of CAFs is crucial for advancing CAF-based research and therapeutic development.

Single-cell technologies have profoundly refined our understanding of CAFs by revealing their cellular heterogeneity within the tumor microenvironment. Techniques like single-cell RNA sequencing (scRNA-seq) have identified distinct CAF subtypes with unique gene expressions and roles, enhancing our insight into their contributions to tumor progression and potential as therapeutic targets. Future research should integrate single-cell data with spatial transcriptomics to explore the dynamic interactions of CAFs with the TME across tumor development and therapy response, aiming to develop targeted treatments that disrupt crucial CAF-driven pathways.

The resistance mechanisms mediated by CAFs, particularly in the context of chemotherapy, immunotherapy, and radiotherapy, further complicate treatment efficacy. CAFs influence drug resistance through several mechanisms, including the promotion of ECM stiffness, secretion of pro-inflammatory cytokines, and modification of immune responses within the TME. Notably, CAFs enhance immune evasion by modulating the activity of immune cells, such as Tregs and NK cells, which significantly impacts the success of immunotherapies. Therefore, strategies that combine CAF-targeted therapies with conventional treatments, such as chemotherapy or immune checkpoint inhibitors, may offer a promising approach to overcome therapeutic resistance.

In addressing the current challenges and gaps identified in CAF research, we propose several specific experimental approaches and therapeutic strategies to advance this field. Firstly, the development of innovative CAF-specific markers through high-throughput screening techniques could greatly refine the targeting of these cells in diverse cancer types. Additionally, leveraging cutting-edge technologies such as CRISPR-Cas9 for gene editing within CAFs offers a promising avenue to dissect their functional roles in tumor progression and resistance mechanisms (295). Therapeutically, exploring bi-specific antibodies that target both CAFs and tumor cells could provide a dual approach to disrupt the supportive tumor microenvironment. Furthermore, employing organoid models incorporating CAFs from patient-derived samples would enhance our understanding of their interaction with tumor cells in a controlled, yet biologically relevant system. These approaches not only aim to fill the existing gaps but also pave the way for novel interventions that could be translated into clinical applications.

However, further research is still essential to gain a deeper understanding of the molecular mechanisms that regulate CAF behavior, particularly their interactions with tumor cells, immune cells, and the extracellular matrix. Investigating the dynamic roles of CAFs in different stages of tumor progression, as well as their involvement in the establishment of pre-metastatic niches, will be crucial for developing more effective therapeutic strategies. Additionally, refining methods to accurately identify and classify CAF subpopulations, along with developing therapies that can specifically modulate CAF function, holds great promise in advancing cancer treatment. Combining CAF-targeted therapies with current immunotherapies and other treatment modalities could significantly improve clinical outcomes and provide more effective treatment options for cancer patients.

## Author contributions

XL: Methodology, Writing – original draft. WL: Validation, Writing – original draft. KZ: Formal Analysis, Writing – original draft. JW: Visualization, Writing – original draft. SL: Methodology, Writing – original draft. HZ: Conceptualization, Writing – review & editing.

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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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# Effect of bispecific recombinant oncolytic adenovirus carrying apoptin on apoptosis of MCF-7 cells

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**Objective:** In this study, breast cancer cell line MCF-7 was infected with recombinant oncolytic adenovirus Ad-VT expressing apopsin protein, and its anti-tumour pathway was detected to determine its possible anti-tumour signalling pathway.

**Method:** In this study, the inhibitory effect of recombinant oncolytic adenovirus Ad-VT on breast cancer cells was investigated through cell activity experiment and establishment of tumour bearing model in mice. Subsequently, in order to determine the apoptosis-inducing effect of recombinant oncolytic adenovirus on breast cancer cells, the effects of three recombinant oncolytic adenovirus on the apoptosis-inducing level of breast cancer cells were further analysed by Annexin V-FITC/PI detection, Hoechst staining, JC-1 staining and transmission electron microscopy. Then the differentially expressed proteins associated with apoptosis and possible signalling pathways were identified by proteomics and WB experiments.

**Results:** *In vivo* and *in vitro* experiments showed that recombinant oncolytic adenovirus Ad-VT expressing apoptosis protein could induce apoptosis and inhibit the growth of MCF-7 cells. Proteomic analysis showed that differential genes were enriched in mTOR, MAPK and other pathways after Ad-VT infection of breast cancer cells, and the expression of S6K genes related to mTOR pathway was significantly increased in differential gene analysis, subsequently, the high expression of phosphorylated mTOR and S6K proteins was also determined by WB experiment, suggesting that Ad-VT may regulate the apoptosis of breast cancer cells through mTOR/S6K signalling.

**Conclusion:** Ad-VT can significantly increase the apoptosis level of breast cancer cells, which may be induced by the mTOR/S6K signalling pathway. The results of this study provide a theoretical basis for the development of anti-tumour drugs based on Ad-VT in the future.

KEYWORDS

**MCF-7 cells, apoptin, proteomics, apoptosis, oncolytic adenovirus**

## Introduction

According to statistics from the World Health Organization, breast cancer is the most common cancer in the world and has the highest incidence rate among all cancers in women (1). Despite the continuous progress in the treatment of breast cancer, metastatic breast cancer is still incurable, and the overall 5-year survival rate is still less than 25% (2). An effective treatment strategy is urgently needed. Traditional treatments, such as radiotherapy, chemotherapy or surgery, exert direct inhibitory effects on tumours, but most of these treatments have side effects and cause significant damage to normal human tissues (3). Moreover, improvements in the survival rate and quality of life of most cancer patients are limited. Therefore, a treatment that has few side effects and can inhibit the metastasis of breast cancer must be developed.

Apoptosis is a gene-regulated cell suicide process mediated by the activation of caspases 3, 6 and 7. Apoptosis is a basic biological phenomenon of cells that plays a necessary role in the removal of unnecessary or abnormal cells in multicellular organisms (4–6). With the development of molecular biology technology, the process of apoptosis has become well understood, but the exact mechanism of apoptosis is not completely clear. The target of rapamycin (mTOR) signalling pathway plays an important role in tumorigenesis and development; thus, it has received an increasing amount of attention. mTOR is an atypical serine/threonine protein kinase belonging to the phosphatidylinositol 3-kinase-associated kinase (PIKK) family. In mammals, there are two main target molecules downstream of mTOR: ribosome S6 kinase (S6K) and eukaryotic translation initiation factor (eIF4E)-binding protein 1 (4E-BP1) (7, 8). The activation of mTOR leads to the phosphorylation of S6K, which induces apoptosis and inhibits tumour growth. However, there are few reports on whether Apoptin can induce the apoptosis of tumour cells by affecting the mTOR/S6K signalling pathway. In this study, proteomic sequencing was used to explore whether Ad-VT affects changes in the mTOR/S6K signalling pathway and induces the apoptosis of breast cancer cells.

Apoptin was one of the first tumour-selective anticancer genes isolated. Apoptin is derived from chicken anaemia virus (CAV) (9–11), which is a single-stranded DNA virus and is considered an apoptosis-inducing protein. The CAV genome contains three partially overlapping open reading frames that encode viral proteins from a single polycistronic mRNA: VP1, VP2 and VP3. The expression of VP3 alone is sufficient to trigger the death of chicken lymphoid T cells and myeloid cells but does not affect chicken fibroblasts; thus, the VP3 protein was renamed Apoptin.

Apoptin is a 14kD small molecular protein abundant in basic amino acids like serine and threonine, comprising two nuclear localisation signal sequences (NLS) and one nuclear export signal sequence (NES) (12–14). These domains are vital for Apoptin's nuclear shuttling. In tumour cells, Apoptin undergoes phosphorylation and subsequently translocates to the nucleus, triggering apoptosis. Conversely, in normal cells, apoptin harbouring NLS may not be adequately activated or could be impeded by inhibitory mechanisms, such as phosphorylation status or chaperone protein binding, preventing an elevation in apoptosis rates. This underscores that mere nuclear localisation alone is insufficient to induce cell death in normal cells (15).

Therefore, in addition to nuclear localisation, the activation of apoptotic proteins also requires one or more additional events, such as the ability of apoptotic proteins to interact with multiple proteins in tumour cells (16), induce tumour cell apoptosis, and exert anti-tumour effects. In this process, they are usually activated by relevant signalling protein pathways in tumour cells, leading to their accumulation in the nucleus, while in normal cells, they remain in the cytoplasm. Elucidating the proteins and signalling pathways involved in the interaction between apoptotic factors and tumour cell apoptosis will contribute to the advancement of targeted tumour therapy mediated by apoptotic factors. Therefore, in addition to nuclear localisation, the activation of apoptotic proteins necessitates one or more supplementary events, such as their capacity to interact with various proteins in tumour cells (16), thereby inducing tumour cell apoptosis and exerting anti-tumour effects. Throughout this process, they are usually activated by pertinent signalling pathways in tumour cells, resulting in their accumulation within the nucleus, whereas in normal cells, they reside in the cytoplasm. Elucidating the proteins and their signalling pathways that interact with apoptins during the apoptosis of tumour cells will facilitate advancements in apoptin-mediated targeted tumour therapy.

Du et al. (17) efficiently transfected mesenchymal stem cells with apoptin using a lentiviral expression system, enabling them to synthesise and secrete apoptin, which in turn effectively activated caspase-3 and the mitochondrial/cytochrome C signalling pathway, thereby inducing apoptosis in lung cancer cells. Basse et al. (18) demonstrated that, in cervical cancer cell lines, the combination of antimicrobial peptides (ABPs1) and apoptin significantly enhanced caspase-3 activity, suggesting that apoptin induced cell death involves the caspase-dependent mitochondrial pathway. Song et al. (19) validated that recombinant adenovirus carrying apoptotic factors targets AMPK and subsequently inhibits glycolysis, migration, and invasion in lung cancer A549 cells via the AMPK signalling pathway, playing a pivotal role in tumour cell energy metabolism. Zhou et al. (20) demonstrated that apoptotic peptide-derived peptides reverse cancer cell resistance to cisplatin by inhibiting the PI3K/AKT/ARNT signalling pathway, downregulating MDR1 expression, and inhibiting cancer cell invasion and metastasis. In this study, we observed that the recombinant oncolytic adenovirus delivering apoptin induced apoptosis in MCF-7 tumour cells, accompanied by significant alterations in key proteins of the mTOR/S6K signalling pathway. These findings suggest a potential role of this pathway in apoptin-mediated breast cancer cell apoptosis.

Apoptin mainly induces tumour cell death in the form of apoptosis (21), but further research has revealed that apoptin can also promote cancer cell death through other cell death mechanisms or signalling pathways, such as autophagy-dependent death (22) and pyroptosis (23).

Ad-VT (Ad-Apoptin-hTERTp-E1A), a novel oncolytic adenovirus, has the following excellent characteristics. (1) As an oncolytic adenovirus, Ad-VT can replicate in tumour cells and kill tumour cells. (2) The addition of the apoptin gene, namely, the so-

called bispecific oncolytic adenovirus, has a targeted killing effect on tumours. (3) In combination with other antitumour drugs or chemotherapy drugs, Ad-VT has a synergistic effect on reducing the toxicity of chemotherapy drugs. (4) Due to its low production- and treatment-related costs, oncolytic adenovirus can be prepared in large quantities, and it is safe and reliable. The purpose of this study was to transfer apoptin to tumour cells to study whether apoptin can promote apoptosis in the human breast cancer cell line MCF-7 *in vivo* and *in vitro*. Apoptin can induce the apoptosis of many types of tumour cells but does not damage normal cells.

A better understanding of the signalling pathway through which apoptin inhibits tumour cell survival is necessary for apoptin to be an effective anticancer drug. In this study, proteomics and Western blot experiments were performed to explore the apoptosis signalling pathway induced by apoptin and the changes in the levels of key proteins.

In this study, MCF-7 tumour cells were labelled with firefly luciferase and inoculated into immunodeficient mice to establish a tumour model. The growth and metastasis of tumour cells in a tumour model can be observed and measured continuously and intuitively using an *in vivo* imaging system for small animals, and even slight changes can be detected, suggesting that these animals constitute an ideal model for more intuitively evaluating the therapeutic effects of anticancer drugs.

# Materials and methods

## Viruses, cells and laboratory animals

Human breast cancer (MCF-7) and Normal human mammary epithelial (MCF-10A) cells were purchased from the Cell Bank of the Shanghai Institute of Biology, whereas human embryonic kidney cells (HEK-293) were purchased from the CCTCC (Cell Bank of the Chinese Academy of Sciences). MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin. MCF-10A cells were cultured in DMEM supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin. HEK-293 cells were cultured in DMEM supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin.

Luciferase-labelled human breast cancer cell lines (MCF-7-luc), Ad-hTERTp-E1a-Apoptin (Ad-VT), Ad-Apoptin (Ad-VP3) and Ad-MOCK were constructed and preserved in our laboratory.

In this study, 6–8-week-old female BALB/c nude mice were purchased from Beijing Weitong Lihua Company.

## Methods

### Amplification and virulence determination of recombinant oncolytic adenovirus

HEK-293 cells were cultured in DMEM supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin. Add $5 \times 10^5$ HEK-293 cells to each well of a 6-well cell culture plate and culture them at 37 °C and 5% $CO_2$ for subsequent experiments.

(1)Viral amplification

The recombinant adenoviruses (Ad-VT, Ad-VP3 and Ad-Mock) were generated using the RAPAd.I packaging system (Figure 1). Ad-VT (Ad-Apoptin-hTERTp-E1A) contains a tumour-specific promoter (hTERTp, human telomerase reverse transcriptase) that activates the E1A gene (essential for viral replication) (24) and a cytomegalovirus (CMV) promoter to initiate the expression of the apoptin gene.

Add 50 moi recombinant oncolytic adenovirus to each well of a 6-well cell culture plate for cultivation until the cell morphology is completely diseased. The cells were collected in culture bottles, repeatedly freeze-thawed to completely break the cells, and stored at -80red

(2)Poison value determination

Perform virus titre determination on the amplified virus, dilute the virus solution in a 1:10 gradient, and transfer it to a 96 well cell culture plate with 5000 HEK-293 cells per well for cultivation. During this period, continuously observe the cell pathology, and observe the pathological phenomenon under an optical microscope for about 6–7 days. The complete pathological morphology of the cells is swelling and rounding, high transparency, and the appearance of floating clusters.

Calculate TCID50 and calculate the PFU value based on the relationship between TCID50 and PFU (Karber formula): lgTCID50=L-d(s-0.5)

L=logarithm of the highest dilution; D=difference between dilution logarithms; S=total positive tube ratio

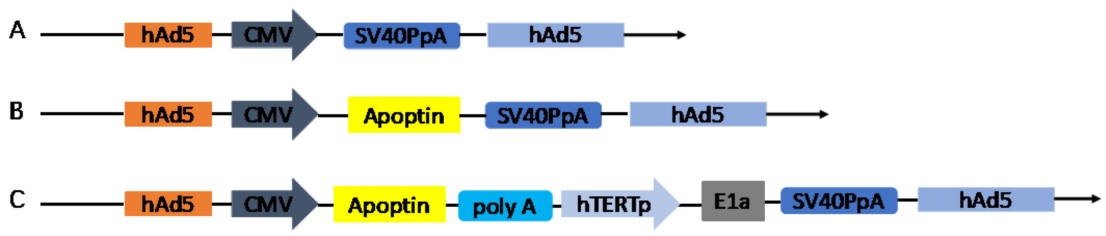


FIGURE 1
Schematic diagram of four recombinant adenovirus vectors constructed using shuttle vectors in our laboratory: **(A)** MOCK, **(B)** Ad-Apoptin (Ad-VP3), **(C)** Ad-Apoptin-hTERTp-E1a (Ad-VT).

## MTS assay

MCF-7 and MCF-10A cells were added to 96-well plates (5000 cells/well) for 24 hours. Virus handling: The recombinant adenoviruses Ad-VT, Ad-VP3 and Ad-MOCK were diluted with serum-free medium (MOIs of 50, 100, and 200). The culture medium in the 96-well plate was discarded, diluted recombinant oncolytic adenoviruses (50 μL/well) were added, and the control well was established. The 96-well cell culture plate was removed at 24 h, 48 h and 72 h. The culture medium was discarded, and 110 μL of MTS (Promega, USA)(diluted at 1:10) was added to each well. The mixture was incubated for 80 min in an incubator in the dark. The absorbance of the MCF-7 and MCF-10A cells in each well was measured with an enzyme labelling instrument (Tecan Trading AG, Switzerland) at a wavelength of 490 nm for 20 s, and the cell activity inhibition rates of three recombinant oncolytic adenoviruses and control were calculated. Cell proliferation inhibition rate (%) = (absorbance value of control well-absorbance value of treatment well)/absorbance value of control well × 100%

## Effects of recombinant adenoviruses on the migration of MCF-7 cells

The insert (Culture-Insert 2 Well, ibidi) was placed in a 6-well plate, and then the MCF-7 cell suspension (3.5ens$^4$ cells/well) was added. The cells were cultured at 37tC in a 5% $CO_2$ incubator until the cells formed a monolayer, after which the insert was removed. The samples were rinsed repeatedly with serum-free RPMI 1640 medium 3 times to ensure that no suspended free cells were present in the scratches, and the initial images were captured with an optical microscope (BX-60, Olympus, Tokyo, Japan). The recombinant adenoviruses Ad-VT, Ad-VP3 and Ad-MOCK were diluted with RPMI 1640 medium, and the PFU was 3.5ium$^5$ $TCID_{50}$/100 μ10 After capturing the first photograph, the diluted solution (1 ml, MOI of 5) was added to the 6-well plate and incubated for 2 hours, after which each well was filled with 1 mL of medium. The 6-well plate was removed at 0 h, 8 h, 16 h and 24 h. The culture medium was removed from the 6-well plate and placed in an EP tube before each images was captured and then placed under the microscope as soon as possible. The width of the scratches at different time points was analysed using ImageJ, and the mobility of the MCF-7 cells was calculated as follows: cell mobility = (0 h scratch width-0/8/16/24 h scratch width)/0 h scratch width.

## Observation of the effects of recombinant adenoviruses on the invasion of MCF-7 cells using BioCoat chambers

The MCF-7 cell suspension (2spe$^5$ cells/well) was added to a 24-well plate and cultured at 37tC in 5% $CO_2$ incubator for 24 hours. For virus treatment, the three recombinant adenoviruses, Ad-VT, Ad-VP3 and Ad-MOCK, were diluted with serum-free RPMI 1640 medium, and the PFU were 2ere$^7$ $TCID_{50}$/100 μ10 1100$^7$ $TCID_{50}$/100 μ1 and 5nd0$^6$ $TCID_{50}$/100 μ10

The culture medium in the 24-well plate was discarded, 250 μ5 (MOIs of 200, 100 or 50) of recombinant adenoviruses was added to the 24-well plate, and a negative control was prepared. After 2 hours of culture in the incubator, 250 μ5 of RPMI 1640 medium was added to each well, and the cells were incubated for 24 hours or 48 hours. The BioCoat (Corning,USA) chambers preserved at -20eC were removed and incubated at room temperature. Serum-free RPMI 1640 medium preheated at 37hC was added to each chamber and hydrated in the incubator for 2 hours. A new 24-well plate was used, 750 μ5 of RPMI 1640 medium was added to each well, sterile tweezers were used to place a BioCoat chamber each well, with one culture plate used for each of the two periods to be tested. The samples from each well were collected in an EP tube by centrifugation at 2000 rpm for 5 min, the liquid was discarded, and 500 μ0 of serum-free RPMI 1640 medium was added to suspend the cells. The samples were transferred to the chamber, and the cells were cultured in an incubator for 24 hours. A new 24-well plate was used, and 825 μ2 of WST-1 (Sigma-Aldrich, USA) (diluted at 1:10) was added to each well. The culture medium in the upper chamber was discarded, the matrix mixture and nonmigrated cells were removed from the small ependyma with cotton swabs, WST-1 was added to the BioCoat chamber, and the 24-well plate was placed into the incubator and incubated for 90 min in the dark. After shaking for 15 s, the OD value of each well was measured with an enzyme labelling instrument at a wavelength of 450 nm.

## Detection of metastasis-related proteins

Suspensions of MCF-7 cells were seeded in a 6-well cell culture plate at a density of $3 \times 10^5$ cells/well. The cells were then infected with Ad-VP3 or Ad-VT at an MOI of 100. After 48 hours, we performed Western blotting to determine the levels of key proteins in MCF-7 cells. For this experiment, we incubated membranes containing cell lysates with antibodies against E-cadherin, N-cadherin, SNAIL and vimentin (Cell Signalling Technology, USA) (diluted at 1:1000).

Forty-eight hours after infection, the cells were harvested by centrifugation at 5000 rpm for 5 minutes. Then, 200 μ0 of the cell lysis reagent SD-001 from the MinuteTM Total Protein Extraction Kit (Invent, Germany) was added to the cell pellets. The cell lysates were then homogenised by repeated pipetting and centrifuged at 14000 rpm in a precooled centrifuge tube for 30 sec (4eC). The final concentrations of the protein extracts were then determined using a BCA protein quantification kit (Beyotime Biotechnology, China). The levels of the 4 proteins associated with cell transfer were then analysed using Western blotting.

## Observation of apoptotic bodies via transmission electron microscopy

MCF-7 cells were added to a 6-well plate ($2\times10^5$ cells/well). After 24 hours of culture, Ad-VT, Ad-VP3 and Ad-MOCK(MOI of 100/well) were added. The control group was established. After 48

hours, the culture medium was removed, and PBS (1×) was added to wash the cells twice. The cells were collected in 15 ml centrifuge tubes. After centrifugation at 1000 rpm for 5 min, 5 mL of PBS (1×) was added to wash the cell precipitate, the mixture was centrifuged at 1000 rpm, and the supernatant was discarded. After 1.5 mL of PBS (1×) was added to the suspended cells for precipitation, the cells were transferred to EP tubes and centrifuged at 1500 rpm for 8 min. Glutaraldehyde (700–800 μL) was slowly added to the precipitate, which was subsequently fixed overnight at 4°C. The samples were washed 3 times with PBS (1×) for 10 minutes each, after which they were fixed again for 2 hours with osmic acid. The samples were washed with PBS (1×) three times for 10 min each, after which they were embedded in epoxy resin overnight. After the samples were cut, the samples were sliced with a 200 mesh copper mesh and stained, and the results were observed via transmission electron microscopy.

## Hoechst staining

MCF-7 cells were added to 6-well plates ($2\times10^5$ cells/well) and cultured for 24 h, Ad-VT, Ad-VP3 and Ad-MOCK (MOI of 10/well) were added, and control wells were established. One 6-well plate was removed at 24 h, 48 h and 72 h, and the cells in each well were diluted with 1 mL of Hoechst (Life Technologies, USA) dye solution (Hoechst (5 mg/ml) 1:1000 dilution), incubated in an incubator for 15 minutes and washed repeatedly with PBS (1×) for 15 minutes. Then, 500 μL of serum-free medium was added to each well, and the cells were removed, placed on slides, and photographed under the same conditions with a fluorescence microscope (BX-60, Olympus, Tokyo, Japan).

## JC-1 staining

1. Qualitative detection of the mitochondrial membrane potential

After the clean tablets were spread into 6-well plates, MCF-7 cells ($2\times10^5$ cells/well) were added, and after 24 h of culture, Ad-VT, Ad-VP3 and Ad-MOCK (MOI of 100/well) were added. Control wells were set up, and a 6-well plate was removed at 24 h, 48 h and 72 h after exposure. The medium in the 6-well plate was discarded, 1 mL of JC-1 (Life Technologies, USA) staining solution (diluted 1:1000) was added to each well, and the samples were incubated for 15 min in an incubator in the dark and washed twice with PBS (1×). Five hundred microlitres of serum-free medium was added to each well, and the coverslips were removed and photographed under the same conditions using a fluorescence microscope (BX-60, Olympus, Tokyo, Japan).

2. Quantitative detection of the mitochondrial membrane potential

MCF-7 cells (5,000 cells/well, 100 μL) were added to a 96-well plate and cultured for 24 h, and Ad-VT, Ad-VP3 and Ad-MOCK (MOI of 100, 50 μL) were added to the cells in the virus and control groups. A 96-well plate was removed at 24 h, 48 h and 72 h, and the medium was discarded. Then, 100 μL of JC-1 staining solution (diluted 1:1000) was added to each well, and the samples were incubated for 20 min in an incubator in the dark and washed twice with PBS (1×). After 50 μL of serum-free medium was added, an enzyme labelling instrument (Tecan Trading AG, Switzerland) was used for detection. The detection conditions were as follows: JC-1 monomer, excitation wavelength of 490 nm and emission wavelength of 530 nm; JC-1 polymer, excitation wavelength of 525 nm and emission wavelength of 590 nm.

## Annexin V-FITC/PI detection

1. Laser confocal microscopy observations of fluorescence staining to assess apoptosis

MCF-7 cells ($2\times10^5$ cells/well) were added to a 6-well plate. After 24 hours of culture, the three recombinant adenoviruses, Ad-VT, Ad-VP3 and Ad-MOCK (MOI of 100), were added, and a control well was established. The cells were collected into EP tubes at 24 h, 48 h and 72 h and centrifuged at 2000 rpm for 5 min. Then, 500 μL of binding buffer (1×) was added to each tube, 5 μL of Annexin V-FITC (Cell Quest Pro, Becton Dickinson) and 5 μL of PI (Cell Quest Pro, Becton Dickinson) were added, and the mixture was incubated for 20 min at room temperature and photographed with a laser confocal microscope(Leica, Germany).

2. Detection of apoptosis by flow cytometry

MCF-7 monollayers were prepared in a 6-well plate with a cell density of 2.0s 105 cells/well and cultured in a 5% $CO_2$ incubator at 37c for 24 h. MCF-7 cells were infected with 100 MOI recombinant tumour-lytic adenovirus (Ad-VP3 and Ad-VT).MCF-7 cells were treated with fitc Annexin-V apoptosis kit (Cell Quest Pro, Becton Dickinson) for 24h, 48h and 72h. Meanwhile, negative control group, Annexin V-FITC single staining control group and PI single staining control group were established. Apoptosis was detected by flow cytometry (C6 Plus and FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

## Proteomics and WB verification of the changes in the levels of key proteins in MCF-7 cells induced by the recombinant oncolytic adenoviruses

1. Proteomic analysis of changes in protein levels

MCF-7 cells were inoculated in 6-well plates ($2\times10^5$ cells/well). After 24 hours of incubation in the incubator, the cells were inoculated with the recombinant adenoviruses Ad-VT (MOI of 100). 48 hours after recombinant oncolytic adenovirus infection, cells were collected for proteomic analysis, searching for significantly different proteins by using hypergeometric test analysis.

2. Verifying the credibility of the proteomic data via Western blot experiments

The previous experiment was the same as that described in (1). Western blotting was performed to detect the expression levels of the p-mTOR and p-S6K proteins in MCF-7 cells that were incubated with the recombinant adenovirus Ad-VT at an MOI of

100 for 48 hours. Cells that were not infected with the virus during the same period were used as the control group.

## Detection of luciferase activity and stability

MCF-7-luc cells ($5\times10^3$ cells/well) were added to a 96-well plate and cultured for 48 h. After 3 min, the cells were lysed with an ONE-Glo™ Luciferase Assay System (Promega Corporation, USA) (100 μL/well), and luciferase activity was detected with an enzyme labelling instrument (Tecan Trading AG, Switzerland). The clone with the highest fluorescence value was continuously cultured for 8 weeks, and the luciferase activity was measured using a luciferase detection kit (Promega Corporation, USA) regularly (5 generations) to observe the stable expression of the luc gene.

## *In vivo* imaging of MCF-7-luc cells *in vitro*

The density of the MCF-7-luc cells was diluted in a gradient of 10 dilutions ($5\times10^5$ cells/mL–$9.8\times10^2$ cells/mL). A total of 100 μL/well was inoculated into a 96-well plate, and the control group was established. After 24 hours of culture, the fluorescein substrate (Promega Corporation, USA) (luciferin, *in vivo* grade, 150 μg/mL) was added at 100 μL/well, and the relationship between the number of cells and the bioluminescence intensity was analysed.

## Determination of the cell growth curve

MCF-7 cells and MCF-7-luc cells (5000 cells/well) were added to a 96-well plate. One 96-well cell culture plate was removed at 1 d–7 d, and the culture medium was discarded. The growth of the cells was tested with an MTS (Promega Corporation, USA) assay, and the cells were incubated in the dark and shaken for 20 s. The OD values of the two types of cells were measured with an enzyme labelling instrument (Tecan Trading AG, Switzerland) at a wavelength of 450 nm.

## Establishment of a mouse model of breast cancer xenotransplantation

The hormone treatment prior to the formation of breast tumours included an intramuscular (I.M.) injection of oestradiol valerate (2 mg/kg/week) once a week one week before the tumour cell injection. MCF-7-luc cells were collected, the cell density was adjusted to $1\times10^7$ cells/mL, and 100 μL of cells was injected into each mouse. After successful tumour cell loading, the mice were randomly divided into 5 groups (Ad-VT treatment group, Ad-VP3 treatment group, Ad-MOCK treatment group, normal saline treatment group and control group), with 6 mice in each group. The size of the tumours was measured every week, and the mice were photographed with a small animal imager (0–6 w). When the tumour has grown to a volume greater than $10mm^3$, treatment is initiated. After continuous treatment for 5 weeks, recombinant adenovirus ($1\times10^9$ $TCID_{50}$/100 μL) was injected into the tumours every 3 days.

Volume=(Length×$Width^2$)/2

## Detection of the luminescence value, tumour volume and life cycle of luciferase in tumour-bearing nude mice

After the tumours were successfully implanted into the nude mice, the tumour site was photographed continuously for 4–6 weeks with an *in vivo* imager (Merck KGaA, Darmstadt, Germany). An intraperitoneal injection of 200 μL of the fluorescein substrate luciferin D-potassium salt (Promega Corporation, USA) (15 mg/mL) was performed in each nude mouse. After 3–5 min, each nude mouse was injected intraperitoneally with 80–100 μL of 1% pentobarbital sodium. After the mice were completely anaesthetised, they were placed in a tray and put into a dark box for imaging (white light photography and bioluminescence photography). The white light image was superimposed on the bioluminescence image, a ruler was added, and the luminescence of the tumour area was calculated to assess the results. The tumour length and width were measured once a week for 5–6 weeks. Survival was recorded every day, and no significant change in the survival of the nude mice in each group (3–5 weeks) was observed.

## Statistical analysis

Statistical significance between groups was determined using GraphPad Prism, Version 8.0 (GraphPad Software, San Diego, CA). Data were presented as mean ± SEM in all experiments and analysed using Student's t test or one-way analysis of variance (ANOVA) followed by a Dunnett's *post hoc* test. Comparisons were significant when $P < 0.05$, $P < 0.01$ or $P < 0.001$.

# Results

## Recombinant adenoviruses inhibit the proliferation of MCF-7 and MCF-10A cells

The concentration gradient revealed that the inhibitory effects of MOIs of 50, 100 and 200 of Admuri VT on cells significantly increased with increasing infection time, with an obvious effect of time observed in the following order: 72 h > 48 h > 24 h ($P<0.05$, $P<0.01$, and $P<0.001$; Figures 2A–C). At 72 h, an MOI of 200 of Ad-VT had the strongest inhibitory effect on MCF-7 tumour cell activity (60.336 ± 0.756%). Because the constructed recombinant Ad-VP3 adenovirus did not have the ability to be continuously amplified, the inhibitory effect on tumour cells was not obvious after 24 hours. When cells were infected with Ad-VP3 for 48 hours, the inhibition of MCF-7 tumour cell activity was the most obvious at an MOI of 200 (26.108 ± 2.005%). Over time, the inhibition rates of Ad-VT and Ad-VP3 on MCF-7 cells increased with increasing

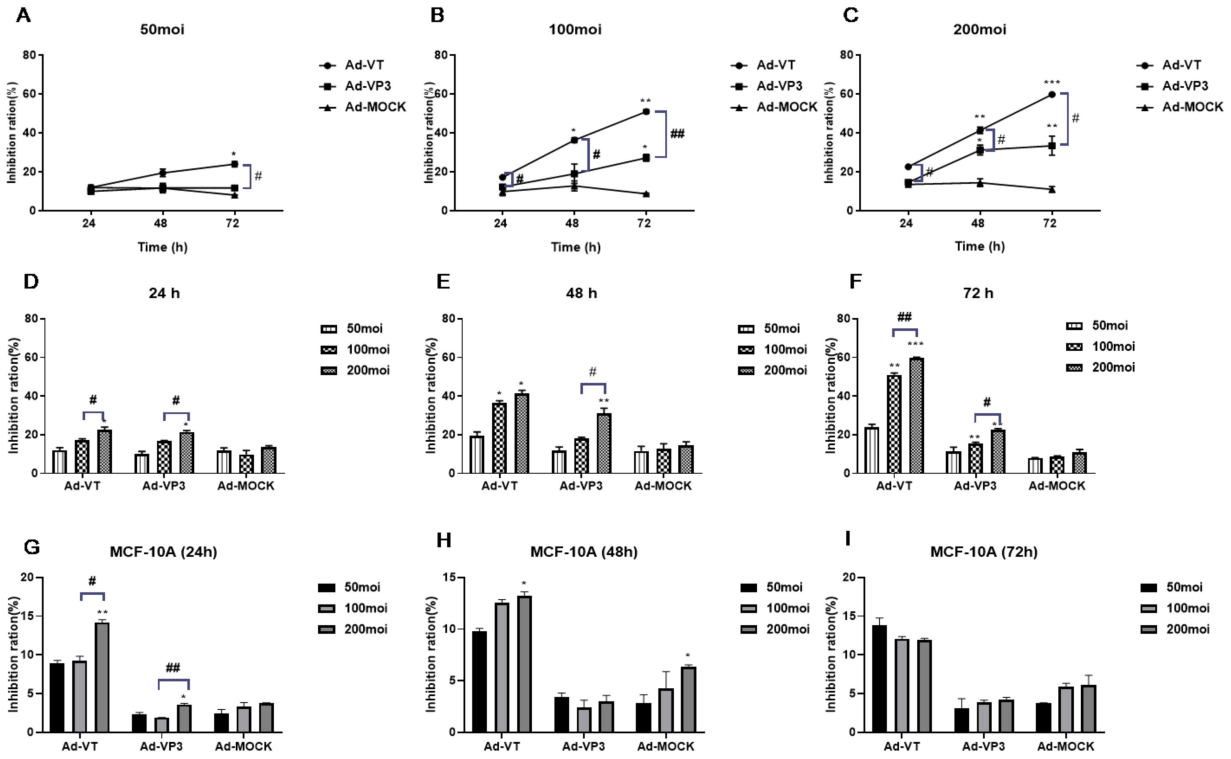


FIGURE 2
The effect of recombinant oncolytic adenovirus on MCF-7 and MCF-10A cell viability was detected by MTS assay. **(A-C)** Rates at which different concentrations (MOIs of 50, 100, and 200) inhibited MCF-7 cell activity. *P < 0.05 compared with the same recombinant adenovirus group at 24 h, **P < 0.01 compared with the same recombinant adenovirus group at 24 h, and ***P < 0.001 compared with the same recombinant adenovirus group at 24 h. **(D-F)** Inhibitory effects of the recombinant adenoviruses on MCF-7 cell activity at different times (24 h, 48 h, and 72 h). **(G-I)** Inhibitory effects of the recombinant adenoviruses on MCF-10A cell activity at different times (24 h, 48 h, and 72 h). *P < 0.05 compared with the same recombinant adenovirus administered at an MOI of 50, **P < 0.01 compared with the same recombinant adenovirus administered at an MOI of 50, and ***P < 0.001 compared with the same recombinant adenovirus administered at an MOI of 50. (#p<0.05, ##p<0.01, ###p<0.001) when compared with Ad-VT.

concentration (Figures 2D–F), with an MOI of 200 > MOI of 100 > MOI of 50. In summary, the inhibitory effects of Ad-VT and Ad-VP3, which carry apoptin, on MCF-7 cells increased with increasing infection time, but the effect of Ad-VP3 began to decrease at 72 h. A dose–effect relationship was observed between Ad-VT and Ad-VP3 (MOI of 200>MOI of 100>MOI of 50), and the strength of the inhibitory effect was as follows: Ad-VT>Ad-VP3. For normal mammary epithelial cells MCF-10A, three recombinant adenoviruses at three time periods of 24, 48h, 72h and three different concentrations of 50MOI, 100MOI and 200MOI did not show significant cell inhibition, and the inhibition rate was less than 15% (Figures 2G–I).

## Recombinant oncolytic adenoviruses inhibit the migration and invasion of MCF-7 cells

Images were captured at the same location at 0 h, 8 h, 16 h and 24 h after scratch formation (Figure 3A). The migration rate of cells infected with Ad-VT and Ad-VP3 was significantly lower than that of the Ad-Mock group and Control group, and Ad-VT had the strongest inhibitory effect on cell migration. At 8 h, the migration rate of the MCF-7 cells was 15.01%. The migration rate of MCF-7 cells in the Ad-MOCK VP3 group was 26.5%, which was significantly lower than that in the Ad-MOCK group (32.27%) and the control group (34.26%). The inhibitory effects of the three recombinant adenoviruses on MCF-7 cell migration were in the following order: Ad-VT > Ad-Vp3 > Ad-Mock.

Both Ad-VT and Ad-VP3 inhibited the invasion of MCF-7 cells (Figure 3B). Compared with that of the control, the inhibitory effect of Ad-VT on invasion was greater than that of Ad-VP3 at 24 h or 48 h ($P < 0.001$) and was time-dependent because the value measured at 48 h was greater than that at 24 h. However, Ad-MOCK had no inhibitory effect on the invasion of MCF-7 tumour cells. In addition, at 48 h, the inhibitory effects of Ad-VT and Ad-VP3 on cell invasion were as follows: MOI of 50 < MOI of 100 < MOI of 200 (Figure 3C). The invasion rates of the three recombinant adenoviruses were in the following order: Ad-VT < Ad-VP3 < Ad-Mock. Ad-VT and Ad-VP3, which carry apoptin, also exhibited a time-dependent relationship. The invasion rate of cells infected for 48 hours was lower than that of cells infected for 24 hours.

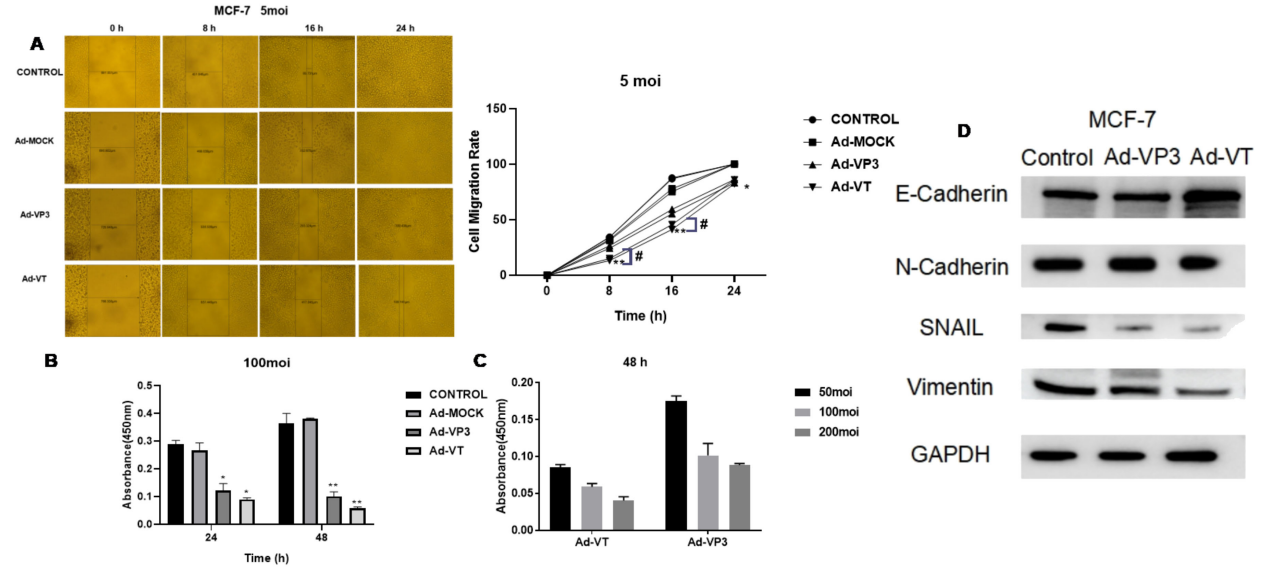


FIGURE 3
Effects of the recombinant oncolytic adenoviruses on the migration and invasion of MCF-7 cells detected using migration and BioCoat™ Matrigel® invasion chamber assays. **(A)** The administration of three recombinant oncolytic adenoviruses at an MOI of 5 (Ad-VT, Ad-VP3, and Ad-MOCK) inhibited MCF-7 cell migration. **(B)** Three recombinant oncolytic adenoviruses were used to infect MCF-7 cells at an MOI of 100, and invasion was detected after 24 and 48 hours. All the measurements were performed in triplicate, and the means ± standard deviations were compared with those of the control group (*P<0.05 and **P<0.01). **(C)** After infecting MCF-7 cells with different concentrations of recombinant adenoviruses (MOIs of 50, 100, or 200) for 48 h, the invasion results were detected with a BioCoat™ Matrigel® invasion chamber. **(D)** After 48 hours, we extracted total protein from the cells and performed Western blotting to detect the protein expression levels of E-cadherin, N-cadherin, SNAIL and vimentin. All measurements were performed in triplicate, and the means ± standard deviations were compared with those of the MOI group (*P<0.05 and **P<0.01). MOI, multiplicity of infection. #p<0.05 when compared with Ad-VT.

Western blotting was performed to detect changes in the ability of the recombinant oncolytic adenoviruses to induce MCF-7 cell-associated metastasis (Figure 3D). After 48 hours of stimulation, the recombinant oncolytic adenoviruses increased the expression of the E-cadherin protein and decreased the expression of the HIF-1, VEGF-C, MMP-3, MMP-9, N-cadherin, SNAIL and vimentin proteins in MCF-7 cells. The above results showed that recombinant oncolytic adenoviruses could inhibit the migration and invasion of breast cancer cells, reduce the expression of metastasis-related proteins, and reduce the metastatic ability of breast cancer cells.

## Observation of apoptotic bodies via transmission electron microscopy

Apoptotic bodies were distributed in MCF-7 cells treated with Ad-VT (MOI of 100) (Figure 4A). Typical apoptotic bodies were also detected in Ad-VP3-infected MCF-7 cells. Compared with those in the control group and Ad-MOCK group, nuclear fragments and apoptotic bodies formed by nuclear fragmentation were observed 48 hours after Ad-VT and Ad-VP3 infection, especially in the Ad-VT group.

## Changes of nucleus induced by recombinant oncolytic adenovirus in breast cancer

After the MCF-7 cells were infected with Ad-VT and Ad-VP3 (Figure 4B), the nuclear staining was uneven, some of the nuclei presented bright blue staining, and some cell swelling or nuclear fragmentation was observed in the Ad-VT group, whereas the nuclei of the Ad-MOCK group and control group presented uniform blue fluorescence, indicating that apoptosis occurred in both the Ad-VT and Ad-VP3-infected cells and that the degree of apoptosis was Ad-VT > Ad-VP3. In addition, we observed cells at different time points (0 h, 24 h, 48 h, and 72 h) and found that the nuclei of MCF-7 cells infected with Ad-VT became increasingly brighter with prolonged treatment (72 h > 48 h > 24 h > 0 h) in a time-dependent manner (Figure 4B).

## Recombinant oncolytic adenovirus induces apoptosis of breast cancer cells

After the MCF-7 cells were infected with the recombinant adenoviruses Ad-VT and Ad-VP3 for 48 h and 72 h, the cells

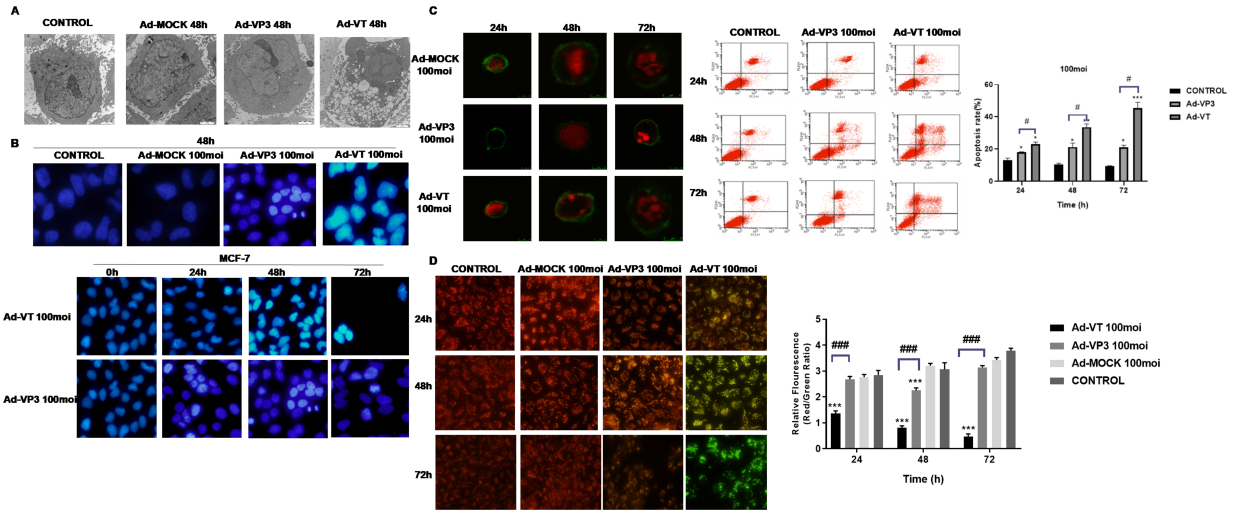


FIGURE 4
The apoptosis of MCF-7 cells induced by recombinant adenoviruses was detected via transmission electron microscopy, Hoechst staining, JC-1 staining, Annexin V staining, laser confocal microscopy and flow cytometry. **(A)** TEM revealed apoptotic structures in the enlarged images of cells treated with Ad-VT, Ad-VP3 or Ad-MOCK for 48 h. **(B)** MCF-7 cells were treated with recombinant oncolytic adenoviruses, and changes in the morphology of breast cancer cell nuclei were observed via Hoechst staining at 24 h, 48 h, and 72 h. **(C)** Apoptotic breast cancer cells were analysed by laser confocal microscopy and flow cytometry after staining with Annexin-V FITC/PI at 24 h, 48 h and 72 h. **(D)** Changes in the mitochondrial membrane potential of breast cancer cells induced by recombinant oncolytic adenovirus were analysed via JC-1 staining. All the measurements were performed in triplicate, and the means ± standard deviations were compared with those of the control group (*P<0.05 and ***P<0.001). (#p<0.05 and ###p<0.001) when compared with Ad-VT.

with green fluorescence on the cell membrane and red fluorescence in the nucleus were detected via laser confocal microscopy, and some of them exhibited typical characteristics of apoptosis (Figure 4C): cell membrane blebbing, cell membrane damage, nuclear fragmentation, and cell swelling. No obvious characteristics of apoptotic cells were observed in the Ad-VP3 group at 24 h. Confocal laser scanning microscopy revealed that Ad-VT- and Ad-VP3-infected MCF-7 cells underwent apoptosis in a time-dependent manner: 72 h > 48 h > 24 h. However, a very small number of apoptotic cells was occasionally observed in the control group and Ad-MOCK group, which were difficult to capture via laser confocal microscopy. We further observed the apoptosis of MCF-7 cells induced by apoptin and found that Ad-VT and Ad-VP3, which carry apoptin, could induce the apoptosis of MCF-7 cells at three different time points (24 h, 48 h and 72 h) (Figure 4C). However, the degree of apoptosis was different at different time points as follows: Ad-VT > Ad-VP3, 72 h > 48 h > 24 h. The apoptosis rate in the Ad-VT and Ad-VP3 groups was significantly higher than that in the control group at 48 h and 72 h, and the apoptosis rate reached a maximum at 72 h as the duration of Ad-VT treatment increased. The rate of apoptosis induced by Ad-VP3 was also significantly higher than that in the control group [48 h group (23.268 ± 1.103)% vs. (10.680 ± 1.104)%, $P < 0.05$; 72 h group (22.002 ± 0.317)% vs. (9.670 ± 1.102), $P < 0.05$; Figure 3C]. The rate of apoptosis induced by Ad-VP3 was the highest at 48 h and decreased at 72 h. We further observed concentration dependence of the effect and found that the concentration was positively correlated with the degree of apoptosis, and the apoptosis rate of MCF-7 cells treated with Ad-VT for 48 h changed in the order of MOIs of 200 > 100 > 50. These results suggest that Ad-VT and Ad-VP3 induce apoptosis by causing changes in the cell membrane.

## Recombinant oncolytic adenovirus induces apoptosis by changing mitochondrial membrane potential of breast cancer cells

MCF-7 cells were inoculated with the recombinant adenoviruses Ad-VT, Ad-VP3 and Ad-MOCK. After 24, 48 and 72 hours of treatment, the inhibitory effects of Ad-VT and Ad-VP3 on MCF-7 cells were determined by monitoring changes in the mitochondrial membrane potential (Figure 4D). The three recombinant adenoviruses had different abilities to induce apoptosis and different levels of membrane depolarisation. The ability of Ad-VT to induce apoptosis increased with increasing infection time, and the number of apoptotic cells increased gradually. JC-1 gradually changed from an initial red aggregate to a green monomer. Compared with the control group, the Ad-VP3 group also presented obvious colour changes, but the colour comparison was not obvious at different time points. The ability of Ad-VT to induce apoptosis in MCF-7 cells was greater than that of Ad-VP3 (Figure 4D), the number of apoptotic cells was greater at 72 h in the Ad-VT group, and the ratio of red fluorescence to green fluorescence was significantly lower than that in the control group ($P < 0.001$). The ability of Ad-VP3 to induce apoptosis was greater than that of the control group ($P < 0.01$), as the ratio of red fluorescence to green fluorescence decreased; however, because Ad-VP3 could not replicate and proliferate, the ratio of red fluorescence to green fluorescence increased at 72 h. At any of the three different time

points, the ratio of red fluorescence to green fluorescence in the infected cells was in the order of Ad-VT < Ad-VP3 < Ad-MOCK.

## Changes of apoptosis-related protein expression levels induced by recombinant oncolytic adenovirus in breast cancer cells

Proteomic analysis revealed that, compared with control cells, MCF-7 cells infected with recombinant adenovirus (Ad-VT) presented significant differences in multiple signalling pathways and protein expression. Forty-eight hours after exposure, GO enrichment analysis revealed that the q values of the genes associated with organelles organization and nucleic acid metabolic processes were small (close to 0), and the difference was significant. Among the cell components, the nuclear part and nuclear lumen genes had significant changes, and the differences were significant. In molecular function, the q values of poly(A) RNA binding and protein binding genes were small and close to 0, and the differences of genes were significant(Figure 5A). According to the functional annotation and classification results of differential genes, we conducted enrichment analysis of the KEGG pathway(Figure 5B), and found that the Rich factor value of the signalling pathway with changes in MAPK and mTOR pathways gradually increased, and the closer Pvalue was to 0, it showed that the enrichment degree gradually increases and the enrichment becomes more obvious. Subsequently, differential protein heat maps showed that M3K1, S6K, MYC, STK3, KS6A6, FLNA, GBG12, PA24A, RICTR, STRAA and IF4B were highly expressed in MCF-7 cells after infection with Ad-VT, while TSC1, RS6, AKTS1 and KS6A1 were low(Figure 5C). According to the results of KEGG analysis, cell cycle, MAPK, mTOR pathway and other top ranked pathways are related to apoptosis, and S6K was found to have a significant difference in differential proteins, and S6K is a key gene in the mTOR pathway. Some studies have shown that activation on the mTOR pathway can cause apoptosis, in which S6k activation plays an important role (7, 8). Therefore, we suspect that the elevation of S6K is the key to promoting apoptosis.

In order to determine the proteomic results(Figure 5D), WB detection was performed after infection with Ad-VT, and it was found that phosphorylated mTOR was significantly up-regulated at 48h after Ad VT was treated on MCF-7 cells compared with the control group, phosphorylated S6K proteins was upregulated at 48h. The above results are consistent with the results of labelfree proteomic experiments, namely, the promotion of apoptosis of MCF-7 cells by recombinant adenovirus Ad-VT carrying apoptin may be related to the increase of S6K. In summary, the results of protein immunoblotting are basically consistent with the quantitative results of proteomic experiments, with good repeatability.

## MCF-7-luc cells can stably express luciferase

A luciferase assay system was used to detect the luciferase activity of different cell clones (Figure 6A). Among the 15 groups of cloned cell lines, the luciferase activity of Clones 9 and 15 was the highest.

The two selected clones with the highest fluorescence values were amplified and cultured to observe whether passage had any effect on luciferase activity. The luciferase activity was measured every 5 generations, and no significant change in the fluorescence value at the initial detection up to the 40th generation was detected (Figure 6B, $P > 0.05$), which showed that the two screened cell lines did not affect the luciferase activity due to cell passage and still maintained a stable and high level of luciferase activity.

Clone 15 was seeded in 96-well plates at a density of 50,000 cells/100 μl in the first row of wells. The cell concentration was gradually reduced by double dilution, and then, fluorescein substrate was added and detected with a small animal living imager. The bioluminescence intensity of the MCF-7-luc cells was proportional to the number of cells, with a correlation coefficient of $R^2 = 0.9903$, and no fewer than 400 cells were detected (Figure 6C). MCF-7-luc cells expressing the firefly luciferase gene were successfully generated from this cell line.

## Comparison of the growth curves of MCF-7 cells and McF-7-luc cells

The growth trends of MCF-7 cells and McF-7-luc cells were not significantly different, and the growth curves were basically consistent (Figure 6D), indicating that the growth characteristics of McF-7-luc cells expressing the firefly luciferase gene were not significantly different from those of human MCF-7 breast cancer cells.

## Recombinant oncolytic adenovirus inhibited tumour bioluminescence intensity

Female BALB/c (nu/nu) mice were subcutaneously inoculated with MCF-7-luc cells, and the bioluminescence intensity of the tumour cells was observed with a small animal *in vivo* imager beginning at 0 W after inoculation (Figures 7A, B). We found that the average bioluminescence intensity of the control group, the normal saline treatment group and the Ad-MOCK treatment group increased weekly, and the intensity increased rapidly. The luminescence intensity of the three groups was essentially the same at 0–2 w, and the average luminescence intensity of the normal saline treatment group began to be slightly lower than that of the control group and the Ad-MOCK treatment group beginning in the 3rd week; however, no significant difference in the average weekly bioluminescence intensity was observed among the three groups ($P > 0.05$). The average bioluminescence intensity of the Ad-VT and Ad-VP3 treatment groups decreased slowly, and the average bioluminescence intensity of the two groups was not significantly different ($P > 0.05$). Starting from the third week of photography, namely, the first week after treatment, the luminescence intensity of the Ad-VT treatment group and the Ad-VP3 treatment group differed from that of the control group

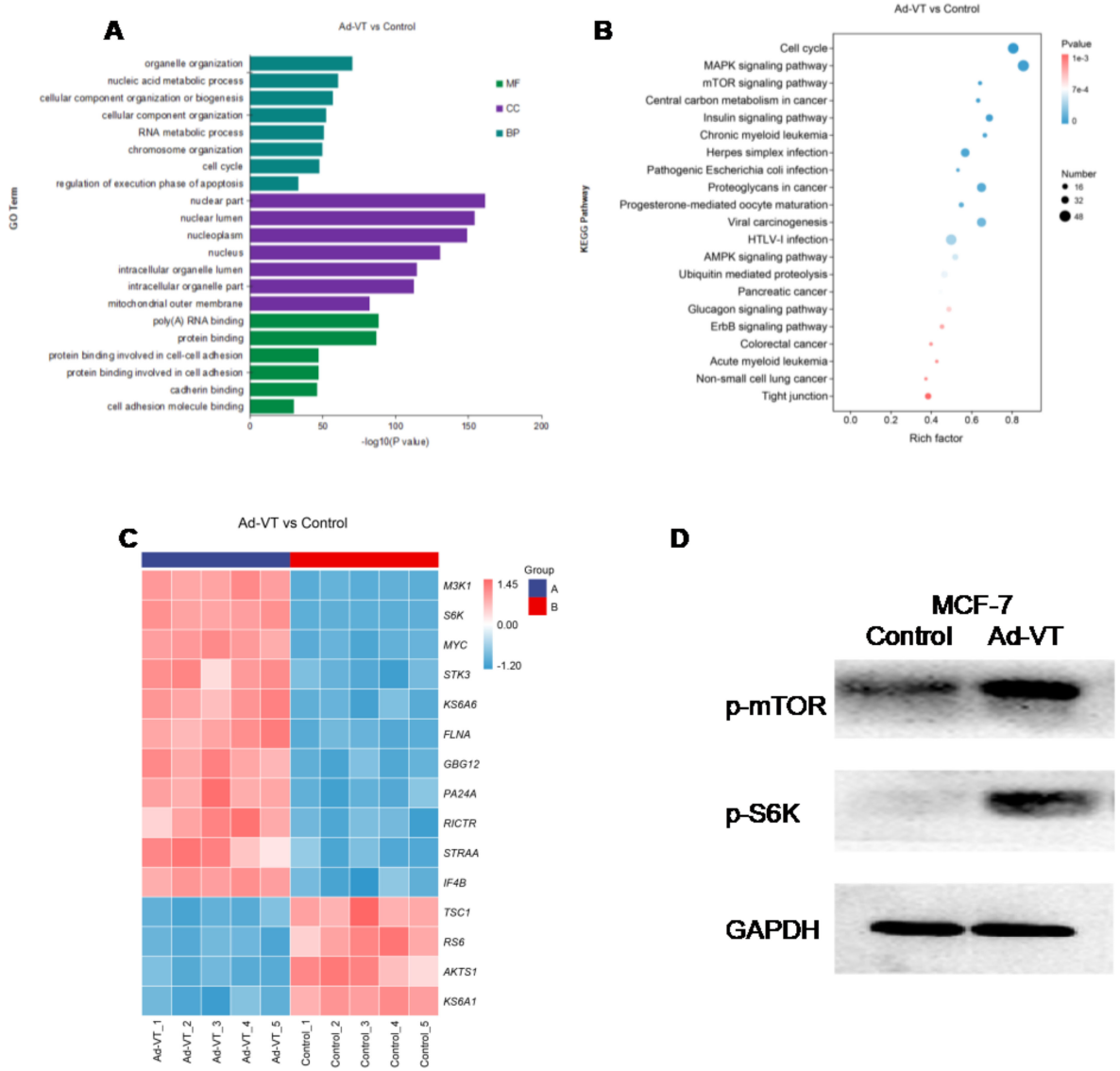


FIGURE 5
Proteomic analysis of the effects of recombinant adenoviruses carrying apoptin on MCF-7 cells. **(A)** MCF-7 cells were infected with recombinant oncolytic adenoviruses (Ad-VT ) for 48 h, and the changes in cell biological processes, cell components and molecular functions were analysed via proteomics. **(B)** After infecting MCF-7 cells with recombinant oncolytic adenoviruses (Ad-VT ) for 48 h, the differentially expressed genes were subjected to KEGG analyses. **(C)** After recombinant oncolytic adenovirus (Ad-VT) infected MCF-7 cells for 48 hours, Ad-VT induced MCF-7 cells to express differential genes. **(D)** After infecting MCF-7 cells with recombinant oncolytic adenoviruses for 48 h, the changes in the levels of three proteins were analysed by Western blotting. All measurements were performed in triplicate.

and gradually increased (Figure 6B), and significant differences were observed at 3 w and thereafter ($P < 0.05$).

## Recombinant oncolytic adenovirus inhibited tumour growth in BALB/c nude mice

After the MCF-7-luc cells were inoculated subcutaneously near the backs of the hind limbs of the nude mice, the tumour size was measured once a week for 4–6 weeks (Figure 7C). The tumour volumes of the control group, the normal saline group and the Ad-MOCK treatment group all increased significantly over time, increasing from approximately 10 $mm^3$ to more than 100 $mm^3$ at 4 w, and no significant difference in the average tumour growth rate was observed among the three groups ($P > 0.05$, Figure 7C). Neither normal saline nor Ad-MOCK inhibited tumour growth, but the tumour volume of the Ad-VT treatment group and the Ad-VP3 treatment group tended to decrease after treatment (Figure 7C), and the rate of reduction in the average tumour volume of the two treatment groups was slower than that of the control group. We found that before treatment (0 W and 1 W), the average tumour volume of the five groups of mice was not significantly different, but after different toxic treatments (2 W), the tumour volume began to change differently. Due to the different trends,

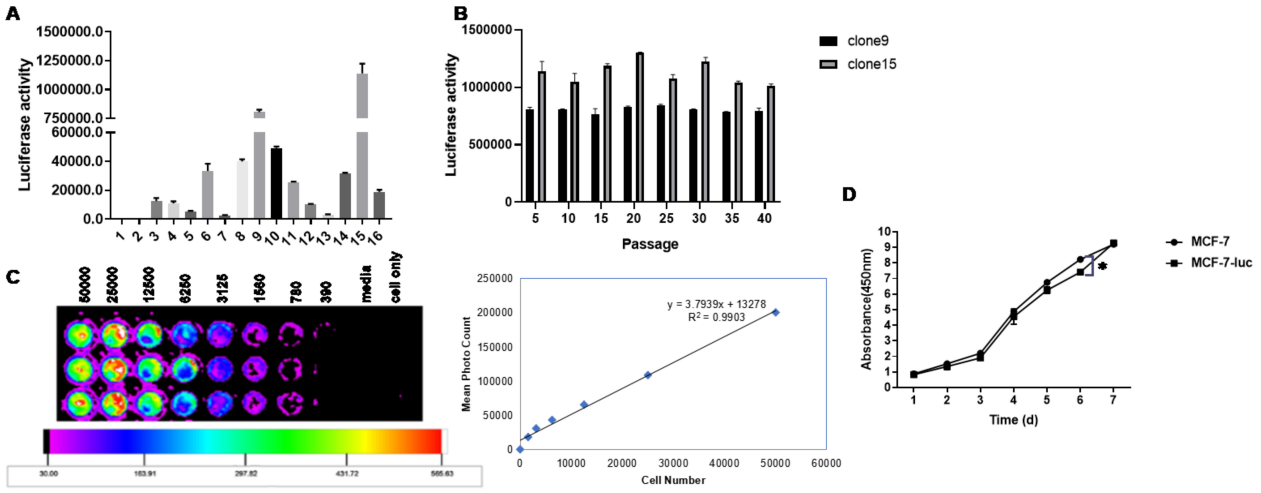


FIGURE 6
Enzyme activity of luciferase in a human breast cancer cell line (MCF-7-Luc) and comparison of their biological characteristics before and after the test. **(A)** Luciferase activity of different cell clones. **(B)** Luciferase stability in MCF-7-luc cells from different generations. **(C)** MCF-7-luc cells were inoculated into 96-well plates according to the principle of multiple dilutions. After the addition of luciferase, the luminescence intensity was observed. The luminescence intensity increased as the number of cells increased. **(D)** MCF-7 and MCF-7-luc cells were cultured in a 96-well plate. The cell growth rates were detected on days 1, 2, 3, 4, 5, 6 and 7. All the measurements were performed in triplicate, and the means ± standard deviations were compared with those of the MCF-7-luc group (*P<0.05).

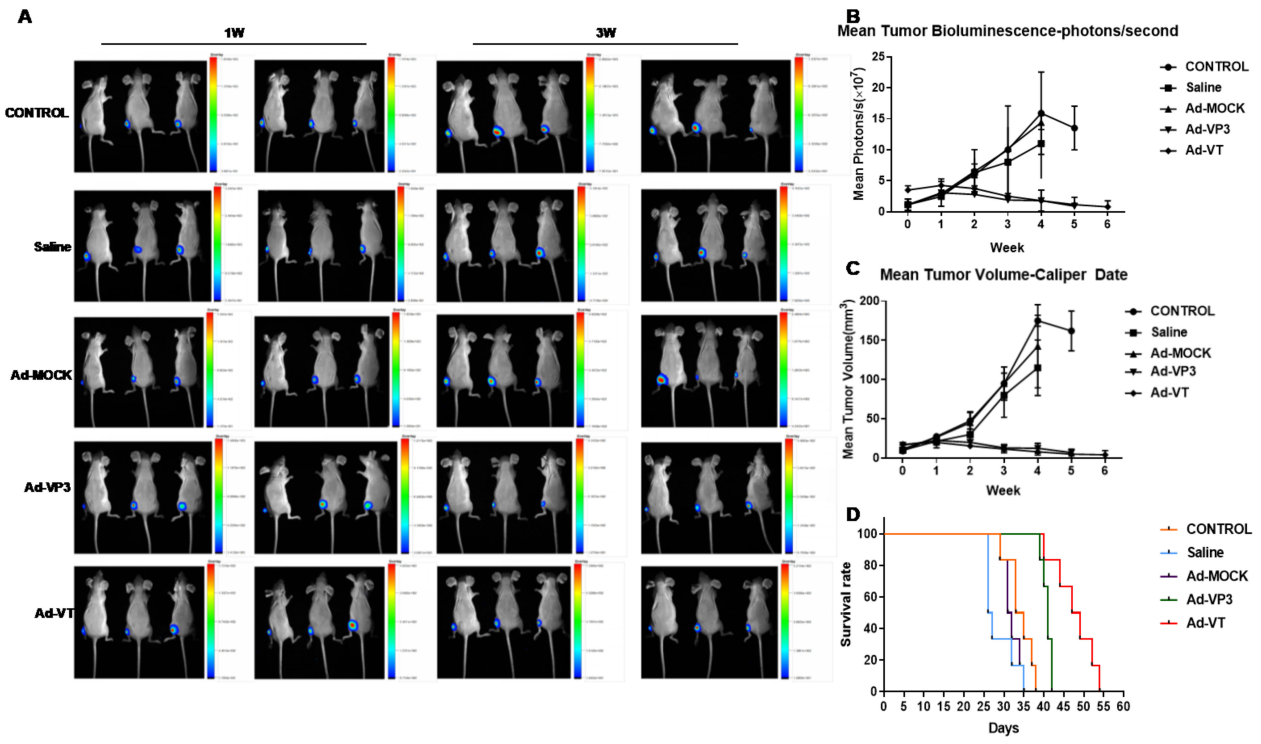


FIGURE 7
Effect of the recombinant oncolytic adenoviruses on breast cancer in a BALB/c nude mouse model. **(A, B)** MCF-7-luc cells ($1\times10^7$/100 litres) were injected subcutaneously into the near back of the right hind limb of the mice (6 mice in each group) to establish the xenograft model. An in vivo imaging luminescence system was used to monitor changes in tumour bioluminescence intensity continuously. **(C)** After the xenograft model was successfully established in nude mice, the survival of the mice was recorded daily for 5 weeks. Vernier callipers were used to measure the diameter of the tumours in the nude mice, the tumour volume was calculated once a week, and continuous measurements were performed for five weeks. **(C)** The average tumour inhibition rate of the 1x109 PFU/100 μL Ad-VT treatment group was significantly higher than that of the other groups. The survival rate of the $1\times10^9$ PFU/100 μL Ad-VT treatment group was also the highest, and the average survival rate of the nude mice exceeded 60%. **(D)**The survival time of the $1\times10^9$ PFU/100 μL Ad-VT treatment group was significantly prolonged compared with the control group or the $1\times10^9$ PFU/100 μL MOCK treatment group.

the tumour volume of the two treatment groups, Ad-VT and Ad-VP3, decreased, whereas the tumour volume of the other three groups increased continuously, and the differences between them became increasingly obvious. In summary, Ad-VT and Ad-VP3, which carry apoptin, have inhibitory effects on the MCF-7 human breast cancer cell line.

### Survival curves of tumour-bearing BALB/c nude mice after treatment

After the subcutaneous inoculation of MCF-7-luc cells into BALB/c female mice, the survival times of the nude mice in the different groups were observed (Figure 7D). The mice in the saline group died at approximately 25 days, and the mice in the control group and the Ad-MOCK treatment group died at 29 days. In the next 10 days, 6 mice in each of the three groups died, and the average survival time was 34.17 days in the control group, 28.67 days in the normal saline group, and 32.00 days in the Ad-MOCK treatment group. No significant difference in mean survival was observed between the Ad-MOCK group and the control group ($P > 0.05$). The first mouse in the Ad-VP3 group and Ad-VT group died at approximately the 40th day, and the average survival times were 40.83 d and 47.67 d, respectively. The survival time of both groups was significantly longer than that of the control group; in particular, the survival time of the Ad-VT group was longer than that of the Ad-VP3 group. The survival rate of both groups was 100% after 38 days of tumour cell inoculation, and all of the mice in the control group died. These results indicated that the Ad-VP3 and Ad-VT treatments significantly prolonged the average survival time and improved the survival rate of tumour-bearing mice.

## Discussion

Breast cancer is the most common type of cancer among women, and its incidence is increasing worldwide. Each year, approximately 1400000 women are diagnosed with breast cancer, and approximately 500000 people die (25). Breast cancer has become the tumour with the highest incidence worldwide (26–28). At present, surgical treatment for primary breast cancer is combined with targeted therapy or chemotherapy, endocrine therapy, and immune therapy (3, 29). With continuous improvements in technology, these treatments have achieved good curative effects, but the current treatment strategies for patients still have greater side effects, with a certain negative effect on patients' quality of life.

Gene therapy is one of the most promising cancer treatments and is now in a stage of rapid development. The selection of appropriate therapeutic genes is the first stage in the development of gene therapy. Due to its potential application for treatment with a variety of genes, adenovirus is a promising tool for cancer treatment because of its ability to manipulate genes and exert a variety of anticancer effects (30–33). Apoptin, a highly conserved protein derived from chicken anaemia virus (CAV), can selectively induce the apoptosis of many malignant tumour cells without affecting normal cells and has become an important latent gene therapy for cancer (34–37). Apoptin is the first isolated tumour-selective anticancer gene. The independence of the small virus protein p53 in tumour cells can be induced by specific types of cell death (21, 38–40). Our laboratory developed a method of gene therapy to observe the effects on apoptosis and the mechanism of action in MCF-7 human breast cancer cells and to inhibit tumour growth *in vivo* and *in vitro*.

Oncolytic viruses (OVs), which evolved and are engineered for cancer specificity, are gaining momentum as a new class of drugs in the fight against cancer. This research used the MTS test to preliminarily evaluate the effects of recombinant adenovirus infection on the proliferation of MCF-7 cells. Both Ad-VT and Ad-VP3 had significant inhibitory effects on MCF-7 cells because Ad-VT not only carried apoptin but also carried a peculiar tumour promoter (hTERTp, human telomerase reverse transcriptase) and expressed the E1A gene (viral replication essential gene). After infection in breast cancer cells, replication and amplification, the strength of the MTS signal within 24 h is significantly greater than that of Ad-VP3 infection after 72 h. Ad-VT (200 MOI) inhibited the proliferation of MCF-7 cells with a strength of 60.34% and was positively correlated with the time and dose. Ad-VP3 was not as potent as Ad-VT in MCF-7 cells because of its lack of a sustained expansion capacity and its effect was not time sensitive; however, it also affected MCF-7 cells with increasing infectious concentrations, and the inhibition rate was significantly increased. Ad-MOCK had no significant inhibitory effect on MCF-7 cells.

After MCF-10A cells were infected with Ad-VT, the inhibition rate was less than 15%. Although the inhibition rate was very low, it could not completely damage normal mammary epithelial cells. Although Ad-VT has been genetically modified to become a bispecific oncolytic adenovirus, its targeting is still insufficient, which also reflects the limitations of oncolytic adenovirus as a viral therapy.

Ya Cao et al. reported that CYT997 induces autophagy and apoptosis in gastric cancer by triggering mitochondrial ROS accumulation to silence the JAK2/STAT3 pathway. In another study, Wenjin Liang et al. suggested that miR-644a promotes apoptosis in HCC cells by inhibiting HSF1. This study aimed to further clarify the effects of this virus on promoting the apoptosis of MCF-7 cells 48 hours after Ad-VT (MOI of 100) or Ad-VP3 (MOI of 100) infection, and transmission electron microscopy was used to observe typical cytoplasmic organelles and nuclear fragments of the apoptotic body, confirming that the recombinant adenoviruses can cause MCF-7 cell apoptosis. By performing Annexin V-FITC/PI staining, flow cytometry and laser confocal microscopy experiments, the three recombinant adenoviruses, Ad-VT, Ad-VP3 and Ad-MOCK, were found to be effective in the treatment of MCF-7 cells at 48 h after infection. Both Ad-VT and Ad-VP3 induced obvious apoptosis in MCF-7 cells, with Ad-VT > Ad-VP3 and an MOI of 200>an MOI of 100>an MOI of 50. However, the cell apoptosis rate was not consistent with the results of the cell inhibition rate. The apoptosis rate induced by Ad-VT at an MOI of 100 (48 h) was 37% higher than the cell suppression rate of 33%. At 72 hours, Ad-VT (MOI of 100) produced a 45% lower apoptosis rate than the cell inhibition rate of 51%, and the difference between the two may be due in part to the use of different assays. Additionally, other death modalities are likely involved.

The results of the JC-1 assay revealed that the ability of Ad-VT to induce apoptosis gradually increased with increasing infection time, as JC-1 gradually changed from an initial red aggregate to a green monomer. Ad-VP3 also obviously changed the colour of JC-1 compared with that of the control group; moreover, no significant difference was observed between the Ad-MOCK group and the control group. Compared with the results of the control group, the results of standard fluorescence microscopy were the same: in the Ad-VT group (the ratio of red fluorescence to green fluorescence), the ratio was the lowest ($P < 0.001$). Compared with the control group, Ad-VP3 induced apoptosis in MCF-7 cells at 48 h.

Hui Zeng et al. showed that lycorine induced apoptosis of A549 cells by regulating AMPK-mTOR-S6K signalling pathway (8). The proteomic analysis revealed that the phosphorylated mTOR protein may be involved in apoptin-induced apoptosis in MCF-7 cells, especially changes in the p-S6K proteins associated with the p-mTOR protein, which provides a new approach for studying the potential signalling pathways involved in apoptin-induced apoptosis.

This study conclusively demonstrates that oncolytic adenovirus-delivered apoptin effectively induces apoptosis in MCF-7 breast cancer cells, with the apoptin response exhibiting dose- and time-dependent characteristics. Label-free quantitative proteomic analysis revealed significant alterations in both the global proteome and phosphoproteome of Ad-VT-infected MCF-7 cells. KEGG pathway enrichment analysis of differentially expressed proteins identified the MAPK and mTOR signalling pathways as being particularly prominent, showing substantial enrichment and marked changes in associated pathway proteins.

Our experiment confirmed that Ad-VT infection in MCF-7 cells leads to altered phosphorylation of mTOR and S6K proteins, suggesting potential involvement of the mTOR/S6K signalling pathway in apoptin-induced apoptosis (Figure 8). However, the exact molecular mechanisms remain to be fully elucidated. These preliminary findings, while indicative, are subject to certain limitations and require more comprehensive investigation to establish definitive mechanistic insights.

Living imaging technology is a new noninvasive technology for obtaining biomedical images of living tissue at the cellular and molecular levels and can be used for continuous monitoring of disease *in vivo*. Compared with other technologies, *in vivo* imaging is noninvasive, highly sensitive and allows dynamic monitoring, which is advantageous for the quantitative evaluation of cell proliferation and tumours in the body.

In this study, the firefly luciferase-labelled human breast cancer cell line McF-7-luc, which has the highest luciferase activity and good stability, was selected for comparison with MCF-7 cells *in vitro*. The biological characteristics of the constructed firefly luciferase-labelled McF-7-luc and MCF-7 cells were not significantly different, and a subcutaneous tumour-bearing model of McF-7-luc cells in nude mice was subsequently constructed. *In vivo* imaging revealed that the average bioluminescence intensity of tumours in the Ad-VP3 treatment group and Ad-VT treatment group was always lower than that in the other

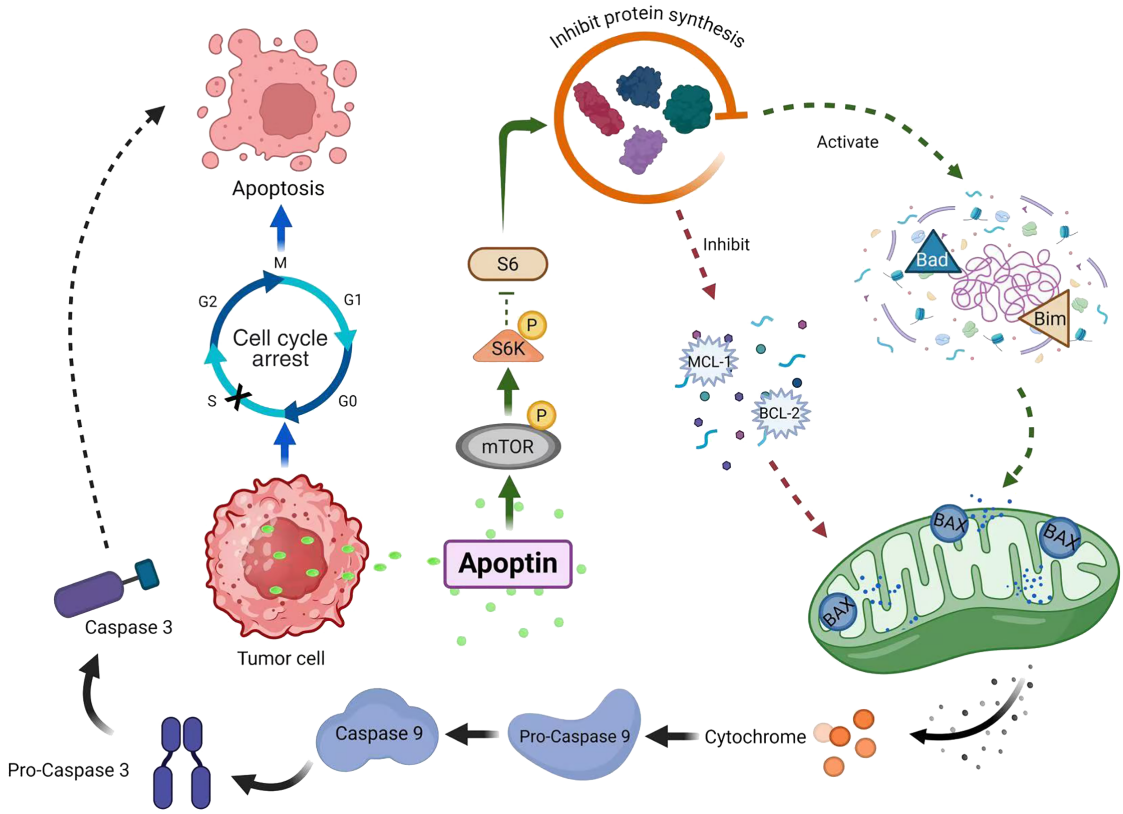


FIGURE 8
Ad-VT promotes the phosphorylation of mTOR/S6K signal, which leads to apoptosis of MCF-7 cells.

treatment groups after treatment and gradually decreased. In the control group, saline group and Ad-MOCK groups, the average tumour bioluminescence intensity was increased. Tumour growth curves revealed that the Ad-VP3 treatment and Ad-VT treatment inhibited tumour growth, whereas the other three treatments did not. Survival curves of nude mice revealed that the Ad-VP3 treatment group and the Ad-VT treatment group exhibited obviously prolonged survival to a significantly greater extent than the control group. The recombinant adenoviruses carrying apoptin, namely, Ad-VP3 and Ad–VT, slowed the tumour growth rate and extended the survival of the mice.

In conclusion, Apoptin can significantly inhibit the growth of MCF-7 human breast cancer cells, mainly by inducing the apoptotic death of MCF-7 cells. Proteomic experiments revealed that Ad-VT promoted the apoptosis of MCF-7 cells via the upregulation of the phosphorylated mTOR and S6K proteins. These findings suggest that Ad-VT can significantly enhance the apoptosis level of breast cancer cells, which is induced by the mTOR/S6K signalling pathway (Figure 8). *In vivo* experiments revealed that the recombinant adenovirus Ad-VT effectively inhibited the growth of tumour cells and prolonged the survival of the mice. This discovery provides a new idea and method for the treatment of breast cancer, and also provides a possibility for individualised combined therapy.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can bedirected to the corresponding authors.

## Ethics statement

The animal study was approved by Animal Ethics Committee, Jilin Medical University (2020-KJT043). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

SC: Writing – original draft, Writing – review & editing. WL: Data curation, Formal Analysis, Writing – original draft. XY: Methodology, Writing – review & editing. CH: Conceptualization, Writing – review & editing. YL: Data curation, Methodology, Software, Writing – review & editing. XS: Data curation, Formal Analysis, Writing – review & editing. XL: Formal Analysis, Software, Writing – review & editing. NJ: Data curation, Supervision, Writing – original draft.

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## Conflict of interest

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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.2025.1530583/full#supplementary-material

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# Case Report: Personalized peptide-based immunization in an advanced-stage prostate cancer patient with bone metastasis

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Neoantigens, which are recognized as non-self and trigger an immune response, are novel antigens generated by tumor cells. Here, we report a *de novo* metastatic hormone-sensitive prostate cancer (mHSPC) case, which benefited from our personalized peptide immunization named BioInformatic Tumor Address Peptides (BITAP) in a monotherapeutic setting. Our in-house bioinformatics pipeline involved identifying somatic variations, analyzing their expression, and computationally predicting novel epitopes from both metastatic and primary tumors, separately. As stand-alone therapy, the patient has been administered multiple injections of two peptide pools (BITAP-1 and BITAP-2). Several months following immunizations, a significant regression of both metastatic and primary tumor lesions was recorded along with low-level of prostate-specific antigen (PSA). Besides mild and short-lasting local and systemic reactions, no serious treatment-related adverse effects were reported by the patient. In conclusion, this case suggests that BITAP immunization is feasible and safe, and may present an immunotherapeutic approach inducing sustainable tumor regressions in mHSPC patients.

KEYWORDS

**immunotherapy, neoantigen, peptide, prostate cancer, case report**

# 1 Introduction

In Western countries, prostate cancer (PrCa) is the most commonly diagnosed cancer and one of the leading causes of cancer-related death among men (1). Over the past years, some progress has been made for the treatment of metastatic PrCa (2), and the 5-year survival for distant stage PrCa improved from 28.7% to 32.3% (3). Although targeting bone metastases in prostate cancer leads to improving clinical outcome (4), currently, there are no effective treatments for these patients (5).

During the last decade, immunotherapy has shown clinical benefit across various tumor entities such as melanoma, lung cancer, and renal cell carcinoma (6). Cancer vaccines have been under development over the past century, and shown promise by priming T cell and prompting antigen-specific responses targeting tumor antigens, including tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). Personalized therapeutic cancer vaccines have recently attracted a lot of attention due to the developments in next generation sequencing technologies and a deeper understanding of neoantigens production (7).

Unfortunately, bone metastatic PrCa is considered an 'immune-cold' tumor, resulting in poor immune checkpoint therapy (ICT) responses (8). Despite advances in the management of mHSPC, the emergence of castration resistance is unavoidable, and many patients develop metastatic castration-resistant disease (mCRPC). Numerous clinical trials have been completed or are ongoing to assess various immunotherapeutic approaches for patients with metastatic PrCa (9). This clinical case report sheds light on the application of a personalized peptide-based immunization approach, referred to as BITAP, in a PrCa patient with bone metastasis.

# 2 Case description

A 72-year-old Caucasian male was admitted to the urologist on 04$^{th}$ October 2022, complaining of urinary urgency, interrupted urination, nocturia, and erectile dysfunction. At this time, prostate-specific antigen (PSA) level was measured to be 99.8 ng/mL. The Magnetic Resonance Imaging (MRI) examination showed a moderately enlarged prostate, with an extensive, organ-crossing and infiltrating mass on the left side of prostate organ, highly suspicious for PrCa. The prostate-specific membrane antigen positron emission tomography (PSMA-PET) examination revealed significant involvement of the middle of the prostate gland and the apex on the left periphery, with infiltration extending to the left seminal vesicles. No evidence of PSMA-positive lymph node metastases was documented. However, a total of 32 multilocular sclerotic bone metastases were reported. To finally confirm the suspected diagnosis of advanced PrCa, biopsies were taken from bone metastases in the area of the lateral mass of the left sacrum. Pathological assessment of the biopsies from metastatic tumor revealed a strong expression of cytokeratin, NKX3.1 and P504S which prove malignant prostatic origin of the metastatic tumors. Secondary biopsies were also taken from both the primary tumors (according to the pathologists letter; no biopsy data shown) and non-tumor regions of the prostate. The pathological assessment revealed a Gleason pattern of 4 + 4, corresponding to a Gleason score of 8, indicating high-grade prostatic adenocarcinoma.

# 3 Therapeutic intervention

The patient was already symptomatically treated for 4 weeks of Tamsulosin to manage lower urinary tract symptoms associated with prostate enlargement (see Figure 1A). Immediately, following the histological diagnosis of *de novo* mHSPCA, evidence-based first-line systemic therapy with androgen deprivation therapy (ADT) plus abiraterone was strongly recommended to the patient. However, patient explicitly refused this regimen due to his concerns about potential side effects. Instead, the patient opted for lifestyle changes, including diet with addition of fermented soy products, and strictly avoiding smoking and alcohol consumption. Surprisingly, this individual lifestyle approach led to a continuous drop in PSA levels over the following 10 weeks (see Figure 1A). However, follow-up whole-body MRI after the initial diagnosis revealed progressive bone metastases suggesting regulatory effects of the soy products only on PSA kinetics without any anti-tumor activity. It has already been reported that soy products may alter PSA levels in untreated PrCa patients (10, 11). Still explicitly refusing evidence-based treatments, patient reached out then for personalized immunization by BITAP. After being given detailed information about the experimental character of this novel treatment approach, the patient received the first prime dose of BITAP-1 by subcutaneous injection (see Figure 1A). The next prime doses of BITAP-1 were applied 3, 10 and 36 days after the first injection. The patient received two boost injections 3 and 5 months after priming. The immunization process of the prostate cancer patient using multi-peptide BITAP pools in a monotherapeuitc setting is shown in Figure 1B.

# 4 Methods

## 4.1 Next generation sequencing and preparation of immunization pool

Genomic DNA and RNA extracted from metastatic and primary tumor tissues, along with genomic DNA obtained from blood samples, were subjected to next-generation sequencing (NGS) analysis (Supplementary Figure 1). The selection of neoantigen-containing peptides in the immunization peptide pool was performed according to our in-house BITAP analysis pipeline and using sequencing data of tumor, normal and blood samples. Neoantigen-containing peptides refer to synthetic long peptides that include 8–15-mer neoantigenic sequences derived from tumor-specific mutations. These are extended at the N- or C-terminal to

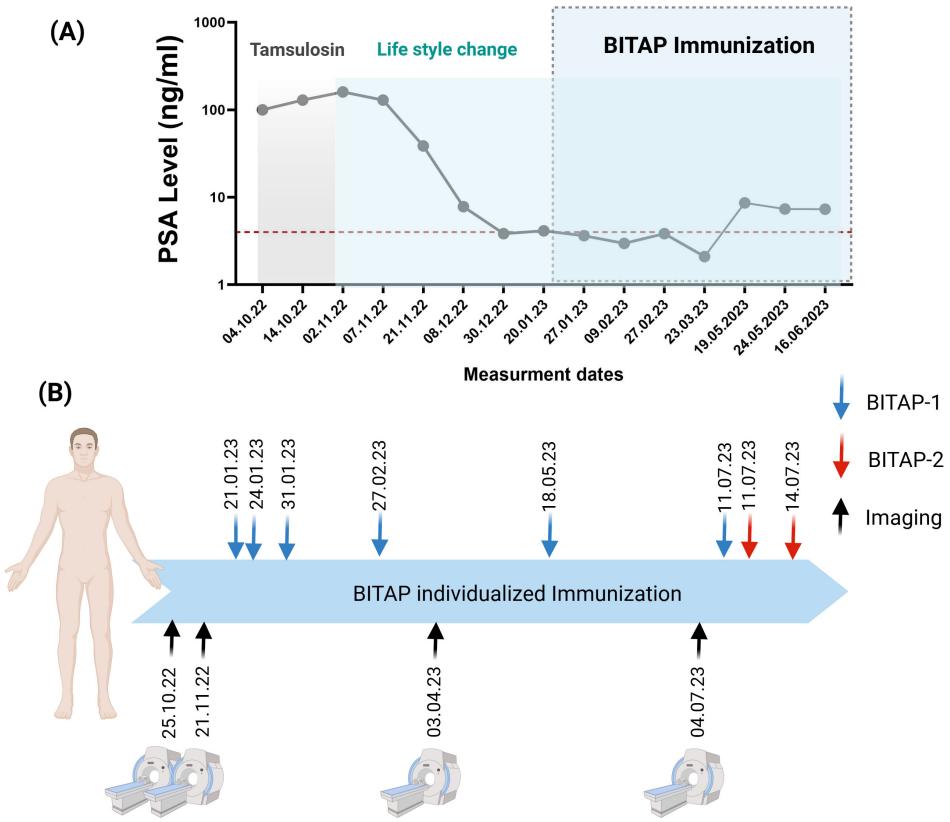


FIGURE 1
**(A)** The PSA levels of the patient during the time of patient diagnosis and treatment. **(B)** Timeline for the immunization process of a prostate cancer patient using multi-peptide BITAP pools. The patient has been receiving different BITAP peptide pools (BITAP-1 and BITAP-2) over several months. Each BITAP pool was developed using a new NGS (next-generation sequencing) analysis: one derived from metastatic tumor (BITAP-1) and the other from primary tumor (BITAP-2). Created with BioRender.com.

enhance immunogenicity and presentation by both MHC class I and II molecules. The peptides were then produced through chemical synthesis at >90% purity to generate the immunization peptide pool (Intavis Peptide Services GmbH, Tübingen, Germany). The details of the immunization peptide pool preparation are explained in the Supplementary Materials.

## 4.2 T-cell responses of BITAP

The immunogenicity of each peptide was analyzed in the patient's peripheral blood mononuclear cells (PBMCs) using an IFN-γ ELISpot assay (11). The ELISpot results were considered positive when the numbers of spots were at least 2-fold above the negative control (medium) and with a minimum of 50 detected spots. Accordingly, strong responses were shown in 7 out of 12 (~ 58% positivity) peptides from BITAP-1 which were prepared by the analysis of bone metastases (Figure 2A). The BITAP-2 peptide pool was prepared by the evaluation of biopsies from primary tumor and non-tumor tissues of prostate. Initially, the 12 predicted epitopes were combined into 7 peptides and tested using ELISpot assays (Figure 2B). Subsequently, these peptides underwent Good Manufacturing Practice-like (GMP-like) production and were incorporated into the BITAP-2 pool for injection into the patient. The dosing regimens for BITAP-1 and BITAP-2 were aligned with those used in our previous case report as well as prior clinical trials investigating personalized neoantigen peptide vaccines (12).

# 5 Clinical outcome

Approximately two months after the first dose of BITAP-1, patient examination using PSMA-PET showed a clear regression of multiple bone metastases compared to the previous examination, which had been performed before starting the BITAP immunization (Figure 3A). The PSMA-expressing metastases still showed a low PSMA expression, which indicates a minimal residual viability. Additionally, no evidence of new bone metastases was apparent. Another imaging analysis of the primary tumor using MRI at five months following the start of BITAP immunization showed an additional regression of 40% in the primary tumor mass (Figure 3B). After the patient had received multiple primary and booster injections of BITAP-1, the BITAP-2 immunization was started (Figure 1B). The patient received routine clinical follow-up during and after the immunization cycles. Monitoring included vital signs, and PSA levels at regular intervals.

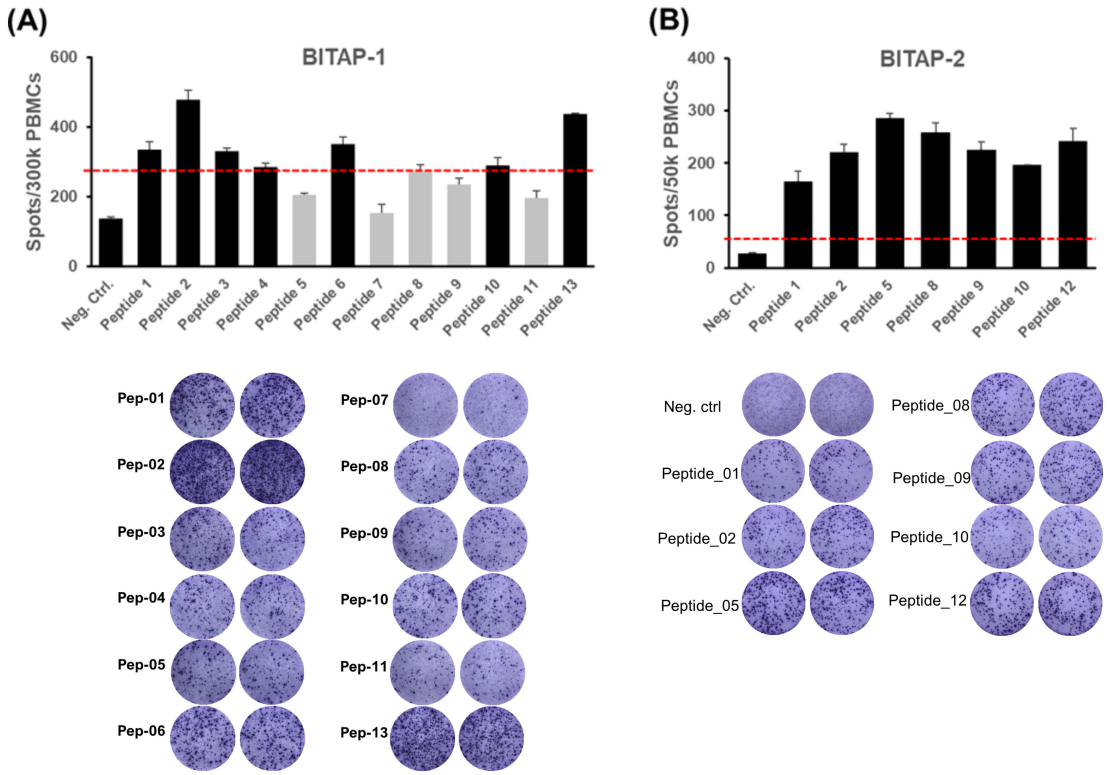


FIGURE 2
Immunogenicity testing of BITAP peptides using ELISpot assay. Blood was taken from the patient prior to the first application of the respective peptide pool, and a 10–12 days ex vivo pre-stimulation was performed. Afterward, the numbers of specific cells against the individual peptides were determined. The red dotted lines indicate the 2-fold negative control values. Seven out of 12 peptides from BITAP-1 were immunogenic **(A)**. Testing of BITAP-2 peptides using ELISpot assay revealed immunogenicity of all 7 **(B)**. The red line represents the threshold line for results to be considered positive. The ELISpot response was considered positive if it showed at least a two-fold increase over the negative control and exceeded 50 spot-forming units (SFUs) per $1\times10^6$ PBMCs. **(A)** shows mean spot counts from duplicate wells per peptide condition. **(A, B)** display representative images of ELISpot wells corresponding to the same experiment. PBMCs were collected before the first BITAP injection for each cocktail.

Due to ethical constraints, no experimental testing beyond standard-of-care diagnostics was performed.

## 6 Treatment-related adverse effects

Beside weekly doctoral follow-ups, the patient was encouraged to actively participate in his treatment journey by promptly documenting and reporting any new or unusual symptoms. The treatment-related adverse effect reported and documented by the patient included only short-term local reactions after BITAP administration such as burning sensation and redness at the subcutaneous injection site. In addition, the patient also experienced mild systemic symptoms, including increased night sweats, during the first two days following each BITAP injection.

## 7 Discussion

The presented case study highlights a promising avenue in metastatic prostate cancer therapy through the application of a personalized neoantigen cancer immunization. The patient's response to the immunization peptide pool (BITAP), as evidenced by the considerable regression of metastatic and primary tumor lesions along with maintenance of low-level PSA, suggests the potential efficacy of this immunotherapeutic approach. The patient implemented individual lifestyle changes during the course of treatment, which may have influenced PSA kinetics; therefore, the observed PSA reduction cannot be attributed solely to BITAP immunotherapy, and future controlled studies will be necessary to define these effects.

The positive outcome of this case study aligns with the growing evidence on neoantigens as key targets for cancer vaccines (13, 14). Emerging from tumor-specific alterations, neoantigens could trigger a highly specific immune response against the cancer cells while minimizing damage to the healthy tissues (15). As shown, the patient's positive response to the BITAP immunization supports the concept that neoantigens can indeed serve as potent antitumor stimulator for immune recognition and activation, while triggering only minimal and short-lasting immune-related side effects. However, it is important to highlight the complexity of the immune system and the potential variability in individual responses to neoantigen-based therapies. Various factors such as tumor heterogeneity, immune status, and the tumor

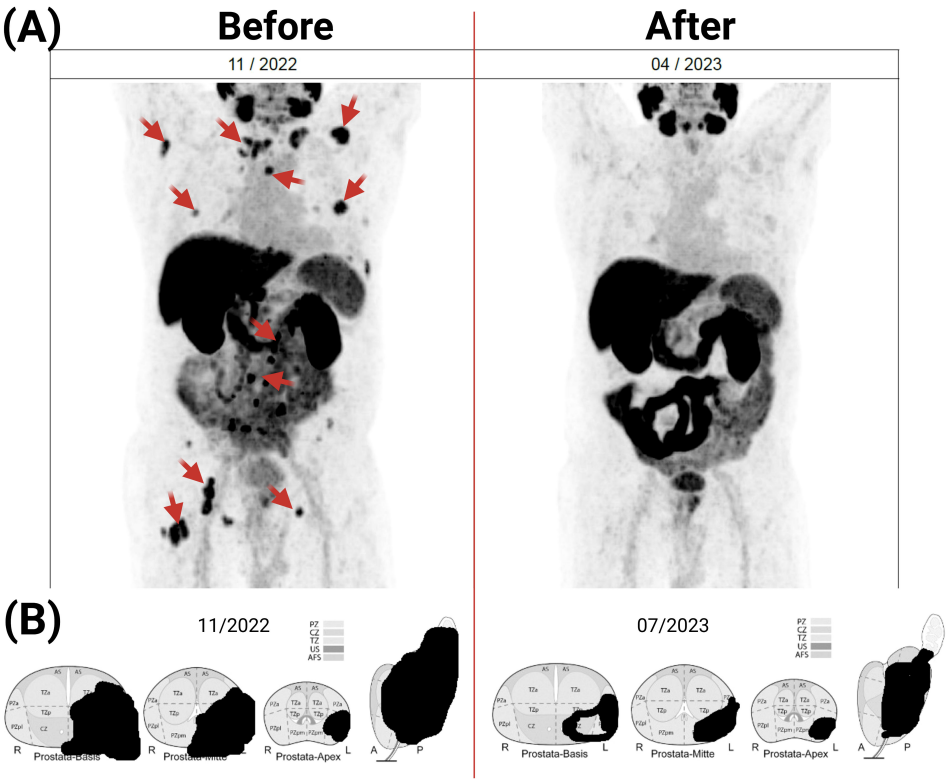


FIGURE 3
Effect of BITAP immunization on the metastatic and primary tumor lesions. The patient examinations before and after BITAP immunization treatment was done by PSMA-PET CT **(A)** and MRI **(B)**. The MRI of the prostate revealed an additional 40% reduction in primary tumor volume at five months after BITAP immunization.

microenvironment can influence treatment outcomes (16). The potential for synergy between BITAP peptide vaccination and immune checkpoint inhibitors as combining these modalities might enhance antitumor immunity by both broadening T cell responses and overcoming immune evasion. Despite the positive outcome in this case, the broader applicability and consistent response rates across a diverse patient population need to be further investigated.

The duration of the observed response and the potential for long-term remission also warrant consideration. Monitoring the patient's progress over a longer period and exploring mechanisms that contribute to the observed immune responses will provide valuable insights into the durability of BITAP immunization efficacy. The durability of immune responses may be limited by tumor escape mechanisms such as neoantigen loss or immune suppression. While no post-treatment biopsy was available to assess these changes in this case, future studies should include longitudinal tissue analyses to better understand resistance and guide re-immunization strategies. Additionally, this case study suggests that a combination of BITAP therapy with other treatment modalities, such as immune checkpoint inhibitors could potentially enhance the therapeutic effect by leveraging multiple aspects of the immune response and tumor biology. As this case study contributes to the evolving landscape of prostate cancer immunotherapy, it prompts the need for larger clinical trials to validate its findings and establish its place within the current treatment paradigm.

## 8 Conclusions

This case illustrates the potential of personalized peptide-based immunization in managing advanced PrCa patients with bone metastasis. The BITAP therapy yielded a positive response in this patient, suggesting it could be an innovative and promising approach for other patients with similar conditions. However, more clinical data is needed to conclusively validate these findings. In addition, identifying patients who would benefit most from BITAP therapy through companion diagnostic markers is essential for clinical translation. Therefore, future studies should focus on defining predictive biomarkers, such as neoantigen load, T cell repertoire, and tumor immune microenvironment to optimize patient selection. The potential benefits and side effects of this individualized immunotherapy approach also need further investigation. Importantly, ethical and regulatory standards must be stringently adhered to in the deployment of such personalized treatments.

## 9 Patient perspective

From the patient's perspective, the decision to undergo the BITAP personalized neoantigen cancer immunization was a significant step in managing his disease. The observed regression of tumor lesions and stabilization of PSA levels were interpreted by the patient as encouraging indicators of progress. These outcomes contributed to a sense of relief and optimism about the potential of this novel intervention. The patient emphasized the importance of open communication and collaborative decision-making with healthcare providers, which he felt was integral to his care experience.

## Data availability statement

The datasets presented in this article are not readily available because due to data protection regulations, we are not allowed to share the sequencing data generated in this study. Access to the data is restricted to authorized personnel involved in the research, and all patient information is anonymized to ensure privacy and confidentiality. Requests to access the datasets should be directed to Meik Kunz (meik.kunz@fau.de) and Mari Carmen Martos-Contreras (mari.martos@immuneo-therapeutics.de).

## Ethics statement

The requirement for ethical review and approval was waived in accordance with local legislation and institutional guidelines applicable to single case studies involving human participants. The case was conducted in compliance with all relevant regulations. Written informed consent was obtained from the individual for participation, as well as for the publication of any potentially identifiable images or data included in this case report.

## Author contributions

NT: Conceptualization, Writing – original draft, Investigation, Project administration, Formal analysis, Writing – review & editing, Methodology, Supervision. MM-C: Methodology, Investigation, Data curation, Writing – review & editing, Software, Formal analysis. MM: Writing – review & editing, Investigation, Conceptualization, Writing – original draft, Visualization, Data curation, Project administration, Methodology, Formal analysis. MN: Methodology, Investigation, Conceptualization, Writing – review & editing. FB: Writing – review & editing, Methodology, Investigation. JD: Data curation, Writing – review & editing, Investigation, Methodology, Formal analysis. JC: Methodology, Writing – review & editing, Resources. MK: Data curation, Software, Methodology, Writing – review & editing, Supervision. WS: Project administration, Formal analysis, Conceptualization, Writing – review & editing, Investigation, Methodology, Supervision.

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## Conflict of interest

NT, MM-C, MM, MN and WS are employed by Immuneo Therapeutics GmbH. JD, JC and MK have consultancy relationship with Immuneo Therapeutics GmbH. MK is founder and shareholder of BioInf4Life UG (haftungsbeschränkt). MM-C, MM, MN, MK and WS have filed patents for peptide-based immunization approaches.

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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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2025.1596315/full#supplementary-material

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# Dual targeting of BCMA and SLAMF7 with the CARtein system: chimeric antigen receptors with intein-mediated splicing elicit specific T cell activation against multiple myeloma

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**Introduction:** Chimeric antigen receptor (CAR) T-cell therapy has demonstrated remarkable efficacy against multiple myeloma (MM). However, several barriers continue to limit the overall effectiveness of this approach, such as high production costs, prolonged manufacturing timelines, safety issues, and the potential for tumor antigen escape due to selective therapeutic pressure. To overcome these challenges, innovative CAR T strategies, such as engineering modular CAR systems, are being explored. These systems utilize adaptor molecules to enable multi-antigen targeting, thereby enhancing specificity, safety, and overall efficiency of CAR T-cell therapy. Notably, CAR T-cells directed against BCMA and SLAMF7 antigens have generated strong and robust antitumor responses in MM therapy.

**Methods:** To address the limitations of conventional CAR T therapy, we developed a novel modular CAR platform targeted against BCMA and SLAMF7. This was achieved using a split intein-mediated protein splicing mechanism, which allows specific covalent peptide bonds to form between CAR modules. This strategy maintains an almost seamless CAR structure, preserving its overall integrity and functionality. The design of the intein-spliced CAR system (termed "CARtein") was further optimized through advanced protein structure prediction software.

**Results:** Cells expressing the spliced CARtein constructs, engineered to target BCMA, SLAMF7, or both antigens simultaneously, demonstrated robust and highly specific activation in response to their respective antigens.

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**Discussion:** These results suggest that the CARtein platform is a promising, versatile, and highly specific approach for the modular design and engineering of CARs, enabling multi-antigen targeting while maintaining structural and functional integrity. This modular strategy addresses key limitations of conventional CAR T-cell therapy and may improve both the safety and effectiveness of future MM treatments.

KEYWORDS

**chimeric antigen receptor, multiple myeloma, inteins, protein splicing, B-cell maturation antigen, SLAMF7, modular CAR, immunotherapy**

## Introduction

Chimeric antigen receptor (CAR) T-cell therapy has demonstrated remarkably effective long-lasting clinical responses in patients with hematological malignancies, including B-cell leukemia, lymphoma and multiple myeloma (1–4). However, this therapy still needs to overcome many limitations, such as high production costs and long manufacturing periods, or hurdles related to potential lethal toxicity such as cytokine release syndrome (CRS) and neurotoxicity due to CAR overactivation. Other factors that may hinder the efficacy of CAR therapy against hematological cancers are the tumor inhibitory microenvironment and other tumor scape mechanisms. Nevertheless, approaches are being developed in order to improve the antitumor response and safety of CAR therapy, as well as to facilitate the manufacturing process (2, 5).

Despite improvements in survival of multiple myeloma (MM) patients in the last decade due to the development of novel therapeutic alternatives such as monoclonal antibodies or immunomodulatory drugs, it remains largely incurable as most patients eventually relapse (5, 6). In this landscape, CAR therapy has shown promising results in the treatment of patients with relapsed or refractory multiple myeloma. B-cell maturation antigen (BCMA), also known as TNFRSF17, has been the preferred antigen targeted by CAR T-cells directed against MM, as it is highly expressed in most MM malignancies and CAR T-Cell therapies directed against this surface antigen have elicited powerful antitumor responses in relapsed or refractory patients (5, 7–9).

However, many patients exhibit resistance mechanisms after short-term efficacy of CAR T-cell therapy directed against BCMA. One of the most concerning evasion strategies by cancer cells is antigen downregulation under therapeutic pressure, resulting in relapses and poor prognosis during CAR therapy. Therefore, targeting more than one surface protein is an effective approach in order to prevent and/or manage antigen escape. Among the candidates for antigen targeting, SLAMF7 (also known as CS1, CD319 or CRACC) is one of the most promising alternatives. SLAMF7 is a surface antigen upregulated in MM that plays a significant role in the uncontrolled proliferation of malignant cells (5, 10–13). Gogishvili et al., showed that CAR T-cells targeting SLAMF7 by an antigen binding domain based on Elotuzumab exhibited a strong antitumor response (14).

New strategies for targeting multiple antigens in CAR therapy are being developed, such as the tandem design, with a single construct containing two distinct antigen-binding domains or the dual CAR T-cell approach, which is based on the co-expression of two individual CARs targeting different surface proteins in a single T cell (15, 16). In this scenario, engineering of modular CARs containing a cytoplasmic signaling and an extracellular moiety that will capture tailored recognition domains with affinity for different surface antigens is an attractive alternative for multiple targeting, as it will decrease construct size, thus improving transduction efficiency and improve safety as recognition domains can be easily discontinued and/or blocked (17, 18). Multiple modular CAR strategies are currently being explored with promising results, including systems like the UniCAR platform or the anti-FITC CAR strategy. However, most of these approaches rely in transient interaction between labeled antibody and antigen in CAR-modified cells. Nonetheless, new systems are emerging, such as the SpyCatcher-SpyTag platform and its derivatives based in the formation of an isopeptide linkage (19–23).

Inteins are proteins capable of performing through successive nucleophilic displacement reactions, an autocatalytic process, known as protein splicing, in which, they can join adjacent residues via a covalent peptide bond that can be considered almost scarless, since inteins are excluded from the final peptide and only exteins (3aa flanking sequences) remain in the mature construct. Split inteins function as orthogonal pairs composed of an N-terminal fragment (IntN fused to N-extein) and a C-terminal fragment (IntC fused to C-extein), which self-catalyze sequence-specific trans-splicing. This process precisely ligates the flanking 3aa exteins through a covalent peptide bond, while excising the intein, enabling scarless fusion of proteins attached to either or both intein fragments (24–27). Intein-mediated protein splicing has already proven to be a promising platform for protein modification in gene and cell therapy. For instance, Han et al., 2017 successfully generated bispecific IgG antibodies through a technology platform based on split inteins. Moreover, a similar system was explored by

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Ray et al., 2023 in which they efficiently modified target membrane proteins for live cell application (28–31).

In this study, we designed a dual CAR approach for targeting BCMA and SLAMF7 in MM cells through the development of a modular CAR platform based on split intein-mediated protein splicing, which we named CARtein system. For this purpose, we separated the chimeric antigen receptor in two distinct modules, each containing orthogonal split inteins as shown in Figure 1A. The signaling CARtein module (SCM) was fused to the C-terminal intein IMPDH-1 (27) while the antigen recognition modules, containing scFv targeting either SLAMF7 or BCMA, were fused to the orthogonal N-terminal part. The spliced anti-BCMA and SLAMF7 CARtein constructs are shown in Figure 1B. As previously described, split inteins are not present in the final CARtein construct, except for the residual exteins, consisting in a short sequence of 3 residues each. Herein, we demonstrate that the CARtein system targeting BCMA, SLAMF7 or both antigens simultaneously, allows Jurkat T cells to elicit a specific activation response against MM cells, which makes the CARtein platform an attractive approach for multiple targeting through modular CAR T-cell therapy, as the junction between modules is mediated by a covalent peptide linkage and the proteins responsible for binding are not present in the final CAR sequence. Moreover, we show that structure prediction software, such as ColabFold based on the deep learning model AlphaFold2, can prove a useful tool for rational CAR design (32, 33).

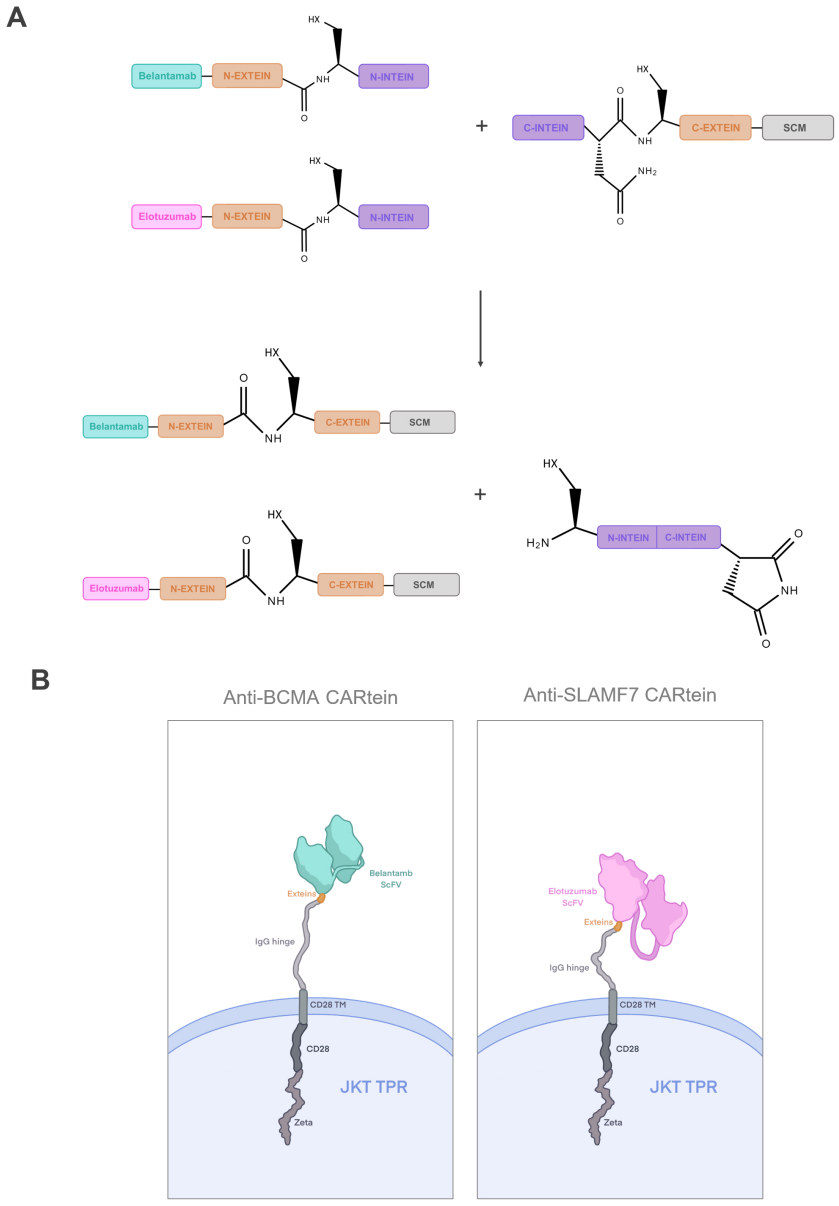


FIGURE 1
Dual targeting CARtein strategy. **(A)** Schematic representation of CARtein modules before and after split intein-mediated protein splicing. **(B)** Representation of Jurkat (JKT) triple parameter reporter (TPR) expressing the final anti-BCMA and anti-SLAMF7 CARtein constructs. JKT TPR, Jurkat-TPR cells.

# Materials and methods

## Protein structure prediction and protein docking

In order to facilitate and simplify the rational design of the CARtein modules, we performed protein structure predictions of these constructs. All protein folding predictions were run with ColabFold (ColabFold (RRID: SCR_025453) open-source software (32). In order to improve the accuracy, the prediction of sequences containing parts of the IMPDH-1 split intein were carried out with the crystal structure of gp41–1 intein (PDB id: 6QAZ) used as template. First, we predicted the tridimensional structure of the extracellular domain of the signaling CARtein module (SCM) containing a CD28 transmembrane (TM) domain and an $IgG_1$ spacer followed by the C-terminal part of IMPDH-1 split intein (including the C-intein and C-extein). Then, we predicted the same structure without the C-terminal part of IMPDH-1 with the aim of assessing how much the split intein impacts on the final structure. The same strategy was employed to predict the antigen recognition modules folding. These modules included either an anti-BCMA (Belantamab) or an anti-SLAMF7 (Elotuzumab) scFv fused to the N-terminal region of IMPDH-1. This analysis was also extended to constructs lacking the split intein.

For the purpose of evaluating whether the partners of IMPDH-1 split inteins in the CARtein modules would have no steric hindrance for performing protein splicing, the antigen recognition modules directed against BCMA or SLAMF7 were docked with the SCM using HADDOCK2.4 (HADDOCK (RRID: SCR_019091) modeling platform (34). Moreover, we performed a prediction of the antigen-CARtein complexes after protein splicing with ColabFold, using the structure of CD28 transmembrane domain as template (PDB id: 7VU5), for comparing the interaction between the scFv and the extracellular domain (ECD) of BCMA (H3BMB5_HUMAN: 1-69aa) or SLAMF7 (SLAF7_HUMAN:23-247aa) without the residual exteins (GGG-SIC).

Additionally, we predicted the structure of two well-established modular CAR approaches using the same scFv and SCM components: (i) the SpyTag/SpyCatcher system, in which the Belantamab or Elotuzumab scFvs were fused to SpyTag and docked to a SpyCatcher domain (22) inserted upstream on the SCM, keeping a similar design to previously described constructs (21); and (ii) the SUPRA CAR-based platform, utilizing SYNZIP1 and SYNZIP2 heterodimeric coiled-coil domains as modular interaction interfaces (35). For the SUPRA constructs, SYNZIP1 was fused to the scFv via a flexible 35-aa glycine/serine linker keeping the same architecture previously described in the SUPRA CAR system (36), and SYNZIP2 was fused to the SCM. SpyTag/SpyCatcher complex predictions were performed with ColabFold, guided using the crystal structure of SpyTag/SpyCatcher covalent complex (PDB id: 4MLI) as a docking template. The anti-SLAMF7 SUPRA CAR-based complex prediction was carried out with ColabFold as well, while the anti-BCMA SUPRA CAR-based construct in complex with the antigen was conducted by AlphaFold server (37). All protein structures and complexes were analyzed and rendered with the 3D protein imager interface (38).

## Cell culture

Human Embryonic Kidney (HEK Lenti-XTM 293T) (Clontech) cell line was used for lentiviral vector production. A Jurkat T JE6.1 (Jurkat, RRID: CVCL_0367) cell subline known as Jurkat-TPR (Triple Parameter Reporter) expressing eGFP, CFP and mCherry, respectively governed by NFAT, NFκB and AP-1 promoters was established by Prof. Steinberger's group (39) and previously used by us and others to study CAR T-cell activation signaling (40). The human multiple myeloma (MM) cell line MM.1S (RRID: CVCL_8792) was used as SLAMF7+/BCMA+ target in activation signaling assays. Lastly, a chronic myelogenous leukemia K562 cell line (RRID: CVCL_K562) expressing BCMA and/or SLAMF7 was generated to be used as targets in activation signaling assays. All human cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA).

Human Embryonic Kidney (HEK Lenti-XTM 293T) (Clontech) as well as Jurkat-TPR cell lines were cultured in DMEM GlutaMax medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, 50 μM 2-mercaptoethanol (Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin–streptomycin (Invitrogen,Carlsbad, CA, USA) and 50μg/ml Gentamicin (Gibco) at 37 °C and 10% $CO_2$. MM.1s cell line was cultured in RPMI 1640 medium (Gibco) equally supplemented at 37 °C and 5% $CO_2$. Genetically modified K562 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented the same way as DMEM GlutaMax and RPMI media at 37 °C and 5% $CO_2$.

## Design of CARtein constructs

The signaling CARtein module (SCM) contained the same spacer, transmembrane and intracellular domains as our previously reported ACE2-CAR, including a CD3ζ intracellular domain, a CD28 transmembrane domain mutated for improved membrane expression, a CD28 co-stimulatory domain, and a human $IgG_1$ heavy chain spacer spaning hinge, $CH_2$ and $CH_3$ domain, mutated to prevent FC receptor activation (40). Upstream SCM, the C-terminal IMPDH-1 split intein (including the C-intein and C-extein) was engineered (27), preceded by a Twin-Strep-tag® (tST) connected through a flexible $(GGGGS)_2$ linker and a hIgKVIII leader sequence. The SCM construct was synthetized and codon-optimized by GeneArt™ (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and cloned via a LR Gateway™ reaction into the lentiviral vector pHRSINcPPT CEW, downstream a SFFV promoter.

Two different antigen recognition modules were generated to interact and bind to the SCM via a peptide bond mediated by split intein protein splicing. Antigen recognition modules contained the complementary N-terminal IMPDH-1 split intein and either an anti-SLAMF7 scFv (Elotuzumab) or anti-BCMA scFv (Belantamab). Both antigen recognition modules were followed by a flexible $(GGGGS)_2$ linker and an in-frame KDEL sequence,

responsible for sequestering these modules in the endoplasmic reticulum (ER) until the interaction of the N-terminal IMPDH-1 with its C-terminal partner in the SCM, occurs ensuing in the mature CAR molecule. These antigen recognition modules were synthetized and codon-optimized by GeneArt™ (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and cloned via a LR Gateway™ reaction into the expression lentiviral vector pLEX_307 (a gift from David Root (Addgene plasmid # 41392; http://n2t.net/addgene:41392; RRID: Addgene_41392), under the control of an EF-1α promoter.

## Design of SLAMF7 and BCMA surface antigens

SLAMF7 and BCMA extracellular and transmembrane domains coding sequences were synthetized by GeneArt™ (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and then cloned via a LR Gateway™ reaction into the expression lentiviral vector pLEX_307, governed by an EF-1α promoter.

## Lentiviral vector production

HEK Lenti-XTM 293T cells were used to produce all lentiviral supernatants. These cells were co-transfected with the corresponding transfer vector, together with plasmids pMD2.G, encoding for the Vesicular Stomatitis Virus G protein (VSVG) (RRID: Addgene_12259) and pCMVΔR8.91 (RRID: Addgene_202687), encoding for HIV-1 GAG and POL proteins. Polyethylenimine (PEI)-mediated transfection was performed in OptiMEM™ medium (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) according to the method optimized by Tang et al., 2015 (41). Lentiviral supernatants were collected at 48 and 72 h, centrifuged for cell debris removal and concentrated with a lentivirus concentrator solution containing 40% (W/V) PEG-8000 and 1.2M NaCl, according to the 4×Lentivirus Concentrator Solution protocol facilitated by the University of Texas M.D. Anderson Cancer Center (RRID: SCR_004699). The high-titer virus-containing pellets were frozen at -80°C until use and viral titers were determined by evaluating transduction efficiency in Jurkat cells.

## Cell transduction

Jurkat-TPR or K562 cells were first cultured for 24h in fresh media, then virus-containing pellets were thawed and resuspended in culture media. 48h after transduction, cells were centrifuged and plated in fresh media. K562 cells ($5x10^5$ cells/ml) transduced with either BCMA or SLAMF7 expressing lentiviral vectors, were selected by addition of 0.5μg/ml puromycin Dihydrochloride (Gibco). SLAMF7+ K562 cells were then transduced and re-selected for BCMA ECD expression. Surface antigens expression in K562 cells was analyzed by FACs 72h after selection.

Jurkat-TPR cells ($3x10^5$ cells/ml) transduced with lentiviral vectors containing SCM constructs, were selected 48h after transduction by culturing in 40 μg/ml of Blasticidin S HCl (Gibco). SCM expression in Jurkat-TPR transduced cells was evaluated by FACs 72 h after selection. SCM+ Jurkat TPR cells were then transduced by either or both antigen recognition modules directed against BCMA and/or SLAMF7. After 48h, transduced cells were selected through the addition of 0.125ug/ml of puromycin Dihydrochloride (Gibco) to the cell culture media at a final concentration of $3x10^5$ cells/ml. CARtein module expression in Jurkat-TPR cells were evaluated by FACs 72h after selection.

## T cell activation signaling assays

Jurkat-TPR cells expressing the SCM and the final CARtein sequences were co-cultured ($10^5$ cells/well) with MM.1s target cells at different target to effector (T:E) ratios (1:1, 1:5 and 1:10) in 96-well flat-bottomed plates in a final supplemented DMEM volume of 200ul. Cells were then incubated at 37°C and 10% $CO_2$. T cell activation signaling was measured at three different time points: 0h (unstimulated), 24h and 48h after stimulation. The signal of Jurkat-TPR promoter reporters as well as CD69 upregulation were analyzed for each time point.

To further assess CARtein specificity, target K562 cells either expressing BCMA or SLAMF7 ECD, or both (SLAMF7+/BCMA+ K562) were co-cultured with Jurkat-TPR cells expressing the SCM and the final CARtein sequences ($10^5$ cells/well) at T:E ratio of 1:1 in 96-well flat-bottomed plates at a final volume of 200ul of supplemented DMEM. Cells were incubated at 37°C and 10% $CO_2$. Jurkat-TPR activation signaling was equally measured at three different time points: 0h (unstimulated), 24h and 48h after stimulation. Signal of NFAT and NFκB promoter reporters as well as CD69 upregulation were analyzed for each measurement.

## Flow cytometry

Expression of SCM and anti-BCMA or anti-SLAMF7 post-splicing CARtein sequence in Jurkat-TPR cells were evaluated by staining with Strep-Tactin®XT DY-649 (Iba-Lifesciences, #2-1568-050), Brilliant Violet 421™ anti-human IgG Fc Antibody (Biolegend, San Diego, CA, USA) and Biotinylated Recombinant Protein L Protein, His,Avitag™ (ACROBiosystems, RPL-P81Q7) followed by PE-conjugated streptavidin™ (Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Zombie Violet™ Fixable Viability Kit (Biolegend, San Diego, CA, USA) was used to assess live cells, except for cells stained with Brilliant Violet 421™ anti-human IgG Fc Antibody, for which Zombie NIR™ Fixable Viability Kit (Biolegend, San Diego, CA, USA) was used.

In order to prevent unwanted binding of antibodies to human FC receptors in K562 and MM.1s cells, they were previously blocked with FcR Blocking Reagent, human (Cat# 130-059-901, RRID: AB_2892112, Miltenyi Biotec, Cologne, Germany). Expression of BCMA and SLAMF7 surface antigens in MM.1s cells and transduced K562 cells were evaluated by staining with APC anti-human CD269 (BCMA) Antibody (Cat# 357506, RRID:

AB_2562889, Biolegend, San Diego, CA, USA) and PE/Cyanine7 anti-human CD319 (CRACC) Antibody (Cat# 331816, RRID: AB_2565237, Biolegend, San Diego, CA, USA) respectively. For T cell activation signaling assays analysis, Jurkat-TPR cells were stained with anti-human CD69-APC (Cat# 310909, RRID: AB_314844) and CD3-PerCP/Cy5.5 antibodies (Cat# 100218, RRID: AB_1595492) both from Biolegend (San Diego, CA, USA), in order to discriminate CARtein-TPR cells from target cells and evaluate CD69 expression. Cells were also stained with Zombie Violet™ Fixable Viability Kit (Biolegend, San Diego, CA, USA) for live cells determination. Flow cytometry was performed on Cytek® Aurora 5L 16UV-16V-14B-10YG-8R spectral cytometer (RRID: SCR_019826). Data was collected using SpectroFlo software (Cytek SpectroFlo RRID: SCR_025494, Cytek Biosciences) and analyzed using FlowJo software V10.8.1 (RRID: SCR_008520, TreeStar Inc., Olten, Switzerland). Flow cytometry gating strategy for both T cell activation signaling assays is shown in Supplementary Figure S1.

## Statistical analysis

Statistical analyses were performed with GraphPad Prism v.9.0.2 (RRID: SCR_002798, GraphPad, La Jolla, CA, USA). Data are presented as the mean ± standard errors of the mean (SEM) from independent triplicates performed on different days to account for batch effects. Two-way ANOVA was used for statistical comparison among multiple groups followed by a Tukey's multiple comparison test. $p$ value of less than 0.05 was considered significant. $p$ value significance levels are indicated in the figures ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

# Results

## CARtein structure prediction

In order to evaluate beforehand whether the integration of split intein modules into mature chimeric antigen receptor would distort protein folding, therefore impairing its functionality, we performed structure and complex predictions with ColabFold software, based on the deep learning model of AlphaFold2 (32, 33). AlphaFold2-based software is capable of performing highly accurate protein structure predictions, and is emerging as a powerful and useful tool in rational protein design, including chimeric antigen receptors development (42, 43).

First, we wanted to evaluate whether the integration of IMPDH-1 split intein parts into our CARtein modules would affect their folding and therefore, functionality. We found that structure predictions of anti-BCMA (Belantamab) and anti-SLAMF7 (Elotuzumab) scFvs contained within the antigen recognition modules were sufficiently well-preserved after the addition of the N-terminal part of IMPDH-1(Figures 2A, B) when compared with the unmodified scFv predicted conformation. Furthermore, the N-terminal section of the split intein was accessible to the split intein partner, and the structural configuration of complementarity-determining region (CDR) loops of both Belantamab and Elotuzumab were well-preserved. Moreover, we performed a folding prediction of the extracellular and transmembrane domains of the signaling CARtein module (SCM), which comprises a CD28 transmembrane (TM) domain and the $IgG_1$ spacer coupled to the C-terminal part of IMPDH-1 split intein (Figure 2C). The SCM folding conformation in the presence of the intein moiety was well conserved when compared to the sequence in the absence of intein. In addition, IMPDH-1 C-terminal part is also accessible to its split intein partner.

The following step was to analyze whether the IMPDH-1 split intein partner embedded within the CARtein modules were sufficiently accessible for protein splicing. For this purpose, the predicted structure of the antigen recognition modules directed against BCMA or SLAMF7 was docked with the predicted conformation of the SCM using HADDOCK2.4 protein-protein docking platform (34). It was noted that in both cases, the anti-BCMA and anti-SLAMF7 antigen recognition modules to exhibited an accessible split intein N-terminal region, thereby facilitating engagement with its C-terminal partner contained within the SCM, with no discernible major steric hindrances (Figures 3A, B).

Following the split intein-mediated protein splicing process, the N-extein (GGG) domain of IMPDH-1, which is embedded within the antigen recognition module, will be covalently linked to the SCM C-extein (SIC) through a peptide bond, resulting in the exclusion of the N-intein and C-intein complex from the final anti-BCMA or SLAMF7 CARtein sequence. Accordingly, to assess the potential influence of residual exteins, encompassing six residues (GGG-SIC), on the spliced anti-BCMA and anti-SLAMF7 CARtein structure on scFv-mediated surface antigen recognition, we conducted a prediction of the antigen-CARtein complexes following protein splicing with ColabFold. Structure prediction was performed on the extracellular domain (ECD) of BCMA and SLAMF7, in complex with post-protein spliced CARtein, directed against either BCMA or SLAMF7. Additionally, CARtein constructs lacking residual exteins were analyzed. (Figures 3C, D). Comparison of the complex structure prediction between CARtein constructs, in the presence or absence of intervening exteins, suggests that antigen proximal residues in Belantamab and Elotuzumab maintain their relative proximity. Consequently, concluded that scFv functionality, and therefore CAR activity, should not be adversely affected.

To further evaluate the structural integrity of mature CARtein constructs and compare them with other modular CAR designs, we performed additional complex predictions. We compared complexes of anti-BCMA and anti-SLAMF7 unmodified CARs with the extracellular domain (ECD) of their respective antigens (Figures 4A, B) and the post-spliced CARtein complexes including the residual exteins (GGG-SIC) resulting from intein-mediated ligation (Figures 4C, D). As mentioned before, structural comparisons indicate that the presence of the six-residue extein linker does not noticeably affect the interaction between the scFvs and their respective antigens.

We then performed structure predictions of alternative modular CAR designs employing either SpyTag/SpyCatcher or coiled-coil

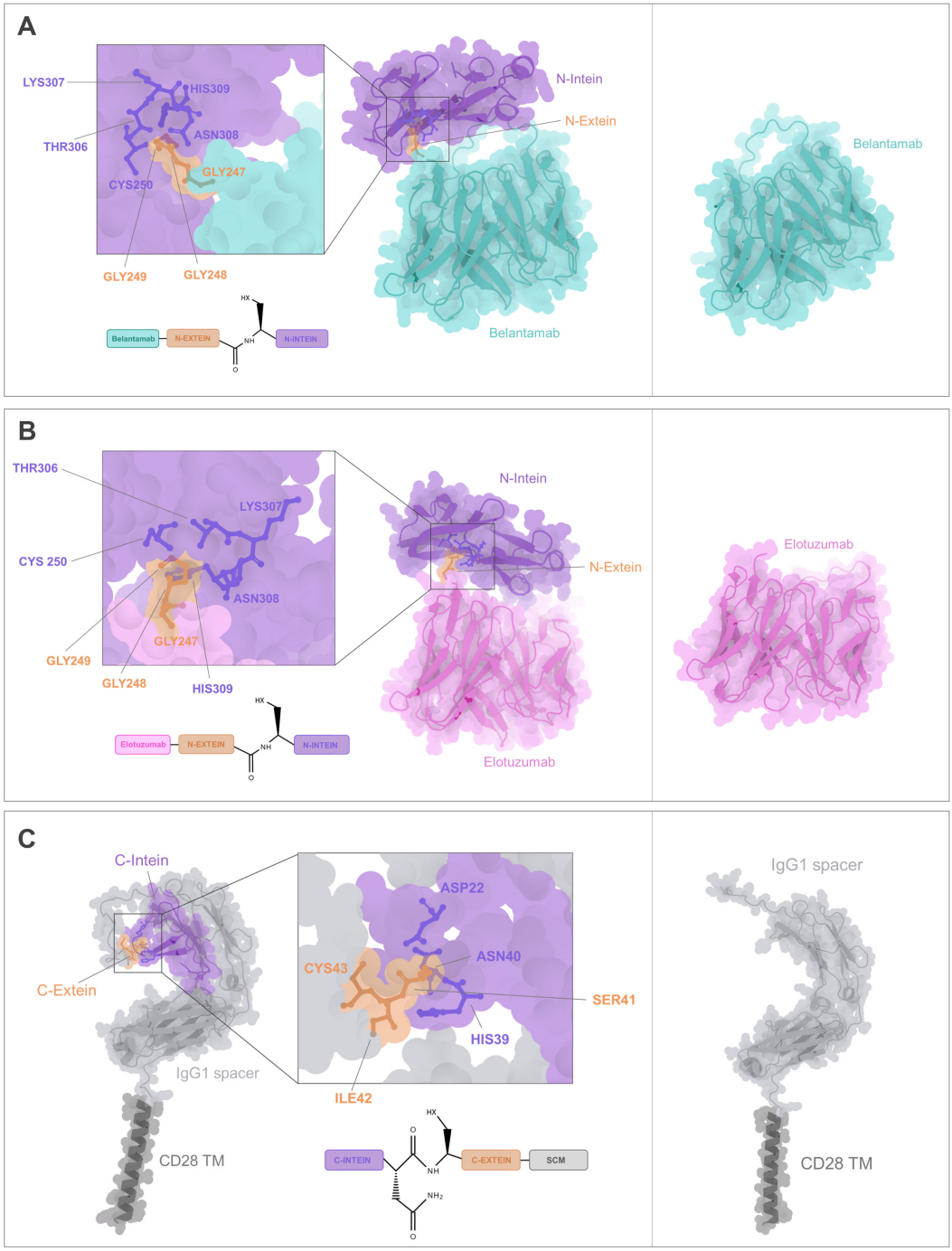


FIGURE 2
CARtein modules structure prediction. **(A)** Folding prediction of anti-BCMA antigen recognition module containing the scFv (Belantamab) fused to the N-terminal part of IMPDH-1 split intein (including the N-intein and N-extein domain) compared to the unmodified anti-BCMA scFv. **(B)** Structure prediction of anti-SLAMF7 antigen recognition module consisting in the scFv (Elotuzumab) fused to the N-terminal part of IMPDH-1 split intein compared to unmodified anti-SLAMF7 scFv. **(C)** Structure prediction of the extracellular domain of the Signaling CARtein module (SCM) spaning C-terminal intein IMPDH-1 (including the C-intein and C-extein domain) followed by $IgG_1$ spacer domains and CD28 TM compared to the SCM without intein. Relevant residues for split intein-mediated protein splicing are shown.

(SUPRA-based architecture) interactions to mediate antigen recognition module attachment. SpyTag/SpyCatcher-based CARs (Figures 4E, F) exhibited an overall bulkier configuration due to the interdomain covalent interaction, while the SUPRA CAR-based complexes (Figures 4G, H), which incorporated SYNZIP1/2 and a flexible linker, displayed elongated and more extended conformations. Importantly, in all modular CAR systems analyzed, the scFv maintained an orientation compatible with antigen binding. However, the compactness and minimal distortion observed in the CARtein constructs relative to the

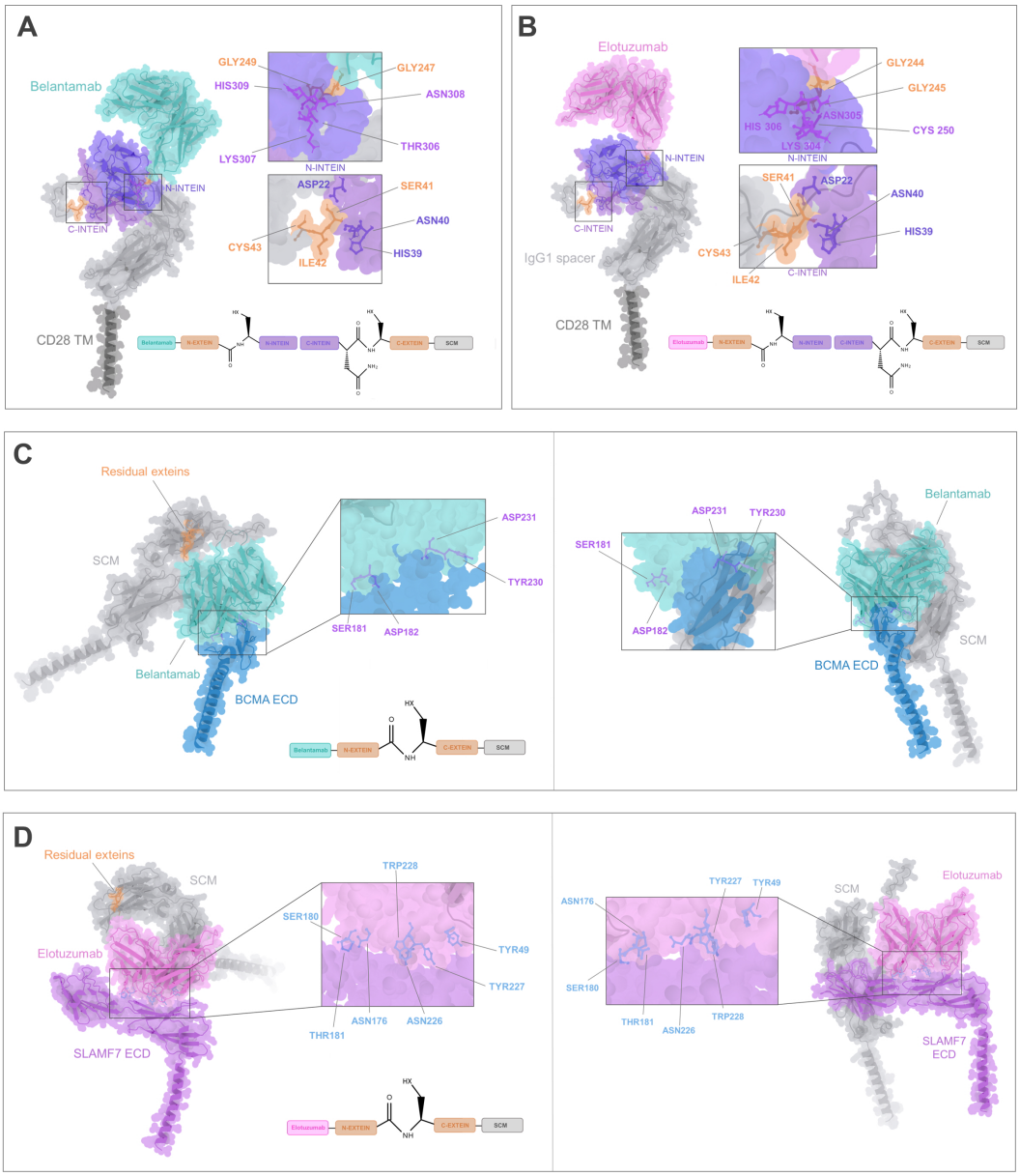


FIGURE 3
CARtein complexes prediction. **(A)** Docking of the predicted structures for anti-BCMA antigen recognition and SCM modules, with key residues for intein-mediated protein splicing being shown. **(B)** Docking of the predicted tridimensional structures of anti-SLAMF7 antigen recognition and SCM modules, with highlighted relevant residues for split intein-mediated protein splicing. **(C)** Complex prediction of post spliced anti-BCMA CARtein bound to BCMA ECD, compared to a CAR without exteins. scFv residues proximal to the antigen are highlighted. **(D)** Complex prediction of post-spliced anti-SLAMF7 CARtein bound to SLAMF7 ECD compared to a CAR devoid of residual exteins. Elotuzumab residues proximal to the antigen are shown.

native CARs suggest that post-splicing CARteins may offer improved structural fidelity.

Collectively, these results support that CARtein constructs retain a highly native-like conformation after intein-mediated assembly, outperforming other modular systems in terms of structural preservation and minimal interdomain interference. Furthermore, the short extein linker, encompassing a GGGS sequence, may contribute favorable flexibility that supports productive antigen binding without compromising CAR stability or recognition geometry.

## CARtein system design and establishment of CARtein-TPR cells and K562 antigen expressing cells

In order to generate split intein-mediated modular chimeric antigen receptors (CARteins) against BCMA and SLAMF7, three different CARtein modules were designed, including two distinct antigen recognition modules targeting each surface antigen, both containing the IMPDH-1 N-terminal sequence (encompassing the N-intein-extein), and one signaling CARtein module (SCM), which

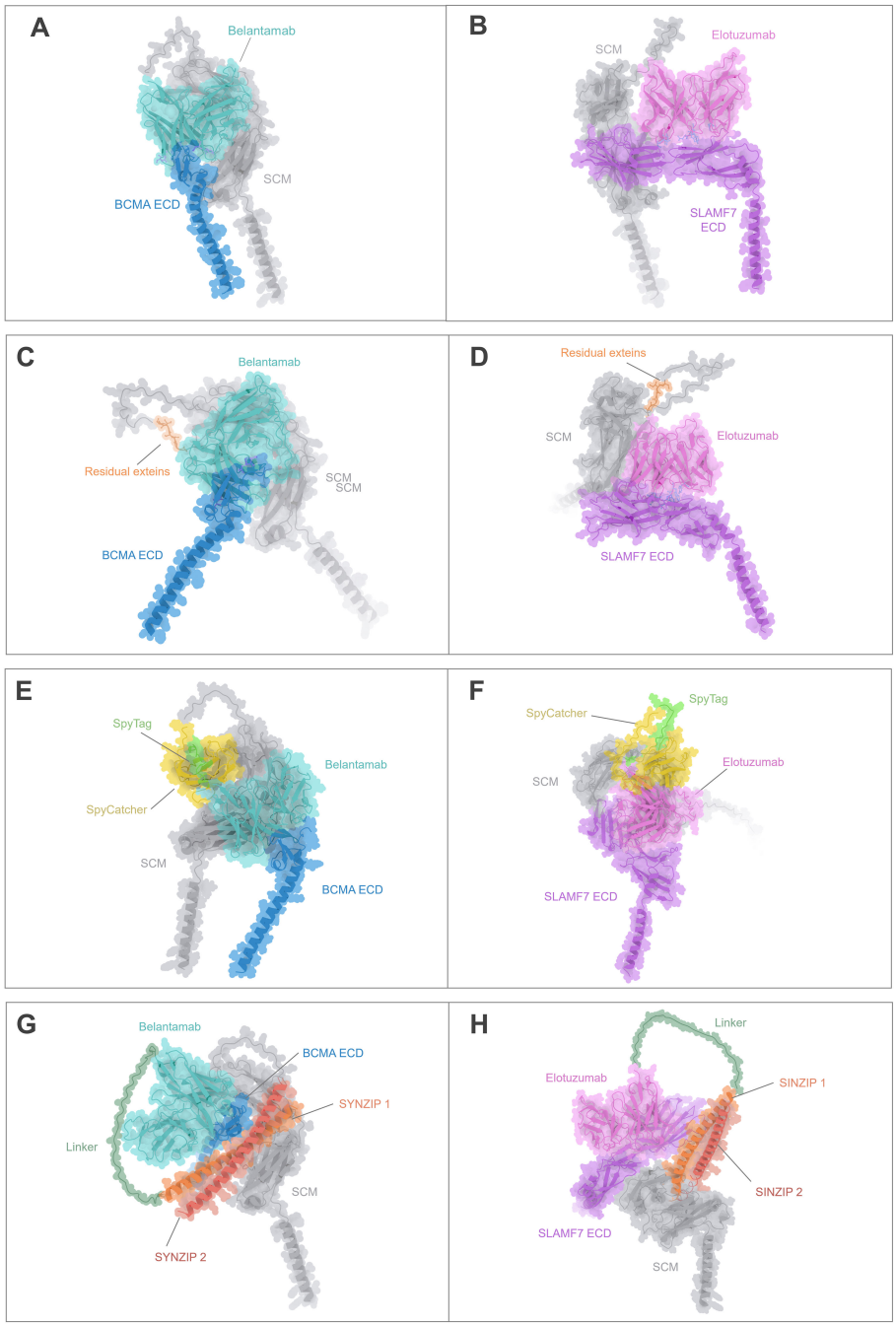


FIGURE 4
Structural comparison of the CARtein design and other modular CAR architectures in complex with the antigen. **(A, B)** Predicted structures of unmodified anti-BCMA and anti-SLAMF7 CARs in complex with their respective extracellular domains (ECDs). **(C, D)** Predicted post-splicing anti-BCMA and SLAMF7 CARtein complexes comprising residual exteins (GGGSIC) fused to the antigen-binding scFvs. **(E, F)** Predicted SpyTag/SpyCatcher-based modular CAR complexes for anti-BCMA and anti-SLAMF7. **(G, H)** Predicted anti-BCMA and anti-SLAMF7 SUPRA CAR system-based complexes depending on SYNZIP1-SYNZIP2 coiled-coil dimerization incorporating a 35-aa glycine/serine linker between the scFv and SYNZIP1. All models preserve the same SCM, scFvs, and antigen sequences.

contained the C-terminal intein (including the C-intein-extein), followed by the $IgG_1$ spacer, CD28 transmembrane, and intracellular domains and a CD3ζ intracellular domain (40). A Twin-Strep-tag® (tST) sequence was positioned right after the hIgKVIII leader sequence, upstream the C-terminal intein, for easy detection of the un-spliced SCM module (Figure 5A).

Antigen recognition modules comprised the scFv (either Belantamab targeting BCMA or Elotuzumab targeting SLAMF7) followed by the N-terminal IMPDH-1 and an in-frame Lys-Asp-Glu-Leu (KDEL) sequence to retain these modules within the endoplasmic reticulum (ER) until protein splicing via split inteins occurred, as KDEL receptors (KDELRs) retro-transport KDEL-

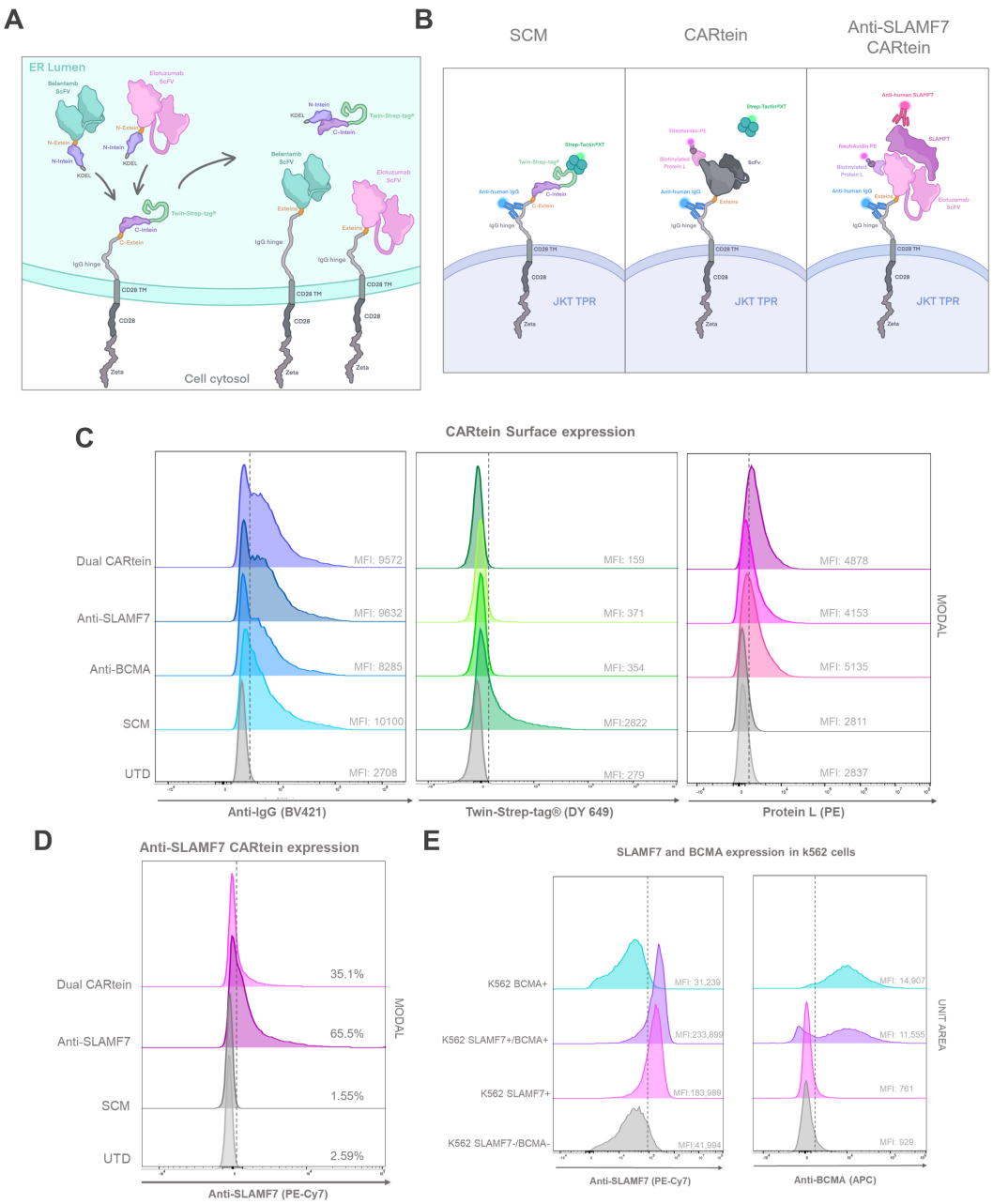


FIGURE 5
CARtein maturation and expression after split intein-mediated protein splicing. **(A)** Representative illustration of anti-BCMA and anti-SLAMF7 CARtein modules before and after intein-mediated CARtein splicing in the ER. **(B)** Illustrative representation of the staining strategy performed for the evaluation of SCM (Streptactin) mature whole CARtein (Protein L) and SLAMF7 CARtein expression. **(C)** Flow cytometry histogram overlays of SCM and spliced CARtein expressing unpermeabilized Jurkat-TPR cells. **(D)** Flow cytometry histogram overlay of Elotuzumab staining in SCM and anti-SLAMF7 or Dual CARtein-TPR cells. **(E)** Histogram overlay of BCMA and SLAMF7 expression in transduced k562 cells with either or both surface antigens. UTD, untransduced cells. JKT TPR, Jurkat-TPR cells.

bearing proteins from the Golgi to the ER (44). They were designed in such a way that the anti-BCMA and SLAMF7 CARtein constructs following intein-mediated protein splicing, would lose the tST, the KDEL sequence and the split inteins, except for the six extein residues (GGG-SIC) conforming the final CARtein sequence. As a consequence of KDEL removal, the spliced CARteins would be able to migrate outside the ER and be expressed on the surface membrane (Figure 5A).

All three constructs were cloned into lentiviral vectors and expressed in JKT-TPR cells to easily measure Nuclear factor-κB (NFκB) and Nuclear factor of activated T-cells (NFAT) promoter activation, as the synthetic promoters respectively control expression of reporter CFP and eGFP (39). Jurkat TPR cell line has been previously validated as an efficient platform for the evaluation of CAR functionality (45–48). Jurkat-TPR cells were transduced with lentiviral vectors containing the SCM construct, and then selected

through the Blasticidin-S resistance gene. SCM expression was evaluated by staining with an anti-IgG antibody recognizing the $IgG_1$ spacer and Strep-Tactin®XT- Twin-Strep-tag® (tST). SCM+ Jurkat-TPR cells were transduced with lentiviral vectors coding for antigen recognition modules directed against BCMA or SLAMF7, or both vectors simultaneously (Dual CARtein), and further selected by means of a Puromycin resistance gene. Since IMPDH-1 splicing reaction reaches its optimal temperature at 37°C, the split intein-mediated protein splicing occurred spontaneously under standard Jurkat cell incubation (27).

In order to analyze SCM, anti-BCMA, anti-SLAMF7 and Dual CARtein expression in Jurkat-TPR cells by flow cytometry, a triple staining strategy was carried out. (Figure 5B). Cells transduced with SCM alone or together with one or both recognition modules were stained with an anti-IgG antibody recognizing the $IgG_1$ spacer, Strep-Tactin®XT or Protein L, which recognizes either Belantamab or Elotuzumab VL, followed by PE-conjugated streptavidin™ (49). As evidenced by IgG-staining, the $IgG_1$ spacer was highly and consistently expressed among both, cells expressing unspliced SCM and those expressing any post spliced mature CARtein. As expected, Mean Fluorescence Intensity (MFI) values corresponding to tST staining in SCM+ Jurkat-TPR cells decreased in anti-BCMA, anti-SLAMF7 or Dual CARtein cells, indicating that intein-mediated protein splicing has occurred in these cells. Moreover, this is supported by the concomitant increase in protein L MFI in post-spliced CARtein cells, in contrast with single transduced cells that only expressed SCM and maintain high tST and low Protein L staining. Notably, anti-SLAMF7, anti-BCMA and Dual CARtein cells exhibited similar MFI values and flow cytometry histogram profiles for anti-IgG, protein L and Strep-Tactin®XT staining (Figures 5B,C). To evaluate ARM occupancy in dual CAR-expressing cells, we incubated them with soluble SLAMF7 antigen followed by a fluorescent anti-SLAMF7 antibody (Figure 5B right panel) targeting an epitope distinct from that recognized by the Elotuzumab-derived scFv in our anti-SLAMF7 ARM (an equivalent non-competing antibody was unavailable for BCMA/Belantamab). This approach allowed detection of unoccupied SLAMF7-binding sites. Dual CARtein cells exhibited significantly reduced anti-SLAMF7 staining intensity compared to cells expressing only anti-SLAMF7 CAR, while total CAR expression (protein-L staining) remained equivalent across samples. The reduced SLAMF7-specific signal confirms that only a subset of SCMs is occupied by the anti-SLAMF7 ARM, while the remainder are bound to the anti-BCMA ARM, demonstrating successful co-assembly of both ARMs and a balanced occupancy with no competitive exclusion.

To assess the specificity of the activation response elicited by CARtein-TPR cells targeting BCMA, SLAMF7 or both surface antigens, K562 cells were transduced with lentiviral vectors expressing BCMA or SLAMF7, and then puromycin selected. To obtain K562 expressing both antigens, after antigen expression and FACS evaluation, SLAMF7+ K562 cells were transduced and re-selected for BCMA expression. As shown in Figure 5E, transduced K562 cells with BCMA (BCMA+ K562), SLAMF7 (SLAMF7+ K562) or both surface proteins (SLAMF7+/BCMA+ K562) consistently expressed the corresponding surface antigen.

## Kinetics of CARtein-TPR cells upon SLAMF7 and BCMA stimulation mediated by transduced K562 cells

In order to validate the functionality of cells expressing the different CARtein constructs after split intein-mediated protein splicing, we performed a T cell activation signaling assay, using transduced K562 cells as target. We would measure specific CARtein-driven signaling upon BCMA or SLAMF7 stimulation by analyzing expression of CD69 activation marker as well as NFAT and NFκB promoter activity in JKT-TPR cells (CARtein-TPR cells) (39, 50).

Transduced K562 cells were co-cultured with anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR cells for T cell signaling activation analysis at a target to effector ratio of 1:1. CD69 expression, as well as NFAT and NFκB activity of CARtein expressing cells were evaluated at different time points after stimulation (0h, 24h and 48h). Response of SCM+ cells was evaluated as a control. Untransduced Jurkat-TPR cells (UTD) were used for baseline GFP, CFP or CD69 MFI values. Untransduced K562 cells (SLAMF7-/BCMA- K562) were also co-cultured to evaluate K562 mediated non-specific activation. Additionally mock (unstimulated) CARtein cells were also cultured under the same conditions.

Consistent with previous results, anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR cells, elicited NFAT and NFκB activation response directed against their respective targets in transduced K562 cells after 24h (Figures 6A, B). Moreover, CARtein cells exhibited relatively high expression of the activation marker CD69 (Figure 6C). On the other hand, SCM+ cells did not show CD69 upregulation or SCM-mediated NFAT or NFκB transcriptional activity when coculture with any target cell. Additionally, we did not observe any unspecific activation nor tonic signaling in CARtein-TPR cells or SCM+ cells when co-cultured with untransduced K562 cells. Nonetheless, Dual-CARtein cells exhibited relatively higher GFP and CFP MFI values when co-cultured with SLAMF7+/BCMA+ K562 cells instead of SLAMF7+ K562 cells.

For the purpose of comparing activation of either single or dual CARtein cells when co-cultured with cells expressing BCMA, SLAMF7 or both tumor antigens, we performed a Two-way ANOVA analysis. We did not find statically significant differences when comparing Jurkat-TPR cells stimulated with K562 cells expressing a single antigen or both antigens simultaneously. As illustrated in Figure 7A, anti-BCMA and anti-SLAMF7 CARtein cells exhibited high CD69 as well as NFAT and NFκB reporters MFI values (****$p < 0.0001$) when co-cultured with K562 cells expressing one or both surface proteins. Dual CARtein cells co-cultured with BCMA+ K562 cells elicited a slightly reduced NFAT and NFκB activity, but similar CD69 expression, while upon co-culture with SLAMF7+ K562 cells a significant decrease of CD69 (***$p < 0.001$), NFAT and NFκB reporters (*$p < 0.05$) MFI values were observed. Interestingly, in the presence of SLAMF7+/BCMA+ K562 cells, the stimulation of Dual CARtein-expressing cells resulted in slightly higher NFAT activity than single CARtein cells, although not statistically significant. Dual CARtein cells displayed similar CD69 and NFκB reporter MFI values when compared with

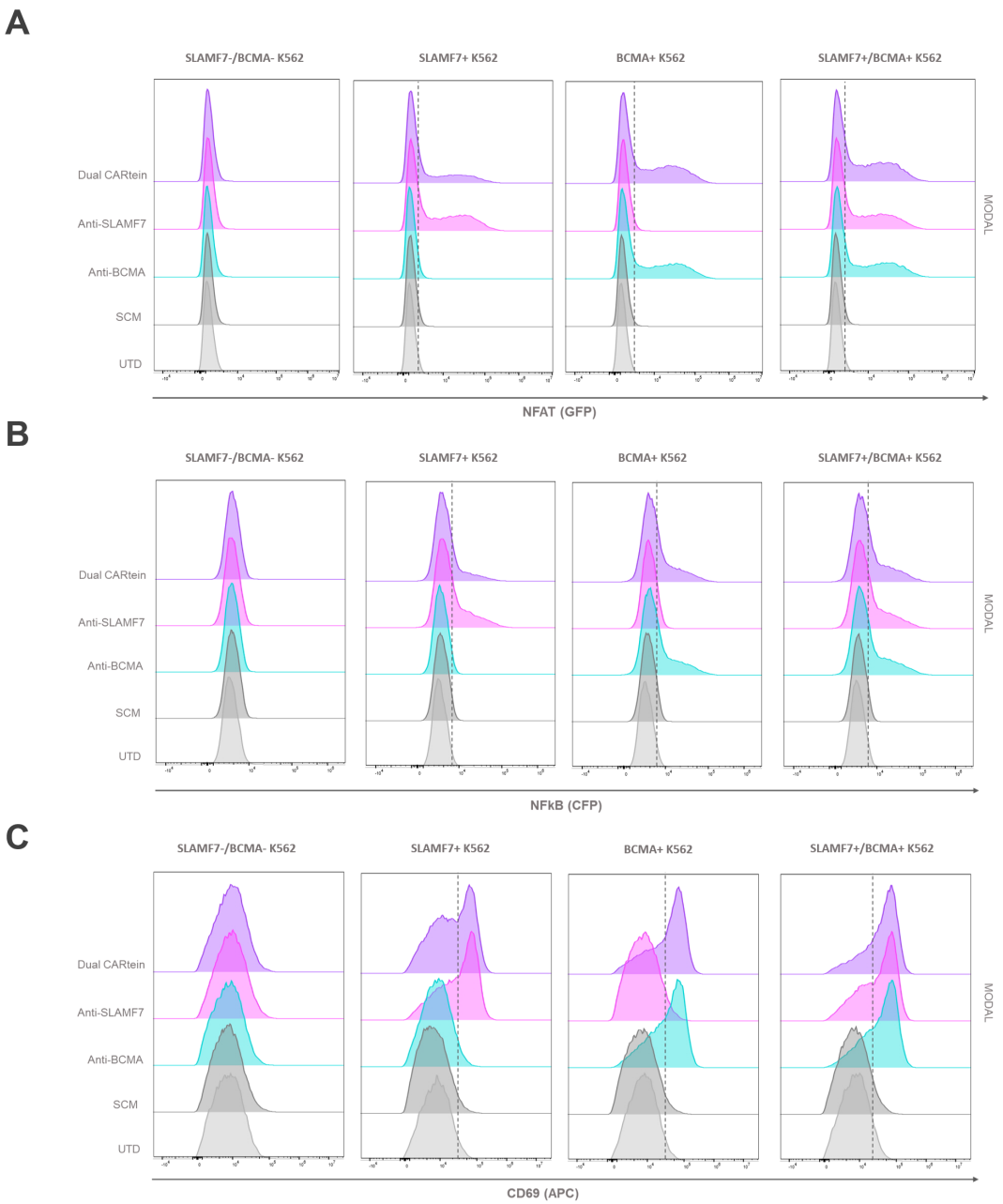


FIGURE 6
Activation signaling assay of anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR cells co-cultured with k562 cells expressing BCMA, SLAMF7 or both surface proteins. Representative flow cytometry analysis of NFAT **(A)** and NFκB **(B)** promoter reporters, or CD69 **(C)** in CARtein-TPR cells 24h after co-culture with either untransduced (UTD) or CARtein transduced k562 cells in a T:E ratio of 1:1.

CARtein cells targeting only one antigen upon SLAMF7+/BCMA+ stimulation. Furthermore, no tonic signaling or SCM+ mediated activation response was observed.

Regarding CARtein activation kinetics, a two-way ANOVA analysis was conducted in order to compare CARtein mediated cell activation at varying time points following stimulation (Figures 7B–D). CARtein-TPR cells targeting only one or both surface antigens exhibited a pronounced and significant (**** $p < 0.0001$) GFP and CFP (NFAT and NFκB reporters) upregulation 24h upon stimulation with K562 expressing BCMA, SLAMF7 or both. After 48h, these high GFP and CFP MFI values decreased, although they remained relatively elevated. A similar trend was observed for the CD69 activation marker at 24h and 48h. However, while anti-BCMA and anti-SLAMF7 CARtein cells elicited similar expression patterns, Dual CARtein-TPR cells maintained elevated CD69 expression for 48h.

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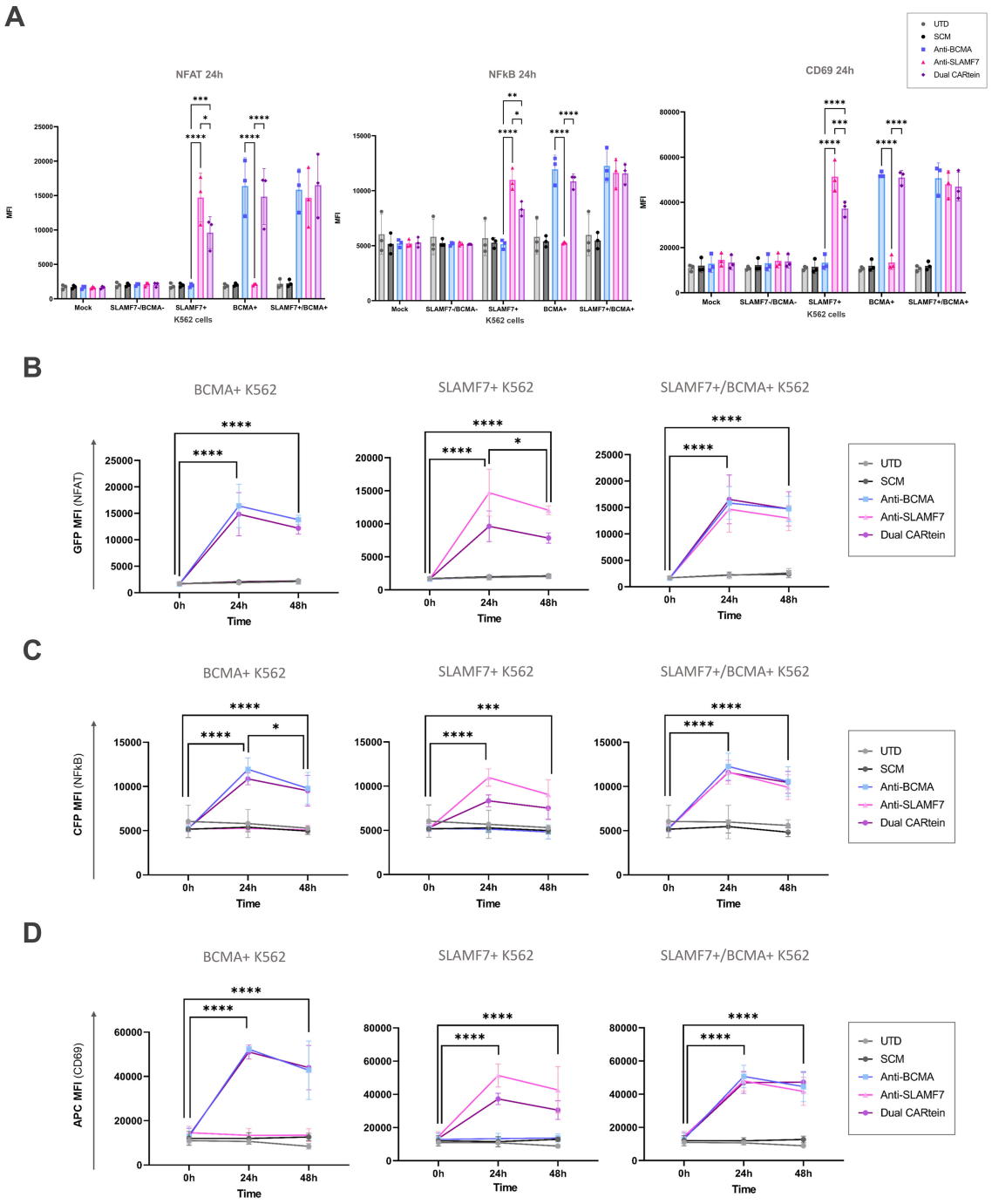


FIGURE 7
Kinetics of anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR cell activation upon co-culture with k562 cells expressing BCMA, SLAMF7 or both. **(A)** Flow cytometry comparison of NFAT and NFκB reporters and CD69 MFI for CARtein-TPR cells co-cultured with k562 cells expressing BCMA, SLAMF7 or both surface proteins. Each dot represents an independent experimental triplicate. Statistical comparison between anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR cells is indicated (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). NFAT **(B)**, NFκB **(C)** and CD69 **(D)** activation kinetics for anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR cells upon co-culture with either, untransduced (UTD) or CARtein transduced k562 cells. Statistical analysis of Dual CARtein cells is shown for each time condition (*$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001, ****$p$ < 0.0001). Data are average ± SEM of three different experimental replicates.

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## Kinetics of T cell activation in CARtein-TPR cells co-cultured with BCMA and SLAMF7 expressing MM.1s cells

In order to analyze CARtein-TPR cells signaling activation upon interaction with a multiple myeloma cell line, we co-cultured them with MM.1S cells. Prior to MM.1s coculture, we conducted a flow cytometry analysis to evaluate the expression of BCMA and SLAMF7 surface antigens using anti-BCMA and anti-SLAMF7 antibodies for staining. As shown in Figure 8A.1s cells exhibited elevated expression of both tumor antigens.

We then co-cultured MM.1s cells, with Jurkat-TPR cells expressing anti-BCMA, anti-SLAMF7 or Dual post-protein splicing CARteins (CARtein-TPR cells) as illustrated in Figures 8B, C. Additionally, Jurkat-TPR cells expressing the SCM (SCM+ cells) were also stimulated, in order to assess any potential SCM-mediated tonic signaling. As a control for baseline eGFP and CFP fluorescence signals, MM.1s cells were also co-cultured with untransduced (UTD) Jurkat-TPR cells [Mock (unstimulated)] We then analyzed by flow cytometry CD69, NFAT and NFκB activity at different time points (0h, 24h and 48h) and for different T:E ratios (1:1, 1:5 and 1:10).

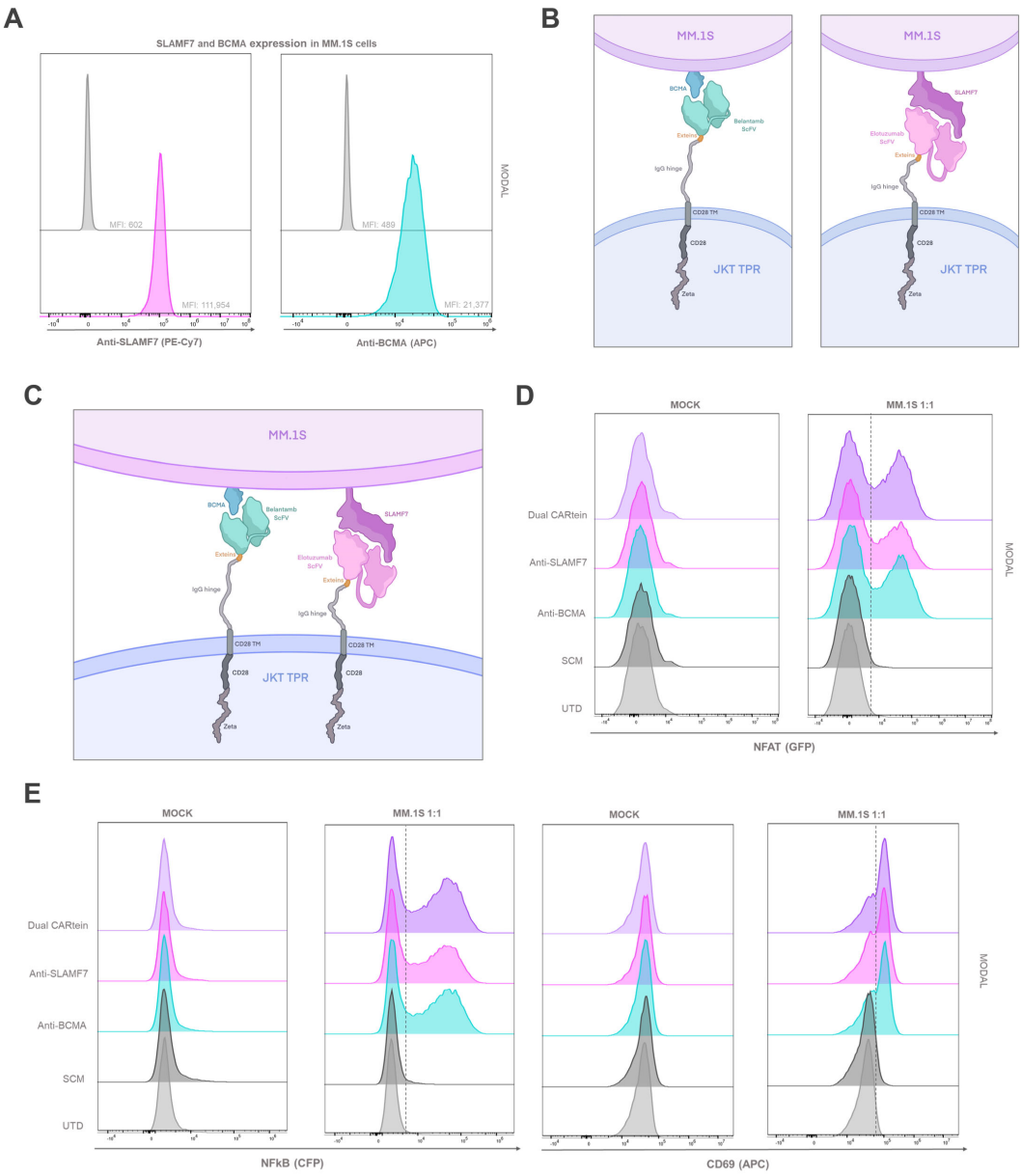


FIGURE 8
T cell activation signaling assay in CARtein Jurkat-TPR cells co-cultured with MM.1s cells. **(A)** SLAMF7 and BCMA expression in MM.1s cells. Schematic illustration of anti-BCMA or anti-SLAMF7 CARtein **(B)** or Dual CARtein **(C)** cells co-cultured with multiple myeloma target cells. **(D)** Representative flow cytometry analysis of NFAT and **(E)** NFκB activity and CD69 upregulation 24h after co-culture in a T:E ratio of 1:1. UTD, untransduced cells. JKT TPR, Jurkat-TPR cells.

As evidenced in Figures 8D, E, anti-CD69 expression as well as NFAT and NFκB activity increased 24h after MM.1s stimulation in anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR, exhibiting similar activation MFI profiles at 1:1. Moreover, minimal or negligible response was observed in SCM+ cells, as well as in mock samples, which suggests that CAR-mediated activation is strongly specific with little to none tonic activation in CARtein-TPR or SCM+ cells. In addition, the biparametric flow cytometry analysis (Supplementary Figure S2) revealed that most cells exhibiting CD69 upregulation, aligned with those showing higher NFAT or NFκB reporters signals, as well as cells displaying an enhanced NFAT activity with those with higher NFκB MFI values, indicating that most activated cells exhibited a complete signal transduction process.

With the aim of comparing anti-BCMA, anti-SLAMF7 and Dual CARtein-mediated T cell activation upon antigen stimulation at different target to effector ratios (1:1, 1:5 and 1:10), we performed a two-way ANOVA analysis. As illustrated in Figure 9A, all CARtein cells elicited a strong, specific and sustained NFAT and NFκB-mediated response as well as CD69 upregulation at different target to effector ratios. We found statistically significant differences among T:E ratios for NFAT reporter (GFP) MFI values in CARtein-TPR cells 24h after stimulation. Anti-BCMA CARtein-mediated NFAT activity moderately decreased at 1:5 (*$p < 0.05$) and 1:10 (**$p < 0.01$) ratios compared to 1:1 ratio. The same effect was observed for Dual CARtein cells (*$p < 0.05$), as well as for anti-SLAMF7 cells (*$p < 0.05$) between 1:1 and 1:10 ratios. However, NFAT activity analysis performed 48h after stimulation, revealed that CARtein cells exhibited similar MFI values for different T:E ratios for each construct. Interestingly NFκB activation is delayed at 48h in 1:5 and 1:10 ratios when compared to 1:1 ratio. Notably, SCM+ cells showed none or insignificant activation response, suggesting that no unspecific SCM-driven activation is exhibited by CARtein cells. Interestingly, MFI values for CD69 and NFAT and NFκB reporters exhibited by Dual CARtein cells exceeded those of CARtein cells targeting a single antigen in all T:E ratios and for both, 24h and 48h after co-culture, although the difference is not statically significant. This tendency may be indicative of a synergistic effect in T cell signal transduction when cells expressing these CAR constructs target both antigens.

To gain further insight into the activation kinetics of these CARtein-TPR cells, a two-way ANOVA analysis was conducted, comparing different time points for a cell expressing a particular intein-mediated CAR (Figures 9B–D). NFAT, NFκB and CD69 MFI values at target to effector 1:1 ratio strongly peak at 24h upon antigen stimulation for anti-BCMA, anti-SLAMF7 and Dual CARtein cells (****$p < 0.0001$). However, NFAT signal slightly decreases, although no significantly, for all CARtein-expressing cells at 1:1 ratio after 48h. A similar but more pronounced effect can be appreciated for NFκB activity. Moreover, we can observe a delayed NFAT response for all CARtein-TPR cells at T:E ratios 1:5 and 1:10, since they reach their highest MFI values at 48h (****$p < 0.0001$). Interestingly, anti-BCMA CARtein cells at 1:10 ratio and Dual CARtein cells at 1:5 and 1:10 ratios maintained high NFκB MFI values up to 48h, but not in the remaining cases, in which the signal declined. Regarding CD69 expression, 24h after co-culture, all CARtein-TPR cells exhibit high CD69 MFI values at different T:E ratios. Nonetheless, cells co-cultured at 1:1 ratio display a slightly higher, although no significant, CD69 expression. CD69 activation marker MFI values remained elevated 48h after stimulation, even though a downward trend is appreciated. NFAT, NFκB and CD69 MFI values remained at background levels for SCM+ cells at any T:E ratio or time point.

To obtain a more comprehensive view regarding CARtein-mediated signaling activation in T-cells, we performed a two-way ANOVA analysis, comparing NFAT, NFκB and CD69 MFI values in CARtein-TPR cells when stimulated with MM.1S multiple myeloma cell line, which endogenously expresses BCMA and SLAMF7 proteins, or K562 cells, which have been engineered to express both simultaneously (Figures 10A, B). For both cases we conducted the statistical analysis at target to effector ratio of 1:1 and 24h after coculture, as NFAT and NFκB reporters, as well as CD69 marker, show the highest MFI values. Dual CARtein cells exhibit significantly enhanced NFAT (*$p < 0.05$), NFκB (***$p < 0.001$) activity and increased CD69 expression (**$p < 0.01$) when stimulated with MM.1s cells. Moreover, anti-BCMA and anti-SLAMF7 CARtein cells also displayed significantly higher CD69, NFκB MFI values (** $p \leq 0.01$) and NFAT after MM.1s coculture.

## Discussion

In this study we have demonstrated successful assembly and antigen-dependent activation of a split intein-mediated CAR approach targeting BCMA and SLAMF7 in MM cells using the Jurkat-TPR cellular model. Moreover, the anti-BCMA scFv Belantamab, which has given promising results for MM treatment as an antibody-drug conjugate (51, 52), has been used for the first time in the context of a CAR construct being highly selective and functional for CAR T cell therapy. For the modular system we have chosen IMPDH-1 intein as it exhibits the highest trans-splicing rates and yields (27, 53), and it has already been shown to function in ER transplicing (53). The dual CARtein strategy proposed herein can be used for the complex integration and expression of two or more independent CAR constructs simultaneously in a single cell, as it has been previously reported that Dual CARs outperform bispecific CARs based on two distinct scFvs linked in tandem or pooled CARs strategies (54), overcoming critical limitations observed in tandem CARs. Tandem CARs tether two scFvs to a single signaling domain forcing co-dependency, where steric hindrance between scFvs may reduce binding efficiency, while for CARtein, each ARM assembles with its own SCM, creating two independent CAR populations per cell, thus preserving full signaling autonomy and allowing for independent spacer optimization, as spacers of different lengths could be included in the ARM instead of SCM when necessary. Although different bispecific CAR approaches targeting BCMA and SLAMF7 have already been developed for multiple myeloma treatment, the Dual CARtein system represents the first modular CAR engineered to target BCMA and SLAMF7 simultaneously, thus offering dual

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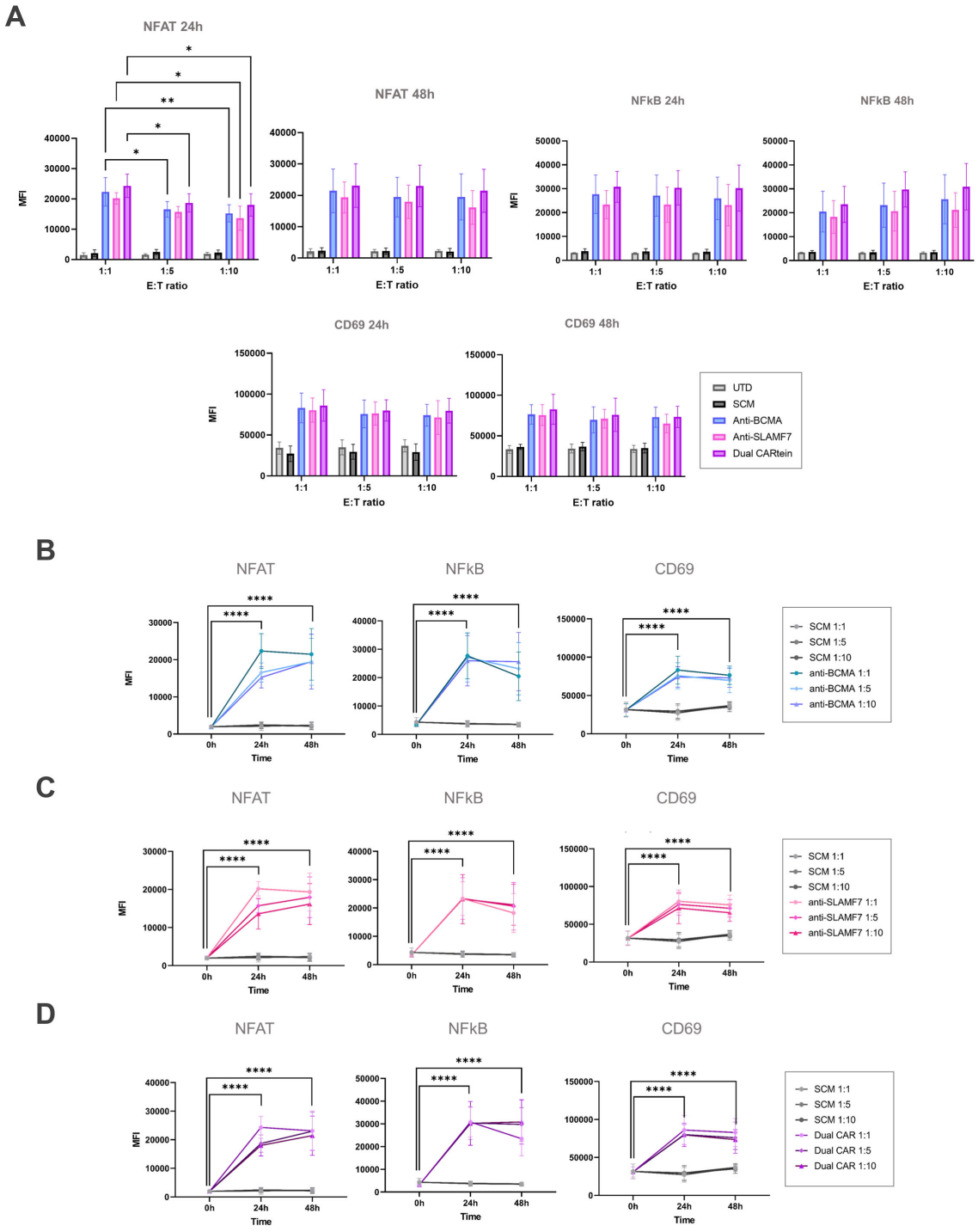


FIGURE 9
Kinetics of anti-BCMA, anti-SLAMF7 and Dual CARtein-TPR cell activation upon MM.1s stimulation. **(A)** Flow cytometry-based statistical comparison of the mean fluorescence intensity (MFI) of NFAT and NFκB activity reporters and CD69 upregulation after co-culture with MM.1S cells at three different T:E ratios. Statistical analysis between T:E ratios is shown (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). **(B)** NFAT, NFκB and CD69 activation kinetics for three T:E ratios of anti-BCMA, **(C)** anti-SLAMF7 and **(D)** Dual CARtein cells upon stimulation. Statistical analysis of CARtein cells in 1:1 ratio is indicated for each time condition (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Data are average ± SEM of three different experimental replicates. UTD, untransduced cells.

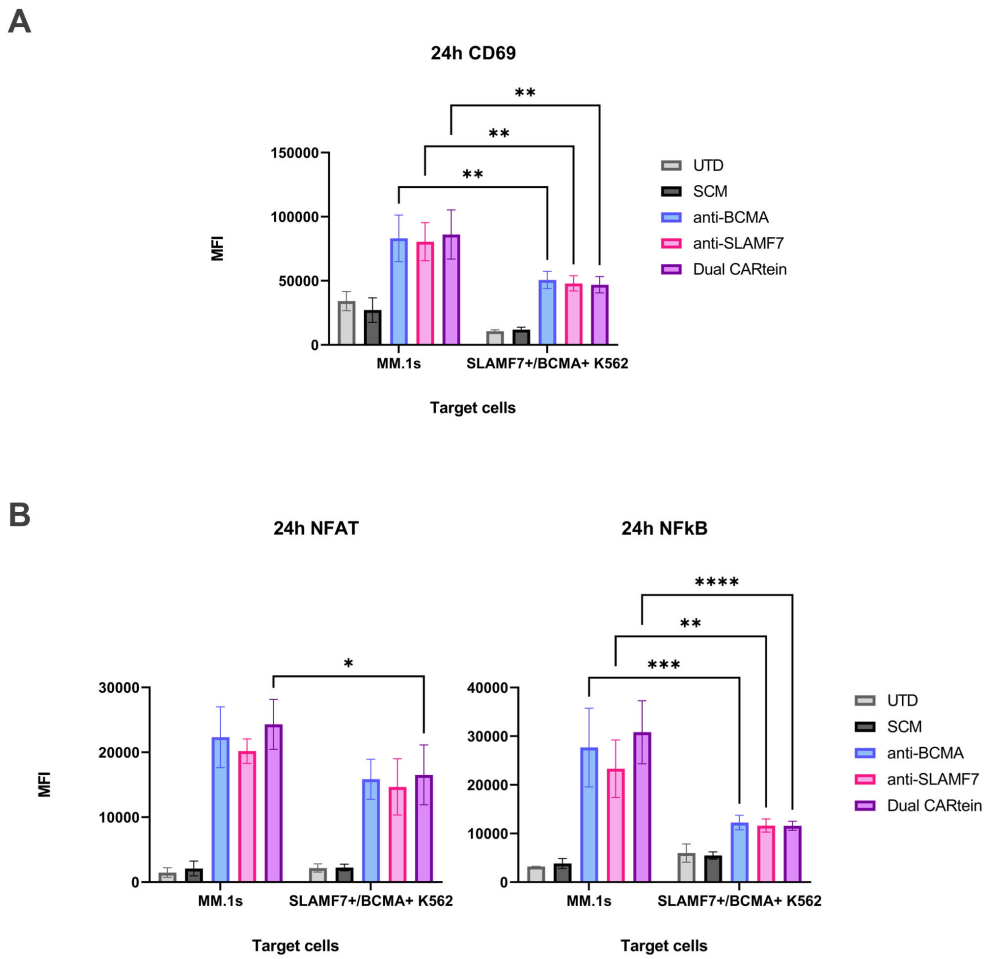


FIGURE 10
Comparison of CARtein-TPR cell activation upon stimulation with MM.1s or K562 cells expressing BCMA and SLAMF7. **(A)** Flow cytometry-based statistical comparison of the mean fluorescence intensity (MFI) of CD69 activation marker and **(B)** NFAT and NFκB reporter activity 24h after coculture at a target to effector ratio of 1:1. Statistical analysis between MM.1s and SLAMF7+/BCMA+ coculture is shown (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Data are ± SEM of three different experimental replicates. UTD, untransduced cells.

functionality without signal dilution. Leveraging intein-mediated protein splicing, this platform offers unprecedented adaptability, irreversible action, compact design, and minimal toxicity (55–59). The CARtein system overcomes limitations of existing modular CAR platforms by providing irreversible stability with minimal scarring. This is achieved through a permanent peptide bond, chemically identical to native protein bonds, leaving only a 6-amino acid scar (GGGSIC). This scar is comparable in size and impact to cloning artifacts in conventional CARs and can even be further shortened (although such optimization may risks reduced splicing efficiency). Importantly, the residual split intein sequences are no longer part of the CAR construct and exhibit negligible immunological impact, as confirmed by unsuccessful attempts to generate antibodies in animal immunization studies. As for the Twin-Strep tag, it has been used experimentally but is removable in a therapeutic construct. To further enhance safety, we are developing proprietary clearance strategies to eliminate residual intein-derived sequences from circulation. On the other hand, non-covalent systems (SUPRA CAR, UniCAR, etc.) rely on antibody-epitope, receptor-ligand, or leucine zipper interactions that risk dissociation and require continuous adapter infusion, while also leaving bulky scars (e.g., leucine zippers, scFv domains) that may disrupt immune synapse formation and increase immunogenicity. The spycatcher/tag systems: form irreversible covalent bonds but leave a large bacterial-derived scar (138 aa spycatcher + 13 aa spytag) linked by an isopeptide bond, a foreign domain that significantly elevates immunogenicity risk. Biotin-binding cars: introduce non-covalent streptavidin/biotin interactions (bbir/msa2-cars) with risks of interference from endogenous biotinylated proteins, variable valency, and a bulky (~53 kda) and potentially immunogenic avidin/streptavidin scar (60).

The CARtein system also offers versatility in assembly modes, with trans-assembly based on external adapter (ARM) administration for flexible targeting and/or ARM optimization, but also with the possibility of intracellular assembly such as the one shown in our work that is unique to the CARtein system, where transduced ARMs enable self-contained functionality, eliminating adapter infusion entirely, ensuing in a mature CAR with a minimal

scar that minimizes anti-CAR immune responses and avoids steric hindrance at the immunological synapse. We have shown that in cells co-expressing both SCM as well as ARM modules, mature spliced CARtein receptors are expressed in the cell surface as staining shown in Figure 5 are performed in unpermeabilized cells.

The Jurkat-TPR cell line, a cellular model for T cell signaling activation studies, was used to evaluate the functionality and specificity of the split intein-mediated CAR (CARtein) system directed against multiple myeloma. This cellular model, in which the expression of eGFP, CFP and mCherry proteins is respectively governed by NFAT, NFκB and AP-1 transcription factors, has been previously validated for the functional analysis of CAR constructs (45, 47, 61). In this study we show NFAT and NFκB activity, given that AP-1 activation can better be monitored in T cells the human distal ARRE-2 site from the IL-2 promoter used in Jurkat-TPR cells as NFAT reporter as it is dependent on NFAT and AP-1 cooperative binding (62, 63). Additionally, we further evaluated CD69 expression upon antigen stimulation, as CD69 is an early activation marker rapidly expressed on lymphocyte surface upon stimulation (64).

Overall, a strong and specific T cell signaling activation response was elicited upon antigen stimulation of Jurkat-TPR cells expressing the post-spliced anti-BCMA or anti-SLAMF7 CARtein individual constructs, as well as in those expressing both CARtein constructs simultaneously (Dual CARtein-TPR cells). High CD69, NFAT and NFκB reporters MFI values were reported in CARtein-TPR cells upon stimulation with MM.1s and K562 cells transduced with one or both target antigens. Interestingly, when reducing the number of target cells (MM.1s) relative to the number of effector cells (T:E ratios 1:5 and 1:10), CARtein-TPR cells displayed delayed NFAT and NFκB activation compared to the one displayed at 1:1 ratio. However, these cells still exhibited a relatively strong signal transduction despite the significant reduction in the number of target cells, suggesting that our CARtein system is highly sensitive and selective, even at low concentrations of cancer cells, which will allow strong responses against low or moderate tumor burdens. Nonetheless, an exacerbated response of CAR T cells against cancer cells can lead to toxic systemic cytokine levels, and may result in significant adverse effects, such as the cytokine-release syndrome (CRS) or immune effector cell-associated neurotoxicity syndrome (ICANS) (65, 66). However, previous studies have reported that CAR-mediated activation in T cells upon antigen binding can be tuned by optimizing CAR molecular density, which may decrease CAR T cells activation response if necessary (48, 67) a feature that can be optimized by means of the modular CARtein system either by controlling the expression of the recognition module by means of a inducible promoter or by adding in trans proteins spanning the signaling module.

Regarding signaling activation kinetics in CARtein-TPR cells, when co-cultured with MM.1s cells at 1:1 ratio, JKT-TPR cells expressing the anti-BCMA, anti-SLAMF7 or both CARtein molecules exhibited similar kinetics trend for NFAT reporter and CD69 expression as previously reported for ACE2-CAR-Like cells, although NFAT activation follow a faster kinetic with maximal stimulation at 24h instead of 48h. However, NFκB kinetics were closer to one previously described to be exhibited by JKT-TPR cells after anti-CD3+ anti-CD28 stimulation, with a strong peak 24h after antigen engagement followed by a decline in MFI values at 48h. Nonetheless CARtein cells at target to effector 1:5 and 1:10 ratios displayed closer NFAT, NFκB and CD69 kinetics to the one exhibited by ACE2-CAR cells (40). These findings may indicate that, although our CARtein cells exhibit activation kinetics similar to ACE2-CAR-like expressing cells, with delayed and sustained NFAT activity and CD69 expression at 1:1 ratio, CARtein cells may elicit an activation response that more closely resembles that of T-cell receptor (TcR) than CAR-like cells, as it is widely accepted that anti-CD3+ anti-CD28 stimulation mimics TcR engagement and signal transduction (68). Nevertheless, when stimulated with a reduced number of target cells (1:5 and 1:10 ratios), CARtein-TPR cells elicited a delayed and maintained NFAT and NFκB activation kinetics, similar to the activation response reported by Rydzek et al., using ROR1-specific CAR cells bearing two reporter genes that induce the expression of reporter proteins under the government of NFAT and NFκB (45).

Remarkably, when co-cultured with K562 cells expressing either BCMA, SLAMF7, or both antigens, CARtein cells exhibited a decreased NFκB activity compared to those stimulated with MM.1s. Moreover, CD69 MFI values were also significantly reduced, while NFAT MFI values remained relatively similar. In addition, Dual CARtein-TPR cells stimulated with MM.1s cells, exhibited higher NFAT, NFκB and CD69 MFI values than cells expressing only one of the spliced CARtein molecules. This tendency may be indicative of a synergistic effect due to the total antigen density obtained by targeting both antigens on target cells because on the effector cell the total CARtein density is the same regardless of the signaling module. This enhanced effect in T cell signaling activity could also explain the fact that Dual CARtein-TPR cells exhibited higher NFAT and NFκB reporters MFI values when co-cultured with SLAMF7+/BCMA+ K562 cells compared to K562 cells expressing only BCMA or SLAMF7 surface antigens. Fernández Larrea et al., reported that an anti-BCMA and anti-GPRC5D dual CAR approach based on bicistronic vectors encoding two independent CARs outperformed the anti-myeloma efficacy of a mono-targeted pooled CAR approach (54). However, further experimentation is required to elucidate the exact mechanisms underlying this enhanced antitumor response.

MM.1s-mediated co-stimulation could explain the enhanced synergetic effect when CARtein cells are stimulated with the multiple myeloma cell line rather than genetically modified K562 cells expressing target antigens. This theory is further reinforced by the fact that Jurkat T cell line JE6.1 consistently express CD28 receptor and the multiple myeloma cell line, MM.1s expresses CD86 (B7-2) ligand. It is described that CD28 activation is mediated by the binding to its ligands, CD80 and CD86, which eventually leads to the induction of NFκB-dependent expression of the anti-apoptotic protein BCL-XL, which is a key survival mechanism in T-cells (69–71). Moreover, it has been reported that in the absence of co-stimulation, TCR signaling induces a PLCγ-dependent and prolonged increase in cytosolic Ca2+ concentration and the

transcriptional induction of NFAT, which is consistent with the reduction of NFκB activity in CARtein cells when co-cultured with transduced K562 cells instead of MM.1s cells, while NFAT-mediated activation remained relatively similar (72).

One of the current limitations in CAR T cell therapy is ligand independent tonic signaling, which can lead to poor antitumor response, impaired survival and T cell exhaustion. One of the mechanisms that could be responsible for tonic signaling is the instability of scFvs in CAR molecules, promoting self-aggregation and therefore signaling via CD3ζ (73–76). $IgG_1$-derived spacers have also been reported to mediate CAR tonic signaling, as cells expressing FcγR I and II could interact with $IgG_1$ $CH_2$ region (73, 77). However, neither CARtein-TPR cells nor SCM+ cells have exhibited evidence of significant tonic signaling, which implies that none of the mentioned tonic signaling mechanisms are triggering an off-target response. These results are consistent with the absence of significant tonic signaling in our previously reported ACE2-CAR-like cells, which shares with the CARtein constructs a mutated $IgG_1$ spacer that does not bind Fc receptors (40, 78). As for the off-tumor toxicity risks for CARtein constructs targeting SLAMF7 and BCMA, they are expected to be identical to conventional CAR-T therapies using the same humanized scFvs, as the mature CARtein receptor is structurally equivalent except for a 6-amino acid scar.

It is also noteworthy that in this study we have employed *in silico* analysis based on protein and complex structure prediction software in order to evaluate whether the integration of split inteins into the scFv or CAR signaling module would negatively impact their structure and hence, functionality. For this purpose, we have used ColabFold software, based on the highly accurate deep learning model AlphaFold2 (32, 33). Not only were we able to predict the structure of the CAR signaling and antigen-binding modules comprising split inteins moieties, but also the ability of said inteins to interact with each other and thus catalyze their reaction. Moreover retention of antigen binding capacity, of spliced CARteins bearing residual exteins was also predicted, as the scFv-binding pocket exhibited minimal alteration. These findings, together with previous results, suggest that protein structure prediction is a valuable and powerful tool for the rational design of modular CARs and other chimeric proteins (23, 42, 79).

Furthermore, unlike conventional modular CAR designs that depend on transient scFv-tag conjugate interactions with modified CAR constructs, the innovative CARtein system employs an intein-mediated protein splicing mechanism that establishes a covalent peptide bond between the N- and C-exteins, involving only three specific residues (19, 80). While alternative modular CAR approaches—such as the SpyTag-SpyCatcher system or the SNAP-CAR platform—also utilize covalent linkage strategies through isopeptide bonds, they present significant limitations (20, 81–84). The CARtein system's distinct advantage lies in its unique modular attachment mechanism that leaves an exceptionally minimal residual "scar" of merely six amino acids after interaction. This remarkable characteristic substantially reduces the probability of interference with antigen binding, a critical concern in CAR functionality. The split intein-mediated CAR platform proposed in this study effectively addresses these limitations while maintaining optimal binding efficiency (27, 85). In addition, Tornabene et al., reported no signs of intein-related toxicity in retinal gene therapy performed on mice, pigs and human retinal organoids, in which they used adeno-associated viral (AAV) vectors encoding fragments of target proteins followed by split intein sequences in order to overcome issues related to limited AAV cargo capacity (28).

The CARtein® system enables creation of "off-the-shelf" SCM-expressing T-cell banks that can be cryopreserved and rapidly armed with disease-specific ARMs for optimization. Additionally, already optimized ARMs can either be infused or permanently transduced. The system allows for manufacturing efficiency, decoupling a general SCM production from specific ARM design. It will also allow for sequential re-targeting as patients could receive multiple courses with different ARMs without additional T-cell harvests, including ARMs against escape antigen administered to existing $SCM^+$ cells. Simultaneous dual targeting to address tumor heterogeneity is also possible as well as tapered dosing, gradually reducing ARM frequency to manage chronic toxicity while preserving $SCM^+$ cells. The CARtein system is also relevant for CRS mitigation as system-level activity can be dynamically regulated by halting ARM administration, as mature CARs in the surface are cleared within 24–48 hours and replaced by inactive SCM. For rapid inactivation, the SCM can be engineered to include a secondary C-terminal intein module positioned downstream of the primary intein. This module remains intact post-splicing. Administration of a cognate N-intein effector (lacking scFv domains) then triggers excision of the ARM via intein-mediated splicing, enabling rapid CAR disassembly, a safety switch unattainable with conventional CARs. In conclusion, the CARtein system presents a flexible, versatile, and powerful platform for assembling modular CARs, offering an extensive range of potential combinations that can be further expanded and integrated with diverse strategies, enabling optimization of CAR constructs and the treatment of a broader number of malignancies, thus, providing a valuable resource for universal CAR T cell therapy. While this study establishes proof-of-concept for CARtein® assembly, signaling, and modular functionality in engineered cell lines, demonstrating tumor-cell lysis and cytokine release profiles in primary human T cells remains a critical next step for translational validation that we are currently pursuing. Additionally, SCM transgenic animals or SCM expressing human cells in humanized mice will streamline ARM and CAR optimization processes for preclinical studies in tumor models, while in parallel "off-the-shelf" SCM-expressing T-cell banks that could be further manipulated for allogeneic therapy can be used as a universal CAR strategy and approved in clinical trials, with an independent approval for specific ARM that can be either infused or co-transduction for fixed dual-targeting.

## Data availability statement

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

## Author contributions

NM: Visualization, Writing – original draft, Writing – review & editing, Data curation, Investigation, Conceptualization. PG-G: Conceptualization, Writing – review & editing, Writing – original draft, Data curation, Investigation, Visualization. WY-H: Writing – review & editing, Formal Analysis. JM-M: Writing – review & editing, Resources. AG: Writing – review & editing. RL-E: Writing – review & editing, Resources. JO: Writing – review & editing. RF-C: Writing – review & editing, Formal Analysis. CF-P: Validation, Writing – review & editing. FG-C: Project administration, Conceptualization, Funding acquisition, Validation, Supervision, Writing – review & editing.

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## Conflict of interest

All authors are inventors of the patent application EP25382080.7, filed by the University of Cádiz and INIBICA. The patent pertains to modular CARs, and uses thereof, which is directly related to the findings reported in this manuscript. No financial or non-financial benefits have been realized from this patent at the time of publication. The authors confirm that no other competing interests exist.

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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.2025.1613222/full#supplementary-material

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# Unlocking new horizons in oncology: ivonescimab's dual-target approach to anti-VEGF/PD-1(L1) therapy

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Dual blockade of the PD-1/PD-L1 axis, enabling tumor immune evasion, and the VEGF pathway, driving immunosuppression, represents a promising cancer immunotherapy strategy. Combining immune checkpoint inhibitors (ICIs) with antiangiogenics faces toxicity and cost limitations. Bispecific antibodies (BsAbs) targeting both pathways offer a solution. Preclinical and clinical studies demonstrate that simultaneous inhibition enhances antitumor immunity by reversing T-cell exhaustion, normalizing vasculature, and countering immunosuppression. Ivonescimab, a first-in-class PD-1/VEGF BsAb, exemplifies this approach. Approved in China (NMPA, May 2024) for EGFR-mutant non-squamous NSCLC post-TKI failure and included in national insurance (November 2024), it is under global evaluation in solid tumors. PD-1 (L1)/VEGF BsAbs like ivonescimab represent a novel therapeutic strategy with potential for improved efficacy and mitigated toxicity compared to combination therapies. Ongoing trials will define broader applications.

KEYWORDS

**bispecific antibodies, ivonescimab, immune checkpoint inhibitors, vascular endothelial growth factor, dual-target**

## Introduction

Programmed death-1 (PD-1) is an immune checkpoint receptor and that regulates T cell activity by inhibiting immune responses and promoting self-tolerance. Programmed Death Ligand 1 (PD-L1), the natural receptor for PD-1, predominantly expressed on tumor cells, binds PD-1 to activate downstream signaling pathways and suppresses T cell activation, cytokine secretion, and induces apoptosis; Critically, the PD-1/PD-L1 axis is a key mechanism enabling tumor immune evasion (1). Targeting this axis with immune checkpoint inhibitors (ICIs) revitalizes exhausted T cells in the tumor microenvironment

(TME), demonstrating significant clinical efficacy in multiple malignancies with manageable toxicity (2). It is well-established that vascular endothelial growth factor (VEGF) can induce local immunosuppressive effects through diversexb mechanisms; Antiangiogenic agents (e.g., VEGF/VEGFR pathway inhibitors) can remodel the immunosuppressive TME, enhancing ICI efficacy by normalizing tumor vasculature to improve perfusion, blocking VEGF-mediated immunosuppression, and preventing endothelial cell-induced T cell apoptosis (3–5).

Preclinical evidence further indicates that VEGF inhibition may upregulate PD-L1, suggesting bidirectional crosstalk between these pathways in sustaining a pro-tumorigenic microenvironment (6, 7). Despite the promise of ICI-antiangiogenic combinations, high costs and toxicity necessitate strategies to optimize therapeutic outcomes (8). One of the most effective strategies to improve overall efficacy is to develop a bispecific antibody simultaneously targeting PD-1/PD-L1 and VEGF could potentially gain higher target binding specificity and enhanced anti-tumor activity, while also improving safety profiles (9). Several such BsAbs, including ivonescimab, PM8002, and HB0025, are now in clinical development. Notably, ivonescimab received approval from China's National Medical Products Administration (NMPA) in May 2024 for EGFR-mutated advanced non-squamous NSCLC following tyrosine kinase inhibitor failure and was subsequently included in China's national medical insurance in November 2024 (10). In addition, Global clinical trials of ivonescimab are ongoing. This review explores recent advances and therapeutic potential of ivonescimab, alongside the development of other PD-1(L1)/VEGF BsAbs in solid tumors.

# Ivonescimab: a novel anti-PD-1/VEGF bispecific monoclonal antibody

## Chemistry and mechanism of ivonescimab

Bispecific antibodies (BsAbs) are a class of engineered proteins that resemble immunoglobulin (IgG) or antibody fragments (Fab), combining various antigen-binding sites into one structure. Their primary goal is to simultaneously block two targets involved in pathological processes, thereby enhancing therapeutic efficacy (11). BsAbs can be created by fusing a single-chain variable fragment (scFv) with the C-terminal Fc fragment of an IgG (i.e., IgG-scFv) (12). Ivonescimab is a pioneering, IgG1-scFv humanized, bispecific monoclonal antibody developed by Akeso Biopharma. It targets both PD-1 and VEGF and is in development for the treatment of non-small cell lung cancer (NSCLC) and other advanced solid tumors, such as breast, liver, and gastric cancer (Figure 1). The underlying mechanism of Ivonescimab is as follows (Figure 2): 1) In the presence of VEGF, the tetravalent structure of Ivonescimab facilitates the formation of a soluble complex with VEGF dimers. This complex formation accelerates the binding kinetics of Ivonescimab to PD-1, thereby potentiating its blockade of the PD-1/PD-L1 signaling axis and further alleviating PD-1/PD-L1 pathway-mediated immunosuppression, leading to the activation

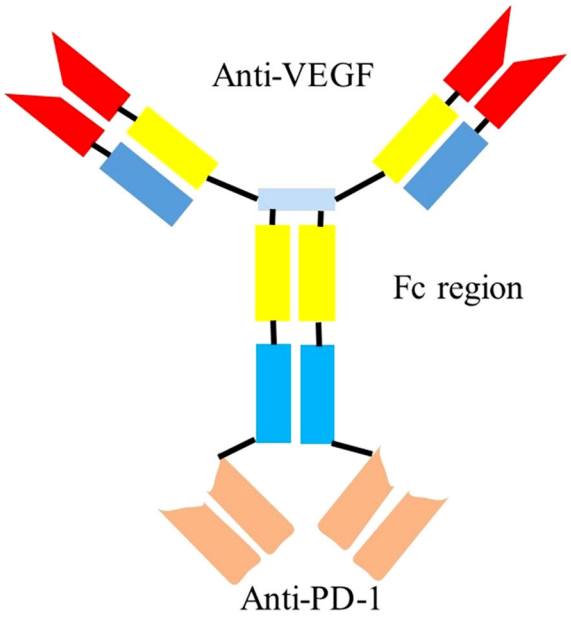


FIGURE 1
This figure illustrates the tetravalent binding configuration of ivonescimab, an IgG-scFv fusion BsAb developed by Akeso Biopharma. This figure is created with BioRender.com.

of cytotoxic T lymphocytes (CTLs) and an amplified T-cell response. Furthermore, the antigen-binding activity of Ivonescimab to PD-1 exhibits slower dissociation kinetics in the presence of VEGF, resulting in an 18-fold higher binding affinity compared to penpulimab (11).

2) The binding of ivonescimab to PD-1 reciprocally augments its affinity for VEGF, by effectively sequestering VEGF-A and VEGF-B, ivonescimab inhibits their interaction with VEGFR, and this inhibition disrupts the downstream PI3K/AKT/mTOR signaling cascade, suppressing the proliferation of tumor cells and vascular endothelial cells, and attenuating neovascularization. Concomitantly, this mechanism promotes the infiltration of CTLs into tumor tissues, reprogramming the immunosuppressive tumor microenvironment into an immunologically active state (12).

3) As is well known, the Fc fragment forties the solubility and stability of the protein increases its pharmacokinetic profile, and improves avidity via Fc dimerization, resulting in increased local (near target) antibody concentrations (13). However, for immune checkpoint inhibitors (ICIs), Fc-mediated effector functions can confer detrimental effects. Immune-related adverse events (irAEs) are known to be associated with the recruitment of immune cells expressing Fc receptors. Importantly, the Fc region of ivonescimab has been modified to minimize binding to Fcγ receptors (FcγR), that was engineered to incorporate the double mutation L234A/L235A (termed "DM"), conferring Fc silencing properties. This design eliminates the binding capacity of ivonescimab to FcγRI/IIIa, thereby substantially abrogating Fc-mediated effector functions. These eliminated functions include antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, antibody-dependent cellular phagocytosis, antibody-dependent cytokine

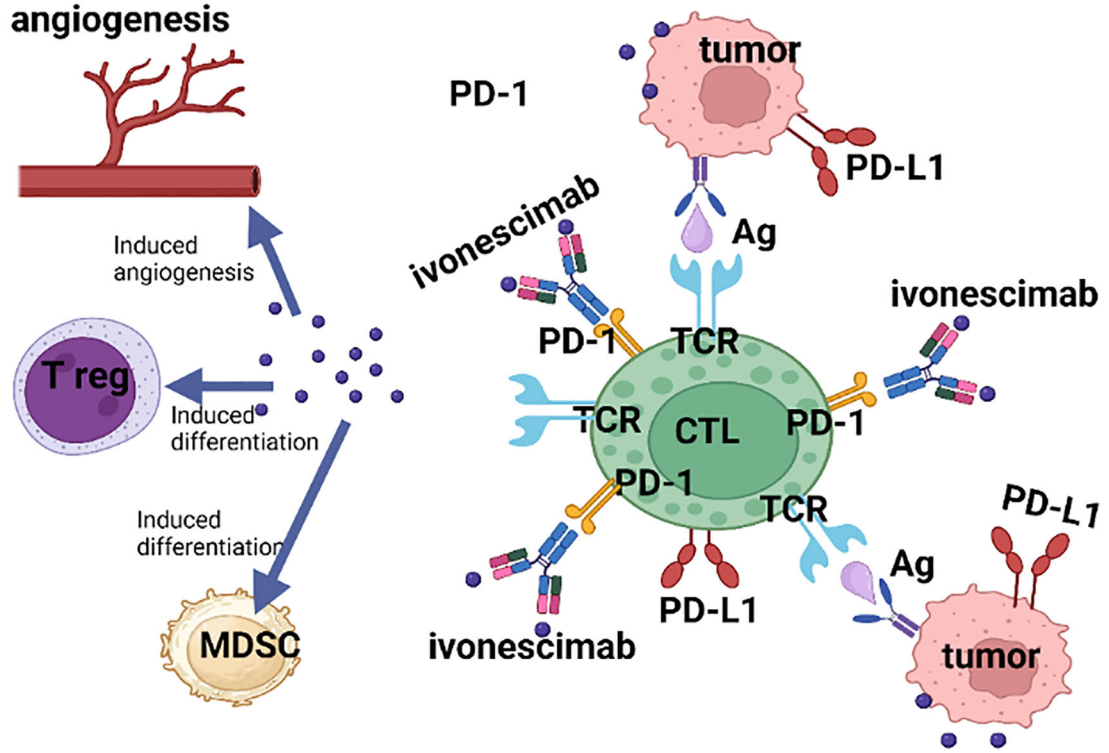


FIGURE 2
Diagram of the mechanism of action of PD-1/VEGF BsAb (such as Ivonescimab). This figure is created with BioRender.com.

release, and cytokine release syndrome. This attenuation of Fc functionality may contribute to a reduced risk of irAEs (11).

VEGF and PD-1 are typically co-expressed within the tumor microenvironment. As a PD-1/VEGF BsAb, Ivonescimab not only maintains the activity of the PD-1 monoclonal antibody but also functions as an immune checkpoint inhibitor, further eliminating tumors through effector T cells. Moreover, the combination with VEGF can suppress and block the multiple immunosuppressive effects of VEGF and induce various vascular-modulating responses that enhance immunity. This includes normalizing blood vessels to increase intratumoral blood perfusion and flow, as well as restricting VEGF to promote the differentiation of bone marrow-derived inhibitory T cells. In essence, the dual-target combination increases Ivonescimab's retention in the tumor microenvironment, further enhancing its anti-tumor activity and achieving an effect where 1 + 1 > 2.VEGF: vascular endothelial growth factor; PD-1: programmed death-1; PD-L1:programmed death ligand 1; Treg: T regulatory cell; MDSC: myeloid-derived suppressor cell; CTL: Cytotoxic T lymphocytes; TCR: T cell receptor; Ag: antigen.

## Preclinical studies of ivonescimab

The results of preliminary research demonstrated that Ivonescimab exhibits high affinity and specific binding to human PD-1 and VEGF; Notably, through SEC-HPLC analysis, it has been detected that Ivonescimab forms soluble complexes with VEGF; Moreover, VEGF efficiently strengthened the binding of Ivonescimab to PD-1, which led to enhanced PD-1 internalization and better potency on blockade of PD-1/PD-L1 signaling. Intriguingly, ivonescimab significantly inhibits tumor growth in a dose-dependent manner in the HCC827 xenograft mouse model (12).

## Pharmacokinetics

Pharmacokinetic (PK) data for ivonescimab were derived from, a multicenter, open-label, dose-escalation phase I study, conducted across five hospitals in China, evaluated ivonescimab monotherapy, with doses ranging from 0.3 to 30 mg/kg administered every two or three weeks (10, 14). After a single intravenous dose, the serum levels of ivonescimab showed a dose-proportional increase within the 3–30 mg/kg range, with steady-state concentrations achieved by the 15th week following repeated administrations. In addition, following single-dose and multiple-dose intravenous administrations of ivonescimab, serum concentration of ivonescimab increased in a dose-dependent manner (9). The maximum concentration ($T_{max}$), mean half-life ($t_{1/2}$), and mean clearance (CL) did not show obvious dose dependence, and The average accumulation ratio results of Cmax and AUC after multiple dosing indicated that there was a slight accumulation of ivonescimab exposure at steady state (14). Both the initial and fifth doses demonstrated a dose-dependent rise in Cmax for the 0.3,

1, 3, 10, 20, and 30 mg/kg groups dosed biweekly (9). Over time, the clearance rate of ivonescimab declined, starting at 0.461 L/day and stabilizing at 0.334 L/day, marking a 26% reduction. The drug's elimination half-life was calculated at 9.85 days (10, 14). A population PK analysis indicated that factors such as body weight (ranging from 31 to 155 kg), age, and sex did not significantly influence the drug's pharmacokinetics. Additionally, total bilirubin levels within the normal range and elevated AST levels did not have a clinically meaningful impact on the drug's PK profile (14).

### Pharmacodynamics

The pharmacodynamic biomarkers evaluated included PD-1 receptor occupancy (RO) on peripheral CD3+ T cells and serum-free VEGF levels. Following repeated administrations of ivonescimab, high levels of RO were maintained across all dose groups (0.3, 1.0, 3.0, 10.0, 20.0, and 30.0 mg/kg every two weeks), with saturation (>80%) achieved at doses of 3.0 mg/kg and above. Serum-free VEGF concentrations dropped significantly by 80%–95% within 24 hours after the initial dose in all cohorts (9). More importantly, following the first ivonescimab dose, serum VEGF levels decreased relative to baseline in all cohorts except 10 mg/kg Q3W. A consistent pattern of postdose decline followed by gradual recovery was observed after each administration. These VEGF fluctuations showed no clear dose-response relationship. In contrast, the 10 mg/kg Q3W cohort exhibited increased VEGF levels from baseline after the initial dose (14).

## Therapeutic efficacy of Ivonescimab in the clinical study

### Advanced solid tumors

A multicenter, phase I, open-label dose escalation and expansion study (NCT04047290) (9) was conducted in advanced solid tumors that are resistant/refractory to standard therapies, patients received Ivonescimab (0.3 mg/kg to 30 mg/kg) administered IV every 2 weeks (Q2W) and using accelerated titration followed by 3 + 3 + 3 dose escalation design, and results demonstrated that the 47 participants who had at least one postbaseline assessment, the confirmed objective response rate (ORR) was 25.5% and disease control rate (DCR) was 63.8%. Furthermore, results showed that Ivonescimab achieved good antitumor activity and safety in patients with platinum-resistant/refractory epithelial ovarian cancer (PROC), with an ORR of 29.4% and a DCR of 76.5%. The median follow-up duration was 4.5 months, and the median duration of response (DOR) had not yet been reached. Among the four responders, one patient with endometrial cancer had no prior exposure to ICIs or bevacizumab, one patient with ovarian cancer and another with mesothelioma had previously received ICIs, and one patient with microsatellite stable colorectal cancer had undergone prior bevacizumab treatment. Notably, 68.4% of the patients had undergone three or more lines of prior therapy. PR was achieved in five patients, including three with high-grade serous pathology and two with clear cell pathology, resulting in an ORR of 26.3%. The 20 mg/kg dose group demonstrated a higher ORR compared to the 10 mg/kg group (30.0% vs. 14.3%). Additionally, four patients who had previously received bevacizumab experienced stable disease (SD) lasting over 12 months. Promising efficacy signals were also observed in patients with mismatch repair proficient colorectal cancer, non-small cell lung cancer (NSCLC), and both mismatch repair deficient and proficient endometrial cancer. These findings suggest that ivonescimab is well-tolerated at doses up to 20 mg/kg every two weeks (Q2W), with doses of 3 mg/kg Q2W or higher demonstrating significant antitumor activity, achieving an ORR of 23.5% in patients with advanced solid tumors resistant to or relapsed after standard therapies (9).

### Non-small cell lung cancer

A multi-center, open-label Phase Ib/II study (NCT04900363) (15) enrolled 96 patients diagnosed with stage IIIB/C or IV non-small cell lung cancer (NSCLC) to assess the safety and efficacy of Ivonescimab as a first- or second-line monotherapy in advanced NSCLC patients without prior immunotherapy. Participants were administered Ivonescimab intravenously at doses of 10 mg/kg every three weeks (Q3W), 20 mg/kg every two weeks (Q2W), 20 mg/kg Q3W, or 30 mg/kg Q3W. Among the cohort, 66 patients (68.8%) exhibited PD-L1 positivity (tumor proportion score, TPS ≥1%), and 81 patients (84.4%) were treatment-naïve. The study was structured into two phases: dose selection (Phase Ib) and randomized control (Phase II). Across all participants, the overall response rate (ORR) and disease control rate (DCR) were 39.8% and 86.1%, respectively. ORR stratified by TPS was 14.7% for TPS <1%, 51.4% for TPS ≥1%, and 57.1% for TPS ≥50%. In the subset of 67 PD-L1 positive patients receiving first-line Ivonescimab, ORR varied by dose: 33.3% at 10 mg/kg Q3W, 52.6% at 20 mg/kg Q2W, 60.0% at 20 mg/kg Q3W, and 75.0% at 30 mg/kg Q3W.

A phase II, open-label, multicenter clinical trial (NCT04736823) (16) was conducted to assess the safety and efficacy of AK112 combined with chemotherapy in patients with metastatic non-small cell lung cancer (NSCLC). Participants were divided into three cohorts based on their clinical characteristics. Cohort 1 included treatment-naïve patients with advanced NSCLC lacking EGFR or ALK gene alterations. These patients received Ivonescimab alongside pemetrexed (500 mg/m$^2$) for non-squamous (non-sq) NSCLC or paclitaxel (175 mg/m$^2$) for squamous (sq) NSCLC, combined with carboplatin (AUC 5 mg/mL/min) for four cycles. Maintenance therapy involved continued Ivonescimab with pemetrexed for non-sq NSCLC or Ivonescimab monotherapy for sq NSCLC. Cohort 2 comprised patients with EGFR-sensitive mutations who had progressed after EGFR-TKI therapy. They were treated with pemetrexed, Ivonescimab, and carboplatin for four cycles, followed by pemetrexed plus Ivonescimab as maintenance. Cohort 3 included patients with advanced NSCLC who had failed platinum-based chemotherapy and anti-PD-1/PD-L1 therapies, receiving Ivonescimab combined

with docetaxel (75 mg/m$^2$). Two Ivonescimab doses (10 or 20 mg/kg) were tested across cohorts, yielding confirmed ORRs of 53.5%, 68.4%, and 40.0% in cohorts 1, 2, and 3, respectively. Additionally, in cohort 1, the median progression-free survival (m-PFS) was not reached, with a 12-month PFS rate of 59.1%. For cohorts 2 and 3, the m-PFS was 8.5 months (95% CI, 5.5–not estimable [NE]) and 7.5 months (95% CI, 2.3–NE), respectively, with corresponding 12-month PFS rates of 35.5% and 44.5%. In summary, among 135 patients with advanced or metastatic NSCLC treated with Ivonescimab plus chemotherapy, 63 had small cell lung cancer (SCLC) and 72 had non-small cell lung cancer (non-SCLC). SCLC patients achieved an ORR of 75%, a median duration of response (DOR) of 15.4 months, a disease control rate (DCR) of 95%, and 9-month PFS and overall survival (OS) rates of 67% and 93%, respectively. Meanwhile, NSCLC patients demonstrated an ORR of 55%, a DCR of 100%, and 9-month PFS and OS rates of 61% and 81%, respectively, though the DOR was not reported.

A global, multicenter, randomized, double-blind Phase III trial (NCT05184712) (17, 18) was conducted to assess the efficacy of Ivonescimab combined with chemotherapy compared to chemotherapy alone in patients with EGFR-mutated, locally advanced, or metastatic NSCLC who had progressed following third-generation EGFR-TKI therapy. In this study, 322 participants were randomized in a 1:1 ratio to receive either Ivonescimab (20 mg/kg) plus pemetrexed (500 mg/m$^2$) and carboplatin or placebo plus chemotherapy every three weeks for four cycles. Among the participants, 86.3% in the Ivonescimab group and 85.1% in the placebo group had previously received third-generation EGFR-TKI therapy, while 21.7% had brain metastases. Stratification was based on prior third-generation EGFR-TKI treatment (received vs. not received) and the presence of brain metastases. Maintenance therapy involved Ivonescimab with pemetrexed or placebo with pemetrexed. After a median follow-up of 7.89 months, the Ivonescimab plus chemotherapy group showed a significant improvement in m-PFS compared to the chemotherapy-alone group (7.06 months vs. 4.80 months, HR 0.46). The m-PFS (95% CI) was 7.06m in the Ivonescimab arm versus 4.8m in the chemotherapy arm. The ORR was 50.6% in the Ivonescimab group (vs 35.4% in the placebo group). Subgroup analyses revealed that nearly all subgroups treated with Ivonescimab exhibited superior PFS compared to placebo. Specifically, patients who had progressed after third-generation EGFR-TKI therapy (HR 0.48, 95% CI 0.35–0.66), those with brain metastases (HR 0.40, 95% CI 0.22–0.73), and individuals with EGFR 19 deletion mutations (HR 0.48, 95% CI 0.35–0.66) or T790M mutations (HR 0.22, 95% CI 0.09–0.54) showed significant benefits. Additionally, the intracranial response rate (IRR) and complete response (CR) rate for the Ivonescimab-chemotherapy combination were 39% and 25%, respectively, while the IRR and CR rate for Ivonescimab monotherapy were both 14%. The median intracranial progression-free survival reached 19.3 months. Therefore, ivonescimab plus chemotherapy significantly improved PFS in the intention-to-treat population. However, the median overall survival data were not mature; at the data cutoff, 69 patients (21.4%) had died.

The randomized, multicenter Phase III clinical trial (NCT05499390) (19) of Ivonescimab vs pembrolizumab in the first-line treatment of PD-L1-positive locally advanced or metastatic NSCLC with PD-L1 positive was performed, and a total of 398 subjects were enrolled, among them PD-L1 tumor proportion score (TPS) 1-49% accounted for 57.8% and PD-L1 TPS ≥50% accounted for 42.2%, and interim analysis revealed that ivonescimab significantly prolonged m-PFS (11.1 vs. 5.8 months), results demonstrated that the primary clinical endpoint of PFS was achieved; The PFS benefit of ivonescimab was observed across patient subgroups, including those with PD-L1 low expression [PD-L1 TPS 1-49%], PD-L1 high expression (PD-L1 TPS > 50%), squamous and nonsquamous histologies, as well as other high-risk clinical features (12). Additionally, Ivonescimab demonstrated higher response rates than pembrolizumab, with ORR (50% vs 39%) and DCR (90% vs 71%); Patients with hepatic metastases demonstrated the most pronounced improvement in the Ivonescimab group (mPFS: 7.1 vs 2.7 months), indicating that adding VEGF inhibition to PD-1/PD-L1 blockade enhances efficacy in this subgroup, and median DoR was not reached in either group.

## Biliary tract cancer

At the 2024 American Society of Clinical Oncology Annual Meeting, a study evaluating the safety and preliminary antitumor activity of ivonescimab combined with chemotherapy in patients with locally advanced or metastatic biliary tract cancer (BTC) was presented, this ongoing, open-label, multicenter, phase 1b/2 study (NCT052114482) (20) was conducted in 22 patients with advanced biliary tract cancer (BTC) in China, including 12 patients with intrahepatic cholangiocarcinoma, one with extrahepatic cholangiocarcinoma and nine with gall bladder cancer, and aimed to evaluate the safety and efficacy of AK112 combined gemcitabine and cisplatin as first-line therapy. The results demonstrated that the ORR and DCR were 63.6% and 100%, respectively after a median duration of 13.8 months (31 January 2024), with eight patients having stable disease; Furthermore, the ORR of gallbladder cancer patients was 77.8%.In addition, the m-PFS and m-OS were 8.5m and 16.8m, respectively.

## Head and neck squamous cell carcinoma

During the ESMO Congress 2024 (Barcelona, Spain), preliminary safety and efficacy data from an investigator-initiated study led by Prof. Chen Xiaozhong were presented as a poster. The study evaluates the novel dual-immunotherapy combination of ivonescimab plus ligufalimab (anti-PD-L1) in the first-line setting for PD-L1-positive recurrent/metastatic head and neck squamous cell carcinoma (HNSCC), this open-label, multicenter, Phase 2 study (NCT04736823) (21) was conducted to assess the safety and efficacy of ivonescimab in combination with Ligufalimab as first-line treatment for PD-L1 positive recurrent/metastasis HNSCC, that

enrolled 30 eligible patients with recurrent/metastatic HNSCC who had PD-L1-positive disease (CPS ≥ 1), encompassing oropharyngeal, hypopharyngeal, laryngeal, and oral cancers. These patients received Ivonescimab (10 mg/kg Q3W) as monotherapy or in combination with Ligufalimab (45 mg/kg Q3W). All patients (100%) had an ECOG performance status score of 1, and among them, 56.7% had a CPS≥20. Efficacy data showed: that in the Ivonescimab monotherapy group, among 10 patients, the ORR was 30.0% the DCR was 80.0%, the median DoR was not reached, and the median PFS was 5.0 months. In addition, in the Ivonescimab combined with Ligufalimab group, among 20 patients, the ORR and DCR were 60.0% and 90.0% respectively, the median DoR was not reached, the median PFS was 7.1 months, and the 6-month PFS rate was 71.8%. Among the 11 patients with CPS ≥ 20 in the Ivosidenib + Lifralimab group, the ORR and DCR were 72.7% and 81.8% respectively, while among the 9 patients with 1 ≤ CPS < 20, the ORR and DCR were 44.4% and 100.0% respectively.

## Small cell lung cancer

A phase Ib trial (22) enrolled 35 treatment-naïve patients with extensive-stage small cell lung cancer (ES-SCLC) to evaluate the safety and efficacy of Ivonescimab in combination with etoposide and carboplatin. Participants received intravenous Ivonescimab every three weeks at doses of 3 mg/kg, 10 mg/kg, or 20 mg/kg, with the treatment regimen lasting up to four cycles, followed by Ivonescimab monotherapy as maintenance. After a median follow-up of 13.3 months, the ORR was 80%, with measurable tumor lesions shrinking by over 30%, and the DCR reached 91.4%. Among the three dose groups, the ORRs were 66.7% for 3 mg/kg, 90.9% for 10 mg/kg, and 76.2% for 20 mg/kg, with the 10 mg/kg dose demonstrating the highest efficacy.

## Triple-negative breast cancer

Building on the favorable outcomes observed with prior combinations of immunotherapy, anti-angiogenic therapy, and chemotherapy in breast cancer, Professor Wang Xiaojia presented preliminary findings from a study investigating first-line ivonescimab plus chemotherapy for advanced triple-negative breast cancer (TNBC) in an oral presentation at the 2024 European Society for Medical Oncology (ESMO) Congress, this the open-label, multicenter, Phase 2 study (NCT05227664) (23) was conducted to assess the safety and efficacy of ivonescimab in combination with chemotherapy as a first-line treatment for TNBC. A total of 30 patients were enrolled, with 80% of them having a PD-L1 combined positive score (CPS) <10. Additionally, 60% of the patients had previously undergone neoadjuvant or adjuvant therapy based on taxoid drugs. The patients were administered ivonescimab at a dosage of 20 mg/kg every two weeks in conjunction with paclitaxel 90 mg/m$^2$ or nab-paclitaxel 100 mg/m$^2$ on days 1, 8, and 15 of each four-week treatment cycle. The median follow-up duration was 7.2 months, during which 29 patients underwent at least one post-baseline tumor evaluation. The investigator-assessed ORR was 72.4% (21/29), with a DCR of 100% (29/29). Among the patients, those with a PD-L1 CPS of ≥10 had an ORR of 83.3% (5/6), while those with a PD-L1 CPS of <10 had an ORR of 69.6% (16/23). Median PFS and OS data are not yet available, but the 6-month PFS rate was 68.4%.

## Metastatic colorectal cancer

At the 2024 ESMO Congress, specifically within the Gastrointestinal Cancers track, Professor Deng Yanhong, serving as the leading investigator, presented an oral report on the latest findings from an open-label, multicenter, randomized phase II clinical trial (NCT05382442) (24) was conducted to evaluate the efficacy and safety of Ivonescimab in the treatment of metastatic colorectal cancer (mCRC), that untreated mCRC patients were randomly assigned in a 1:1 ratio to two treatment groups: Group A received FOLFOXIRI combined with Ivonescimab, while Group B received the same regimen as Group A with the addition of Ligufalimab. Both groups underwent a maximum of 8 cycles of treatment, followed by maintenance therapy with 5-fluorouracil plus Ivonescimab (Group B) or 5-fluorouracil alone (Group A), with or without Ligufalimab. A total of 40 mCRC patients were enrolled and randomly assigned to Group A (n=22) and Group B (n=18). Notably, 11 patients in both Group A and Group B had KRAS/BRAF mutations. Except for one patient with an unknown status, all other patients were microsatellite stable (MSS). The median follow-up duration was 9 months for Group A and 9.6 months for Group B, of Group A and Group B were 81.8% and 88.2%, respectively. More impressively, the DCR was 100% in both groups. Median PFS and OS data are not yet mature. However, the 9-month PFS rate was 81.4% (95% CI:52.1%-93.7%) for Group A and 86.2%(95% CI:55%-96.4%)for Group B.

# The safety and tolerability of ivonescimab

Ivonescimab demonstrated a higher incidence of treatment-related adverse events (TRAEs) versus pembrolizumab or control groups. TRAEs with ivonescimab were consistent with known toxicities of PD-L1 and VEGF inhibitors (19). The predominant TRAEs included proteinuria, elevated aspartate aminotransferase, hypercholesterolemia, hyperbilirubinemia, neutropenia, leukopenia, thrombocytopenia), anemia, and hypertension. The incidence of grade ≥3 TRAEs was higher with ivonescimab versus pembrolizumab. Serious AEs occurred more frequently in the ivonescimab group, with disease progression, thrombocytopenia, anemia, pneumonitis, COVID-19 infection, and hepatic dysfunction being the most prevalent. Treatment-emergent AEs leading to death were reported in 10.6% of ivonescimab-treated patients (all attributed to disease progression), versus 7.5% (12 cases) in the placebo group (11/12 due to disease progression). Consequently, SAEs were more common with ivonescimab than

with placebo or pembrolizumab. Immune-related adverse events (irAEs) occurred at higher rates with ivonescimab (24.2% vs 6.2% placebo). The most frequent grade ≥3 irAEs were rash, dermatitis, interstitial lung disease, pneumonitis, hepatic dysfunction, and hypothyroidism. VEGF inhibition-related adverse events predominated in the ivonescimab group, notably grade ≥3 hypertension (5% vs 1%) and proteinuria (3% vs 0%). Importantly, grade ≥3 hemorrhage rates were comparable (1% vs 1%). Notably, grade ≥3 irAEs and overall grade ≥3 AEs occurred at similar frequencies between ivonescimab and placebo. Safety profiles were consistent across histologic subtypes (squamous vs non-squamous NSCLC) and comparable to pembrolizumab in squamous NSCLC. Overall, ivonescimab's safety profile appears manageable, particularly given its substantial progression-free survival benefit (18, 19, 25).

## The advantages and disadvantages of PD-1(L1)/VEGF BsAbs

These PD-1(L1)/VEGF BsAbs aim to block two protumor signaling pathways, potentially producing synergistic anti-cancer effects or minimizing drug resistance, and represent a novel class of drugs that have made significant advancements in recent years. Hence, we summarize the structure characteristic and clinical maturity (Table 1), and the status and efficacy of several PD-1 (L1)/VEGF BsAbs entering clinical trials (Table 2, Supplementary Table 1). In addition, PD-1(L1)/VEGF BsAbs will provide several superiority over other anti-PD-1(L1) and anti-VEGF antibodies drug as follows: 1) Enhanced efficacy: Compared with ordinary monoclonal antibodies, bispecial antibodies can bind two specific epitopes or target proteins simultaneously, so they can play a special function to play a biological function that is difficult to achieve with monoclonal antibodies; 2) BsAbs containing Fc region: Have good solubility and stability, a long half-life, play antibody-dependent cell-mediated cytotoxicity effect to further enhance the tumor killing effect; 3) Prevention of drug resistance: simultaneously blocking two different signal pathways with unrelated or overlapping pathogenic functions; 4) Low toxicity: Compared with monoclonal antibody, BsAbs have stronger specificity and targeting, reducing off-target toxicity; 5) Low cost: Compared with the combination therapy of monoclonal antibody, it can also effectively reduce the cost of treatment (5, 16). As is well known, the inhibition of the VEGF–VEGFR axis has numerous favorable effects that can, in principle, enhance the efficacy of immunotherapy. However, PD-(L)1/VEGF BsAbs exhibit certain limitations, as exemplified by the clinically approved agent ivonescimab: 1) in subgroup analyses, patients with TPS ≥50% consistently demonstrated superior response rates compared to those with TPS 1%-49%, this observation aligns with the dual targeting mechanism of ivonescimab against both PD-1 and VEGF. Notably, squamous NSCLC patients exhibited a 13% higher ORR than non-squamous counterparts, whereas non-squamous NSCLC showed prolonged m-PFS. These discordant outcomes underscore the complexity of treatment responses across histological subtypes and warrant further investigation to elucidate underlying mechanisms; 2) The exclusive reporting of PFS without corresponding OS data precludes comprehensive assessment of therapeutic benefit, particularly given the documented dissociation between PFS and OS in the immunotherapy era. 3) the study's confinement to a single Chinese cohort raises concerns regarding generalizability across ethnic and geographic populations. Consequently, while demonstrating therapeutic promise, ivonescimab's global applicability requires validation through multinational trials—specifically examining OS in diverse populations.

Ivonescimab reactivates dysfunctional T cells through PD-1 blockade, concurrently remodels the tumor vasculature, and reverses immunosuppressive signaling via VEGF inhibition. However, it is worth noting that althoughIvonescimab can induce long-lasting responses in some refractory tumors, they paradoxically trigger elevated PD-L1 expression within the tumors and increase the population of regulatory T cells (Tregs) in the TME, thereby attenuating their antitumor efficacy (28). Furthermore, it is widely accepted that non-exhausted T cells within the tumor microenvironment constitute the key effector

TABLE 1 Comparison of Ivonescimab with other anti-VEGF/PD-1(L1) BsAb in molecular architecture and clinical maturity.

| Comparison dimension | Ivonescimab | PM8002 | IMM2510 | HB0025 |
|---|---|---|---|---|
| molecular architecture | Heterotetramer structure, anti-PD-1 ScFv is attached to the C-terminal of each anti-VEGF antibody heavy chain | anti-PD-L1 single-domain antibody was fused onto an anti-VEGF-A IgG1 antibody containing the FC-silencing mutation | mAb-Trap structure, It is composed by the fusion of anti-PD-L1 antibodies with VEGFR | second Ig-like domain outside the VEGFR1 membrane is connected to the N-terminal of the IGG1-type anti-PD-L1 mAb heavy chain through a flexible linker |
| FC region | mutation sites (L234A and L235A) were introduced into the fc region. leads to a silent mutation in Fc, block the binding of FcγRI/IIIA | FcγRIIB binding enhanced mutation | No Fc silencing mutation<br>exerts the ADCC effect by binding to the Fcγ receptor | No Fc silencing mutation |
| Clinical Maturity | NSCLC Indication Approval (China)<br>Three Phase III projects completed<br>Data of over 2,000 patients | Phase Ib/II is in progress<br>Solid tumor<br>• Public data: n=87 | Phase I dose expansion<br>• Advanced solid tumor<br>Only disclose the security of n=24 | Phase I completed<br>Solid tumor<br>• Publish n=24 |

TABLE 2 Comparison of Ivonescimab with other anti-VEGF/PD-1(L1) BsAb in clinical trials.

| Drug | Phase | Tumor type | subclassification | ORR (%) | DCR (%) | m-PFS (months) | m-DOR (months) | AEs (%) | ≥grade 3 AEs (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Ivonescimab | Ia | Solid tumor | | 25.5 | 63.8 | – | – | 74.5 | 27.5 | (9) |
| | Ia | PROC | | 26.3 | 76.5 | 13.5 | NR | 88.5 | 15.8 | (9) |
| | II | NSCLC | | 39.8 | 86.1 | – | | 91.6 | 51.8 | (16) |
| | Ib | NSCLC | TPS<1% | 14.7 | – | – | – | – | | (15) |
| | | | 1%≤TPS<50% | 51.4 | – | – | – | – | | |
| | | | TPS>50% | 57.1 | – | – | – | – | | |
| | II | NSCLC | sq-NSCLC | 75 | 95 | – | 15.4 | – | – | (16) |
| | | | Non-sq-NSCLC | 55 | 100 | – | NR | | | |
| | III | NSCLC EGFR Variant | | 50.6 | | 7.1 | – | – | 61.5 | (18) |
| Ivonescimab | II | BTC | | 63.6 | 100 | 8.5 | NR | – | 86.4 | (16) |
| Ivonescimab | II | SCLC | | 80 | 91.4 | – | – | – | | |
| Ivonescimab | II | TNBC | | 72.4 | 100 | 9.3 | 7.49 | – | - | |
| Ivonescimab | II | HNSCC | Ivonescimab | 30 | 80 | 5 | – | – | – | |
| | | | Ivonescimab +ligufalinab | 60 | 90 | 7 | – | – | – | |
| Ivonescimab | II | mCRC | Ivonescimab +FOLFOXIRI | 81.8 | 100 | – | | | | |
| | | | Ivonescimab +FOLFOXIRI+ ligufalinab | 88.2 | 100 | – | | | | |
| HB0025 | I | Solid tumor | | 9.1 | 50 | – | – | 83.3 | 20 | (26) |
| IMM2510 | I | Solid tumor | | 75.8 | – | – | – | 97 | 27.4 | (27) |
| PM8002 | I | Solid tumor | dose escalation | 19.7 | 72.7 | – | – | 95.4 | 35.6 | (28) |
| | | | dose expansion | 20 | 70 | – | – | | | |
| | Ib/II | NSCLC | | 79 | 77 | – | – | 85.2 | 18 | (29) |
| | II | SCLC | | 72.7 | 81.8 | 5.5 | – | 93.8 | 62.5 | (30) |
| | Ib/II | TNBC | | 78.6 | 95.2 | 9.2 | 7.2 | 95.2 | 38.1 | (31) |

ORR, the objective response rate; DCR, disease control rate; PR, partial responses; SD, stable disease; PD, progressive disease; SCLC, small cell lung cancer; sq-NSCLC, squamous non-small cell lung cancer; Nonsq-NSCLC, nonsquamous non-small cell lung cancer; DOR, duration of response; TRAEs, treatment-related adverse events; ICI, immune checkpoint inhibitor; NK, ADCC/ADCP; TNBC, triple-negative breast cancer; CC, cervical cancer; PROC, Platinum-resistant ovarian cancer; NR, not reached. Cohort 1, patients receiving first-line Ivonescimab in combination with platinum-based chemotherapy; Cohort 2, patients with EGFR-sensitive mutations who failed previous targeted therapy; Cohort 3, patients who failed previous systemic platinum-based chemotherapy and PD-1/L1 inhibitor treatments; HNSCC, Head neck squamous cell Carcinoma; mCRC, metastatic colorectal cancer.

mechanism of immunotherapy, while T cell exhaustion and senescence represent critical pathways enabling cancer cells to evade immune surveillance, sustain an immunosuppressive microenvironment, and drive resistance to cellular immunotherapies (28).

The core mechanism of action of ivonescimab involves blockade of key inhibitory signaling pathways, primarily the PD-1/PD-L1 axis, and this blockade aims to reinvigorate exhausted T cells and restore their anti-tumor functionality. However, prolonged exposure to the antibody may paradoxically promote the progression of T cells into a state of deeper exhaustion (29). For instance, under conditions of persistent antigen exposure, a sustained immunosuppressive TME, and adaptive tumor evolution, T cells may compensate by upregulating alternative

inhibitory receptors. This compensatory upregulation can maintain and drive T cells towards a terminally exhausted phenotype, while simultaneously facilitating the development of novel immune evasion mechanisms, ultimately leading to acquired resistance (30). Furthermore, following successful T cell activation by ivonescimab, the continued presence of unresolved tumor antigens subjects these T cells to chronic activation. Such sustained, high-intensity stimulation is intrinsically a critical driver of T cell exhaustion. Additionally, profoundly exhausted T cells undergo stable epigenetic reprogramming, which functionally locks them into an irreversible dysfunctional state (32).

In addition, from our perspective, some potential issues also deserve attention if the antiangiogenic function of drugs reduces tumor vascularity and thus overall tumor blood vessel perfusion and flow, one might expect at least two unfavorable functional outcomes that, alone or together, could impair the immunotherapeutic properties of the drug: (1) increased levels of tumor hypoxia — a known mediator of resistance to immunotherapy; and (2) impaired intratumoral antibody biodistribution, thus reducing immunoefficacy (5, 33–35). Therefore, the final clinical benefits of anti-VEGF/PD-1(L1) BsAb will depend on the net balance between the opposing effects of VEGF signaling and its inhibition on the antitumor immune response in a given treatment situation.

## Ongoing clinical trials

Ivonescimab has been approved in combination with pemigatinib and carboplatin for the treatment of patients with advanced or metastatic NSCLC who have progressed after receiving treatment with an EGFR tyrosine kinase inhibitor and have tested positive for EGFR gene mutation. In addition, there are 5 Phase III clinical studies underway (Table 3), including 2 international multi-center Phase III clinical studies conducted overseas, and 4 Phase III clinical studies using PD-1 monoclonal antibodies as positive control drugs.

## Further perspective and challenges

The development of BsAbs, including anti-VEGF/PD-1(L1) and similar constructs, represents a focused effort to improve anti-tumor immune responses, counteract immune evasion mechanisms, and address the limitations of single-agent therapies. Although early clinical successes highlight the potential of BsAbs, their effective incorporation into cancer treatment regimens demands careful planning.

Key areas for future investigation include enhancing the precision, effectiveness, and safety profiles of BsAbs. This involves optimize the design of BsAb with lower immunogenicity, improved tumor-targeting capabilities, and optimized pharmacokinetic properties, as well as ensuring selective accumulation in the TME to reduce systemic toxicity. Additionally, identifying predictive biomarkers for BsAb therapy is critical. Comprehensive analyses of tumor genomics, proteomics, and immunology may uncover biomarkers that guide personalized treatment and reduce adverse effects; furthermore, comprehensive multi-omics analyses are critical to identify predictive biomarkers for patient stratification, mitigating off-target effects and maximizing therapeutic efficacy. Combining BsAbs with other therapies, such as chemotherapy, targeted agents, radiation, and additional immunotherapies, also

TABLE 3 Ongoing clinical trials of Ivonescimab.

| Clinical registration number | Disease | Regimen | Phase | Single/ multicenter | Number of participants |
|---|---|---|---|---|---|
| NCT06396065 | nsq-NSCLC | ivonescimab+ Pemetrexed + carboplatin | III | International multicenter | 420 |
| NCT05899608 | sq-NSCLC | ivonescimab vs Keytruda + chemotherapy | III | International multicenter | 400 |
| NCT05840016 | sq-NSCLC | ivonescimab vs tislelizumab + chemotherapy | III | Single center | 396 |
| NCT05499390 | NSCLC | ivonescimab vs Keytruda | III | Single center | 388 |
| NCT05184712 | nsq-NSCLC | ivonescimab | III | International multicenter | 470 |
| NCT04870177 | gynae-ecological tumours | ivonescimab | II | multicenter | 270 |
| NCT05229497 | malignant tumors | ivonescimab +ligufalimab | Ib/II | multicenter | 114 |
| NCT05432492 | HC | ivonescimab | II | multicenter | – |
| NCT05247684 | NSCLC | ivonescimab | II | multicenter | – |

shows significant potential. Evidence-based combination strategies can amplify anti-tumor effects and expand the therapeutic scope of BsAbs. However, rational combination strategies with other therapies require mechanistic evidence to amplify anti-tumor responses without compounding toxicity; cost-effectiveness analyses must further guide clinical adoption. Crucially, the TME plays a pivotal role in the efficacy of BsAbs in solid tumors. Thus, advancing BsAbs that modulate suppressive TME components, enhance antigen presentation, and promote T cell infiltration and activation is essential. Given the TME's pivotal role, next-generation BsAbs should actively remodel immunosuppressive elements (e.g., Tregs, MDSCs), enhance antigen presentation, and promote T-cell infiltration/activation. Spatial heterogeneity of the TME warrants dedicated investigation. Finally, further research into the PK and PD of BsAbs, (particularly organ-specific toxicities like hepatotoxicity or cytokine release syndrome), and real-world evidence generation are essential to refine dosing and safety monitoring, along with elucidating their mechanisms of action and potential organ toxicity, is necessary to improve targeting accuracy and minimize off-target effects.

## Conclusion

The China National Medical Products Administration approved ivonescimab, in combination with pemetrexed and carboplatin for the treatment of patients with EGFR-mutated locally advanced or metastatic non-squamous NSCLC who have progressed after tyrosine kinase inhibitor therapy on May 23, 2024. This therapy targets two distinct antigen-binding sites involved in immune regulation, representing a pivotal evolution in the treatment landscape for advanced or metastatic NSCLC patients who have progressed after receiving treatment with an epidermal growth factor receptor tyrosine kinase inhibitor and have EGFR gene mutation positivity. Looking ahead, it is highly likely that more anti-VEGF/PD-1(PD-L1) BsAbs will also demonstrate potential for approval in treating other cancers, such as TNBC and SCLC.

## Author contributions

FS: Writing – original draft, Conceptualization. LY: Data curation, Formal analysis, Writing – review & editing. YG: Writing – review & editing, Formal analysis, Data curation. YH: Writing – review & editing, Project administration. QL: Writing – review & editing. FC: Writing – review & editing. XC: Writing – review & editing. JZ: Writing – review & editing. LW: Writing – review & editing.

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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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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1599181/full#supplementary-material

SUPPLEMENTARY TABLE 1
Comparison of Ivonescimab with other anti-VEGF/PD-1(L1) BsAb in clinical trials.

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