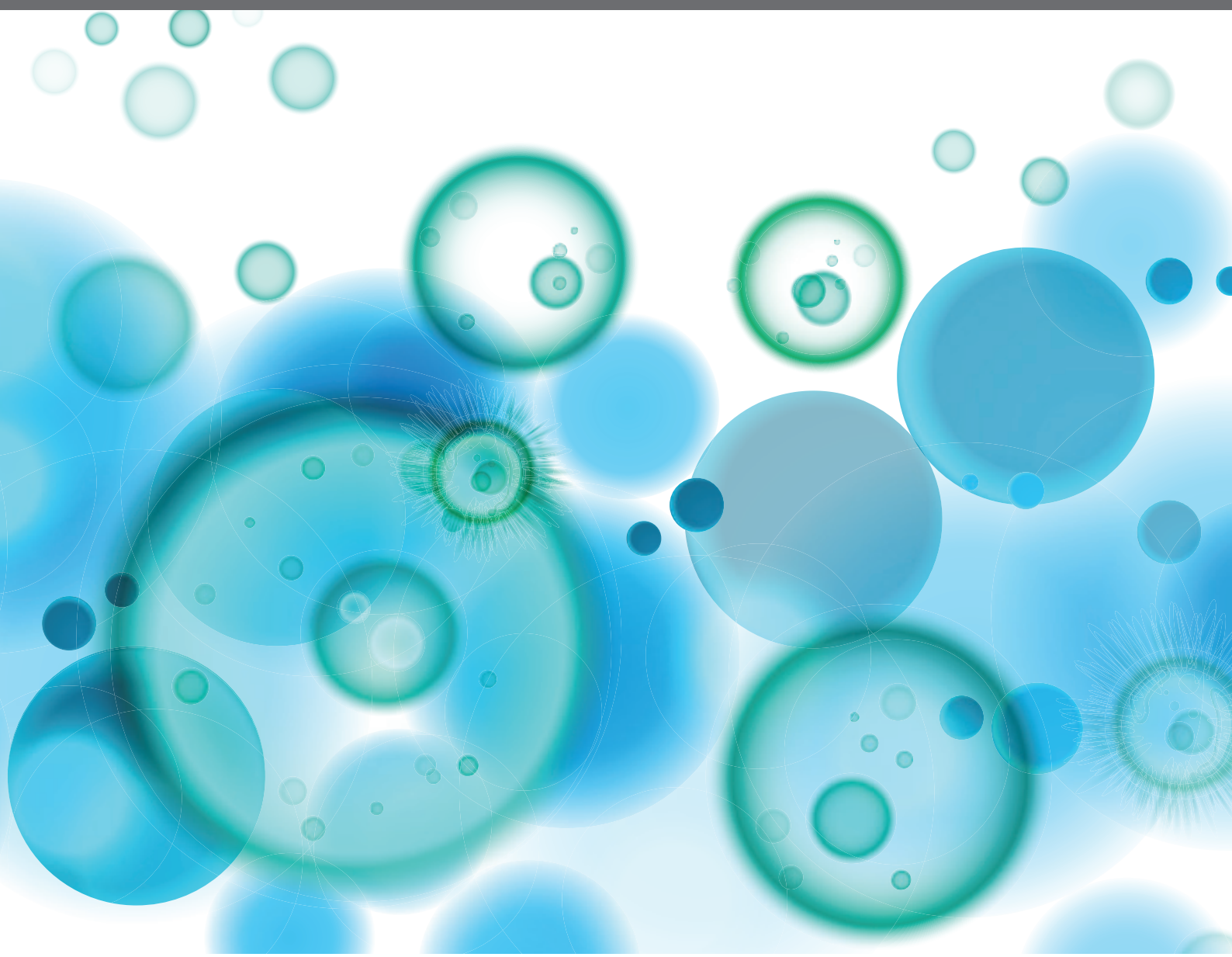
MECHANISMS OF LYMPHOCYTE EXCLUSION IN THE TUMOR MICROENVIRONMENT

EDITED BY: Sarah E. Church, Carlo Bifulco, Rieneke Van De Ven and Jason J. Luke

PUBLISHED IN: Frontiers in Immunology and Frontiers in Oncology



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ISSN 1664-8714
ISBN 978-2-88976-206-4
DOI 10.3389/978-2-88976-206-4

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MECHANISMS OF LYMPHOCYTE EXCLUSION IN THE TUMOR MICROENVIRONMENT

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Citation: Church, S. E., Bifulco, C., Van De Ven, R., Luke, J. J., eds. (2022). Mechanisms of Lymphocyte Exclusion in the Tumor Microenvironment. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-206-4

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frontiers | Frontiers in Immunology

EDITORIAL
published: 29 April 2022
doi: 10.3389/fimmu.2022.908612

Editorial: Mechanisms of Lymphocyte Exclusion in the Tumor Microenvironment

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Keywords: tumor micreoenvironment (TME), lymphocyte exclusion, anti-tumor immune response, immune cell infiltration, immune suppression

Editorial on the Research Topic

Mechanisms of Lymphocyte Exclusion in the Tumor Microenvironment

OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *30 March 2022*
Accepted: *12 April 2022*
Published: *29 April 2022*

Citation:
van de Ven R, Bifulco CB, Luke JJ and Church SE (2022) Editorial: Mechanisms of Lymphocyte Exclusion in the Tumor Microenvironment. Front. Immunol. 13:908612. doi: 10.3389/fimmu.2022.908612

Solid tumors which have an abundance of lymphocytes penetrating the tumor fields, so-called immune inflamed tumors, often have a prognostic benefit over non-immune inflamed tumors, which generally respond less to treatment. Non-inflamed tumors can be classified as immune deserts, with no lymphocytes present within the tumor microenvironment (TME), or immune excluded, where lymphocytes are unable to penetrate the tumor core from the stromal areas. This Research Topic includes 14 articles that explore the mechanisms that drive lymphocyte exclusion and provide insight how to attract the lymphocytes into the tumor nests.

Zhang et al. elaborately review the chemokines and cytokines that are needed to recruit, expand, differentiate and nurture T cells within the TME and those that promote T cell absence, exclusion, exhaustion and apoptosis. Pietrobon et al. provide an informative review on the conventional and next generation imaging technologies available to interrogate the TME as a means to better understand the mechanical, functional and dynamic barriers that underlie immune exclusion. In an original research article, Campisi et al. studied one of these barriers, using an *in vitro* microfluidics system to mimic a vascularized tumor environment. They show that tumor-derived 2'3'-cGAMP was able to, through STING, activate endothelial cells. This promoted production of lymphocyte-recruiting chemokines and adhesion molecules on the endothelium required for lymphocyte extravasation and recruitment. Several cancers, among which KRAS-LKB1 mutant lung cancers, are irresponsive to immune checkpoint treatment with anti-programmed death 1 (PD-1) agents. The authors show that loss of LKB1 activity impaired tumor 2'3' cGAMP production, blocking T cell recruitment *via* the vasculature.

Not only the vasculature can prevent T cells from entering the TME, growing scientific evidence is pointing towards an obstructive role of suppressive myeloid cell populations within the TME. Asiry et al. extensively review the role of M2-type tumor-associated macrophages (TAMs) in regulating T cell trafficking into the tumor area and supporting the formation of so-called Tumor MicroEnvironment of Metastasis (TMEM) doorways, which aid in cancer cell dissemination and

metastatic spread. These TMEM are characterized by the presence of tumor cells highly expressing the actin-regulatory protein Mammalian enabled (MENA), perivascular macrophages expressing the M2-linked tyrosine kinase receptor TIE2, and endothelial cells. *Via* a process that requires the interaction of a tumor cell with an M2-polarized TAM, the tumor cells acquire invasive features and are "streamed" towards the TMEM doorways, where they can metastasize. This concept is referred to as the "*dissemination trajectory*". While T-cell directed immunotherapies may potentiate the attack of the bulk of the proliferating tumor, there is a possibility that cancer cells within a dissemination trajectory are shielded from T-cell mediated killing. Sticking with myeloid cells, Mehta et al., elegantly review macrophage biology and their role in immune suppression in breast cancer. Breast cancer cells secrete multiple factors that recruit monocytes and promote the skewing towards M2-like TAMs. Also in breast cancer, specific TIE2+ TAMs have been linked to promotion of angiogenesis and inhibition of T cell specific immune responses. In their review Mehta et al. discuss the current toolbox at hand, and those required in the future, to effectively target TAMs to promote T cell infiltration and functionality in breast cancer. A comprehensive review by the same group (Goldberg et al.) discusses the TME composition, novel therapeutic targets and potential combination therapies, focused at hormone receptor positive (HR+) breast cancer. Compared to HER2+ breast cancer and triple negative breast cancer (TNBC), HR+ tumors appear to have a lower level of TILs infiltrating into the tumor. These HR+ tumors are also characterized by high myeloid cell contents and low tumor HLA-I and PD-L1 expression and generally respond poorly to immune checkpoint inhibitor therapies. An interesting combination treatment, which was shown effective in preclinical *in vivo* models, and showed promising results in small cohorts of HR+ breast cancer patients in clinical studies, is the combination of a CDK4/6 inhibitor with PD-(L)1 inhibition.

Immune checkpoint therapy targeting PD-(L)1 and/or CTLA-4 barely induces responses in patients with advanced stage pancreatic ductal adeno carcinoma (PDAC). In a mini review, Vonderheide and Bear address that the chemokines secreted by PDAC cells are often driven by tumor-cell intrinsic factors such as KRAS mutations, and promote a myeloid-rich TME. In patients, targeting suppressive myeloid cells has not yet been very successful, and the authors discuss whether targeting tumor-intrinsic oncogenic pathways that drive chemokine production might be a more effective strategy to take forward. In their original research article, Raphael et al. identified PD-1 and TIGIT as the main immune checkpoint molecules related to poor survival in glioblastoma (GBM). Using a syngeneic GBM mouse model, they show that combination treatment of anti-TIGIT and anti-PD-1 inhibitors reduced tumor burden and improved survival. This coincided with increased CD8+ and CD4+ TIL frequencies in the tumor. Myeloid-derived suppressor cells (MDSCs) in GBM were found to express high levels of the ligands activating PD-1 and TIGIT. Combined blockade of TIGIT and PD-1 reduced polymorphonuclear (PMN)-MDSCs and increased the CD8 TIL/MDSC ratio in GBM tumors. Obviously myeloid cells are not the only type of cells within the TME that prevent the infiltration and functioning of effector T cells. In a detailed mini review, Scott et al. discuss the suppressive barriers that T regulatory cells (Treg) create at multiple levels at different sites: within the tumor by secreting suppressive cytokines and expressing multiple checkpoint molecules, altering the stromal compartment preventing effector T cells from reaching the tumor nests, deregulation of vasculature and obstructing dendritic cell (DC) activation or inducing DC apoptosis in tumor-draining lymph nodes (tdLNs), thereby preventing T cell priming and activation.

Liver cancer, including hepatocellular carcinoma (HCC), has seen unprecedented responses (30%) in the advanced disease setting since the introduction of immune checkpoint inhibitors. Lindblad et al. review the tumor-intrinsic mechanisms that may underlie immune cell exclusion, and unresponsiveness to checkpoint inhibition in the remaining 70% of non-responding patients. An important player in HCC, which has also been shown to regulate T cell exclusion in other cancer types, appears activation of the wnt/β-catenin (CTNNB1) pathway, which results in a paucity of cross-presenting DCs in the TME, preventing efficient downstream priming, activation and recruitment of effector T cell subsets. In a mini review, van Pul et al. emphasize the importance of DCs in shaping an effective anti-tumor T cell response and promoting T cell infiltration into tumors, focusing on the importance of tdLNs in generating the right T cells for tumor infiltration. Recent studies have elegantly shown that the effect of anti-PD-(L)1 immunotherapies relies on blockade of the PD-1/PD-L1 axis in the tdLNs in addition to the primary tumor site. This improves the priming of stem-cell like, progenitor exhausted T cells by LN-resident DCs, which can home to and infiltrate the TME and kill tumor cells upon anti-PD-1 therapy. In a comprehensive review, Blair et al. showcase the current literature on the relevance of antigen-specific interactions, T cell (re)circulation and T cell retention within the TME. Production of T-cell attracting chemokines by stromal cells, rather than by tumor cells, may contribute to a T cell excluded TME and increased recirculation of antigen-specific T cells, that may never reach the tumor cell nests to recognize and kill the tumor cells. While many, non-tumor specific T cells may be present within an immune inflamed TME, the ability of a TME to retain the antigen-specific T cells in close proximity to tumor cells, rather than allowing them to leave the tumor *via* lymphatic- or blood vessels may ultimately determine the effectiveness of an anti-tumor T cell response, as well as therapies that aim at reinvigorating such a response.

Since patients with T-cell excluded TMEs in general respond less well to the current immunotherapy strategies, these patient may benefit from combination treatments rather than monotherapy. In a mini review, Kacew and Sweis discuss the rationale for combining immune checkpoint inhibitors with agents inhibiting the fibroblast growth factor receptor-3 (FGFR3) in patients with urothelial bladder cancer. In patients irresponsive to checkpoint inhibitors, due to a non-immune

inflamed TME, or patients with acquired resistance upon initial treatment, inhibition of FGFR3 may overcome T cell exclusion. An ideal way to stratify patients for immunotherapy would be by using a blood-based assay, rather than requiring a tumor biopsy to assess protein or gene expression. In an original research article, Younis et al. show for head and neck squamous cell carcinoma, that patients with high levels of the soluble glycoprotein semaphorin 4D (SEMA4D) in plasma displayed an immune excluded or desert TME type whereas patients with low soluble SEMA4D levels often displayed an immune inflamed phenotype, linked with high interferon immune signatures. Whether this can be used as a predictive biomarker of response to checkpoint inhibitors, requires further assessment.

We hope the readers of this Research Topic, focusing on mechanisms of lymphocyte exclusion from the tumor microenvironment, will appreciate the comprehensive overview of the different barriers T cells encounter in order to reach the tumor nests. With this overview, we would like to encourage the field to explore the suggestions provided by the contributing authors, working towards more means to overcome immune exclusion.

AUTHOR CONTRIBUTIONS

SC initiated the Research Topic. All authors edited the Research topic. RV wrote the editorial. All authors contributed to the editorial article and approved the submitted version.

Conflict of Interest: RV receives research support from Genmab BV. CBis in the Scientific Advisory Boards of: PrimeVax (stock), BioAI (stock) and Lunaphore (no stock). JL discloses the following: member within Data and Science Monitoring Boards: Abbvie, Immutep, Evaxion; Scientific Advisory Board: (no stock) 7 Hills, Bright Peak, Exo, Fstar, Inzen, RefleXion, Xilio (stock), Actym, Alphamab Oncology, Arch Oncology, Kanaph, Mavu, NeoTx, Onc.AI, OncoNano, Pyxis, STipe, Tempest; Consultancy with compensation: Abbvie, Alnylam, Bayer, Bristol-Myers Squibb, Castle, Checkmate, Codiak, Crown, Day One, Duke St, EMD Serono, Endeavor, Flame, Genentech, Gilead, HotSpot, Kadmon, Janssen, Ikena, Immunocore, Incyte, Macrogenics, Merck, Mersana, Nektar, Novartis, Partner, Pfizer, Regeneron, Servier, STINGthera, Synlogic, Synthekine. Research Support: (all to institution for clinical trials unless noted) AbbVie, Astellas, Astrazeneca, Bristol-Myers Squibb (IIT & industry), Corvus, Day One, EMD Serono, Fstar, Genmab, Ikena, Immatics, Incyte, Kadmon, KAHR, Macrogenics, Merck, Moderna, Nektar, Next Cure, Numab, Palleon, Pfizer (IIT & industry) Replimmune, Rubius, Servier (IIT), Scholar Rock, Synlogic, Takeda, Trishula, Tizona, Xencor; Patents: (both provisional) Serial #15/612,657 (Cancer Immunotherapy), PCT/US18/36052 (Microbiome Biomarkers for Anti-PD-1/PD-L1 Responsiveness: Diagnostic, Prognostic and Therapeutic Uses Thereof). SC is a stockholder and employee of NanoString Technologies, Inc.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 04 September 2020
doi: 10.3389/fimmu.2020.02090

Tumor-Derived cGAMP Regulates Activation of the Vasculature

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Intratumoral recruitment of immune cells following innate immune activation is critical for anti-tumor immunity and involves cytosolic dsDNA sensing by the cGAS/STING pathway. We have previously shown that KRAS-LKB1 (KL) mutant lung cancer, which is resistant to PD-1 blockade, exhibits silencing of STING, impaired tumor cell production of immune chemoattractants, and T cell exclusion. Since the vasculature is also a critical gatekeeper of immune cell infiltration into tumors, we developed a novel microfluidic model to study KL tumor-vascular interactions. Notably, dsDNA priming of LKB1-reconstituted tumor cells activates the microvasculature, even when tumor cell STING is deleted. cGAS-driven extracellular export of 2′3′ cGAMP by cancer cells activates STING signaling in endothelial cells and cooperates with type 1 interferon to increase vascular permeability and expression of E selectin, VCAM-1, and ICAM-1 and T cell adhesion to the endothelium. Thus, tumor cell cGAS-STING signaling not only produces T cell chemoattractants, but also primes tumor vasculature for immune cell escape.

Keywords: LKB1, 2′3′-cGAMP, STING, KRAS, T cell, endothelial cells, microfluidic culture

OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *15 June 2020*
Accepted: *31 July 2020*
Published: *04 September 2020*

Citation:
Campisi M, Sundararaman SK, Shelton SE, Knelson EH, Mahadevan NR, Yoshida R, Tani T, Ivanova E, Cañadas I, Osaki T, Lee SWL, Thai T, Han S, Piel BP, Gilhooley S, Paweletz CP, Chiono V, Kamm RD, Kitajima S and Barbie DA (2020) Tumor-Derived cGAMP Regulates Activation of the Vasculature. Front. Immunol. 11:2090. doi: 10.3389/fimmu.2020.02090

INTRODUCTION

Immune recognition of tumor cells in the tumor microenvironment (TME) requires effector T cells and other immune cells to extravasate from the vasculature and migrate through the extracellular matrix (ECM) to recognize tumor antigens. Indeed, resistance to PD-1 immune checkpoint blockade (ICB) has been linked to an "immune cell excluded" phenotype in many tumor

types (1–3). For example, KRAS mutant non-small cell lung cancers that inactivate the STK11/LKB1 tumor suppressor are strongly resistant to anti-PD-(L)1 therapy and exhibit T cell exclusion (4, 5).

Stimulation of Interferon Genes (STING), an ER-resident protein encoded by *TMEM173*, is an important mediator of the innate immune response to pathogens and in cancer (5–10). Cyclic GMP-AMP synthase (cGAS) recognizes double-stranded DNA (dsDNA) in the cytosol and binds it to generate 2′3′-cGAMP, a cyclic dinucleotide and soluble second messenger that binds STING, which causes activation of the kinase TBK1 via phosphorylation and its downstream substrate, the transcription factor IRF3 (8). Thus, phosphorylated TBK1 (pTBK1) and production of specific cytokines downstream of IRF3, such as CXCL10, can be measured as a function of STING activation, which plays important roles in tumor cells, antigen presenting cells, and potentially other cell types (10). Recently our group identified epigenetic silencing of STING in KRAS-LKB1 (KL) mutant cancer cells, due to an autophagic defect and consequent cytosolic accumulation of mitochondrial DNA, resulting in the inhibition of STING-TBK1-IRF3 mediated type I interferon signaling and impaired production of T cell chemoattractants such as CXCL10 (5). These findings were supported by *in vivo* quantitative IHC data from patient biopsies that demonstrated impaired intratumoral T-cell infiltration from KL tumors lacking STING expression, and instead, retention of T cells in the stroma (5). STING silencing has also been reported in other tumor types with high tumor mutational burden (TMB) such as melanoma, where loss of STING also mediates escape from recognition of tumor antigens (11).

Communication between cancer cells and the vasculature can modulate infiltration of immune cells and regulate the composition of the TME, though the role of cGAS-STING signaling in this process has not been characterized (12). Cancer cells are known to communicate with neighboring cells, such as astrocytes in the brain TME, which can activate STING via 2′3′-cGAMP in a paracrine manner and promote metastasis (7). Emerging work also reveals that tumor derived 2′3′-cGAMP can act as an immunotransmitter and directly influence anti-tumor immunity (8, 13, 14). Given the problem of immune cell exclusion in many tumor types there is an increasing need to understand how the subcomponents of the TME and especially the tumor vasculature regulates immune extravasation. Importantly, tumor vascular endothelial cells have been identified as a major source of type 1 interferon production in the TME following intratumoral injection of 2′3′-cGAMP-based STING agonists, which promote T-cell-mediated therapeutic antitumor immunity (15). These studies suggest that endogenous 2′3′-cGAMP could also influence the tumor vasculature and regulate its activation in a paracrine manner.

We have also previously reported the use of microfluidic devices to support 3-dimensional (3-D) culture of perfusable microvascular networks (MVNs), comprised of human umbilical vein endothelial cells (HUVECs) and human lung fibroblasts (hLFBs) in a supportive ECM-like gel (collagen or fibrin), which self-organize into vasculature after 5 days of co-culture (16, 17). The same microfluidic devices also enable 3-D culture of cancer cells in similar hydrogels using tumor cell aggregates (spheroids) previously formed in ultra-low attachment plates for 24 h (18–20). This system enables more detailed study of the biological interactions between KRAS mutant cancer cells and the tumor microvasculature. Thus, we developed a microfluidic model that could support culture and formation of vascularized KL lung cancer spheroids, with the specific goal of studying how tumor cell dsDNA sensing via cGAS-STING might modulate innate immune signaling in this more physiologically relevant milieu.

MATERIALS AND METHODS

Immunohistochemical (IHC) Staining and Data Analysis

Brain tumor and brain metastasis tissue microarrays (GL2082, GL861) were purchased from US Biomax, Inc and IHC was performed on the Leica Bond III automated staining platform. The antibody for phospho-TBK1 (Cell Signaling Technology #5483, clone D52C2) was run at 1:50 dilution using the Leica Biosystems Refine Detection Kit with EDTA antigen retrieval. The antibody for STING (Cell Signaling Technology #13647, clone D2P2F) was run at 1:50 dilution using the Leica Biosystems Refine Detection Kit with citrate antigen retrieval. Staining was visually scored in a binary manner (presence/absence) in endothelial cells identified using the hematoxylin counterstain marking a circumferential layer of nuclei surrounding red blood cell fragments. These results were confirmed by a board-certified anatomic pathologist (NRM), who also quantified infiltrating lymphocytes by morphology on hematoxylin-counterstained per high power field (HPF = 40× objective), averaged across confidently identified endothelial lumens in 1–4 HPF per specimen. Average tumor infiltrating lymphocytes per HPF was compared for pTBK1+ and pTBK1− blood vessels in each tumor specimen.

Cell Lines

H1355 were cultured in RPMI-1640 (Thermo Fisher Scientific, Cat.# 11875-119) supplemented with 10% FBS, 1× penicillin–streptomycin, and 2.5 (g/mL plasmocin prophylactic (InvivoGen, Cat.# ant-mpp). Cells were originally obtained from the Broad Institute and authenticated by short tandem repeat genotyping. Human umbilical vein endothelial cells, HUVECs (Lonza, C2519AS) were cultured in vascular medium (VascuLife® VEGF Endothelial Medium Complete Kit, #LL-0003). NHLF-human Lung Fibroblasts (Lonza, CC-2512) were cultured in fibroblast growth supportive medium (FibroLife® S2 Fibroblast Medium Complete Kit, # LL-0011). Culture medium was replaced every 2 days, and all experiments were performed before reaching 10 passages. Mycoplasma infection was regularly checked by PCR using the conditioned media derived from each cell line. The sequences of the primers used for checking mycoplasma infection are listed in **Supplementary Table S1**.

CRISPR/Cas9 System

Target sequences for CRISPR interference were designed using the single-guide RNA (sgRNA) designer[1]. A non-targeting sgRNA from the Gecko library v2 was used as a scramble sgRNA. sgRNA target sequences are listed in **Supplementary Table S1**.

Generation of Lentivirus

HEK293T cells (3×10^6) were plated onto a 60-mm dish and transected using X-tremeGENE HP DNA Transfection Reagent (Roche, Cat.# 06366236001) with 1 μg of lentivirus-based expression vectors together with 1 μg of pCMV-dR8.91 and 1 μg of pCMV-VSV-G. After 48-h incubation, the media containing lentivirus particles were collected, passed through a 0.45 μm filter, and concentrated using Lenti-X Concentrator (Clontech, Cat.# 631231). For selection of virally infected cells, 1 μg/mL of puromycin (pCRISPR-v2 sgRNAs) or 6 μg/mL of blasticidin (plx304-NanoLuc or plx304-hLKB1) was used 24 h after infection.

dsDNA Stimulation

Cells (2×10^5 to 5×10^5) were plated onto a 6-well plate and transfected using X-tremeGENE HP DNA Transfection Reagent (Roche, Cat.# 06366236001) with the indicated amount of poly (dA:dT) (*Invivo*- Gen, Cat.# tlrl-patn). Cells utilized for 3D culture in microfluidic devices were transfected for 24 h, then transferred into ultra-low attachment dish to form spheroids as described below.

Immunoblotting

Cells were lysed in RIPA buffer containing 1× protease inhibitors (Roche, Cat.# 11-836-145-001) and phosphatase inhibitors (50 mmol/L NaF and 100 mmol/L Na3VO4). Immunoblotting was performed as previously reported (5). Briefly, protein was isolated from cell lines and measured by BCA (Pierce Biotechnology). Protein extracts were subjected to polyacrylamide gel electrophoresis using the 4–12% NuPAGE gel system (Invitrogen), transferred to PVDF (Millipore) membranes, and immunoblotted using antibodies that specifically recognize STING (#13647, Cell Signaling Technology), cGAS (#15102, Cell Signaling Technology), LKB1 (#3047, Cell Signaling Technology), phospho-TBK1 (#5483, Cell Signaling Technology), TBK1 (#3013, Cell Signaling Technology), and β-actin (#3700, Cell Signaling Technology). Secondary antibodies were from LI-COR Biosciences: IRDye 680LT Goat anti-Mouse IgG (#926-68020) and IRDye 800CW Goat anti-Rabbit IgG (#926- 32211). LICOR blocking buffer (no. 927-40000) was used to dilute primary and secondary antibodies, with the exception of phosho-specific antibodies, which were diluted in HIKARI Signal Enhancer Solutions 1 and 2 (Nacalai United States, Inc., no. NU00101). Imaging of blots and quantitation of bands were performed using the LI-COR Odyssey system.

[1]https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design

Quantitative RT-PCR and PCR Profile Array

Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Cat.# 74106) according to manufacturer's instructions. RNA samples (1 μg) were reverse-transcribed using Super- Script III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, Cat.# 1683483). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Cat.# 4367659), and the Applied Biosystems 7300 Fast real-time PCR system and software. The relative expression was normalized with the expression of the housekeeping gene 36B4.The sequences of the primers used for qRT-PCR are listed in **Supplementary Table S1**. Values represent the average of four technical replicates from at least two independent experiments (biological replicates). The profile expression of 84 genes related to endothelial cell biology were performed using the RT (2) Profiler PCR Array for human endothelial cell biology (Cat. # PAHS-015ZC, Qiagen), reverse transcribed and quantitative real-time PCR was performed using RT (2) First Strand Kit (Cat.# 330404, Qiagen), QuantiTect Reverse Transcription Kit (Cat.# 205313, Quiagen), RT (2) SYBR Green ROX qPCR Mastermix (Cat.# 330523, Quiagen) and Applied Biosystems 7300 Fast real-time PCR system and software.

3-D Microfluidic Device Design and Fabrication

Tumor-vascular interactions were evaluated using a commercial 3-D cell culture chip (DAX-1, AIM Biotech, Singapore) as previously described (16–18, 20) (**Supplementary Figure S5A**), and custom microfluidic devices composed of poly-dimethylsiloxane (PDMS; Sylgard 184; Dow Corning, MI, United States). Custom microfluidic device design and fabrication was conducted by standard soft-lithography techniques. Briefly, elastomer and curing agents were mixed (10:1 vol ratio), degassed, and poured onto a silicon master and cured overnight at 60°C. Access ports for hydrogel injection and media channels were created with biopsy punches and then the devices were taped to remove dust and sterilized in an autoclave. PDMS devices were treated with oxygen plasma (Harrick Plasma), bonded to a glass coverslip (Fisher Scientific) and finally placed in oven until use. Micro-devices have a central gel channel 2200 μm wide and 150 μm high, flanked by two medium channel 1340 μm wide (**Supplementary Figure S5B**). Macro-devices have a central gel channel 3 mm wide and 0.5 mm high, flanked by two fluidic channels 3 mm wide (**Supplementary Figure S5C**). 3-D cell culture chips and micro-devices were used for biological cellular studies. Macro-devices were used for permeability measurements.

3-D Microfluidic Culture

Cancer cell spheroids were generated by seeding 5×10^5 cells in suspension in an ultra-low attachment dish (Corning, Cat.# 3471) for 24 h. Samples were pelleted and resuspended in type I rat tail collagen (Corning) at a concentration of 2.5 mg/mL following the addition of 10 × PBS with phenol red with pH adjusted

using NaOH. pH of 7.0 to 7.5 was confirmed using PANPEHA Whatman paper (Sigma-Aldrich). All pelleted spheroids were resuspended in 250 μL of collagen hydrogel. The spheroid-collagen suspension was then injected into the central gel region of the 3D microfluidic device. After injection, devices containing spheroid-collagen mixture were incubated for 40 min at 37°C in humidity chambers, then hydrated with culture media, and refreshed daily for 7 days.

Microvascular networks (MVN) were created by detaching HUVEC and hLFB cells from cell culture flasks and resuspending them in cold vascular medium (Vasculife, Lifeline #LL-0003) with 2 U/ml thrombin from bovine plasma (Millipore Sigma, #T7326). The two cell types were combined with cell densities of 12×10^6/ml HUVECs and 2×10^6/ml hLFB. Cell suspensions were mixed 1:1 volume ratio with 6 mg/ml fibrinogen (Millipore Sigma, #341573) and gently injected into microfluidic devices. After allowing several minutes of fibrin polymerization (15–30 min) in a 37°C incubator, warm vascular medium was added to the flanking media channels and refreshed each day of culture. MVN self-assembled over several days refreshing media daily. To generate the MVN + Spheroids samples, MVN were co-cultured with tumor spheroids and protocols were combined. To maintain the same conditions used in each individual protocol, cell densities of 24×10^6/ml HUVECs and 4×10^6/ml hLFB were mixed 1:1 volume ratio with 6 mg/ml fibrinogen. This cell-gel suspension was mixed 1:1 with collagen-spheroids mixture previously generated by resuspending spheroids in 125 μL of collagen hydrogel, resulting in the same final cell density as MVNs or Spheroids alone. To maintain consistency for cytokine analysis, all microfluidic devices conditions were cultured in vascular medium (Vasculife, Lifeline #LL-0003).

MVN alone in microfluidic device were treated with 1 μg/ml 2′3′-cGAMP, or 100 ng/ml IFN-β, or both in combination after 6 days of culture, after which conditioned medium was collected or device were imaged with confocal microscopy 24 h later.

3D Permeability Measurements and Quantification

To measure the vascular permeability of fluorescent dextran in 3D, MVN alone in microfluidic device were cultured for 7 days and treated with 1 μg/ml 2′3′-cGAMP or 100 ng/ml IFN-β. Firstly, medium was removed from both media channels of a macro-device. A solution of fluorescein isothiocyanate (FITC)-conjugated dextran (Sigma-Aldrich) (70 kDa, 0.1 mg/ml) was added to each media channel in succession, and the device was transferred to a confocal microscope (Olympus FV1000). Three regions of interest (ROI) were chosen randomly along the gel channel to ensure non-biased sampling of the microvasculature, and z-stacks approximately 50 μm high were acquired immediately after addition of the fluorescent tracer and 15 min later (512 × 512 pixels, 20× magnification). Microvascular permeability was quantified by considering the increase in fluorescence intensity of FITC-dextran within the extra-vascular gel region as previously described (21). Briefly, Vascular network permeability, P, was quantified by measuring the average fluorescence intensity of the vascular (I_v) and matrix (I_m) compartments at two different time points t_1 and t_2 ($t_2 - t_1 = \Delta t$):

$$P = \frac{V_m}{SA\ \Delta I} \frac{\Delta I_m}{\Delta t}$$

$\Delta I_m = I_{m,2} - I_{m,1}$ is the increase in mean fluorescence intensity, in the matrix of volume V_m between time points and $\Delta I = I_{v,1} - I_{m,1}$, is the difference in fluorescence intensity, therefore solute concentration, between the vasculature (with surface area SA) and matrix at the start of the measurement. Reconstruction and segmentation was performed with Fiji (22) using the 3D Trainable Weka Segmentation plugin for quantification of parameters such as surface area (SA) and volume of the vascular network and matrix. The fluorescent intensity values were computed using Fiji.

3D Perfusion and Adhesion Assay

To test adhesion of T cells to the vascular endothelium, Jurkat T cells were perfused through microvascular networks on day 7 after loading the microfluidic devices. Jurkat cells (Clone E6, ATCC TIB-152) were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin–streptomycin and dyed with CellTracker Green CMFDA Dye (Thermo Fisher Scientific) before experiments. Half of the networks were treated with 1 μg/ml of 2′3′-cGAMP in vascular medium on day 6, and incubated for 24 h. On day 7 devices were perfused with fresh medium and then incubated with sterile rhodamine Ulex Europaeus Agglutinin I (Vector Laboratories) for 20 min to label the endothelium and washed again with fresh vascular medium before introducing Jurkat T cells. The Jurkat cells were pelleted and suspended in vascular medium at 106 cells/ml. Each device received 40 μl of the cell suspension in one channel of the microfluidic device, and cells were allowed to flow through the vascular networks for 30 min before perfusing fresh medium to wash away unbound cells. Jurkat cells that remained bound to the vascular networks were imaged with confocal microscopy (Olympus FV1000) and counted in FIJI. The number of cells retained in untreated networks and those treated with 2′3′-cGAMP were compared using a 2-sided student's T-test.

Immunofluorescence and Confocal Imaging

Mature microvascular networks were rinsed with warm PBS followed by the addition of approximately 100 μl of 4% paraformaldehyde (Electron Microscopy Sciences, # 15700) to the media channels and left at room temperature. After 15 min of fixation, devices were rinsed twice with PBS, and blocking solution (4% bovine serum albumin, 0.5% goat serum) (Sigma-Aldrich) was added. Devices were incubated for 1 day at 4°C, washed with PBS, and stained with primary antibodies: ICAM-1 (Biolegend, 4453320),VCAM-1 (Abcam, ab134047), CD31 (Abcam, ab28364), conjugated Alexa Fluor 647 anti-human CD326 (EPCAM) (BioLegend, 324212), Acti-stain 555 phalloidin, F-actin (Cytoskeleton, PHDH1-A) and incubated at 4°C for another day. Devices were again

washed with PBS and secondary antibodies (Thermo Fisher Scientific, A-11070, A-11011, A-21052) DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, Invitrogen) or DyLight 649 labeled Ulex Europaeus Agglutinin I (Vector Laboratories) were added, followed by incubation at 4°C protected from light. Finally, samples were washed again with PBS and 3D images were acquired with a confocal microscope (Olympus FV1000) at 20×. Z-stacks were collapsed with maximum intensity projections for viewing (800 × 800 pixels) using FIJI (22).

Multiplexed Cytokine/Chemokine Profiling

Multiplexed assays were performed utilizing the bead-based immunoassay approach Bio-Plex Pro Human Cytokine 40-plex Assay (Cat.# 171AK99MR2) on a Bio-Plex 200 system (Bio-Rad Laboratories, Cat.# 171000201) and the Human Cytokine/Chemokine Magnetic Bead Panel (Cat.# HCYTMAG-60K-PX30) on a Luminex MAGPIX system (Merck Millipore). Conditioned media concentration levels (pg/mL) of each protein were derived from 5-parameter curve fitting models. Fold changes relative to the corresponding control were calculated and plotted as log2FC. Lower and upper limits of quantitation (LLOQ/ULOQ) were imputed from standard curves for cytokines above or below detection. The degree of cytokine/chemokine modulation (D) in the MVN + Spheroids co-culture samples was calculated from absolute concentration levels (pg/mL) of the values from isolated MVN culture were subtracted to the MVN + Spheroids co-culture and results normalized to spheroid-only results as represented by the following equation:

$$D = \frac{((\text{MVN} + \text{Spheroids}) - \text{MVN})}{Spheroids}$$

Where the resulted degree cytokine/chemokine modulation is simply additive (D = 1) versus supra-additive (D > 1) or antagonistic (D < 1).

ELISA

Human IFN-β (Thermo Fisher Scientific, Cat.# 414101), CXCL10 (R&D systems, Cat.# DIP100) and 2′3′-cGAMP (Cayman Chemical, Cat.#501700) were detected with ELISAs according to the manufacturer's instructions. Conditioned media from each cell line were collected after 24-, 48-, or 168-h culture. Values from 2-D cell culture represent the average of two replicates from at least two independent experiments. Values from 3-D cell culture devices represent the average of four replicates from at least three independent experiments (biological replicates).

Cell Sorting by CD31

Cells (1×10^6) resuspended in 100 μL PBS containing 3% FBS were stained by APC-conjugated anti–CD31 antibody (R&D Systems, Cat.# FAB3567A-025) for 30 min at room temperature, washed by PBS containing 3% FBS, and then analyzed by FACSCanto ll (BD Biosciences). PE/Cy7-conjugated mouse IgG2b (BioLegend, Cat.# 400325) was used as isotype control antibody. Flow sorting for CD31-positive cells was then confirmed with CD31 gene expression by RT-PCR.

Statistical Analysis

All data are plotted as mean ± SD. Sample size (n) is equal to 2 biological replicates or otherwise stated. Unpaired student's t-test was used for significance testing between two conditions. One-way ANOVA with pairwise comparisons by the Tukey *post hoc* test was used to determine whether three or more data-sets were statistically significant. Statistical tests were performed using PRISM7 (GraphPad software) and R (23). P values less than 0.05 were considered significant, $^{**}P < 0.01$, $^{*}0.01 < P < 0.05$.

RESULTS

LKB1 Reconstitution Promotes STING-Driven Cytokine/Chemokine Production in 3-D KL Spheroids

Since LKB1 modulates STING expression in KL cells, and the KL non-small-cell lung cancer (NSCLC) cell line NCI-H1355 (H1355) potently attracts T cells following LKB1 reconstitution in 3-D microfluidic culture (5), we utilized this system to study additional interactions between tumor cell dsDNA sensing and the vasculature. H1355 cells stably expressing a luciferase control (H1355-LUC) or reconstituted with LKB1 (H1355-LKB1) were cultured in ultra-low-attachment dishes for 24 h to allow self-aggregation into 3-D spheroids. Spheroids were cultured in a microfluidic device within a collagen/fibrin hydrogel with or without the self-assembling microvascular network (MVN) using our established HUVEC and hLFB co-culture method (16) (**Figures 1A,B**). HUVECs self-organize into stable and perfusable MVNs, sustained by hLFBs, which provide paracrine support for MVN formation, reduce the diameter of capillary structures, and produce extracellular matrix (ECM) for stability in long-term culture (16).

Consistent with the tumor suppressive nature of LKB1, H1355-LKB1 spheroids grown after 7 days in microfluidic culture without MVNs exhibited decreased proliferation as compared with H1355-LUC spheroids, but remained viable (**Figure 1C**). We first validated the direct role of LKB1 in modulating cancer cell-intrinsic dsDNA sensing using both 2-D and 3-D cell culture systems. As expected, introduction of cytoplasmic DNA via poly(dA:dT) transfection (1 μg/ml) followed by continued 2D culture resulted in significantly more robust TBK1 activation following LKB1 reconstitution, consistent with its ability to restore STING expression downstream of AMPK activation (5) (**Supplementary Figure S1A**). Similarly, poly(dA:dT) transfection of cancer cells followed by spheroid formation and microfluidic 3-D culture revealed that LKB1-reconstituted 3-D spheroids uniquely responded to transfection of cytoplasmic DNA, significantly upregulating multiple immune cell chemoattractants including CCL5, CCL2, and CXCL10, after 7 days (**Figures 1D,E** and **Supplementary Figure S1B**). Notably, IL-6 was suppressed by LKB1 reconstitution as previously

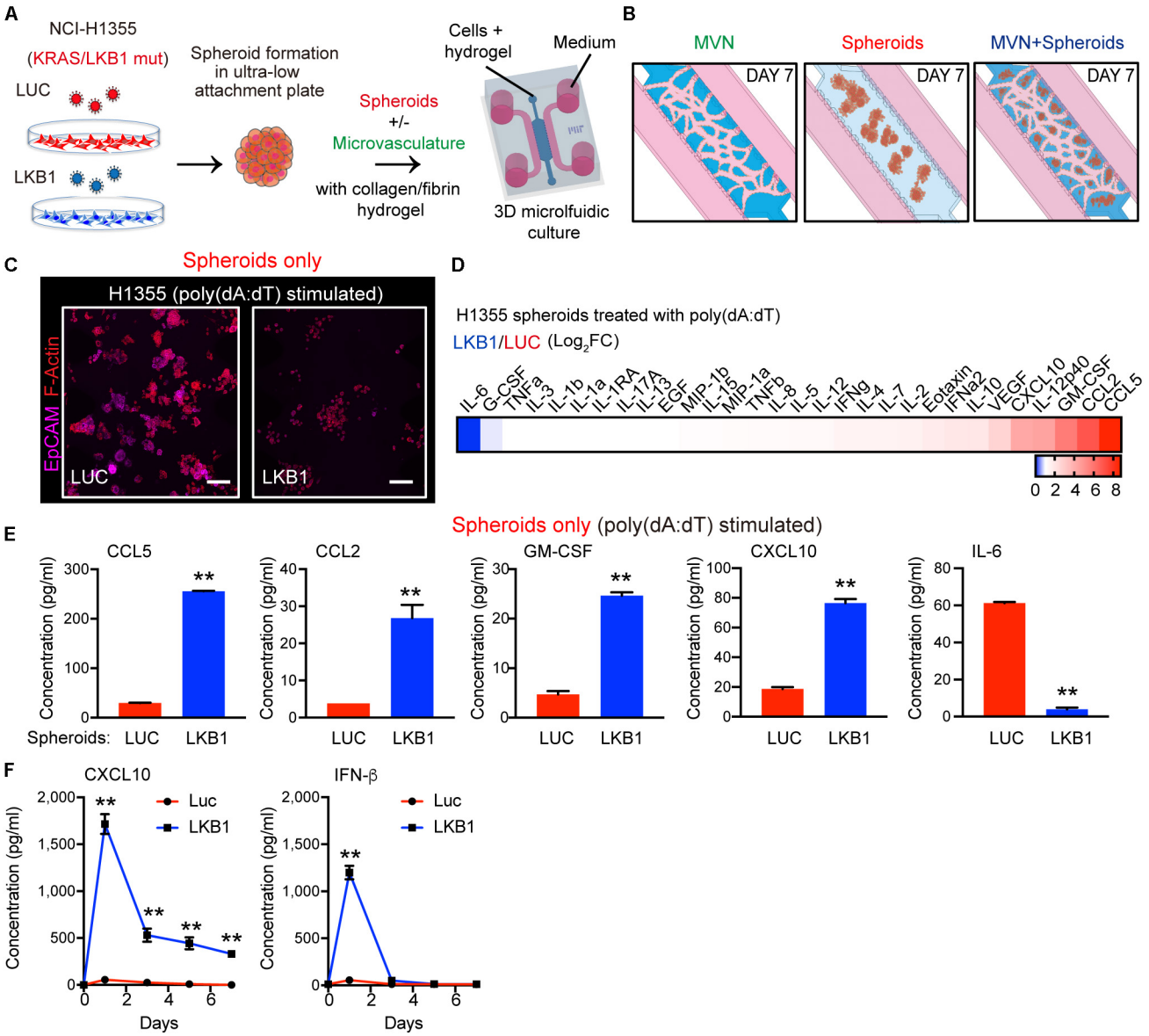


FIGURE 1 | LKB1 reconstitution of 3-D KL spheroids and response to dsDNA in microfluidic culture. **(A)** Schematic of H1355 tumor spheroids formation and their *in vitro* dynamic coculture with or without the microvasculature (MVN) in a 3D microfluidic device within a collagen/fibrin hydrogel. **(B)** Schematic of the dynamic culture of microvasculature only, spheroids only and the combination of spheroids and microvasculature in the microfluidic culture. **(C)** Confocal image of luciferase (LUC) control expressing (left) and LKB1 reconstituted (right) H1355 spheroids in 3D microfluidic culture after 7 days, pre-stimulated with poly 1 μg/mL poly (dA:dT), immunostained for F-actin (red) and EpCAM (CD326) (violet). Scale bar, 150 μm. **(D)** Heat map of cytokine profiles in conditioned medium (CM) 7 days from 3D microfluidic culture of H1355 spheroids. CM was collected 7 days after pre-stimulation with 1 μg/mL poly (dA:dT). Values represent log2 fold change of LKB1 reconstituted H1355 spheroids relative to control. **(E)** Absolute values of cytokine release of human CCL5, CCL2, GM-CSF, CXCL10, and IL-6 produced from 3D microfluidic culture of H1355 LKB1-reconstituted spheroids versus control. **(F)** ELISA of human CXCL10 and IFN-β over 7 days of 2D culture, treated ± 1 μg/mL poly(dA:dT), (n = 3 biological replicates). CM was collected and refreshed daily. P values were calculated by unpaired two tailed student t-test; $^{**}P < 0.01$. Data shown as mean values, error bars ± SD.

described (24, 25) (**Figure 1E** and **Supplementary Figure S1B**). In contrast, control MVNs expressed high levels of CCL2 and IL-8, as well as IL-6 (**Supplementary Figures S1C,D**).

Since these studies were conducted at the 7-day endpoint, we next sought to understand the kinetics of cGAS-STING regulated cytokine/chemokine production over time, focusing on CXCL10, the major T cell chemokine downstream of STING and TBK1-IRF3 signaling. We also compared results with IFN-β, another critical TBK1-IRF3 effector cytokine that was not included on the multiplexed array. Notably, poly(dA:dT)-induced IFN-β secretion was also potently restored by LKB1 reconstitution, but fell off by day 3, whereas CXCL10 production was maintained

over the 7 day period (**Figure 1F**) consistent with its known feed-forward activation by IFN (26–28). Thus, LKB1 reconstitution of KL tumor spheroids restores sensitivity to dsDNA sensing in this 3-D culture model.

A 3-D Microfluidic Co-culture System Captures Changes in Innate Immune Signaling During Tumor-Vasculature Interaction

We next considered the possibility that LKB1 expression in tumor cells might influence innate immune signaling in the neighboring vasculature, especially following dsDNA stimulation of tumor cells. We therefore co-cultured HUVECs/hLFB with H1355 spheroids in microfluidic devices for 7 days, which resulted in effective formation of MVNs and encapsulation of 3D tumor spheroids that are randomly distributed in the perivascular space and in contact interaction with the MVNs (**Figure 2A**).

To capture differences in dsDNA-stimulated cytokine production that occurred in co-culture with the microvasculature, tumor spheroids were exposed to poly(dA:dT) prior to loading in the device, followed by multiplexed profiling of conditioned media with or without co-culture of MVN with spheroids (H1355-LKB1 vs. LUC). To assess the degree to which the combination of MVN and spheroids modulated cytokine/chemokine production in a simply additive versus supra-additive or antagonistic manner, values from isolated MVN culture were subtracted, and results normalized to spheroid-only results (**Figure 2B** and **Supplementary Figures S1B,D**). This calculation revealed supra-additive upregulation of many of the same cytokines/chemokines in LKB1-reconstituted tumor spheroids co-cultured with MVNs, including CCL2 and CXCL10, while there were some differences such as IL-6, which was amplified, and CCL5, which was suppressed (**Figures 2C,D**). Thus, activation of cGAS-STING signaling by dsDNA in tumor cells alone strongly cooperated with MVNs to amplify innate immune production of specific cytokines and chemokines.

Cooperative Production of Innate Immune Cytokines Induced by Tumor-Vasculature Interaction Is Not Dependent on Cancer Cell Intrinsic STING

To dissect how dsDNA activated tumor cells cooperate with MVNs to enhance cytokine release, we knocked out STING via CRISPR/CAS9 deletion in H1355 cells with or without LKB1 reconstitution. As expected, STING deletion prevented LKB1 restoration of STING expression and suppressed activation of TBK1, as measured by S172 phosphorylation after poly(dA:dT) treatment (**Figure 3A**). In consonance with this result, STING knockout in LKB1-reconstituted H1355 tumor spheroids also inhibited downstream production of CXCL10 (**Figure 3B**). We then conducted tumor spheroid/MVN co-culture experiments with STING knockout cell lines, hypothesizing that the cooperative increase in innate immune cytokine production seen in co-culture would be similarly blunted. Surprisingly, we observed comparable induction of cytokines/chemokines following poly(dA:dT) treatment in LKB1 reconstituted H1355 spheroids and MVNs even in the absence of tumor cell STING (**Figure 3C**). Specifically, we again observed CXCL10 and IL-6 production that was significantly higher in co-culture compared to the vasculature alone, regardless of tumor cell STING (**Figure 3D**). Production of CCL2 was further enhanced in LKB1 reconstituted spheroid co-culture without tumor cell STING activity as compared to STING intact spheroid co-culture or vasculature alone (**Figure 3D**). Interestingly, in contrast to STING knockout in LKB1-reconstituted H1355 spheroids, STING knockout in HUVECs consistently resulted in impaired production of CXCL10 in 3D MVN co-culture, despite only treating tumor cells with poly-(dA:dT) (**Figure 3E**). These data revealed that dsDNA-mediated cooperative induction of cytokines/chemokines in tumor spheroid/MVN co-culture does not rely on tumor cell STING, and instead might rely on endothelial STING activation.

Endogenous Activation of TBK1 in the Tumor Vasculature

To understand the potential relevance of this finding, we next examined expression of STING and activation of its immediate downstream target, phosphorylated TBK1 (pTBK1), across different tumor microenvironments in tissue microarrays (TMA) of patient samples. We focused on brain tumors and brain metastases, given the low baseline neuroinflammation and absent STING activation in normal brain. Notably, the most prominent and consistent areas of TBK1 activation in primary brain tumors and metastases were in cross-sections of tumor vasculature (**Figures 4A,B**). We scored STING expression and activation in a binary manner based on the presence or absence of STING and pTBK1 stain, in endothelial cells lining the lumen of blood vessels. Endothelial cells were identified histologically using the hematoxylin counterstain marking a circumferential layer of nuclei surrounding red blood cell fragments. IHC demonstrated endothelial STING staining in nearly every sample from either tumor- or normal brain-associated microvasculature (**Figures 4A,C**). Interestingly, pTBK1 IHC revealed specific activation of STING in the tumor endothelium of both primary GBM and metastatic NSCLC (**Figures 4A,C**), whereas normal brain samples stained uniformly negative for pTBK1 (**Figures 4B,C**).

Taken together with our *in vitro* co-culture findings, these data suggested a potential influence of tumor cells on activation of STING in the microvasculature, potentially in the form of tumor-derived 2′3′-cGAMP. Indeed, consistent with the human tumor data, both HUVECs and fibroblasts exhibited 4-fold higher expression of basal STING levels relative to H1355 (**Figure 4D**). Therefore, we next considered the effect of extracellular 2′3′-cGAMP on these different cell types and on established MVNs. Notably, HUVECs were significantly more sensitive to 2′3′-cGAMP treatment as compared to H1355 tumor cells and fibroblasts, producing significantly higher levels of IFN-β and especially CXCL10 [**Figure 4E**. As expected,

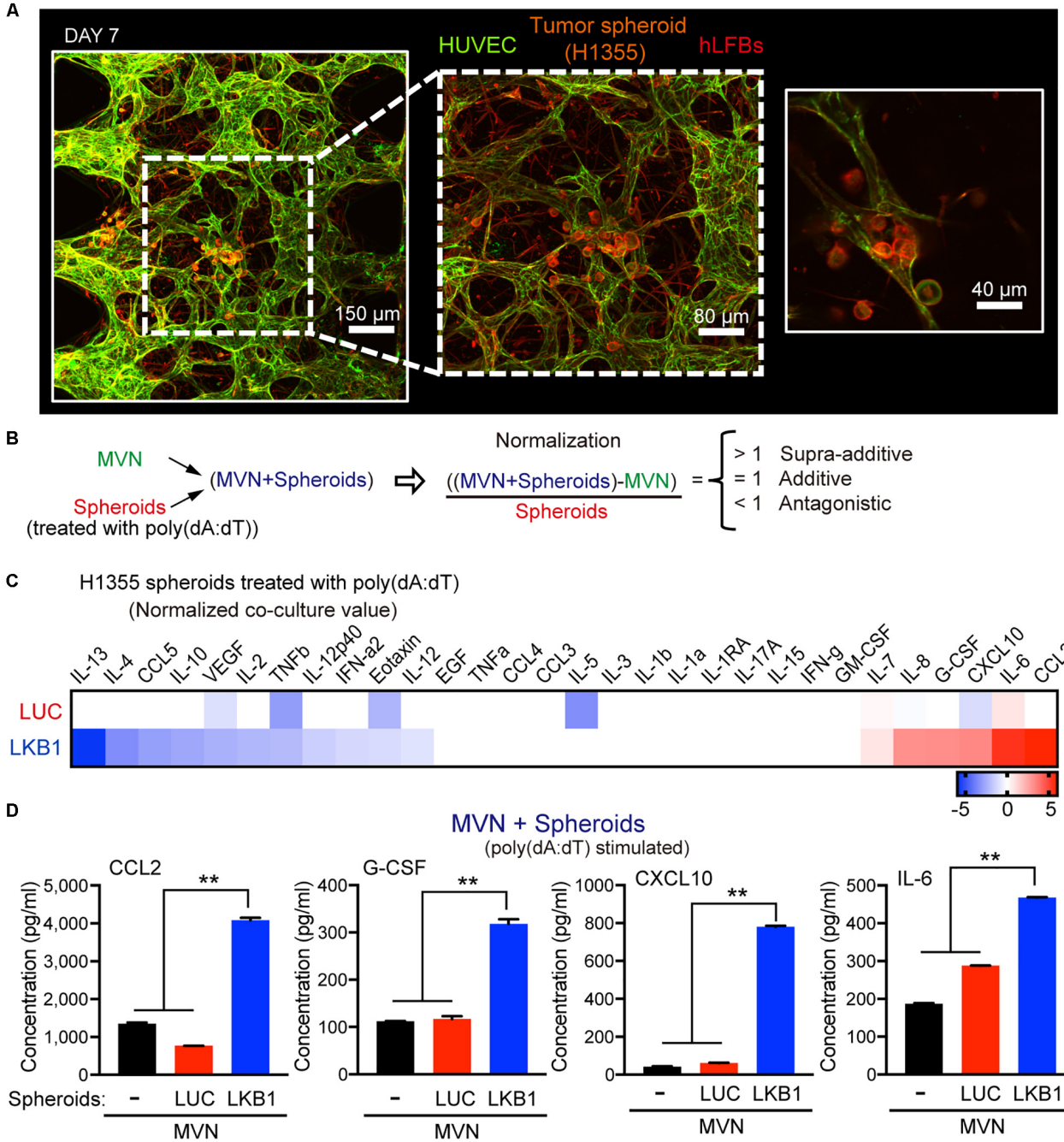


FIGURE 2 | Impact of LKB1 reconstitution on dsDNA sensing in co-culture with MVNs. **(A)** Confocal images at 10×, 20×, and 40× magnification of MVNs formed from HUVECs and hLFBs co-cultured with H1355 tumor spheroids within collagen/fibrin hydrogel after 7 days, immunostained for F-actin (red) and EpCAM (CD326) (orange) and CD31 (green). Scale bars, 150, 80, and 40 μm. **(B)** Schematic equation illustrating normalization of cytokine production from combination of MVN and H1355 tumor spheroids. Cytokine production is considered supra-additive (>1), additive (=1) or antagonistic (<1). **(C)** Heat map of log2 fold change cytokine/chemokine profiles in conditioned medium after 7 days of 3D microfluidic culture of MVN with H1355 LUC and LKB1 spheroids pre-stimulated with 1 μg/mL poly (dA:dT). **(D)** Absolute values of supra-additive cytokine release of human CCL2, G-CSF, CXCL10, and IL-6, from 3D microfluidic culture of combination of MVN + spheroids of H1355 LKB1 and LUC control spheroids and MVN only after 7 days of culture in 3D microfluidic devices. *P* values were calculated by one-way ANOVA followed by Tukey *post hoc* test; $^{**}P < 0.01$. Data shown as mean values, error bars ± SD.

STING knockout in HUVECs markedly reduced sensitivity to 2′3′-cGAMP treatment, especially as measured by CXCL10 production (**Figure 4F** and **Supplementary Figures S3A,B**)]. Cytokine/chemokine profiling analysis of established MVNs revealed that treatment of the vasculature with 1 μg/mL of 2′3′-cGAMP led to strong upregulation of multiple cytokines

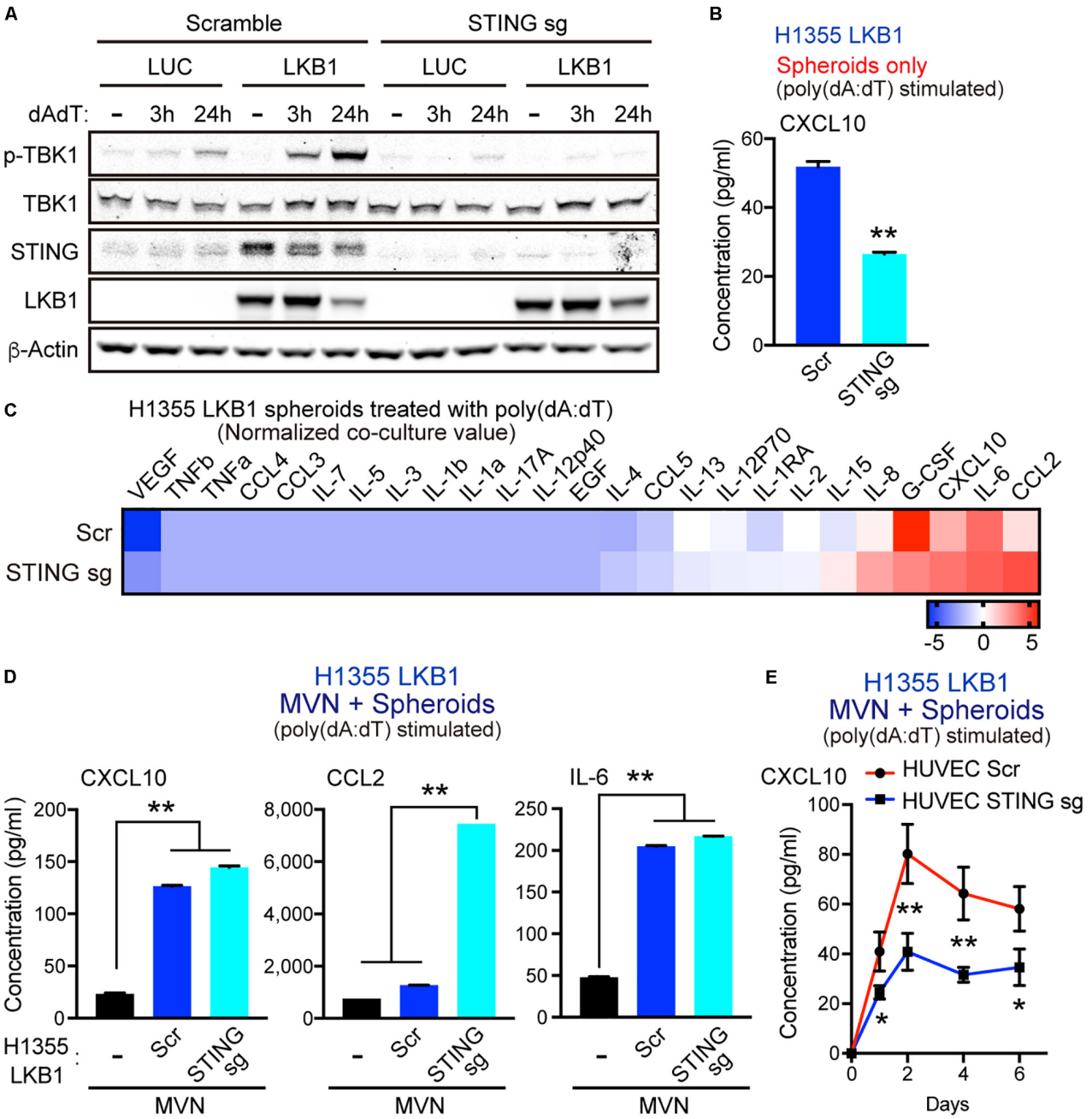


FIGURE 3 | MVN co-culture enhances dsDNA-induced cytokines/chemokines even in the absence of tumor cell STING. **(A)** Immunoblot of the indicated proteins in H1355 cells transduced with LUC and LKB1 ± scramble (control sgRNA) or STING knockout (STING sgRNA) in 2D culture. **(B)** CXCL10 production in H1355-LKB1 spheroids after STING knockout, (n = 3 biological replicates). **(C)** Heat map of log2 fold change cytokine/chemokine profiles in conditioned medium (CM) after 7 days of 3D microfluidic culture of MVN with H1355 LUC and LKB1 spheroids with scramble or STING knockout. Spheroids were pre-treated with 1 μg/mL poly (dA:dT). **(D)** Absolute values of cytokine/chemokine release of CXCL10, CCL2, and IL-6 production. **(E)** ELISA of human CXCL10 over 6 days of 3D microfluidic culture of HUVEC STING knockout (STING sgRNA) or HUVEC scramble (control sgRNA) with H1355 LKB1 spheroids. Spheroids were pre-treated with 1 μg/mL poly (dA:dT). CM was collected after 1 day and every 2 days of 3D culture. P values were calculated by unpaired two tailed student t-test or one-way ANOVA followed by Tukey *post hoc* test; $^{**}P < 0.01$, $^{*}0.01 < P < 0.05$. Data shown as mean values, error bars ± SD.

downstream of STING, such as CXCL10, CCL5, IFN-α, as well as CCL2 and IL-6 (**Figure 4G** and **Supplementary Figure S2A**).

To elucidate the potential impact of these findings on immune cell extravasation, we next quantified lymphocytes by morphologic criteria and examined their proximity to pTBK1+ versus pTBK1- vessels. We observed significantly greater perivascular lymphocyte infiltrates around pTBK1+ vessels, suggesting a potential relationship

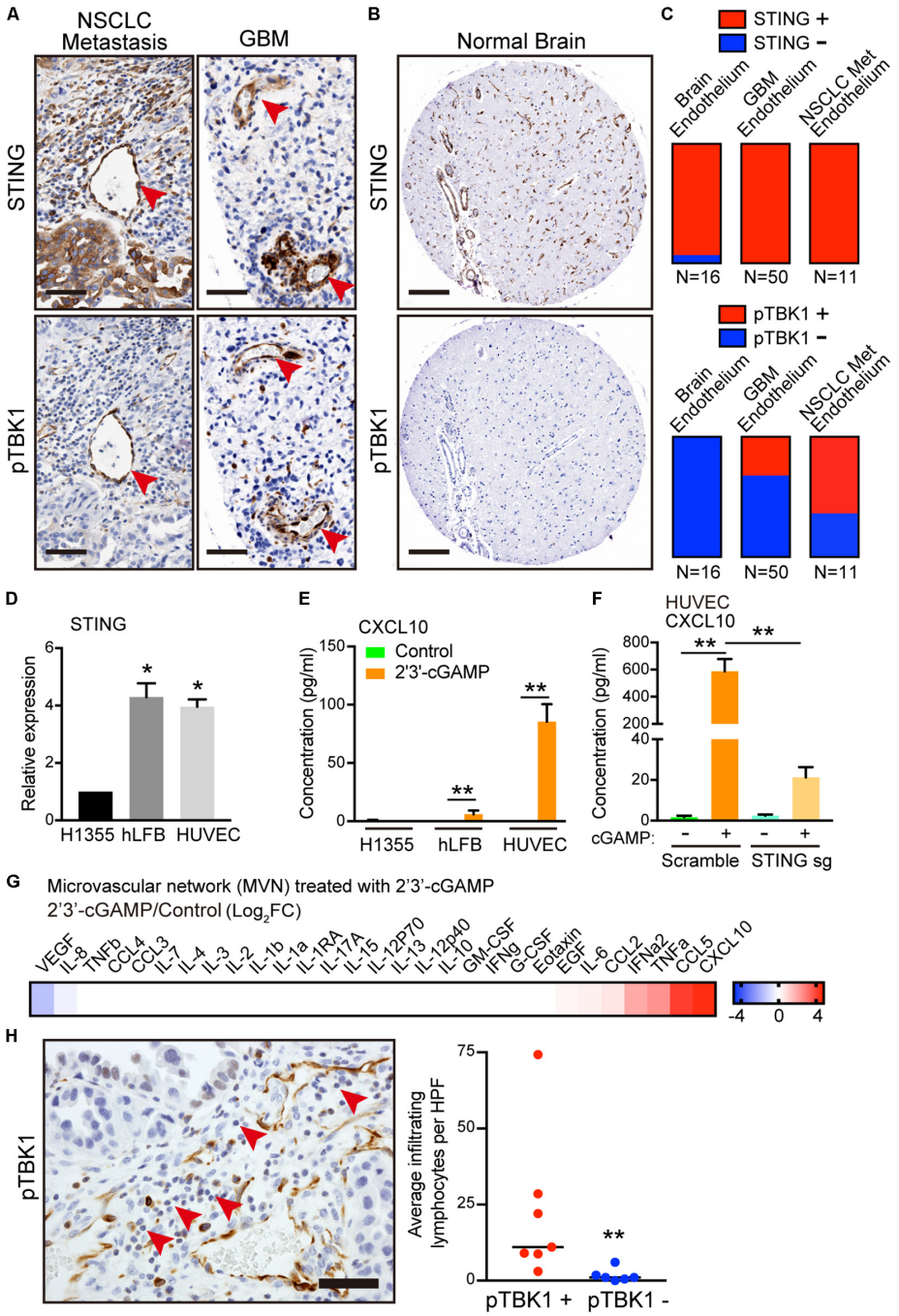


FIGURE 4 | Activation of STING-TBK1 signaling in tumor vasculature and lymphocyte infiltration. **(A)** Representative IHC images from metastatic non-small cell lung cancer (NSCLC) and primary glioblastoma multiforme (GBM) patient brain tissue samples. Red arrows highlight STING (upper) and phospho-TBK1 (pTBK1, lower) staining. Scale bars, 50 μm. **(B)** Representative STING (upper) and pTBK1 (lower) IHC images from control patient brain tissue. Scale bars, 100 μm. **(C)** Endothelial STING and pTBK1 IHC was scored in a blinded manner for each sample on a binary scale based on the presence (positive, +) or absence (negative, -) of staining in cells surrounding the presumed endothelial lumen. **(D)** qRT-PCR of basal STING in parental H1355, hLFB, and HUVEC. **(F)** qRT-PCR of IFN-β and CXCL10 in HUVECs after exogenous 2′3′-cGAMP treatment. Cells were treated with 1 μg/mL 2′3′-cGAMP for 24 h. **(E)** ELISA of human CXCL10 levels in conditioned medium derived from parental H1355, hLFB, and HUVEC after exogenous 2′3′-cGAMP treatment (1 μg/mL) for 24 h. **(F)** ELISA of human CXCL10 levels in conditioned medium derived from HUVECs transduced with scramble (control sgRNA) or STING knockout (STING sgRNA), after exogenous 2′3′-cGAMP treatment (1 μg/mL) for 24 h. **(G)** Heat map of log2 fold change cytokine/chemokine profiles in conditioned medium (CM) after 7 days of 3D microfluidic culture of MVN treated with 1 μg/mL 2′3′-cGAMP treatment over MVN control. MVN were treated for 2 days after MVN formation. **(H)** Representative IHC image from metastatic non-small cell lung cancer (NSCLC) with pTBK1+ endothelial microvessels. Red arrows highlight infiltrating lymphocytes. Scale bars, 50 μm (left). Quantification of infiltrating lymphocytes per high power field (HPF) surrounding pTBK1+ or pTBK1- endothelial lumens, (right). *P* values were calculated by unpaired two tailed student *t*-test, one-way ANOVA, or two-way ANOVA followed by Tukey *post hoc* test; $^{**}P < 0.01$, $^{*}0.01 < P < 0.05$. Data shown as mean values, error bars ± SD, ($n = 3$ biological replicates).

between activation of STING-TBK1 signaling in the tumor microvasculature and its ability to promote immune cell extravasation (**Figure 4H**). These findings thus prompted further examination of whether tumor derived 2′3′-cGAMP might directly activate STING in endothelial cells and prime the tumor vasculature for lymphocyte extravasation.

Export of 2′3′-cGAMP by Tumor Cells Activates STING in Neighboring Endothelial Cells

To determine whether 2′3′-cGAMP produced by cancer cells could activate STING/TBK1/IRF3 signaling in the neighboring tumor vasculature (**Figure 5A**), we first measured intracellular and extracellular 2′3′-cGAMP levels in H1355-LUC and H1355-LKB1 cells using the same concentration of double-stranded DNA used in co-culture experiments. Transfection of poly(dA:dT) not only increased levels of intracellular 2′3′-cGAMP, but also increased 2′3′-cGAMP levels in the media, which was significantly enhanced by LKB1 re-constitution (**Figure 5B**). As expected, knockout of STING failed to suppress either 2′3′-cGAMP intracellular production or extracellular export, regardless of LKB1 status (**Figure 5B**).

To directly assess whether 2′3′-cGAMP generated from tumor cells could activate STING in endothelial cells, we next generated cGAS knockout H1355 cells and treated them with poly(dA:dT). As expected, recognition of cytosolic DNA and subsequent downstream activation of STING was abolished in cGAS null H1355-LUC and H1355-LKB1 cell lines, even after the de-repression of STING following LKB1 re-constitution (**Figure 5C**). Consistent with this, intracellular 2′3′-cGAMP production was significantly diminished, and there were virtually undetectable levels of 2′3′-cGAMP in the media from the cGAS knockout line (**Figure 5D**). We next investigated the impact of co-culturing poly(dA:dT) transfected H1355-LKB1 cells following STING or cGAS knockout with HUVECs, sorting CD31 positive cells to measure HUVEC specific cytokines/chemokines (**Figure 5E**). Consistent with our results in 3D culture, dsDNA stimulation of H1355-LKB1 reconstituted cells potently activated CXCL10 as well as IFN-β expression in HUVECs, even following STING knockout in H1355-LKB1 cells (**Figure 5F**). Meanwhile, CXCL10 production was dramatically reduced by STING knockout in HUVECs co-cultured with H1355-LKB1 cells, further substantiating a direct contribution of endothelial cell STING to induction of CXCL10 secretion in the 3D microfluidic culture system (**Figure 5G**). Importantly, we also observed that cancer cell cGAS deletion significantly decreased expression of both CXCL10 and IFN-β in co-cultured HUVECs, providing direct evidence that ablation of 2′3′-cGAMP export from tumor cells suppresses STING signaling in endothelial cells (**Figures 5H,I**). Of note, expression of CXCL10 and IFN-β in HUVECs was not completely abolished following cGAS knockout, suggesting the potential for additional paracrine mediators of this response downstream of alternate dsDNA sensors. Regardless, these data confirm that cGAS-driven 2′3′-cGAMP export from cancer cells is directly involved in activation of STING signaling in neighboring endothelial cells, prompting us to examine whether it might play additional roles in priming the vasculature to promote immune cell recruitment.

2′3′-cGAMP and IFN-β Prime the Endothelium for Immune Cell Extravasation

To determine whether 2′3′-cGAMP might alter the vasculature to promote immune cell recruitment, we first assessed its impact on microvascular permeability. Permeability of MVNs in microfluidic culture can be measured via flux of a fluorescent tracer from the luminal compartment of the vasculature to the interstitial space, as recently described (21). We assessed the effects of 2′3′-cGAMP or downstream type I IFN, which can also impact tumor vasculature (29), and observed that both 2′3′-cGAMP and IFN-β treatment individually enhanced permeability of the 3-d MVNs (**Figures 6A,B**). We next tested a quantitative PCR array of endothelial activation markers to identify whether expression of specific adhesion molecules or other related genes are also upregulated after treatment of endothelial cells with 2′3′-cGAMP or IFN-β. Notably, analysis of this list to identify common targets shared by both 2′3′cGAMP and IFN-β treatment revealed prominent upregulation of multiple genes involved in T cell trafficking, including E-selectin, ICAM-1, and VCAM-1 (**Figure 6C**). We therefore examined expression of these specific genes in HUVECs in response to 2′3′-cGAMP, IFN-β, or the combination. Indeed, all three genes were significantly induced by 2′3′-cGAMP or IFN-β, treatment, and further increased by the combination (**Figure 6D**). In contrast, we observed negligible changes in expression of genes involved in tight junctions, including ZO-1, Occludin and Claudin-5 (**Supplementary Figure S4C**), suggesting that the observed changes in vascular permeability are likely occur at the post-transcriptional level. We therefore focused on differences in expression of adhesion molecules in established MVNs in 3D microfluidic devices. Treatment with 2′3′-cGAMP and/or IFN-β increased expression of membrane-bound ICAM-1 and VCAM-1 on MVNs by immunofluorescence (**Figure 6E** and **Supplementary Figure S4B**).To determine the functional consequences of this adhesion molecule upregulation, we perfused Jurkat T cells through the MVNs and observed significantly increased attachement of Jurkat cells to the endothelial walls of the microvasculature in the presence of 2′3′cGAMP pre-treatment (**Figure 6F**). Taken together, these findings reveal that tumor derived 2′3′-cGAMP not only amplifies cytokine production in the adjacent vasculature, but also increases permeability and upregulates adhesion molecules that can facilitate T cell escape (30, 31) (**Figure 6G**).

DISCUSSION

Here, we utilize a novel microfluidic co-culture system to probe the impact of LKB1 inactivation on cancer cell intrinsic cGAS-STING signaling in relationship to the microvasculature, a critical gatekeeper of immune cell extravasation. Cytokine profiling of

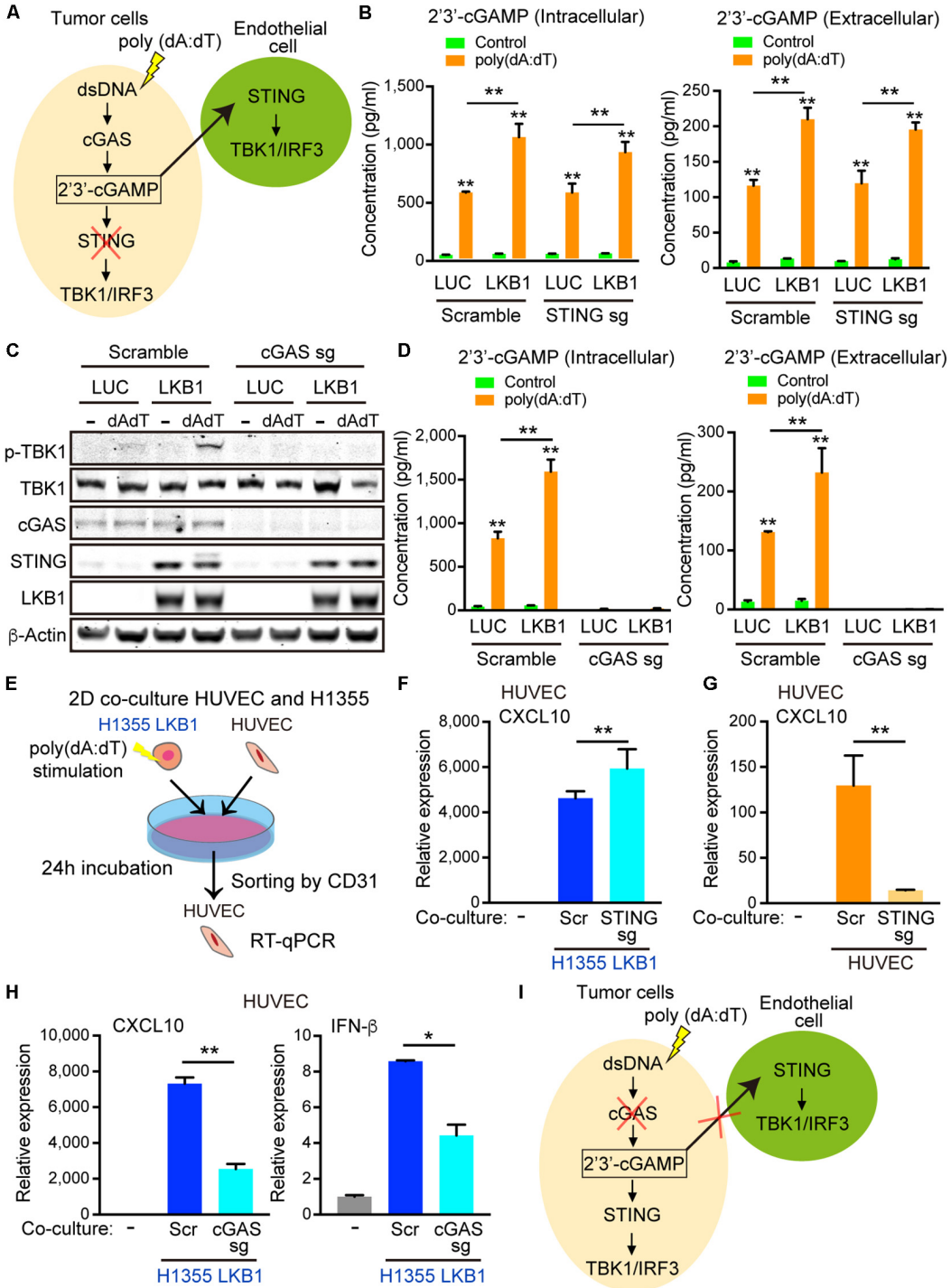


FIGURE 5 | 2′3′-cGAMP exported by cancer cells activates STING signaling in endothelial cells. **(A)** Schematic of cGAS-STING signaling in tumor cells after poly (dA:dT) stimulation and hypothesized export of 2′3′-cGAMP activating STING in the endothelial cells, which would be unaffected by tumor cell STING knockout. **(B)** Intracellular and extracellular 2′3′-cGAMP ELISA of transduced LUC and LKB1 H1355 ± scramble or STING knockout. **(C)** Immunoblot of the indicated proteins in H1355 cells transduced with LUC and LKB1 with scramble or cGAS knockout (cGAS sgRNA) in 2-D culture. **(D)** Intracellular and extracellular 2′3′-cGAMP ELISA of transduced LUC and LKB1 H1355 with scramble or cGAS knockout. **(E)** Schematic of the 2-D co-culture experiment and sorting by CD31 + cells. **(F)** qRT-PCR of CXCL10 of HUVECs after 2-D co-culture with H1355 cells for 24 h with scramble or STING knockout. **(G)** qRT-PCR of CXCL10 of HUVECs scramble or STING knockout after 2-D co-culture with H1355 cells for 24 h. **(H)** qRT-PCR of CXCL10 and IFN-β of HUVECs after 2-D co-culture with H1355 cells for 24 h with scramble or cGAS knockout. **(I)** Schematic of cGAS-STING pathway in tumor cells after poly (dA:dT) stimulation demonstrating that silencing of cGAS impairs accumulation of 2′3′-cGAMP and export from the cells, limiting the STING activation in endothelial cells. *P* values were calculated by one-way ANOVA, or two-way ANOVA followed by Tukey *post hoc* test; $^{**}P < 0.01$, $^{*}0.01 < P < 0.05$. Data shown as mean values, error bars ± SD, ($n = 3$ biological replicates).

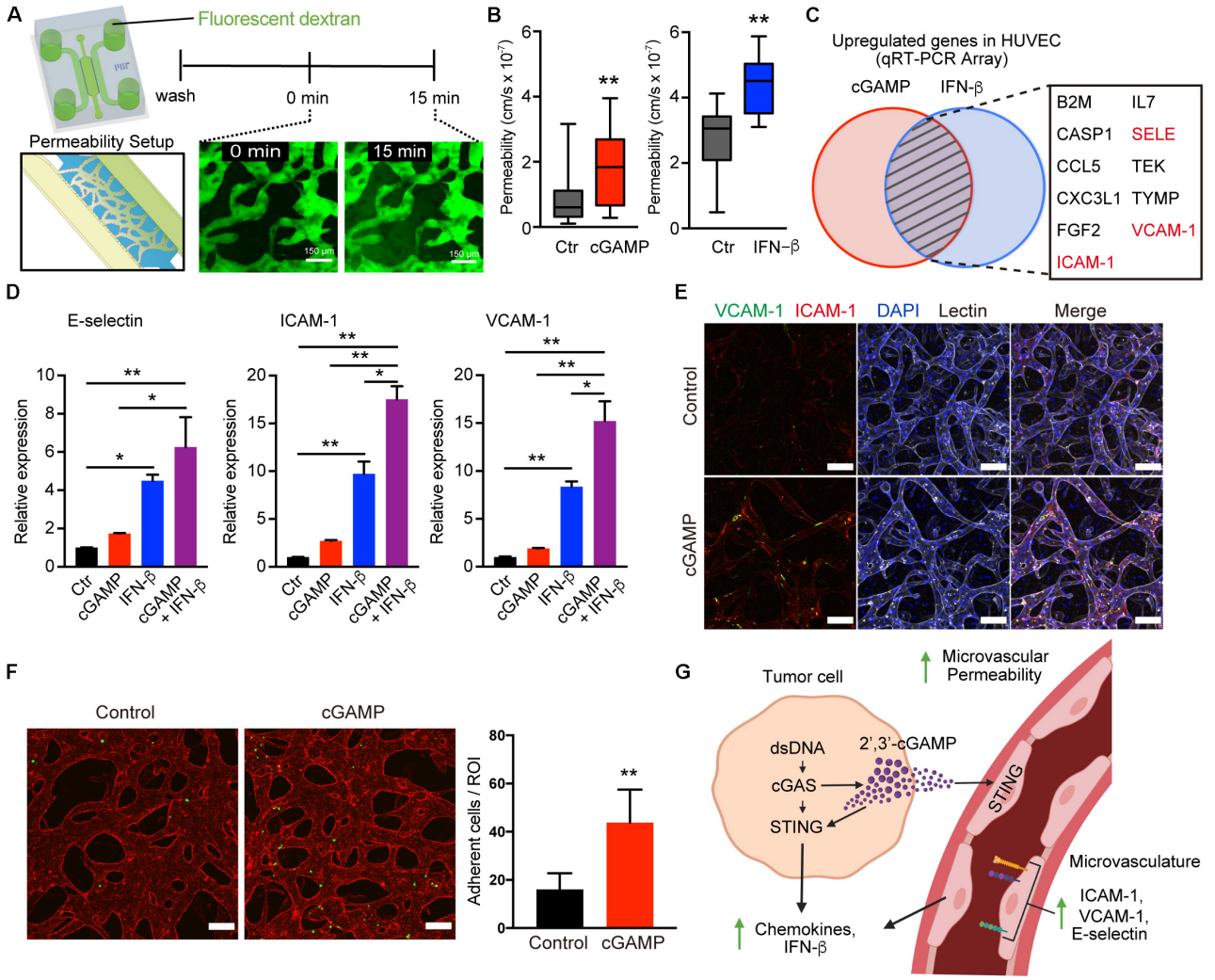


FIGURE 6 | 2′3′-cGAMP and IFN-β promote vascular permeability and upregulation of adhesion molecules. **(A)** Schematic of permeability experiments and analysis. Dextran dye was injected, and image stacks were captured at time 0 and at 15 min. **(B)** Permeability coefficients for different conditions (control, cGAMP, IFN-β), (n = 9 biological replicates). **(C)** Venn diagram of top genes upregulated in HUVEC after treatment with 2′3′-cGAMP or IFN-β. **(D)** qRT-PCR of E-selectin, ICAM-1, VCAM-1 in HUVEC treated with 2′3′-cGAMP, IFN-β, or combination of 2′3′-cGAMP + IFN-β (n = 3 biological replicates). **(E)** Confocal images of microvasculature treated ± 2′3′-cGAMP immunostained for ICAM-1, VCAM-1, and lectin. Scale bars, 100 μm. **(F)** Confocal images of microvasculature (red) treated ± 2′-3′-cGAMP and Jurkat cells (green) (left). Quantification of the number of Jurkat cells adherent to microvasculature per Region of Interest (ROI) (right). Scale bars, 100 μm. **(G)** Schematic of tumor-derived cGAMP (via cGAS) and IFN-β (via STING) influencing the vascular permeability, adhesion molecules, and chemokines. Schematic was created with BioRender. P values were calculated by unpaired two tailed student t-test or one-way ANOVA followed by Tukey $post\ hoc$ test; $^{**}P < 0.01$, $^{*}0.01 < P < 0.05$. Data shown as mean values, error bars ± SD.

conditioned media from the co-culture of 3-D MVNs with tumor spheroids revealed a cooperative production of multiple chemo-attractants downstream of STING such as CXCL10, CCL5, and CCL2, which was surprisingly independent of cancer cell STING. Furthermore, examination of primary tumors and metastases from patients demonstrated STING-TBK1 activation principally in the tumor vasculature, in contrast to the healthy vascular endothelium. These data suggest paracrine signaling from cancer cells to promote STING activation in adjacent vasculature. Further investigation revealed that 2′3′-cGAMP preferentially activates endothelial cells, that LKB1 reconstitution enhances 2′3′-cGAMP export, and that cancer cell-intrinsic cGAS activity contributes directly to endothelial cell activation. Finally, we show that STING activation in the endothelium by 2′3′-cGAMP can be further enhanced by downstream type 1 IFN, resulting in functional changes to the vasculature that favor immune cell extravasation.

Several recent studies have highlighted an emerging role of cancer cell derived 2′3′-cGAMP in the tumor microenvironment (TME). For example, tumor cells have been shown to transfer 2′3′-cGAMP to astrocytes directly via gap junctions, activating NF-κB signaling and promoting adaptation of the TME to facilitate brain metastasis (7). More recently, multiple groups have unveiled a direct role for exported cancer cell 2′3′-cGAMP

in activating anti-tumor immunity. For example, using murine syngeneic cancer lines, tumor-derived 2′3′-cGAMP was shown to activate STING in neighboring host cells to produce type I interferons in the TME and subsequently prime NK cells for tumor cell lysis (8). In two very recent studies, the role of extracellular 2′3′-cGAMP as an immunotransmitter has been further solidified. Inhibition of tumor-associated macrophages has been shown to drive type 1 interferon production in the TME via accumulation of extracellular cancer cell derived 2′3′-cGAMP (13). Furthermore, inhibition of the 2′3′-cGAMP hydrolase ENPP1 was reported to enhance export and accumulation in the media both at steady state and following DNA damage, promoting tumor associated immune infiltration (14). However, to date, a role for endogenous 2′3′-cGAMP in promoting vascular activation, a pre-requisite for this immune cell influx into tumors, has yet to be described.

Intratumoral administration of 2′3′-cGAMP or other cyclic dinucleotide STING agonists promotes therapeutic immune response in several mouse tumor models dependent on host STING but only partially dependent on host T-cell activity (32). Furthermore, tumor endothelial cells have been shown to represent the major source of type I interferon production in the TME following intratumoral injection of 3′3′-cGAMP in the B16F10 melanoma model (15). We now provide evidence that tumor-derived 2′3′-cGAMP can activate endothelial STING, and that 2′3′-cGAMP and downstream IFN-β enhance vascular permeability. Immune cell effectors must receive cues to adhere and extravasate (33) from the vasculature in order to enter the tumor microenvironment and we observed that 2′3′-cGAMP and IFN-β co-operate to upregulate E-selectin, ICAM-1, and VCAM-1. In this regard, endogenous tumor-derived 2′3′-cGAMP could play a gatekeeper role in determining the ability of immune cells to infiltrate by regulating vascular expression of ICAM-1 (27) and VCAM-1 (34), though this possibility needs to be further validated *in vivo*, including animal studies. Regardless, our data suggests that, in addition to silencing STING, downregulation of 2′3′-cGAMP through mutations such as LKB1 could also promote immune cell exclusion from tumors.

STK11/LKB1 mutation has been identified as a main driver of anti-PD1 resistance in KRAS-mutant lung adenocarcinoma, and loss of LKB1 has been linked to decreased numbers and function of T cells in the TME (5, 24, 35). We have previously reported *in vivo* quantitative IHC data from patient biopsies with KL tumors showing that T cells were retained in the stroma rather than infiltrating the cancer epithelium, suggesting that LKB1 is important for T cell recruitment (5). Our new data offer a more refined explanation for the phenomenon of defective T cell chemotaxis seen in the KL TME. The recognition of tumor-derived 2′3′-cGAMP and resultant aberrant STING activation by neighboring tumor vasculature, along with attenuated vascular activation and decreased production of T cell chemokines such as CXCL10 by the KL cancer cells, may contribute to the disruption of T cell chemotaxis and resistance to anti-PD1 treatment in the KL TME. We also note that our IHC studies relied on basal tumors unexposed to DNA damaging agents, as evidenced by lack of robust tumor cell pTBK1 staining. Thus, active therapy with agents that enhance endogenous tumor cGAMP production either through non-specific DNA damage (chemotherapy or radiation) or more targeted approaches such as TREX1 or ENPP1 inhibition, are likely to prime even more robust vascular activation, T cell recruitment and infiltration in the TME. Additionally, while we focused on tumor-derived 2′3′-cGAMP as the source of vascular activation in our co-culture system, extravasated antigen presenting cells in the TME could represent another source of extracellular 2′3′-cGAMP. In addition, macrophages are known to produce IFN-β in response to extracellular 2′3′-cGAMP, which could also contribute to vascular activation via paracrine signaling (36). Finally, although we did not observe robust cytokine production from fibroblasts in response to extracellular 2′3′-cGAMP in our systems, fibroblasts are critical in the formation of a vascularized tumor niche and it remains to be seen how extracellular 2′3′-cGAMP may influence the secretome from cancer-associated fibroblasts (37).

More generally, this study demonstrates that developing more representative physiological cell culture models of the TME can elucidate important aspects of innate immune signaling that cannot be studied using traditional *in vitro* systems (38). Indeed, microfluidic technologies help develop new tools for cancer diagnosis and treatment. Important future directions to enhance the physiologic relevance of the microfluidic co-culture system include introduction of continuous perfusion to mimic blood microcirculation and enable modeling of immune cell trafficking in a long-term culture system. Furthermore, advances in *ex vivo* modeling may allow long-term culture of patient-derived tumor samples to enable a personalized medicine approach to study drug or immune cell penetration in patient-specific tumor niches (18, 20).

In summary, our data expands upon the increasingly recognized role of endogenous tumor-derived 2′3′-cGAMP in the TME (39), revealing a novel function in vascular activation and immune escape following LKB1 loss. They also suggest that DNA damaging agents or targeted therapeutics that increase endogenous 2′3′-cGAMP production may act to enhance vascular activation and improve immune infiltration and subsequent response to PD-1 blockade in these treatment refractory cancers.

AUTHOR'S NOTE

While critical for tumor immune evasion, the phenomenon of immune cell exclusion remains incompletely understood. Using KRAS-LKB1 mutant lung cancer as a model, we demonstrate that tumor cell cGAS-STING not only regulates chemokine-mediated immune cell recruitment, but also directly influences the gatekeeper function of tumor vasculature via extracellular transfer of 2′3′-cGAMP.

DATA AVAILABILITY STATEMENT

The data are available upon requests to the corresponding authors (DB, SK, and RK).

AUTHOR CONTRIBUTIONS

MC, SKS, SES, RK, SK, and DB designed the research and wrote the manuscript. MC, SKS, SES, SK, EK, EI, IC, RY, and TeT performed and supervised biological and cellular studies. MC, SES, TO, and BP designed and prepared the microfluidic device. MC, SKS, and SES performed cell culture in microfluidic devices. SES, MC, TO, and SL performed permeability measurements, confocal imaging, and computational analyses. EK, NM, EI, and CP obtained samples and performed or supervised immunohistochemistry. SKS, TrT, SH, SG, SK, and RY performed multiplexed arrays, immunoblots, ELISA, and RT-PCR. MC, SKS, SES, SK, EK, NM, and SL analyzed the data. CP, VC, RK, SK, and DB supervised the project. All authors read and approved the manuscript.

FUNDING

This work was supported by the National Institute of Health through grant U01CA214381 to DB and RK. MC was supported by the MIT-POLITO grant (BIOMODE – Compagnia di San Paolo) under the joint "Doctorate of Bioengineering and Medical-Surgical Sciences" of University of Turin and Politecnico di Torino. SES was supported by a fellowship from the National Cancer Institute (K00CA212227).

ACKNOWLEDGMENTS

We thank Dana-Farber/Harvard Cancer Center in Boston, MA, for the use of the Specialized Histopathology Core, which provided histology and immunohistochemistry service. Dana-Farber/Harvard Cancer Center is supported in part by an NCI Cancer Center Support Grant # NIH 5 P30 CA06516. We also thank Calixto Saenz and the Harvard Medical School (HMS) Microfluidics/Microfabrication Facility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02090/full#supplementary-material

FIGURE S1 | LKB1 reconstitution in 3D microfluidic spheroid culture. **(A)** Immunoblot of the indicated proteins in H1355 cells transduced with LUC and LKB1. **(B)** log10 of the absolute values of cytokine release corresponding to the heatmap in **Figure 1D** from 3D microfluidic culture H1355 LUC spheroids and H1355 LKB1-reconstituted spheroids. **(C)** Schematic of human lung fibroblasts (hLFB) and human umbilical vein endothelial cells (HUVEC) in a 3D microfluidic device within a collagen/fibrin hydrogel for 7 days. Confocal images of microvasculature (MVN) immunostained for F-actin (red) and CD31 (green). Scale bar, 150 μm. **(D)** log10 of the absolute values of cytokine production corresponding to the heatmap in **Figure 1D** from 3D microfluidic culture of microvascular networks (MVN) alone.

FIGURE S2 | KL-cGAMP export and sensitivity of HUVECs to cGAMP. **(A)** Log10 of the absolute values of cytokine production corresponding to the heatmap in **Figure 4G**, from 3D microfluidic culture of MVN treated with 1 μg/mL 2′3′-cGAMP treatment over MVN control.

FIGURE S3 | Insensitivity of STING knockout HUVEC to 2′3′-cGAMP. **(A)** Immunoblot of the indicated proteins in HUVEC transduced with scramble (control sgRNA) or STING knockout (STING sgRNA). **(B)** qRT-PCR of CXCL10 and IFN-β of HUVECs transduced with scramble (control sgRNA) or STING knockout (STING sgRNA), after exogenous 2′3′-cGAMP treatment (1 μg/mL) for 24 h. *P* values were calculated by two-way ANOVA followed by Tukey *post hoc* test; $^{**}P < 0.01$. Data shown as mean values, error bars ±SD.

FIGURE S4 | cGAMP/IFN-β affects adhesion molecules. **(A)** Upregulated genes from HUVEC treated with 2′3′-cGAMP or IFN-β. **(B)** Immunostaining of ICAM-1 and VCAM-1 in networks treated with IFN-β (100 ng/ml) or in combination with 2′3′-cGAMP. Scale bars, 100 μm. **(C)** qRT-PCR of ZO-1, Occludin and Claudin-5 in HUVEC treated with 2′3′-cGAMP, IFN-β, or combination of 2′3′-cGAMP + IFN-β.

FIGURE S5 | Design of microfluidic devices. **(A)** The 3D cell culture chip (AIM Biotech) is shown with three independent microfluidic chambers (referred to as "device") per chip, Each device contains a center gel region with posts separating the gel region from the anti-parallel side channels. **(B,C)** Custom PDMS microfluidic devices were designed using Autocad (Autodesk) and are comprised of a central gel channel, two medium channels and four reservoirs. Devices were bonded to glass coverslips.

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Conflict of Interest: RK is co-founder and has a significant financial interest in AIM Biotech, a company that manufactures microfluidic systems, and has received research funding from Amgen and Biogen. DB and CP are co-founders and scientific advisory members of Xsphera Biosciences. DB is a consultant for N-of-One/Qiagen, a scientific advisory board member of Tango Therapeutics, and has received research funding from Bristol-Myers Squibb, Novartis, Lilly Oncology, and Gilead Sciences.

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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frontiers in Immunology

MINI REVIEW
published: 05 November 2020
doi: 10.3389/fimmu.2020.575258

FGFR3 Alterations in the Era of Immunotherapy for Urothelial Bladder Cancer

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FGFR3 is a prognostic and predictive marker and is a validated therapeutic target in urothelial bladder cancer. Its utility as a marker and target in the context of immunotherapy is incompletely understood. We review the role of *FGFR3* in bladder cancer and discuss preclinical and clinical clues of its effectiveness as a patient selection factor and therapeutic target in the era of immunotherapy.

Keywords: bladder cancer, fibroblast growth factor receptor, immunotherapy, pharmacogenetics, targeted molecular therapy

OPEN ACCESS

Edited by:
Sarah E. Church,
NanoString Technologies,
United States

Reviewed by:
Amir Sharabi,
Beth Israel Deaconess Medical Center and Harvard Medical School, United States
Francois Radvanyi,
Institut Curie, France

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *23 June 2020*
Accepted: *14 October 2020*
Published: *05 November 2020*

Citation:
Kacew A and Sweis RF (2020) FGFR3 Alterations in the Era of Immunotherapy for Urothelial Bladder Cancer. Front. Immunol. 11:575258. doi: 10.3389/fimmu.2020.575258

INTRODUCTION

Cytotoxic chemotherapy had been the only standard-of-care treatment for advanced urothelial bladder cancer, which is the world's 10th most common cancer and thirteenth most deadly (1). Cisplatin-based regimens are associated with objective responses in up to 45% of patients, but these responses are generally not durable (2, 3). Cisplatin-based therapies are associated with toxicities, including treatment-related mortality in rare cases. Beginning with the regulatory approval of atezolizumab, an inhibitor of programmed death-ligand 1 (PD-L1), in 2016, a total of five immune checkpoint inhibitors (ICIs), including the programmed cell death protein 1 (PD-1) inhibitors pembrolizumab and nivolumab and the PD-L1 inhibitors avelumab and durvalumab, gained regulatory approval for advanced urothelial cancer. These therapies are associated with durable responses in a minority of patients (roughly 15% among patients selected based on immune infiltration) and comparatively favorable side effect profiles (4). They have now been used in the first line alone and in combination with chemotherapy and are the preferred choice in the second line after chemotherapy (5–7).

In spite of the great therapeutic potential of ICIs, only a minority (approximately 20%) of patients experience tumoral response to ICIs and median survival with second line immunotherapy remains shorter than 1 year (8). It follows that the identification of biomarkers is a critical step in improving therapy for advanced urothelial bladder cancer. Recognition of characteristics associated with ICI response can help clinicians and researchers optimize patient selection, appreciate new combination or sequencing strategies, and identify mechanisms or targets for development of novel therapeutics. Tumoral PD-L1 expression is only modestly useful as a marker, as tumoral responses to ICI have been observed regardless of PD-L1 status (albeit at a numerically higher rate among those with greater PD-L1 expression) (9). Consensus molecular classifications, which define

luminal, basal/squamous, stroma-rich, and neuroendocrine-like subgroups of muscle-invasive bladder cancer, although useful in understanding the biology of tumors, similarly fall short in helping to guide ICI therapy (10). The goal remains to discover tumor characteristics, drivers, and markers that can offer greater therapeutic and instructive value in the context of ICI therapy. Overactivity in the *ErbB* family (including *EGFR* and *Her2/neu*), which is associated with luminal and basal/squamous classifications, has only demonstrated utility as a drug target or predictive marker in a small proportion of clinical trials related to that pathway (11). Similarly, although *VEGF* activation portends poor outcomes, *VEGF* has not proved to be particularly promising as a therapeutic target (11). Mutations in DNA damage response genes, including *ERCC1, ERCC2, ATM, FANCC*, and *RB1* can help predict response to platinum-based therapy, but markers for newer immune-based therapies are needed (11). The fibroblast growth factor receptor 3 (*FGFR3*) gene has long been associated with bladder cancer oncogenesis and recently become a therapeutic target (12). It has become particularly important in the context of immunotherapy given its inverse relationship with an anti-tumor immune response due, at least in part, to its association with a lymphocyte-excluded phenotype (13). We review the current knowledge of *FGFR3* in the context of both modern therapies such as anti-PD-1 immunotherapy and FGFR blockade.

FGFR3 IN BLADDER CANCER

The chromosome 4 gene *FGFR3* encodes the FGFR3 protein, a tyrosine kinase that has classically been known to play important roles in development, osteogenesis, and bone maintenance (14, 15). *FGFR3* is highly expressed in chondrocytes and osteoblasts, and germline mutations are associated with bone growth disorders such as achondroplasia, chondrodysplasia, and thanatophoric dysplasia (16–20). Curiously, while activating mutations curb growth in bone, the same mutations are associated with excess growth in other tissues (e.g., nevi in skin) (21). Germline *FGFR3* mutations are paternally inherited and are associated with advanced paternal age (22). The introduction of improved clinical genetic testing techniques in oncology has facilitated the discovery that *FGFR3* gene alterations are implicated in a wide range of cancers [**Figure 1A**, (23, 24)]. The prevalence of *FGFR3* gene aberrations is highest in urothelial carcinomas (18% of cases), followed by uterine carcinosarcoma (14%), esophageal (5%), ovarian (5%), and endometrial (4%) cancers (23–25). FGFR3 signaling has been observed to overlap with known oncogenic pathways such as RAS/PI3K/ERK/AKT/EGFR and has been implicated in tumoral epithelial-to-mesenchymal transition (26, 27). The role of *FGFR3* gene in oncogenesis may even be at the pre-translational level: Has_circ_0068871, a circRNA product of *FGFR3* gene transcription, is overexpressed in bladder cancer, and is associated with cancer cell proliferation and migration (28). Expression of the antisense transcript FGFR3-AS1, which increases stabilizes and promotes expression of *FGFR3* mRNA, and which is overexpressed in urothelial tumors, is associated with tumor invasiveness, proliferation, and motility (29). The most common *FGFR3* mutation, S249C, likely develops through an apoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-mediated mutagenic mechanism (30). FGFR3-transforming acid coiled coil 3 (TACC3) fusions, which result in constitutive signaling, represent another frequent source of *FGFR3* gene aberration (31).

As prognostic indicators, *FGFR3* gene alterations are generally associated with lower grade and stage among all urothelial bladder carcinomas (32). Among non-muscle invasive cases, 49-84% express *FGFR3*, compared to 18% of muscle-invasive cases, and *FGFR3* mutations are associated with lower disease-specific survival (32–34). Among American Joint Committee on Cancer (AJCC) 8th edition T1 tumors, *FGFR3* expression is associated with lower grade tumor and lower risk of cancer progression (35). *FGFR3* gene mutations, amplifications, and fusions are associated with luminal-papillary subtype of urothelial cancer, which itself is associated with non-muscle invasive disease and favorable prognosis compared with other subtypes (13, 36, 37). However, in spite of the general association of *FGFR3* alterations with favorable characteristics, there is no evidence to suggest that *FGFR3* gene alterations correlate with a less aggressive phenotype once urothelial carcinoma has become advanced. In fact, *FGFR3* gene alterations are associated with less favorable outcomes in the context of chemotherapy for advanced disease (38, 39).

The identification of *FGFR3* as an oncogenic driver in urothelial cancer has led to the development of FGFR3-targeting therapeutics [**Table 1**, (40)]. While the dovitinib, which targets FGFR3, among other tyrosine kinases, showed poor single-agent activity in an unselected urothelial cancer patient population, using pan-FGFR inhibitors with greater target affinity in genomically selected populations has proven to be a more promising approach (41, 42). This observation may reflect a compensation of other FGFR isotypes when therapeutics target FGFR3 on its own. The FGFR1-4 inhibitor erdafitinib is the sole FGFR-targeting agent to which the United States Food and Drug Administration has granted regulatory approval to date. Erdafitinib is indicated for patients with *FGFR2* or *FGFR3*-altered, platinum-treated urothelial cancer (43). Infigratinib, a FGFR1-3 inhibitor, has also demonstrated promising activity (44, 45). Rogaratinib, another pan-FGFR inhibitor is under investigation using FGFR1 or FGFR3 RNA expression levels, rather than genetic mutational status, as a patient selection criterion (46). The most common treatment-emergent toxicities among these agents are hyperphosphatemia, stomatitis, diarrhea, elevated creatinine, fatigue, hand-food syndrome, and decreased appetite. Although the FGFR-inhibitors are undoubtedly becoming a valuable component of the oncologist's armamentarium for advanced bladder cancer treatment, a greater understanding is needed of how best to combine and sequence these medications with other therapies in the treatment paradigm.

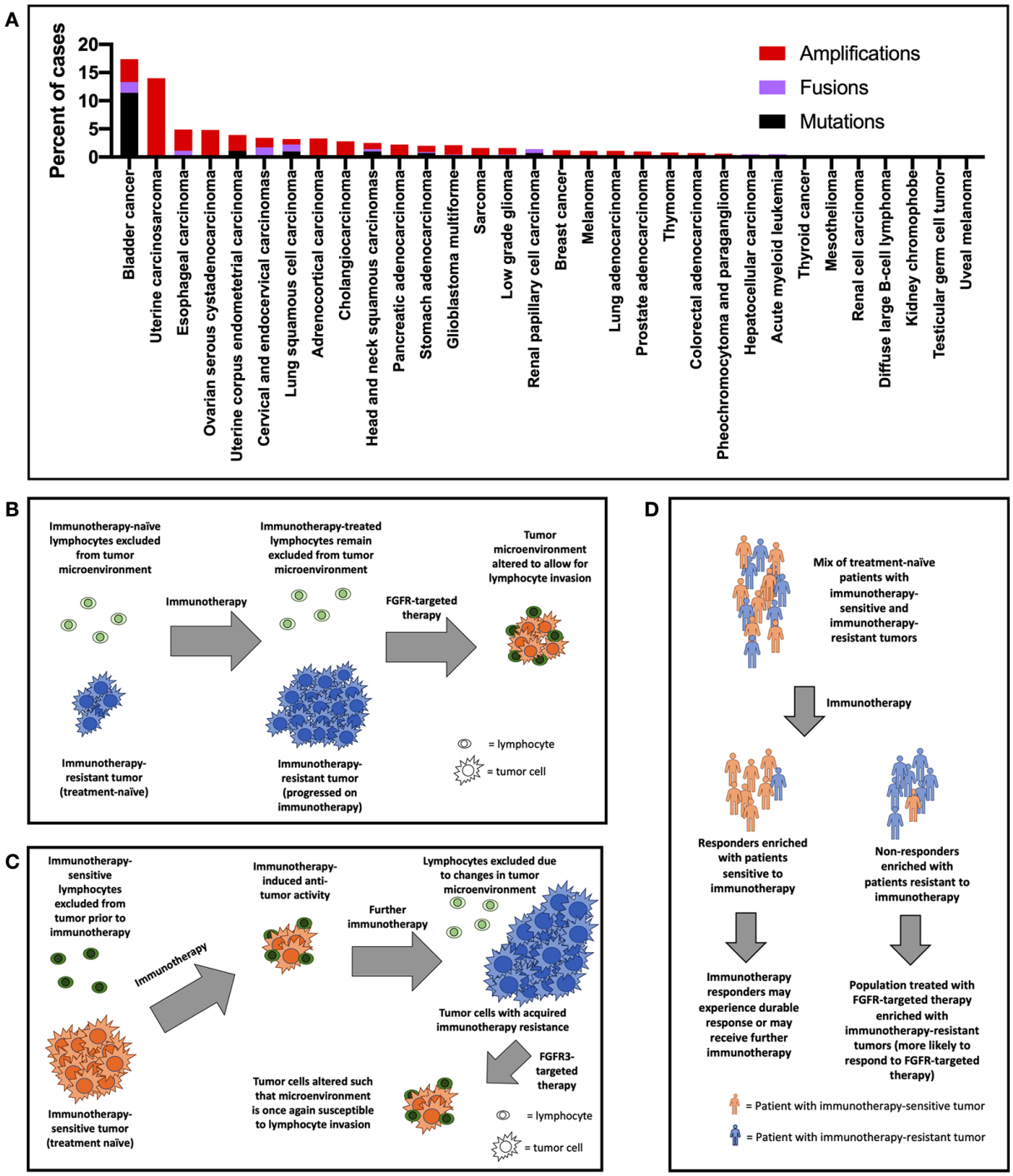


FIGURE 1 | **(A)** *FGFR3* gene alterations by cancer type based on available data from The Cancer Genome Atlas (TCGA) (only recurrent mutations and fusions—those comprising in >1% of mutations/fusions—were included). Potential mechanisms of improved response rate to FGFR3-targeted therapy in the post-immunotherapy setting include **(B)** primary immunotherapy resistance, **(C)** secondary immunotherapy resistance, and **(D)** enrichment of patients with immunotherapy-resistant tumors in trials of FGFR3-targeted therapy.

TABLE 1 | FGFR inhibitors marketed or in development for bladder cancer.

Medication name	Target	Manufacturer	Phase of development	Patient population	Combination	NCT identifier
Erdafitinib (Balversa)	FGFR1-4	Johnson & Johnson	Marketed	FGFR2/3 mutation or fusion	–	NCT02365597
			Ib/II	FGFR2/3 mutation or fusion	Cetrelimab (PD-1 inhibitor)	NCT03473743
Infigratinib (BGJ398)	FGFR1-3	BridgeBio Pharma	III	Adjuvant, FGFR3 altered[1]	–	NCT04197986
			Pilot	Non-muscle invasive, FGFR mutation or fusion	–	NCT02657486
Rogaratinib (BAY 1163877)	FGFR1-4	Bayer	II/III	high FGFR1 or 3 expression	–	NCT03410693
			Ib/II	cisplatin-ineligible, high FGFR1, or three expression	Atezolizumab (PD-L1 inhibitor)	NCT03473756
Pemigatinib (Pemazyre)	FGFR1-3	Incyte	II	FGF or FGFR alteration[2]	–	NCT02872714
			II	platinum ineligible, FGFR3 mutation or rearrangement	Pembrolizumab (PD-1 inhibitor)	NCT04003610
			II	Non-muscle invasive (neoadjuvant)	–	NCT03914794
Derazantinib (ARQ 087)	Pan-FGFR	Basilea	Ib/II	FGFR altered[2]	Atezolizumab (PD-L1 inhibitor)	NCT04045613
Vofatamab (B701)	FGFR3	Rainier Therapeutics	Ib/II		Pembrolizumab (PD-1 inhibitor)	NCT03123055

[1] *"Susceptible" FGFR3 mutations, fusions, or translocations.*
[2] *Definition of "altered" are not specified.*

FGFR3 AS A THERAPEUTIC TARGET AND AS A PATIENT SELECTION TOOL IN CONTEXT OF IMMUNOTHERAPY FOR BLADDER CANCER

The preclinical and correlative literature underpinning the rationale for combining FGFR3-targeted therapy with immunotherapy is substantial. Research in animal models have contributed to an appreciation of the potential synergies between these two mechanisms. Some studies have suggested that FGFR3 has an important role in regulating the innate immune system, including inhibition of interferons and stimulation of tumor necrosis factor-α (47, 48). Others have noted inhibitory effects on a broad range of components of the adaptive immune response, including lymphocyte infiltration, and T-cell CD8A expression, as well as stimulatory effects on the anti-inflammatory TGF-β response signature (13, 49–52). In fact, our previous work has suggested that *FGFR3* mutations and *FGFR3-TACC3* fusions may be associated exclusively with tumors that exhibit a lymphocyte-excluded phenotype. Moreover, the degree of *FGFR3* expression predicts lymphocyte exclusion (13). Wnt/β-catenin signaling, which is associated with non-T-cell-inflamed tumors both in bladder cancers and across most solid cancers, has been shown to overlap with FGFR3 signaling (13, 53–55). In lung cancer models, FGFR3 inhibition enhances the effect of programmed cell death-1 (PD-1) blockade (56). However, evidence that *FGFR3* pathways work in opposition to immune activity is not uniform: *FGFR3* amplifications are associated with decreased anti-inflammatory M2 macrophage bladder tumor infiltration (51). Additionally, some correlative analyses have not detected a difference in ICI response rates among patients with *FGFR3* mutations compared to those with the wild-type allele (52). Additionally, *FGFR3* mutations are associated with lower PD-L1 expression, a marker that has been shown to have some correlation with ICI response in some bladder cancer trials (7, 50).

Investigational approaches studying the most appropriate role for FGFR inhibition in the context of ICI therapy (either through sequencing or combination) are generally in early clinical stages. The most robust experience available are what appear to be post-hoc analyses of FGFR inhibition following ICI therapy. In erdafitinib's pivotal trial, patients who had previously received ICI therapy experienced higher response rates compared with the cohort as a whole (59% vs. 40%) (43, 57). Preliminary data with rogaratinib suggest a similar effect: an interim analysis of its phase I trial demonstrated 30% response among ICI-treated patients compared with 24% across all patients (58). There are several potential reasons for the finding of increased responsiveness to FGFR inhibitors after ICI (**Figures 1B–D**). It may be that previous ICI therapy primes patients for FGFR-targeted therapy – i.e., FGFR inhibition "sensitizes" the tumor to the effects of ICI by altering the microenvironment to allow for lymphocyte invasion (**Figure 1B**). Another related explanation for the clinical trial results is that tumors develop enhanced *FGFR3* pathway (lymphocyte exclusionary) signaling as a resistance mechanism while on immunotherapy. Subsequent FGFR inhibition would disrupt this oncogenic tumoral lymphocyte exclusion (**Figure 1C**). A third possibility is that patients who fail immunotherapy tend to be patients whose tumors exhibit poor lymphocyte exclusion (**Figure 1D**). These may be the exact patients who we might expect to benefit most from FGFR-targeted therapy, which may directly address this immune deficit. These may also be patients whose tumors are driven by mechanisms unrelated to the immune system. Importantly, rogaratinib in combination with atezolizumab for first-line urothelial bladder cancer has now shown an objective response rate of 44% including a 16% complete response rate (59). Future research may provide insight to help identify which of these interpretations (or combination of these interpretations or different interpretation altogether) is most accurate. This research may help us understand to what degree FGFR-targeted therapy is best

considered as a treatment to be sequenced with immunotherapy. Or, alternatively, to what degree patients who will benefit from FGFR-targeted therapies and those who will benefit from immunotherapy represent two distinct categories. Eventual analyses from currently ongoing phase Ib/II trials testing the FGFR inhibitors vofatamab (NCT03123055), erdafitinib (NCT03473743), and rogaratinib (NCT03473756) in combination with ICI therapies in broad (not genetically selected) populations may enhance our ability to evaluate these propositions.

DISCUSSION

The *FGFR3* gene is prevalent in bladder cancers and may hold value as a prognostic marker and as a tool for patient selection. *FGFR3* mutations are associated with less aggressive disease across all bladder cancers, although this is not necessarily the case among advanced tumors. Therapies targeting the FGFR3 protein (and its isoforms) have demonstrated clinical benefit in some patients. However, clinicians still require a greater understanding of how these drugs fit into the treatment paradigm alongside immunotherapies. There is conflicting evidence from preclinical and retrospective correlative studies related to the scientific rationale for combining and/or sequencing FGFR-targeted therapies with immunotherapies. To date, the balance of data suggests that there may be a benefit to combining the two types of approaches. However, an alternate theory is that there may be some patients (perhaps those with tumors termed "immune hot" or "lymphocyte invasive") may be candidates for immunotherapy and not FGFR-targeted therapy, while patients with so-called "immune cold" (or lymphocyte excluded) may be unlikely to benefit from immunotherapy and may be better off with FGFR inhibition earlier on. As FGFR inhibitors become more established in bladder cancer treatment and are studied in earlier lines of therapy, we should gain a more complete view of the best placement of these drugs within therapeutic algorithms.

AUTHOR CONTRIBUTIONS

AK and RS conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This manuscript was supported by the NIH National Cancer Institute award K08CA234392.

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Conflict of Interest: RS reports consulting/honoraria from Aduro, AstraZeneca, BMS, Exelixis, Eisai, Janssen, Mirati, and Puma. Grant/Research support (to institution): AbbVie, Bayer, BMS, CytomX, Eisai, EpiVax Oncology, Evelo, Genentech, Immunocore, Mirati, Merck, Novartis.

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

The handling editor declared a past co-authorship with one of the authors RS.

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frontiers in Immunology

MINI REVIEW
published: 10 November 2020
doi: 10.3389/fimmu.2020.605619

Tumor-Derived Myeloid Cell Chemoattractants and T Cell Exclusion in Pancreatic Cancer

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Like many tumor types, pancreatic ductal adenocarcinoma (PDAC) exhibits a rich network of tumor-derived cytokines and chemokines that drive recruitment of myeloid cells to the tumor microenvironment (TME). These cells, which include tumor-associated macrophages and myeloid derived suppressor cells, block the recruitment and priming of T cells, resulting in T cell exclusion within the TME. Genetic or pharmacologic disruption of this chemokine/cytokine network reliably converts the PDAC TME to a T cell-high phenotype and sensitizes tumors to immunotherapy across multiple preclinical models. Thus, neutralization of tumor-derived chemokines/cytokines or blockade of their respective receptors represents a potentially potent strategy to reverse myeloid immunosuppression in PDAC, enabling benefit from checkpoint inhibition not otherwise achievable in this disease. Inhibition of oncogenic pathways that drive tumor-intrinsic expression of chemoattractants may be similarly effective.

Keywords: myeloid, pancreatic, chemoattractant molecules, T cells, macrophages

OPEN ACCESS

Edited by:
Vassiliki A Boussiotis, Beth Israel Deaconess Medical Center and Harvard Medical School, United States

Reviewed by:
Gregory B. Lesinski, Emory University, United States
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *12 September 2020*
Accepted: *15 October 2020*
Published: *10 November 2020*

Citation:
Vonderheide RH and Bear AS (2020) Tumor-Derived Myeloid Cell Chemoattractants and T Cell Exclusion in Pancreatic Cancer. Front. Immunol. 11:605619. doi: 10.3389/fimmu.2020.605619

INTRODUCTION

A cancer inflammatory reaction dominated by myeloid cells is characteristic of many tumors, especially oncogene-driven invasive adenocarcinomas such as pancreatic ductal adenocarcinoma (PDAC) (1). The immunosuppressive cellular network established by tumor-derived myeloid cell chemoattractants – and the prospect of targeting this network therapeutically – has been increasingly understood, although the initial landmark observations underlying this concept were made by Mantovani and colleagues more than 35 years ago (2).

Experimental scrutiny of genetic mouse models of PDAC has uncovered a network of tumor-derived chemoattractants that promote myeloid cell infiltration from the earliest stages of tumorigenesis, and these observations have been validated to an increasing extent in PDAC patients. Myeloid cells in the tumor microenvironment (TME) block endogenous anti-tumor T cell responses and thwart effective utilization of checkpoint inhibitors. Given multiple redundancies in the myeloid compartment, *in vivo* depletion of myeloid cells in the TME in a clinically relevant fashion has been challenging. Disruption of this chemokine/cytokine network or the respective receptors may be more tractable with neutralizing antibodies or small molecular inhibitors that experimentally lead to loss of myeloid inflammation in the TME. Such strategies have resulted in effector T cell TME infiltration and T cell-dependent tumor regressions that can be further augmented with immunotherapy. Widespread clinical application of this strategy would require

precision profiling of tumors to identify personalized targetable chemokine/cytokines or receptors. A potentially more generalizable and therapeutically effective alternative would be the development of inhibitors of the oncogenes that drive the tumor expression of these chemoattractants, e.g., inhibitors of mutant Kras. This review outlines the role of myeloid chemoattractants in promoting T cell exclusion in PDAC to establish immune privilege as well as suggests opportunities to improve cancer immunotherapy in scenarios where single-agent checkpoint blockade fails.

PDAC RESISTANCE TO CHECKPOINT BLOCKADE

Despite increasing label indications across numerous cancer histologies, antibodies that block CTLA-4, PD-1, and PD-L1 are essentially ineffective in patients with advanced metastatic PDAC, linked mechanistically to a number of tumor-intrinsic and –extrinsic factors in the TME (**Table 1**) (3). Combinations of anti-CTLA-4 and anti-PD-L1 have been equally disappointing (4). One exception are the <1% of all PDAC patients with high microsatellite instability (5), who often can respond to PD-1 checkpoint blockade and for whom pembrolizumab is now FDA-approved. Clinical trials continue to test the combination of checkpoint blockade with chemotherapy in metastatic PDAC, but initial results of gemcitabine, nab-paclitaxel, and nivolumab are discouraging. Thus, PDAC represents one of the most refractory tumors to currently approved checkpoint therapies, a particularly disappointing situation given the dire unmet medical need for a cancer that kills more individuals now than breast cancer and is predicted to become the second-leading cause of cancer death by 2030 (6).

Numerous preclinical studies predicted the poor clinical activity of checkpoint inhibitors in PDAC patients. In the "KPC" genetically engineered mouse model of PDAC (in which mutant Kras and p53 are targeted for expression in the pancreas, resulting in a high fidelity model of the disease), there is no anti-tumor response in spontaneous tumors to single (CTLA-4, PD-1, PD-L1) or combination (CTLA-4 plus PD-1/PD-L1) immune checkpoint blockade (7, 8). In subcutaneous or orthotopic implantable models using KPC-derived, syngeneic tumor cell lines, responses to checkpoint blockade are only rarely observed (7). Classically, KPC tumors exhibit poor T cell infiltration and very low tumor mutational burden (TMB) that translates into few if any neo-epitopes (9–11). In other pancreatic models, where the TMB is higher, responses to checkpoint inhibition are observed at somewhat greater rates (12). These latter observations in non-KPC PDAC tumor models provide a rationale for the clinical evaluation of checkpoint blockade in PDAC patients. Despite the majority of human PDAC having low to very low T cell infiltration and TMB, about 20% of patients do exhibit T cell infiltration and a relatively elevated TMB, although there is no correlation between high T cell infiltration and high TMB (or neo-epitope burden) (13). The objective response rates in PDAC patients with single or dual checkpoint inhibition is far less than 20%; thus, neither T cell infiltration nor TMB serve as adequate predictive biomarkers of response in PDAC. Furthermore, PD-L1 expression as determined by RNA sequencing of primary PDAC tumors is among the lowest for any of other well-described immune checkpoint molecules including CTLA-4, VISTA, TIM3, TIGIT, LAG3, ADORA2A, or IDO1 (13). Moreover, there is no difference in PD-L1 expression among T cell-infiltrated vs. non T cell-infiltrated human PDAC, in contrast to the other checkpoint molecules such as CTLA-4 for which expression is significantly higher in T cell-infiltrated tumors (13). These findings raise the hypothesis that PD-L1 does not serve as the critical immune checkpoint that drives immunosuppression in PDA, consistent with the observation that the addition of nivolumab to a promising cancer vaccine in advanced PDAC patients fails to improve overall survival (14).

TABLE 1 | Obstacles in pancreatic cancer limiting utility of checkpoint blockade.

Category	Factor
Tumor intrinsic	Relatively low tumor mutational burden and neo-epitopes
	Low tumor PD-L1 expression
	Low rate of MSI high tumors (<1%)
T cell response	Relatively low T cell infiltration in most tumors
	Minimal baseline T cell priming against the tumor
Stroma	Dense stroma limiting drug delivery to TME
	Large inhibitory myeloid cell population
	Inhibitory cancer-associated fibroblasts

STRATEGIES TO SENSITIZE PDAC TO CHECKPOINT BLOCKADE

Two primary strategies have been explored to sensitize PDAC patients to checkpoint inhibition (3). The first strategy hypothesizes that PDAC patients exhibit deficient T cell priming and a T cell response must first be mobilized to achieve efficacy with checkpoint inhibition (15). Immune priming strategies explored in combination with immune checkpoint inhibition include chemotherapy or radiation to induce immunogenic tumor cell death or the use of a cancer vaccine. This approach is highly effective in the KPC and other PDAC mouse models (8, 16, 17). To date, in PDAC patients, the combination of chemotherapy or cancer vaccines with PD-1 has not shown synergy. In our experience using the KPC model, the addition of agonistic CD40 antibody, aimed at licensing dendritic cells to activate anti-tumor T cells, has been the single most potent method to sensitize tumor-bearing mice to PD-1, CTLA-4, or combination immune checkpoint blockade – as has been recently reviewed (18). An ongoing national, randomized study is currently evaluating chemotherapy with or without agonistic CD40 mAb, with or without nivolumab, in first-line metastatic PDAC patients, based on promising phase 1b results with chemotherapy and CD40 mAb in the same patient population (NCT03214250). Other immune agonists such as those against stimulator of interferon genes (STING) or toll-like receptors (TLRs) represent additional approaches, as recently reviewed (3).

The blockade of novel checkpoint molecules represent the second strategy to sensitize PDAC tumors to PD-1/PD-L1 inhibition. Many of these novel checkpoints are highly expressed in PDAC and as noted above, these molecules increase in expression in the TME of tumors with higher levels of infiltrating T cells (13). However, evidence for single-agent activity of antibodies targeting these novel checkpoints in PDAC to date been minimal or modest, although preclinical data with select inhibitors (e.g. VISTA) are compelling (19, 20). However, as a sobering reminder, no novel checkpoint inhibitors have been approved by the FDA in oncology since the initial approvals of anti-CTLA-4 and anti-PD-1 aside from variations of PD-1/PD-L1 and combinations with anti-CTLA-4. As a telling example, the novel checkpoint inhibitor epacadostat (an IDO1 inhibitor) failed in combination with pembrolizumab in a randomized study in patients with advanced melanoma (21) despite compelling preclinical data in mice.

THE MYELOID CHECKPOINT IN PDAC

Leukocytes dominate the PDAC microenvironment and among these, myeloid cells are typically the most over-represented, contributing to the well-described picture of cancer inflammation and desmoplastic reaction in this disease (9). This phenotype is reproduced in the spontaneous KPC model, in which infiltration by macrophages and myeloid-derived suppressor cells (MDSC) is evident in neoplastic lesions even before tumor cell invasion (9). The immunosuppressive effect of MDSCs on T cells in particular is striking in the KPC model and can be demonstrated both *ex vivo* and *in vivo* (22, 23). In both the KPC model and human PDAC, there appears to be an inverse relationship between myeloid infiltration and effector T cell infiltration (13). Thus, the question remains - is there a causal relationship between myeloid infiltration and effector T cell paucity? In the KPC model, it has been difficult to discern pharmacologically if myeloid cells are obstructing T cell infiltration because methods to eliminate myeloid cells *in vivo* are at best incomplete and transient (8). An alternative approach is to activate myeloid cells and re-educate (rather than deplete) them away from tumor-promoting activities. Such activation can be accomplished with agonistic CD40 antibody in the KPC model (24). With chemotherapy or radiation therapy, CD40 antibody can produce T-cell dependent tumor regressions (8, 16). Agonism of myeloid cell CD11b also repolarizes tumor-associated macrophages (TAM), reduces intratumoral myeloid cells, and leads to anti-tumor immunity in concert with checkpoint inhibition (25).

Myeloid cells in the TME are highly heterogeneous, and novel techniques such as ultra-high multiplex flow cytometry and single cell sequencing have unearthed significant complexities (26–28) which presents challenges for nomenclature systems to capture (29). Current views of myeloid heterogeneity extend far beyond designations of M1 vs M2 (or N1 vs N2); however, understanding the range of functionalities of various myeloid cells in the TME as either anti-tumor vs pro-tumor has been helpful conceptually. The rich mechanisms and dynamics that regulate myeloid cells and mechanisms of immunosuppression have been reviewed elsewhere (28, 30, 31).

Further indication that myeloid cells represent a major immune checkpoint in PDAC comes from studies in which individual cancer cells from spontaneous KPC tumors were cloned, re-implanted in syngeneic hosts, and upon tumor outgrowth harvested and inspected for T cell and myeloid cell infiltration (32). Although T cell-high tumors are unusual among spontaneous KPC tumors, KPC clones upon re-implantation are frequently T cell-high in this experiment. Interestingly, T cell-high tumors feature unusually poor myeloid infiltration, reproducing the human phenotype. In mixing studies administering T cell-low and T cell-high KPC clones, the T cell-low phenotype is dominant as outgrowing tumors are T cell-low and myeloid-rich (32). T cell-high tumors themselves are strikingly sensitive to anti-PD-1 and anti-CTLA-4 checkpoint therapy, even in the absence of CD40 agonism or chemotherapy (32). On the contrary, T cell-low tumors are refractory to anti-PD-1/anti-CTLA-4 recapitulating observations in the treatment of spontaneous KPC tumors.

MYELOID CHEMOATTRACTANTS IN PDAC

Observations of T cell-high and T cell-low KPC clonal mixing studies led to the hypothesis that a local chemo-attractant factor elaborated by T cell-low tumor clones drives recruitment of myeloid cells to the TME. These myeloid cells in turn inhibit T cells and block their recruitment (**Figure 1**). In addition to the inhibition of T cell recruitment and effector function, myeloid cells may also produce factors that hinder dendritic cells in the TME, thereby preventing effective priming of anti-tumor effector T cell responses. For example, IL-6 has been shown to have such antagonistic effects on dendritic cells in PDAC (33). Consistent with this hypothesis, numerous studies have identified and characterized tumor-intrinsic pro-myeloid cytokines that regulate T cell immunosuppression in mouse models of PDAC. Examples include GM-CSF (22, 23), CXCR2 ligands (32, 34–36), CSF3 (32), CCR2 ligands (37), and CSF1 (38–40), as detailed below. Despite wide variation among cell lines as to which tumor-derived cytokine most prominently drives a myeloid-rich TME, precise ablation of a single, dominant cytokine for any given cell line reliably leads to T cell influx and immunotherapy responsiveness. In each case, the tumor cells themselves (albeit, not necessarily only the tumor cells) elaborate the cytokine or chemokine which are downstream products of either oncogenic mutant Kras or other driver pathways. Thus, oncogenic pathways can also enforce a myeloid-rich TME in addition to promoting oncogenic survival, proliferation and invasion (41). Rapid myeloid domination of the TME occurs from the earliest stages of tumor inception (9). Lineage tracing in the KPC model which permits the identification of metastatic

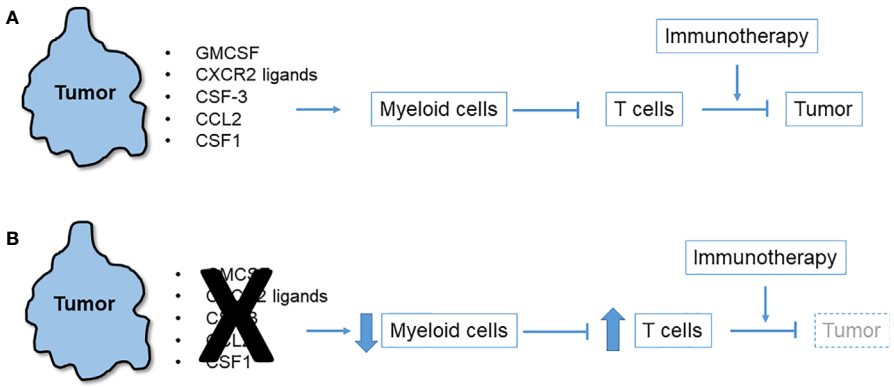


FIGURE 1 | Immunosuppressive network of tumor-derived myeloid cell chemoattractants. **(A)** Multiple chemokines and cytokines released by pancreatic tumor cells trigger influx of myeloid cells to the tumor microenvironment (TME) that in turn suppress T cells that could otherwise attack the tumor or be induced to do so with immunotherapy. **(B)** Blockade or neutralization of tumor-derived chemoattractants in numerous mouse models leads to diminution of myeloid cells in the TME, an upsurge in infiltrating T cells, and tumor regression especially after immunotherapy.

tumor cells as isolated singlets in liver and lung identifies macrophages as accompanying lone tumor cells (42).

GM-CSF

This growth factor is commonly expressed by tumor cells in both KPC mice and humans, even in early lesions (23). GM-CSF drives local proliferation of MDSC, which progressively accumulate in the PDAC microenvironment. When GM-CSF is genetically deleted from tumor cells in mice, T cell influx is triggered and tumors are rejected, but only in mice that are replete of CD8+ T cells (22, 23). GM-CSF is paradoxically understood to be a vaccine adjuvant and key component of promising pancreatic cancer vaccines (43), but as opposed to the low concentrations used in a subdermal vaccine, high concentrations of GM-CSF within the TME are immunosuppressive owing to effects on MDSC recruitment. In PDAC cells, GM-CSF production is downstream from mutant Kras signaling, linking immunosuppression to the driving oncogene (23).

CXCR2 LIGANDS

CXCR2 ligands regulate myeloid trafficking into tumor cells. In humans with PDAC, high expression of CXCR2 is correlated with enrichment of intra-tumoral neutrophils (34). In the KPC model, CXCR2 blockade by genetic or pharmacologic means reduces recruitment of myeloid cells into the PDAC microenvironment (especially neutrophils), permitting T-cell dependent suppression of tumor growth, an effect which can be augmented by PD-1 inhibition to improve survival (34, 35). Although CXCR2 is highly expressed by cells in the tumor stroma, tumor expression of CXCR2 has also been observed in various genetic models and may drive autocrine or paracrine growth (44, 45). In KPC experiments, CXCL5 is the most prominent CXCR2 ligand produced by tumor cells, whereas stromal cells produce CXCL2 (34). Expression of tumor-derived CXCL5 is associated with mutant Kras expression and regulated by tumor NF-kB activation. In comparing T cell-high vs T cell-low KPC clones, CXCL1 – another CXCR2 ligand – is found to be the most differentially expressed (32). In these studies, CXCL2 and CXCL5, although known to be expressed in PDAC genetic models (45), are not differentially expressed. Genetic ablation of CXCL1 in T cell-low KPC clones abrogates the influx of myeloid cells in the TME, enabling infiltration by CD8+ T cells and rendering tumors universally responsive to anti-CTLA-4, anti-PD-1, and CD40 combination therapy (32). In contrast, overexpression of CXCL1 in T cell-high tumors reverses this phenotype and blunts response to immunotherapy. Epigenetic variations acting in concert with MYC in T cell-low vs T cell-high tumors underlie the differential regulation of CXCL1 (32). CXCL1 expression in the KPC system also depends on the necrosome, and in a study of KPC orthotopic tumors, RIP-1/RIP-3 driven necroptosis upregulates tumor-derived CXCL1 production and enhances peritumoral MDSC infiltration (36). RIP deletion reduces MDSC and triggers an influx of T cells and subsequent tumor regression. CXCL1 blockade similarly reduces MDSC in the tumors (36). RIP3 deletion is not, however, protective in B16 melanoma or subcutaneously implanted KPC tumors, indicating needed caution in explanatory models so as not to mistakenly imply that a single chemokine pathway is applicable across models or histologies.

CSF3

Several studies also identify tumor-derived CSF3 (also known as G-CSF) as a cytokine that recruits myeloid cells to the TME, and CSF3 is associated with T cell inhibition and desensitization of PDAC tumors to immunotherapy (22, 32, 45).

CCL2

Also known as MCP-1, CCL2 is a well-known tumor-derived macrophage chemoattractant in the TME (46, 47). Both human PDAC and KPC tumor cells express high levels of CCL2, although normal cells also express this chemokine (22, 37, 48). The elaboration of CCL2 in the TME results in mobilization of CCR2+ monocytes that are immunosuppressive. Although CCR2 inhibitors show promise in depleting TAMS *in vivo* in patients (37, 49), a compensatory mechanism of CXCR2+ neutrophils frustrates anti-tumor efficacy. The combination of both CCR2 and CXCR2 inhibitors in KPC mice prevents this compensatory reaction and results in significantly smaller tumors and improved survival, an effect that can be further enhanced with chemotherapy (50). As with other chemoattractants, the mechanism appears to be tumor-intrinsic. In KPC tumors, the epigenetic regulator HDAC5 inhibits Socs3, a negative regulator of CCL2, promoting CCL2 secretion and the recruitment of tumor-promoting macrophages to the TME (51).

CSF1

Also known as M-CSF, CSF1 is commonly highly expressed by mutant Kras engineered mouse tumors (22, 38) and human PDAC cells. Inhibition of CSF1 or CSF1-R using blocking antibodies or small molecule inhibitors leads to the selective depletion of TAMs in the TME in pancreatic mouse tumor models (38, 39) and promotes tumor regression in combination with anti-PD-1 therapy with or without cancer vaccination (38, 40).

OTHER MYELOID CELL REGULATORS IN THE PDAC TME

Non-cytokine, tumor-intrinsic mechanisms of myeloid cell regulation are also active in PDAC mouse models. The tyrosine kinase EPHA2, for example, is markedly overexpressed in human and murine PDAC and functions to enforce a myeloid-rich, T cell-low phenotype in the TME (52). Knock-out of EPHA2 in tumor cells reverses T cell exclusion, dampens myeloid cell infiltration and sensitizes PDAC tumors to T cell-dependent rejection in response to immunotherapy. Interestingly, prostaglandin endoperoxide synthase 2, the gene encoding COX2, is downstream of EPHA2 and its deletion in KPC mice also reverses T cell exclusion and sensitizes tumors to immunotherapy (52). This genetic phenotype can be largely reproduced with COX2 inhibitors in concert with immunotherapy, revealing a potentially tractable clinically translatable strategy. Another key interaction in the pancreatic cancer TME is the interaction between cancer-associated fibroblasts (CAFs) and stellate cells and myeloid cells. CAFs mold the extracellular matrix (ECM) and provide survival and migration signals to cancer cells, and hinder drug delivery and potentially effector T cell infiltration (53, 54). CAFs themselves secrete cytokines and chemokines and can regulate the immune cell milieu, recruit immune-suppressive cells, and inhibit effector cells (55, 56). There is great phenotypic and functional heterogeneity in PDAC CAFs, including one subpopulation with antigen-presentation function (57). These findings provide new therapeutic opportunities, e.g., administration of vitamin D receptor ligand alters inflammation and fibrosis in pancreatitis and tumor stroma (58).

TARGETING MYELOID CHEMOATTRACTANTS FOR IMMUNOTHERAPY

As illustrated above, tumor-intrinsic chemoattractants recruit myeloid cells into the PDAC TME, block T cell priming, infiltration and effector function, and enable tumor immune escape and growth (**Figure 1**). Importantly, this myeloid network is operative early in neoplastic development, often before invasion – a pathological stage well-characterized in the spontaneous KPC model. Importantly, the tumor promoting effects of myeloid cells are reversible as robust depletion of myeloid cells accomplished with an engineered toxin prevents both initiation and growth of mutant Kras-driven PDAC in mice (59).

Given T cell exclusion is an early and robust feature of PDAC, at least in the KPC model, the paradigm of immune editing is to be reconsidered because there is no or little Darwinian pressure from a T cell attack from which tumors must escape (11). Such a paradigm paradoxically implies tumors remain sensitive to T cells following progression from non-invasive to invasive cancer should the barrier of myeloid immunosuppression be removed (15). The lack of immune editing perhaps explains why PD-L1, upregulated in response to IFN-gamma from T cells, is expressed at low levels in the majority of PDAC tumors (13). In PDAC patients, unleashed T cell immunity may actually be quite powerful as evidenced by the correlation between a PD-L1^{low}/CD8^{high} tumor sub-phenotype and positive prognosis in PDAC (60) as well as improved overall survival in the few resectable patients who naturally develop strong T cell immunity to PDAC compared to patients in whom T responses are minimal to absent (61).

How can the myeloid checkpoint be exploited to unleash anti-tumor T cells of clinical significance? One approach is to eliminate TAMs and other myeloid cells directly (50). In KPC mice, eight methodologies – ranging from clodronate to antibodies to combinations – fail to deeply or durably deplete TAMs (even when *systemic* myeloid cells are successfully depleted in some cases) (8). These disappointing results in mice include the use of antibodies to CSF-1 or CSF-1R. Initial efforts in patients with similar approaches are underway.

A second approach would be to use antibodies that neutralize the dominant cytokine driving myeloid cell accumulation in the TME or the receptor to which it binds. As noted above, this may vary model-to-model, or in the clinic, patient-by-patient – requiring precision immune profiling to select the optimal neutralizing antibody or antibodies. Anti-cytokine antibodies

are increasingly among FDA approved drugs for inflammatory conditions. Among clinical-grade but still experimental antibodies, anti-GM-CSF or anti-CSF1-R antibodies would be logical and reasonable in PDAC patients whose tumors express these cytokines. However, because some cytokines also play other roles in *promoting* immunity including against pathogens (e.g., GM-CSF and CXCL1), cytokine neutralization represents a potential double-edged sword and must be carefully considered.

A third strategy would be to inhibit the cancer-promoting pathway or oncogene that is driving the cytokine or chemokine production, representing a more proximal and potentially more tumor-specific approach. One example would be to inhibit mutant Kras. Mutant Kras has long been an elusive oncologic target, but recently novel Kras inhibitors are providing new hope toward this possibility (62), although the applicability of KrasG12C inhibitors to PDAC patients is largely limited by the rare prevalence of this mutation in this patient population. Nevertheless, inhibition of mutant Kras or its downstream signaling pathways such PI3K may block cytokine production thereby decreasing myeloid cell accumulation in the TME enabling T cell infiltration and responsiveness to immunotherapy. It is also possible that such inhibition might, as an added benefit, also block PD-L1 expression on PDAC cells themselves (63).

There are of course other features of the crosstalk between tumor cells, myeloid cells, and other elements of the stroma that may be therapeutically targetable, as recently reviewed (28, 64, 65). These approaches have been discussed elsewhere and include blockade of CD47 (66) or FAK1 (67) on tumor cells, as well as PD-L1 (7) or TREM2 on TAMs (68). Of interest, TREM2 on TAMs is upregulated by GM-CSF and CSF-1 in certain models (69).

Finally, tumor-derived cytokines represent just one of a growing number of examples of tumor-intrinsic mechanisms of immune suppression. Examples are well-described elsewhere (70–72) and represent immune checkpoints beyond CTLA-4 and PD-1 just like the chemoattractants described here.

CONCLUSIONS

Preclinical studies using mouse models of PDAC uncover a rich network of tumor-derived cytokines and chemokines that drive the recruitment of myeloid cells to the TME, including TAMs and MDSCs. These cells block the influx and priming of T cells, contributing to T cell exclusion. Genetic or pharmacologic disruption of this chemokine/cytokine network converts the TME to T cell-high and sensitizes tumors to immunotherapy. Thus, neutralization of such tumor-derived factors or their receptors – or potentially inhibiting the tumor-intrinsic pathways that drive their production – represents a strategy to address the "myeloid immune checkpoint" not only in PDAC but also potentially other tumor types. Efforts to test this hypothesis in patients remain nascent.

AUTHOR CONTRIBUTIONS

RV conceived the project, and both authors wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Support from NCI grants R01 CA217176, P01 CA210944, K12 CA076931, P30 CA016520, and the Parker Institute for Cancer Immunotherapy.

ACKNOWLEDGMENTS

The author acknowledges valuable discussions with Drs. Katelyn Byrne, Jinyang Li, Nune Markosyan, and Ben Stanger.

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Conflict of Interest: RV reports having received consulting fees or honoraria from Celldex, Lilly, Medimmune, and Verastem; and research funding from Apexigen, Fibrogen, Inovio, Janssen, and Lilly. He is an inventor on licensed patents relating to cancer cellular immunotherapy and receives royalties from Children's Hospital Boston for a licensed research-only monoclonal antibody.

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

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REVIEW
published: 14 December 2020
doi: 10.3389/fimmu.2020.594609

Cytokine and Chemokine Signals of T-Cell Exclusion in Tumors

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The success of cancer immunotherapy in solid tumors depends on a sufficient distribution of effector T cells into malignant lesions. However, immune-cold tumors utilize many T-cell exclusion mechanisms to resist immunotherapy. T cells have to go through three steps to fight against tumors: trafficking to the tumor core, surviving and expanding, and maintaining the memory phenotype for long-lasting responses. Cytokines and chemokines play critical roles in modulating the recruitment of T cells and the overall cellular compositions of the tumor microenvironment. Manipulating the cytokine or chemokine environment has brought success in preclinical models and early-stage clinical trials. However, depending on the immune context, the same cytokine or chemokine signals may exhibit either antitumor or protumor activities and induce unwanted side effects. Therefore, a comprehensive understanding of the cytokine and chemokine signals is the premise of overcoming T-cell exclusion for effective and innovative anti-cancer therapies.

Keywords: T-cell exclusion, cell therapy, immunotherapy, cytokine, chemokine

OPEN ACCESS

Edited by:
Jason J. Luke, University of Pittsburgh Medical Center, United States

Reviewed by:
Gregory B. Lesinski, Emory University, United States
Lubor Borsig, University of Zurich, Switzerland

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *13 August 2020*
Accepted: *30 October 2020*
Published: *14 December 2020*

Citation:
Zhang Y, Guan X-y and Jiang P (2020) Cytokine and Chemokine Signals of T-Cell Exclusion in Tumors. Front. Immunol. 11:594609. doi: 10.3389/fimmu.2020.594609

INTRODUCTION

Cancer immunotherapy aims to treat malignancies by leveraging the human immune system's potential, especially cytotoxic T cells that target tumor-specific antigens. However, T-cell exclusion in tumors presents a significant factor for the adverse outcome of cancer patients and the resistance to cancer immunotherapies (1, 2). CD8 T cells and subgroups of CD4 helper T cells are primary contributors to antitumor immunity (3). Activated T cells need to penetrate the tumor core for their cytotoxic activity. Meanwhile, the infiltrating T cells need to survive, proliferate, and keep active in the hostile tumor microenvironment (TME). Cold tumors, characterized by a lack of effector T cells, can exclude T cells through many mechanisms, such as lack of tumor antigens, defect in antigen presentation, absence of T-cell activation, and the deficit of trafficking signals toward the tumor core (4).

Abbreviations: ICB, immune checkpoint blockade; TME, tumor microenvironment; CRS, cytokine release syndrome; HEV, high endothelial venule; TLS, tertiary lymphoid structure; AICD, activation-induced cell death; EMT, epithelial-mesenchymal transition; ACT, adoptive cell therapy; BiTE, bispecific T-cell; TIL, tumor-infiltrating lymphocyte; DC, Dendritic cell; TSCM, T stem-cell memory cell; TCM, T central memory cell; TEM, T effector memory cell; Th1, T helper type 1; Th2, T helper type 2; Th17, T helper type 17; Tfh, follicular helper T cell; Treg, T regulatory lymphocyte; MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophage; CAR, Chimeric Antigen Receptor; CAF, cancer-associated fibroblast.

Cytokines are small soluble proteins released by the malignant, stromal, and immune cells in the TME. Upon binding to their cognate receptors and triggering the intracellular pathway, cytokines can regulate the growth, apoptosis, activation, and differentiation of target cells (5). Chemokines, a subcategory of cytokines, provide the chemotactic signals for immune cell trafficking to specific destinations (6). Studies have reported significant correlations between the concentration of cytokines and chemokines with the prognosis of cancer patients (7–9).

This review will discuss different cytokines and chemokines that influence effector T-cell exclusion, focusing on T-cell trafficking, survival, and differentiation. We will also summarize cytokine-related therapies to promote T-cell infiltration and enhance antitumor responses. Elucidating the signaling mechanisms in T-cell exclusion and cytokine-mediated strategies to enhance the abundance of effector T cells at tumor sites is of great importance to the development of cancer immunotherapies.

T-CELL TRAFFICKING: T CELLS NEED TO PENETRATE THE BATTLEFIELD

A crucial factor for T-cell antitumor activity is the capabilities of specific and efficient trafficking. Upon primed and activated by antigen-presenting cells such as macrophages and dendritic cells (DC) in tumor-draining lymph nodes, T cells will migrate to tumor sites, exerting antigen-specific cytolytic functions. T-cell trafficking is a dynamic process involving rolling, tethering on the vascular endothelium (10), adhesion, extravasation, and chemotaxis (11) (**Figure 1A**).

Signals for T Cells Crossing the Tumor Vasculature

The activated T cells will first gain the expression of homing molecules, including ligands for E- and P-selectin, which enable the rolling of T cells on the tumor vessel endothelium (12). Activated T cells also gain the expression of leukocyte adhesion protein LFA-1 (ITGAL) and VLA-4 (ITGA4), which bind to the intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) on endothelial cells (11). Then, the T cells may transmigrate through the tumor vessel in response to various environmental stimuli. Cytokines such as IL6 and TNFa can enhance the endothelium adhesion activity by promoting adhesion molecules' expression on tumor vessels (13, 14) (**Table 1**). One study demonstrated that IL6 signaling activated by systemic thermal therapy in a mouse model with the ovalbumin-expressing B16 tumor significantly upregulated E/P-selectin and ICAM1, which enhanced the CD8+ T-cell trafficking (13).

Besides the positive signals discussed above, there are negative signals that block effector T-cell adherence to the tumor endothelium and prevent the T cell recruitment to tumor sites (**Table 2**). The phenomenon is termed as endothelial cell anergy. For example, the angiogenic factors vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) can cause endothelial cell anergy by repressing the expression of adhesion molecules ICAM1 and VCAM1 on the endothelium (76). Meanwhile, high levels of growth factors, such as VEGF, PDGFC (74), and PGF (75), can induce immature tumor angiogenesis. The aberrant vascular permeability and irregular blood flow that come with tumor vessels will cause inefficient effector T cells extravasation.

These observations have suggested therapeutic approaches by targeting proangiogenic molecules. Anti-VEGF therapy produced synergistic antitumor responses with immune checkpoint blockade in phase I clinical trials of many cancer types (77). Also, anti-VEGF therapy resulted in significant clinical efficacy when combined with Chimeric Antigen Receptor (CAR)-T therapies by increasing tumor infiltration in humans (78). However, it is essential to note that the administration dose of anti-VEGF must be carefully considered. A low concentration of therapy could efficiently normalize tumor vasculature and enhance T-cell perfusion (79). However, a high dose of VEGF inhibitor will induce tumor-promoting hypoxic microenvironment by blocking the formation of tumor capillaries and causing insufficient oxygen supply (78, 80).

Notably, positive signals of T-cell extravasation may act as negative signals for T-cell recruitment in a context-specific manner. For example, tumor necrosis factor (TNF), which enhances T-cell adhesion molecules, may impair the effector T-cell infiltration by negatively regulating the formation of high endothelial venules (HEV) (81). HEVs are uniquely organized vessels in proximity to tumor sites and tightly associated with tertiary lymphoid structures (TLS). Tumors with HEV and TLS have a high density of effector T cells infiltration and favorable clinical outcome (82,83).

Chemotaxis for T Cells Trafficking to Tumor Core

After extravasation, T cells need guidance from environmental stimuli to arrive at tumor sites. This process is chemotaxis. The expression of chemokine CXC ligand (CXCL) 9, 10, and 11, secreted by tumor and stromal cells, are highly correlated to T-cell abundance in tumors of melanoma (84), lung (84, 85), and colorectal cancer (86). Moreover, high expression of CXCR3 ligands is significantly related to prolonged overall survival rate in cancer patients (87). Exploiting the therapeutic potential of CXCR3 ligands for antitumor T-cell recruitment has been successful in multiple preclinical models (23–26).

Notably, these CXCR3 ligands are all induced by interferon-γ, the effector cytokine secreted by cytotoxic T cells. However, intratumoral interferon-γ injection, which indeed increased the concentration of CXCL10 and CXCL11 in tumors, failed to induce the recruitment of effector T cells to human melanoma metastases in a clinical trial (88). Interferon-γ may upregulate the expression of PD-L1 and IDO1, leading to T-cell exclusion (89). A high level of Interferon-γ might compromise the combined anti-CTLA-4 and anti-PD-1 therapy in tumors with low tumor burden because Interferon-γ induced the apoptosis of activated antigen-specific T cells (90).

Besides CXCR3 ligands, other chemokines, such as CCL5, CXCL16, CCL21, and CCL27, may positively correlate with effector T-cell density within tumors (27, 37, 91). Recent studies showed that CCL5 may selectively mediate CD4 not CD8 effector T

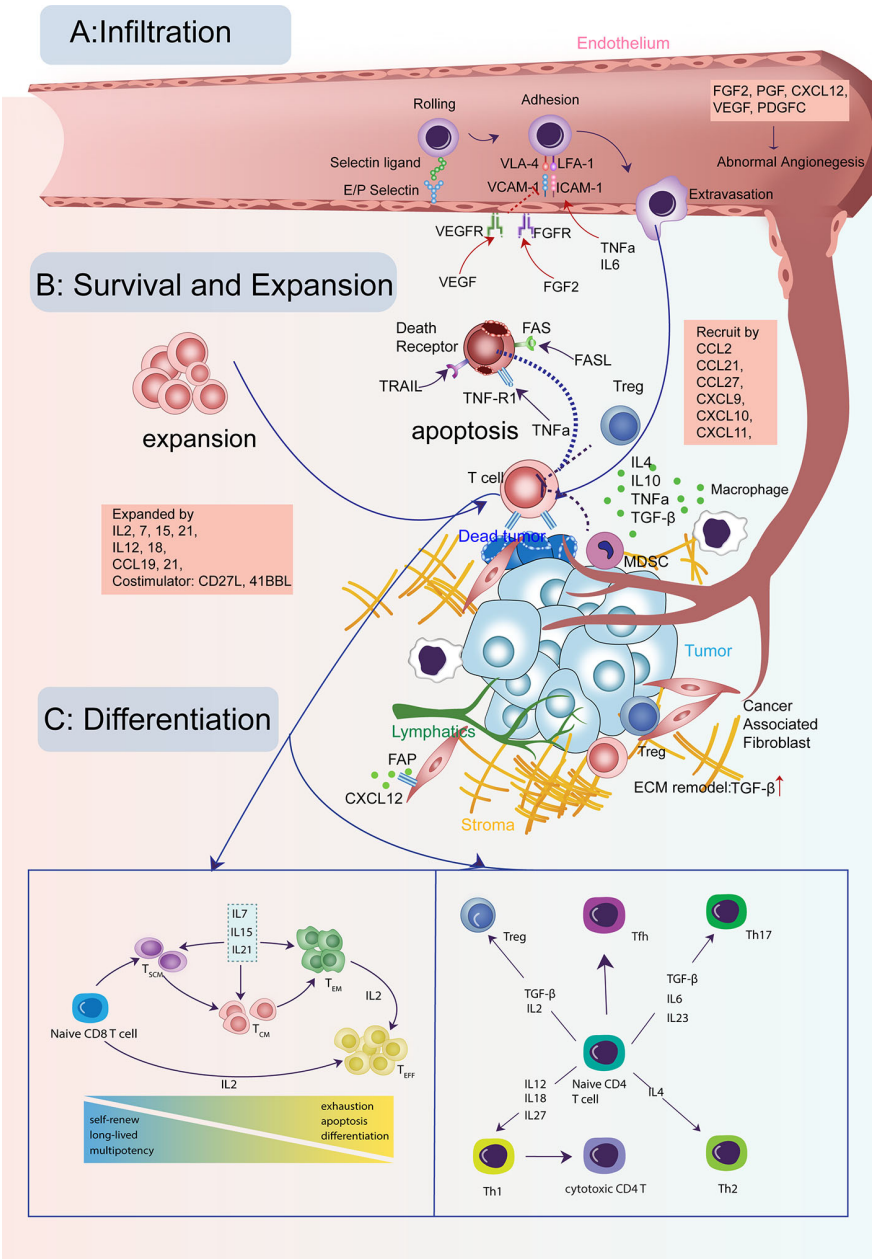


FIGURE 1 | Cytokine signals in the life-span of T cells in tumors. **(A)** T cells need to infiltrate into the tumor for their cytotoxic activity. This process is guided through a set of cytokine, chemokine, and growth factor signals for T cells crossing the tumor vasculature and trafficking to tumor cores. **(B)** The infiltrating T cells need to survive and expand for sufficient numbers with the help from many cytokines and avoid the apoptosis induced by other signals. **(C)** Both CD8 and CD4 T cells need to keep active with the right differentiation path for its long-lasting tumor-fighting capability, modulated by many cytokines and growth factors.

cells infiltration and enhance the therapeutic efficacy of checkpoint blockade in a mouse model of pancreatic cancer (92). However, the role of CCL5 on T-cell recruitment seems to be context-dependent. Another study showed that CCL5 upregulation is positively correlated with CD8 lymphocytes recruitment in the lung adenocarcinoma model (93). CCL27 is expressed in epidermal keratinocytes and mediates CCR10+T-cell homing to cutaneous tumors (40). One study employed an adenoviral vector to deliver chemokine CCL27 into tumors in a mouse model of B16BL6 melanoma and observed considerably increased recruitment of T cells (41) (**Figure 2A**).

Negative Signals of T-Cell Trafficking Into Tumors

Although several chemokines are positive signals of T-cell infiltration as summarized above, the tumor microenvironment is

TABLE 1 | Positive signals of T-cell infiltration.

Signal/Receptor	Producer/Target	Mechanisms	Therapy
IFNG/IFNGR1 +IFNGR2	Cytotoxic T cells/endothelial cell, fibroblasts, tumor cell, monocyte	Interferon-γ induces CXCR3 ligands (CXCL9,10,11), thus enhancing the CXCR3-mediated T-cell recruitment (15).	Intravesical instillation of recombinant interferon-γ inhibits the recurrence of bladder tumors in patients (16).
(fn. 1) IL6/IL6R +IL6ST	T cell, macrophage, endothelial cell, epithelial cell/ endothelial cell	IL6 trans-signaling enhances both E- and P-selectin interactions and ICAM1 dependent T-cell transmigration on tumor vessels (17).	IL6-rich tumor microenvironment provided by systemic thermal therapy improves cytotoxic T cells' delivery to tumor lesions in mouse tumors and patient tumor explants (13).
IL12A+IL12B/ IL12RB1 + IL12RB2	Phagocytic macrophage, DC/ MDSC	IL12 may attenuate the impaired T-cell trafficking mediated by MDSCs by decreasing the percentage of MDSCs in tumors (18).	Treatment of IL12 significantly altered the phenotype and suppressive function of MDSC in mice (18).
(fn. 2) TNF/ITGAV	Lymphoid, mast, endothelial, fibroblast, tumor cell/ endothelial cell (19).	TNF stimulation induces ICAM1 and VCAM1 expression on endothelial cells for T-cell extravasation (14).	The systemic administration of TNF had severe toxicity (20). The fusion of TNF and ITGAV ligand had antitumor effects in mice (21)
CXCL9,10,11/ CXCR3	Endothelial, fibroblast, tumor cell, monocyte/CXCR3+ T cell	These chemokines are induced by interferon-γ and share a receptor CXCR3, directing the migration of activated T and NK cells (22).	Virus-directed expression of CXCL10, 11, or recombinant CXCL10 injection in tumors can recruit anticancer T cells in mice (23–26).
CXCL16/CXCR6	Tumor cell, macrophage, DC, Fibroblast/activated T cell, Th1, NK cell	CXCL16 is chemotactic for cells expressing its receptor CXCR6 (27, 28).	
CCL2/CCR2	Endothelial cells/Th1, CD8 T	Actin fibers beneath the endothelial plasma membranes will dock vesicles storing CCL2. The tight lymphocyte-endothelial synapses will release the chemokine to promote the transendothelial T-cell migration (29).	CAR/CCR2 T cells displayed greater homing and tumor-killing in malignant pleural mesothelioma (30) and neuroblastoma tumors (31) in mice.
CCL3, 4, 5 (fn. 3)/ CCR5	Epithelial cell, fibroblast, monocyte, NK cell, DC, endothelial cell, macrophage, lymphocyte/CCR5+ DC	CCL3,4,5 can indirectly promote effector T-cell recruitment by recruiting the DCs into tumor sites. Then, DC, in turn, recruits cytotoxic T cells into the tumor by producing CXCR3 ligands CXCL9 and CXCL10 (32, 33).	Combining adenoviral delivery of CCL3 with the adoptive transfer of activated effector T cells significantly attracted activated T cells to the murine melanoma tumors (34).
CCL21/CCR7	Lymphatic vessels, fibroblasts, HEV in lymph nodes/CCR7+ T cell, DC (35, 36)	CCL21 can significantly increase the CD4+ and CD8+ T cells, and DCs infiltration to tumors (37, 38).	Intratumoral injection of CCL21 induced DC and T-cell infiltration, causing tumor reduction in a murine lung cancer model (39).
CCL27/CCR10	Keratinocytes/CCR10+ skin-homing T cells	Skin-associated chemokine CCL27 is specifically expressed in epidermal keratinocytes and mediates the recruitment of skin-homing CCR10+ memory T cells to cutaneous sites (40).	The tumor injection of adenoviral vector encoding CCL27 attracted T cells and suppressed tumor growth in a murine melanoma model (41).

a hostile environment containing many negative messages for effector T-cell chemotaxis. VEGF can inhibit NF-κB signaling induced endothelium activation, blocking the induction of chemokines CXCL10 and CXCL11, and impairing the T-cell infiltration (110). Another cytokine IL35, released by T regulatory cells (Treg), macrophages, or B cells (111), can limit antitumor T-cell recruitment and induce a significant decrease in the CD8: Treg ratio in a murine model.

Other factors reshaping the stromal structure will also contribute to reduced T-cell infiltration. Transforming growth factor-beta (TGFβ) is a crucial regulator of epithelial-mesenchymal transition (EMT) in tumor cells. TGFβ plays a critical role in T-cell exclusion since the abundant tumor-stromal compartments induced by EMT will trap effector T cells from penetrating the tumor core (112). One study from a cohort of metastatic urothelial cancer patients receiving anti-PD-L1 therapy found that lack of response was related to activation of TGFβ signaling in fibroblasts (112). Moreover, TGFβ secreted by stromal cells will also promote angiogenic factors like VEGF and recruit immune suppressive cells such as Treg and myeloid-derived suppressor cell (MDSC) in tumors, further creating an unfavorable environment for T-cell trafficking.

TGFβ blockade has demonstrated safety and efficacy in different stages of preclinical models and clinical trials (45, 46, 48). In a EMT6 mouse mammary carcinoma model that exhibited the T cell-exclusion phenotype, combining anti-TGFβ antibody with anti-PD-L1 therapy successfully blocked the TGFβ signaling in stromal cells, which facilitated T cells infiltration and tumor regression (112). Co-expression of a dominant-negative TGFβ receptor II of the CAR-T cells dramatically enhanced its potency of penetration, proliferation, cytokine secretion, and tumor-killing capability in prostate cancer mouse models (49). This therapy has entered Phase I clinical trial status. Additionally, M7824, a fusion compound targeting both TGFβ and PD-L1, is undergoing clinical testing as monotherapy and combination therapy with an adenovirus vaccine encoding the tumor-associated antigen TWIST1 (49, 113).

Many cytokines and chemokines have both well-defined functions and controversial roles in the literature, depending on their cellular context. For example, the tumor endothelium produces CCL2 stored in vesicles of actin fibers beneath the plasma membrane. The stored CCL2 may promote the transmigration capacity of T helper type 1 (Th1) and CD8 T cells after resting on the endothelium (29). However, the extracellular soluble CCL2, produced by variable cell types in tumors, may exert different functions in the antitumor immunity by recruiting macrophages, Tregs, and MDSCs (59–61). Although CCL2 has restricted clinical application, its receptor CCR2 could serve as a useful therapeutic target. CAR T cells engineered with

TABLE 2 | Negative signals of T-cell infiltration.

Signal/Receptor	Producer/Target	Mechanisms	Therapy
IL35 (IL12A+IL27B)/ (fn. 4) IL12RB2+IL6ST	Tregs, macrophages, B cell/T cell	Treg cells derived IL35 can decrease the intratumoral CD4+ and CD8+ T cells infiltration. Also, the infiltrated T cells displayed a less activated, effector memory phenotype (42).	Neutralization of IL35 enhances antitumor immunity in a genetically induced KP mouse model (42).
TGFB1/TGFBR1, 2, 3	Fibroblasts/Tumor epithelial cell	TGFβ is a well-known regulator of EMT. Fibroblast induced TGFβ may reprogram peritumoral stromal fibroblasts and exhibit a fibroblast- and collagen-rich tumor (43), which will decrease the CD8+ T effector cell penetration in the tumor (44).	Inhibitors of TGFβ and receptors have entered clinical trials (45–48). CAR-T cells expressing a dominant-negative TGF-βRII enhance T-cell expansion and prostate cancer eradication in clinical trials (49).
CXCL1, 2, 5/CXCR2	Tumor cell, macrophage, neutrophil/CXCR2+ MDSC	CXCL1, 2, 5- CXCR2 signal promotes the recruitment of MDSC to tumors (50).	Several CXCR2 antagonists and inhibitors have been tested in preclinical models and shown anticancer effects (51, 52).
(fn. 5) CXCL8/CXCR1, 2	Tumor cells, mast cells, TAM, endothelial cells/ CXCR1, 2+ Neutrophil, MDSC	CXCL8 (IL8) enhances the infiltration of immune-suppressive cells expressing receptors (CXCR1, 2), such as tumor-associated neutrophils and MDSCs (53). T cells do not express CXCR1 and CXCR2 (54).	CXCR1 or CXCR2 modified CARs markedly enhance T-cell homing and persistence in murine GBM tumors (54).
CXCL12/CXCR4	FAP+ CAF/CXCR4+ MDSC, Endothelial cell, T cell	CXCL12 has chemo-repulsive effects on T cells (55). CXCL12 promotes angiogenesis by recruiting endothelial precursor cells (56). CXCL12 also recruits MDSCs to tumors (57).	AMD3100, a CXCR4 inhibitor, induced rapid T-cell accumulation around cancer cells in mice (58).
CCL2/CCR2	Endothelial cell, tumor cell, fibroblast, monocyte/CCR2 + TAM, MDSC, Treg	Soluble CCL2 promotes the recruitments of TAM, MDSC, and Treg to the tumor sites (59–61).	
CCL5/CCR5	Epithelial cell, fibroblast, monocyte, NK cell, DC, endothelial cell, macrophage, lymphocyte/ CCR5+ TAM, Treg	CCL5 regulates TAM and MDSC migration (62). It can also recruit Treg to tumors (63).	
CCL17/CCR4, 8	DC, Endothelial cell/CCR4 +, CCR8+ cells.	CCL17 induces chemotaxis in CCR4+ T cells, mainly Th2 and Tregs, generating an immunosuppressive TME (64, 65).	CAR- CD30 coexpressing CCR4 T cells have an improved homing and antitumor activity in the murine Hodgkin tumor model (66).
CCL22/CCR4	TAMs/CCR4+ Treg,	CCL22 enhances the recruitment of Tregs, thus decreasing effector T-cell homing (67).	
CCL28/CCR10	Tumor cells/CCR10+ skin-homing T cells	CCL28/CCR10 signals promote Treg recruitment in hypoxic tumors (68).	
VEGF/VEGFR1, 2, NRP1	Tumor, Macrophage, Endothelial, Fibroblast/ Endothelial cell, Treg, MDSC	VEGF induces FASLG on endothelial cells, leading to T-cell apoptosis during extravasation (69). VEGF recruits the NRP1+ Tregs and VEGFR1,2+ MDSCs (70, 71).	The anti-VEGF/VEGFR is a standard therapy for many tumor types (72).
FGF2/FGFR1,2,3,4	CAF/Endothelial cells	FGF2 significantly blocks the adhesion molecules VCAM1 and E-selectin expression (73).	
PDGFC/PDGFRA	CAF/Endothelial cells	PDGFC acts as a proangiogenic signal (74).	
PGF/FLT1	TAMs/Endothelial cells	TAM production of PGF stimulates angiogenesis (75).	

CCR2 expression displayed satisfactory effector T-cell trafficking and antitumor efficacy in malignant pleural mesotheliomas (30) and neuroblastoma tumors (31) in mice.

Similarly to the application of CCR2, other cytokine-receptor pairs which contribute to immune-suppressive cells recruitment have shown therapeutic potentials in T-cell therapy. For example, CXCL8 recruits myeloid derived suppressor cells (MDSC) and neutrophils into tumors (53). CAR-T cells armed with CXCL8 receptors (CXCR1 and CXCR2) markedly enhanced T cells infiltration and led to complete antitumor responses in murine models ovarian cancer, pancreatic cancer, and glioblastoma (54). Another example is CCR4 receptor for CCL17 and CCL22, which recruit Treg and T helper type 2 (Th2) (64, 67). The CAR-CD30 T cells expressing CCR4 significantly enhanced the CAR T migration to tumor sites and achieved satisfactory tumor control in a Hodgkin tumor model (66).

T-CELL SURVIVAL: T CELLS NEED TO SURVIVE ON THE BATTLEFIELD

Signals Supporting T-Cell Survival and Expansion

Once T cells enter the tumor, the goal is to survive and proliferate in adequate numbers for efficient tumor killing (**Figure 1B**). Tumor infiltrating lymphocytes (TIL) manage to expand in the nutrient-deficient tumor microenvironment with the help of multiple cytokines (**Table 3**). Several studies have supported the pivotal role of the IL2 cytokine family (IL2, IL7, IL15, IL21) for CD8 T-cell expansion. IL2 was one of the earliest FDA-approved immunotherapies for metastatic melanoma and renal cell cancer (94, 117). Autologous TILs were transferred in conjunction with IL2 after *ex vivo* expansion with IL2, which mediated a durable and completed tumor regression in 22% of the heavily pretreated

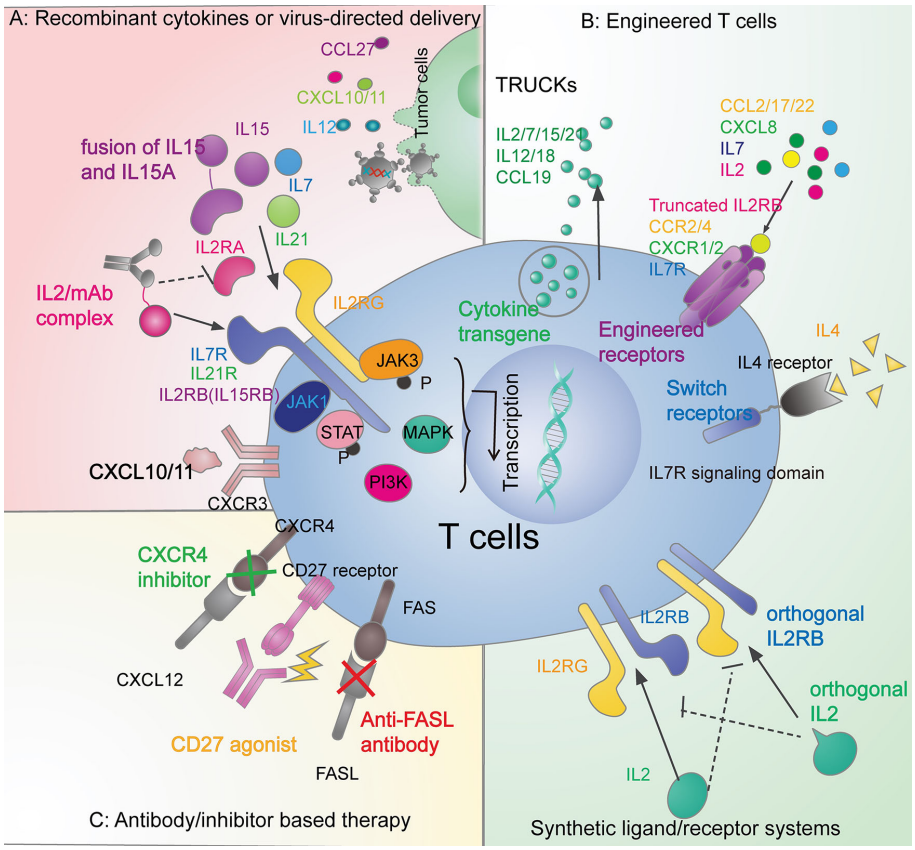


FIGURE 2 | Therapeutic strategies in overcoming the T-cell exclusion by leveraging the cytokine signals. **(A)** Recombinant cytokines or virus-directed delivery. Administration of recombinant IL2 (94), IL7 (95), and IL15 (96, 97) have brought success in clinical trials. Also, there are other recombinant cytokines under research in preclinical models, such as IL21 (98), and CXCL10 (24). Adenoviral, retrovirus, and vaccinia vectors can also deliver cytokines, including CCL27 (41), IL12 (99, 100), CXCL10 (26), and CXCL11 (25). **(B)** Engineered T cells. The fourth-generation CAR T cells (TRUCKs) can release IL2 family cytokines (IL2, IL7, IL15, and IL21) (101), IL12, IL18 (102) and CCL19 (103). CAR T cells can also be engineered with functional receptors such as CCR2 (30, 31), CCR4 (66), CXCR1/2 (54), IL7R (54, 104), truncated IL2B domain (105), and switch receptors to overcome the immune-suppressive cytokines (106). Tumor-specific T cells can also be equipped with synthetic ligand/receptor systems such as IL2 and IL2RB orthogonal pairs (107). **(C)** Antibody/inhibitor-based therapy. Agonistic (105, 108) or antagonistic (109) antibodies and inhibitors (58) are applied to modulate cytokine signaling pathways in the anticancer immune response.

melanoma patients (143). However, reasons preventing IL2 from extensive clinical usage include short half-life *in vivo* (144) and severe toxicity at therapeutic dosage (145). Another barrier is that not only newly-activated CD8 T cells but also Tregs can express the trimeric high-affinity receptor IL-2Rαβγ (IL2RA+B+G complex) for IL2 signaling (116).

Several methods may attenuate IL2's propensity to promote Tregs expansion (146). Besides binding to IL-2Rαβγ, IL2 can stimulate naive and memory T cells expressing an intermediate affinity receptor IL-2Rβγ (IL2RB+G complex). Complexing IL2 with specific anti-IL2 antibodies will present IL2 to the intermediate (T effector) but not high-affinity (Treg) receptors, thereby reducing the Tregs production and causing massive CD8+ T cells expansion in mice (147–149) (**Figure 2A**). Engineered IL2 and IL2RB orthogonal pairs, consisting of the mutant IL2 cytokine and cognate mutant receptor in an engineered T cell, may transmit IL2 signals only to transferred T cells without interacting with the natural counterparts (106) (**Figure 2B**). Transduced CAR-T cells with a truncated IL-2Rβ domain increased STAT3 and STAT5 signaling and improved the CAR-T-cell expansion in mice leukemia and melanoma model (104) (**Figure 2B**).

Other members in the IL2 cytokine family play a similar and synergistic role with IL2 in cancer immunity through a shared gamma chain (CD132) in their receptor and downstream JAK-STAT signaling (133). For example, unlike IL2 produced by immune cells, IL7, mainly secreted by fibroblastic reticular cells in lymph nodes, can support the survival of naive and memory T cells expressing the receptor IL7R. IL7-IL7R signaling regulates the proliferation of target T cells *via* up-regulation of the anti-apoptotic BCL2 (118, 119) and suppression of pro-apoptotic mediators Bad and Bax (120). IL7 cannot trigger the expansion of Tregs because they express low levels of surface receptor IL7R. IL15 is essential for homeostasis and development of effector CD8 T cells and NK cells through interaction with its high-affinity receptor IL15RA. IL15 can also inhibit activation-induced cell

TABLE 3 | Positive signals of T-cell survival.

Signal/Receptor	Producer/Target	Mechanisms	Therapy
(fn. 6) IL2/IL2RA,B, G	DC, activated T cells/ effector T cell, Treg	CD8+ T cells depend on IL2 for sustained expansion (114, 115). However, the high-affinity IL2RA is not only expressed on activated T cells but also on Tregs, which is the primary barrier for the clinical application of IL2 (116).	IL-2 was one of the first FDA-approved immunotherapies for metastatic melanoma and renal cell cancer (94, 117), (fn. 7). Since IL2 expands effector T cells at the cost of Treg proliferation, engineered IL2 is necessary to preferentially target IL2 receptors on effector T cells (107).
IL7/IL7R+IL2RG	Fibroblastic reticular cell/T cell	IL7 promotes the homeostasis and expansion of naive and memory T cells by up-regulating BCL2 (118, 119), and suppressing pro-apoptotic mediators (120).	Coexpressing the IL7R with CAR-GD2 T cells activates STAT5 signaling and shows super antitumor response in metastatic neuroblastoma and glioblastoma mice model (104). Clinical trials using recombinant IL7 as monotherapy showed increases in CD4 and CD8 T cells with a decrease in Tregs in multiple cancer types (95, 121).
(fn. 8) IL10/ IL10RA+IL10RB	Treg, Th1, DC, macrophage, epithelial cell/Tumor-resident T-cell (122).	IL10 directly activates and expands tumor-resident T cells without *de novo* infiltration from secondary lymphoid organs (123).	Treatment with pegylated IL10 restores tumor-specific CD8 T-cell accumulation and controls tumor growth in mice (124).
IL12A, IL12B/ IL12RB1 + IL12RB2	Phagocytic cells, B cells, DC/T cells	IL12 stimulates activated T-cell proliferation (125).	Intratumoral injection of a recombinant retrovirus vector expressing IL-12 induces antitumor and anti-angiogenic effects in murine models of head and neck squamous cell carcinoma (99) and melanoma (100). The phase I trial of (fn. 9) NHS-IL12 in metastatic or locally advanced solid epithelial or mesenchymal tumors showed enhanced antitumor activity with increasing immune cell infiltration (126).
(fn. 6) IL15/IL15RA, IL2RB, IL2RG	Monocytes, macrophages, DC/CD8+ T cell	The IL15-IL15RA signaling triggers the downstream JAK1, JAK3, STAT3, and STAT5, which stimulates T-cell proliferation and survival (127). IL15 inhibits AICD and maintains T cells' homeostatic proliferation.	IL15 has entered the clinical trial in patients with metastatic melanoma, renal cell carcinoma, and non-small cell lung cancer in CAR-T therapy and combination treatment with anti-PD1 antibody (96, 97, 128).
IL18/ IL18R1+IL18RAP	Activated macrophages and Kupffer cells/T cells,	Combined stimulation with IL12 and IL18 can stimulate memory T cells in an antigen non-specific manner (129, 130). IL12 and IL18 may synergize with each other to induce Th1 differentiation (131).	
IL21/IL21RA, IL2RG	Th17, Tfh, NKT cells/T cell	IL21 synergistically works with IL15 to expand CD8+ memory T cells (98). IL21 also suppresses Foxp3-expressing cells (132).	The combined administration of IL21 and IL15 dramatically increased the CD8+ T cells and resulted in tumor regression in mice melanoma models (98). Likewise, the combination treatment of IL21 with IL7 promotes the expansion of CAR-T cells with a TSCM phenotype (133).
CD27L/CD27	T cells/T cells	The CD27L/CD27 signaling plays an essential role in T-cell differentiation, survival, and memory T-cell formation (19, 134).	CD27 agonist showed antitumor efficacy in mice models (135) and phase I and II clinical trials of advanced solid tumors (108, 136),
41BBL/41BB	DC, macrophages, B cells, T cells/activated T cell	41BBL facilitates cell activation, survival, and proliferation upon binding to 41BB on T cells (137).	41BBL armored CAR T-cells showed enhanced *in-vitro* and *in-vivo* efficacy (138).
CCL19/CCR7	Fibroblastic reticular cell/T cell	T zone fibroblastic reticular cells can prevent the death of naive and memory T cells by secreting CCL19 (139, 140).	CCL19 CAR-T achieved superior antitumor activity compared to conventional CAR-T in mice (103).
CCL21/CCR7, CXCR3	Lymphatic vessels, stroma cells, HEV in lymph nodes/CCR7+, CXCR3+ cells (35, 36)	CCL21 promotes the expansion of naive T cells in tumors (141).	Delivery of CCL21 to metastasis tumors enhances the ACT efficacy by promoting the T-cell survival and cytotoxic activity in mice (142).

death (AICD), further facilitating the proliferation of CD8 T cells (150). IL21, produced by T helper 17 (Th17), follicular helper T cells (Tfh), and NKT cells, drives NK expansion and differentiation (133). Also, IL21 inhibits Treg survival by downregulating the FOXP3 expression and favors the development of antigen-specific cytotoxic T cells (132).

In contrast to IL2, other IL2-family cytokines IL7, IL15, and IL21 do not induce Treg expansion. The role of these cytokines in modulating T cell-based cancer immunotherapies is currently being explored. Administration of recombinant IL7 alone showed a dose-dependent increase of T cells along with a decrease of Tregs in patients with lymphopenia (95). In clinical trials, human IL15 as monotherapy promoted proliferation of circulating NK cells and CD8 T cells in patients with metastatic melanoma and renal cancer, with the cost of severe toxicity at the therapeutic dose (96). Combined administration of IL15 and IL21 had synergistically accelerated the growth of both naive and memory CD8 T cells and resulted in tumor regression in a murine model of melanoma. IL21 has also demonstrated cooperative effects with IL7, but not IL2 (98).

Treatment of IL2-family cytokines in combination with TILs, CAR-T, and checkpoint blockade can lead to a broader and stronger antitumor response. Fourth-generation CAR-T cells armed with

inducible cytokines have been defined as T cells redirected for universal cytokine-mediated killing (TRUCK) (**Figure 2B**). TRUCKs, loaded with IL2 family cytokines, enhance the persistence of CAR-T cells for antigen-specific tumor killing, and activate innate immune cells for antigen-negative tumors killing (101).

Besides the IL2 family, IL12 and IL18 secretion by TRUCKs have also been tested (151, 152). IL12 and IL18 can promote the activation and expansion of memory CD8 T cells in an antigen-independent manner (101). TRUCKs with inducible IL12 have dual antitumor activity. Firstly, the activated TRUCKs will lyse tumor cells and secret IL12 upon CAR engagement. Then, the locally released IL12 will not only promote CAR-T cells activation in an autocrine manner but also activate and recruit the innate immune cells (such as NK cells and macrophages) to kill tumor cells without antigens (102). Intratumoral administration of IL12 had antitumor effects in murine models of head and neck carcinoma (99) and melanoma (100). There are advantages of inducible production of IL12 (triggered by CAR signaling) over constitutive IL12 delivery at the tumor sites. As long as the TRUCKs are engaged and stay activated, there will be induction and secretion of IL12 for durable tumor control. It is important to note that the safety of TRUCKs is a concern since cytokine release syndrome happens in most CAR-T therapy (153).

Several cytokines, such as CD27L and 41BBL, which belongs to the tumor necrosis factor family, also act as costimulatory signals for T cells. CD27L is only transiently expressed on activated immune cells. However, the CD27L-CD27 costimulation bolsters T-cell activation, proliferation, and differentiation to a memory T-cell phenotype, thus enhancing anticancer immunity (134, 154). Targeting the CD27L-CD27 axis might be a therapeutic strategy given the observation that patients with CD27L or CD27 mutations and deletions are at a higher risk of developing Hodgkin lymphoma (155). Varlilumab, an anti-CD27 monoclonal agonistic antibody, showed clinical efficacy in a phase I study of refractory solid tumors (109). A combination of Varlilumab with checkpoint blockade therapy also established early success in phase I and II clinical trials (136).

The 41BBL-41BB signal's importance is underscored by the observations that agonistic antibodies against 41BB can significantly promote CD8 T cells expansion and decrease T cells apoptosis, contributing to a robust antitumor immunity in mice (157, 158). However, the low efficiency (Utomilumab) (157) and liver toxicity (Urelumab) (158) severely hampered the clinical application of 41BB antibodies. The most remarkable clinical benefit of 41BB so far comes from the FDA-approved CAR-T cells containing 41BB as the intracellular costimulatory domain. The second-generation CAR-CD19 T cells armed with 41BBL have shown notable antitumor responses in several clinical trials against B-cell acute lymphoblastic leukemia (159, 160).

Interestingly, chemokines may sometimes enhance CD8 T cells' survival and propagation apart from acting as chemoattractants. Both CCL19 and CCL21 can promote naive T cells' survival (161). Secondary lymphoid organs are primary sources for CCL19 and CCL21, and access to the secondary lymphoid organs is crucial for naive T cells survival (139). Intratumoral delivery of CCL21 augmented the tumor-killing efficacy of adoptive cell therapy (ACT) in a murine model of melanoma by promoting T-cell survival rather than recruitment (142). IL10 can also induce the activation and multiplication of tumor-resident T cells without trafficking from the secondary lymphoid organs. IL10R, expressed on CD8 T cells, is necessary for the IL10-mediated tumor regression and the in-situ proliferation of CD8 T cells (123). However, given the suppressive function of IL10 during the T-cell priming and IL10-induced T cells exhaustion (162, 163), the value of IL10 as a therapeutic target needs further investigation.

Signals Triggering T-Cell Apoptosis

There are various cytokine-mediated mechanisms by which the hostile TME triggers T-cell apoptosis (**Table 4**). The best-known one is through activation-induced cell death (AICD). AICD is a process that occurs when CD8 T cells express high FAS and FAS ligand (FASLG) expression levels upon activation, triggering the apoptosis of neighboring CD8 T cells (176). The binding of FAS to FASLG will recruit the FAS-associated death domain (FADD) to the intracytoplasmic death domain (IDD) of the receptor and initiates the caspase 8 activation and the subsequent cascade caspases (177). IL1, IL6, and TNF can also promote FAS and FASLG expression (178, 179). Moreover, the tumor endothelium can release FASLG, leading to apoptosis of T cells when they are trying to transmigrate the tumor vessel (69). Also, tumor-derived VEGF, IL10, and PGE2 can all enhance the FASLG expression (69). Notably, the endothelium-dependent apoptosis hardly works for Tregs because of the activation of anti-apoptotic molecules like BCL2 and CFLAR in Tregs (69, 180). An ovarian cancer study confirmed that the endothelium secreting FASLG induces deficient CD8 T cells infiltration and a predominance of Tregs (69).

Given the immune-suppressive role of FAS-FASLG signaling, different approaches to inhibit FASLG have been tested. For example, treatment with FASLG-neutralizing antibody (**Figure 2C**) markedly reduced T-cell apoptosis and cancer cell migration in a glioblastoma mouse model (109). Genetically engineered T cells with disruption of FAS-FASLG signaling introduced by adoptive T-cell transfer dramatically prevented FASLG-mediated T-cell apoptosis and achieved superior persistence in murine models (167).

Besides FASLG, other death receptors may mediate T-cell apoptosis when triggered by their cognate ligands, such as TNFRSF10A (TRAIL-R1, DR4), TNFRSF10B (TRAIL-R2, DR5), TNFRSF25, TNFRSF1A (TNFR1), and TNFRSF1B (TNFR2). Theoretically, the TNF-related apoptosis-inducing ligand (TRAIL) cannot induce apoptosis of T cells since T cells express low levels of TRAIL-R1/2, which contains the cytoplasmic death domain. TRAIL-R1/2 may recruit FADD, activates caspase 8, and leads to T-cell apoptosis upon binding to TRAIL (181). Interestingly, there was an observation that CAR-T cells did undergo programmed cell death triggered by the FAS-FASLG and TRAIL-DR5 pathway. Also, *ex vivo* combined blockade of FAS and TRAIL signaling significantly rescued the CAR-T cells (169, 170).

TNF receptors are also critical death receptors for activated T-cell apoptosis. Soluble and membrane-bound TNF bind to different receptors to trigger apoptosis. Upon activation, TNFR1

TABLE 4 | Negative signals of T-cell survival.

Signal/Receptor	Producer/Target	Mechanisms	Therapy
TNF/TNFR1, TNFR2	Lymphoid, mast, macrophage, endothelial cell, fibroblast, tumor cell/ Activated T cell	TNF can mediate mature T-cell receptor-induced apoptosis through the TNFR1 (81). Macrophages can induce CD8 T-cell apoptosis *via* the interaction between macrophage membrane-bound TNFa (mbTNF) and TNFR2 on T cells (164).	TNF or TNFR1 blockade synergizes with anti-PD-1 on anti-cancer immune responses against solid tumors in mice (165).
FASLG/FAS	Tumor endothelial cell, NK cell, T cell/Activated T cell.	FASLG is a death ligand for activated T cells. Activated CD8+ T cells are sensitive to FASLG killing (166).	Anti-FASLG antibody treatment before adoptive T cells transfer significantly enhances CD8+ T cells infiltration in mice (69, 167).
TRAIL/TNFRSF10B (DR5)	Tumor cell, Treg cells/CAR- T cell	TRAIL hardly triggers T-cell apoptosis because T cells either lack or express low levels of functional death receptors (DR4 and DR5) (168). Interestingly, CAR-T cells may express DR5 and are prone to TRAIL-mediated apoptosis (169).	In vivo combined blockade of Fas and TRAIL signaling significantly rescued the CAR-T cells in mice (170).
TGFB1/TGFBR1, 2	Leukocyte, fibroblast, tumor cell/T cell	TGF-β impairs the cell cycle progression of CD4+ and CD8+ T cells (171). TGF-β inhibits IL2-dependent T-cell proliferation by suppressing the expression of IL2 and its receptor (172, 173).	Refer to **Table 2**
CCL5/CCR5	Epithelial cell, fibroblast, monocyte, NK cell, DC, endothelial cell, macrophage, lymphocyte/CCR5+ T cell	Tumor cells stimulate TILs to secret CCL5, which activates an apoptotic pathway in TIL involving cytochrome c release into the cytosol and activation of caspase-3 and -9 (174). CCL5 could enhance the Tregs' killing ability on CD8+ T cells through TGF-β signaling (175). Cancer cells might induce CD4+ T cells to secrete CCL5 and activate the Fas-mediated apoptosis in CD8+ cells (47).	

can either trigger cell apoptosis *via* the formation of FADD-IDD complex leading to caspase cascade activation or induce the proliferation pathway through NFkB activation. A study showed that T-cell depletion occurs in mice with FAS and FASLG defects. The inhibition of both FAS and TNF is necessary to eliminate T-cell death, and the TNF-TNFR1 signal mediates most CD8 T cells apoptosis (182). Macrophages can also induce apoptosis of CD8 T cells *via* the interaction between macrophage membrane-bound TNF and TNFR2 on T cells (164). Notably, MDSCs and Tregs also express TNFR2, whose activation will trigger the proliferation through NFkB signaling pathway instead of apoptosis in these cells (183).

Other mechanisms within the tumor microenvironment resulting in T-cell apoptosis include depletion of tryptophan by indoleamine-2,3-dioxygenase (IDO) and generation of Galectin 9, both of which binds to TIM3, which are predominantly mediated by immune-suppressive cells such as Tregs and MDSCs. Unexpectedly, chemokines can sometimes trigger non-classic apoptosis signals in T cells. TILs-secreted CCL5 induces CCR5+ (CCL5 receptor) T cells death through the release of cytochrome c from mitochondria and activation of caspase-3 and 8 (174). CCL5, secreted by tumor-infiltrating CD4 T cells, also facilitates the FAS-FASLG mediated CD8 T-cell apoptosis in gastric cancer (47).

T-CELL DIFFERENTIATION: T CELLS MANAGE TO KEEP IN THEIR PROPER STATES FOR DURABLE FIGHTING

CD8 T Differentiation in Tumors

T-cell differentiation pathway is one of the primary factors determining T cells' prolonged tumor-killing activity. Upon exposure to the cognate tumor-antigens, activated CD8 T cells will differentiate from a naive state into effector T cells. There are disputes regarding the differentiation track of CD8 T cells. The de-differentiation model purports that naive T cells are directly programmed into short-lived terminal effector T cells, followed by de-differentiation into memory cells with increased longevity (184, 185). Weissman and colleagues suggested that naive T cells differentiate along a sequential lineage path into stem-cell memory cells (TSCM), central memory cells (TCM), effector memory cells (TEM), and the terminally differentiated effector cells (184) (**Figure 1C**).

As terminal differentiation proceeds, T cells lose their self-renewal ability, proliferative potential, and lifespan (184, 186). The adoptive transfer of TSCM enhanced antitumor responses compared with TCM and TEM subsets in a humanized mouse model of mesothelioma (187). Given the superior anticancer efficacy of memory CD8 T cells, people have developed various T-cell selection methods for ACT, such as epigenetic and genetic modification (186, 188), reprogramming of induced pluripotent stem cells (189), and cytokine treatment.

The IL2 family members exert their specific roles in CD8 T-cell differentiation and proliferation (**Table 5**). IL2 drives terminal effector T cells differentiation and proliferation by upregulating perforin, granzyme B and IFN-γ and suppressing the memory cell marker BCL6 and IL7RA (199). On the contrary, IL7, 1L21, and IL15 may promote the memory cell phenotype. IL7 can generate TSCMs from their naive precursors (200, 201). CAR-T therapies frequently require IL7 during the ex-vivo expansion phase (202). For example, CAR-CD19 T cells cultured *in vitro* with IL7 and IL15 significantly induced a TSCM phenotype and produced a robust response against B-cell malignancies in phase I clinical trial (203). TRUCKs with constitutive IL7R signaling increased T-cell proliferation, survival, and tumor-killing activity upon exposure to tumor antigens, without stimulating bystander lymphocytes in murine cancer models (103).

TABLE 5 | Positive signals of T-cell effector activity through differentiation.

Signal/Receptor	Producer/Target	Mechanisms	Therapy
IL7/IL7R+IL2RG	Fibroblastic reticular cell/CD8 T cell	IL-7 generated the stem-cell memory T cells from naive CD8 T cells (150).	
IL12A,B/IL12R IFN-α/β/IFNAR1, 2	Phagocytic cells, B cells, DC/ CD8 T cells	IL12 and IFN-α/β provide a third signal, along with Antigen and costimulation, to support CD8 T memory programming, which involves chromatin remodeling and regulation of genes such as *TBX21* (*T-bet*) and *EOMES* (190, 191). IL12 also polarizes naive T cells into Th1 cells (192).	
IL15/IL15RA, IL2RB, IL2RG	Monocytes, macrophages, DC/ CD8+ T cells	IL15 induces the generation of antigen-specific memory T cells (193).	
IL21/IL21R+IL2RG	Th2, NKT cells/CD8 T cell	IL21 suppresses the antigen-induced CD8+ T-cell differentiation from naive T cells to effector T cells and induced stem-like properties, which allows CD8+ T cells for secondary expansion after adoptive transfer (194).	
CD40L/CD40	DC, macrophages, B cells, T cells, mast cells/CD40+ T cell	CD40L/CD40 increases T-cell proliferation, CD8+ T-cell immunity, and memory (195).	
TGFB+IL6+IL23	In-vitro culture treatment.	CD4+ T-cell differentiates into Th17 *via* TGFB and IL6 (196). IL23 maintains the proliferation of Th17. (Fn. 10) Th17 are long-lived cells with stem-like properties. It can also convert into a Th1-lineage over time, switching from IL17 secreting cells to IFNγ producers or IFNγ/IL17A double producers (197).	Transfer of Th17 cells enhances survival and tumor regression better than Th1 cells in a murine melanoma model (198).
CCL21/CCR7, CXCR3	Lymphatic vessels, stroma cells, HEV in lymph nodes/CD4 T cells (35, 36)	CCL21 promotes Th1 polarization (141).	

Antigen-presenting cells secret IL15 bound together with its high-affinity receptor IL15RA (204). The signal will reach target cells that express IL-2Rβγ, including CD8 memory T cells. ALT-803, a fusion complex of IL15 and IL15RA receptor, exhibited a more substantial tumor-killing effect than native IL15 in preclinical models of myeloma through promoting the proliferation of CD8 memory T cells and inducing large amounts of IFN-γ (205). Administration of ALT-803 combined with anti-PD1 antibody showed tumor-killing effects in non-small cell lung carcinoma patients who failed the anti-PD1 monotherapy (97).

IL21 augments ACT therapy by preserving T cells in a younger phenotype but at the cost of less expansion than those expanded with IL2. However, adoptive T cells stimulated with IL21 induced a more robust antitumor response in a murine melanoma model (206). Similarly, IL21 treatment has generated TRUCKs with a more naive phenotype. When transferred into the host, these TRUCKs showed a dramatic propagation upon tumor exposure and achieved improved tumor control (194).

A study compared the therapeutic efficacy of CD19-specific TRUCKs equipped with IL2, 7, 15, and 21 expression cassettes in a murine lymphoma model. The result claimed that IL7 and IL21 were superior to IL2 and IL15 in enhancing tumor eradication, although IL2 and IL15 established increased effector functions. Interestingly, IL21 overexpression best supported the long-term persistence of memory T cells, while IL7-transduced T cells expanded to the greatest extent upon secondary antigen presentation (207). The varying roles of IL2 family members suggest that combinatorial approaches are necessary for ideal T cells-based therapy.

Other cytokines also contribute to CD8 T cell memory programming (**Table 5**). For example, the generation of memory cells needs signals from CD4 T helper cells. The interaction between CD40 expression on CD8 T cells and CD40 ligand (CD40L) expression on CD4 T cells is indispensable for the helper process. However, the CD40L/CD40 signal is not necessary for naive CD8 T cells to differentiate into terminal effector cells (195). IL12 and type I IFN (IFN-α/β) provide a third signal in concert with antigen presentation and costimulation to establish long-term memory CD8 T cells. IL12 and IFN-α/β promoted the memory program of naive CD8 T cells mainly through chromatin remodeling, which involved histone acetylation of genes like EOMES, TBX21, and GRZB (190). In addition, CD4 T helper cells provide a CD40-CD40L stimulus for DCs to produce IL12, promoting the CD8 T cell memory program (190, 209).

Functional Fate of CD4 T-Cell Subsets in Tumors

Unlike CD8 T cells, the differentiation of CD4 T cells is divergent. CD4 T cells differentiate into various T helper (Th) cell and regulatory (Treg) cell lineages to exert their functions in the tumor immunity. Upon exposure to different lineage-determining cytokines during activation, naive CD4 T cells may have several distinct effector fates, such as Th1, Th2, Th17, Treg, and Tfh (**Table 5** and **Figure 1C**). Besides supporting CD8 T cells, CD4 T cells may directly lyse tumor cells in an MHC-II dependent manner through the secretion of perforin and granzyme (209, 210). A recent study demonstrated the occurrence of clonal expanded cytotoxic CD4+ T cells by single cell sequencing, these CD4 T cells possessed lytic capabilities against autologous tumors (210). Multiple studies have identified several origins of CD4 cytotoxic T cells, such as Th2, Th17 and Treg (211). However, the majority of CD4 CTL are thought to come from IFN-γ secreting Th1 cells (212).

Each of the CD4 T subsets has a specific role in antitumor immunity by producing and receiving distinct cytokines. Th1 can provide IFN-γ, TNF, CCL2 and CCL3 to enhance the recruitment of CD8 T cells, NK cells and anticancer macrophages (213–216).

Cytokines IFN-γ, IL12, IL18, IL27 can promote Th1 polarization, thus contributing to tumor control (141, 192). However, the role of Th2 cells in the tumor immunity is contradictory and context-dependent. IL4 is both the inducer of Th2 polarization (**Table 6**) and the effector cytokines secreted by Th2. IL4 has an immune-suppressive role by antagonizing the Th1 response and supporting Tregs (219). CAR-T cells engineered to overcome the immune-suppressive nature of IL4 have brought early success. CAR-MUC1 T cells with an inverted IL4 receptor exo-domain plus an IL7 receptor signal endo-domain (**Figure 2B**) facilitated a potent antitumor response in a mouse model of breast cancer with an abundance of IL4 in the tumor milieu (106). Th2 may also promote angiogenesis and hinder apoptosis of tumor cells by remodeling the cytokine environment for macrophages and eosinophils infiltration (220). However, some studies have demonstrated the antitumor activity of Th2 cells by recruiting the innate cells such as eosinophils to the tumor (221).

The signals of TGFβ and IL10 polarize naive CD4 T cells to Tregs (**Table 6**). Tregs have been regarded as exerting suppression roles in antitumor immunity and high Treg/CD8 ratio in tumor infiltrates correlates with poor prognosis in cancer patients (222). However, recent study showed that IFN-γ production by Tregs is necessary for the therapeutic responses of anti-PD1 in a mice model, which shed light on characterizing the contribution of IFN-γ+ Tregs in tumor immunotherapy (223).

Naive CD4 T cells can also differentiate into Th17 cells with stimulation by TGFβ and IL6 and maintenance with IL23. Characterized by the high production of IL17 and IL22, Th17 cells have a controversial role in the cancer immunity context. Th17 cells may stimulate angiogenesis and promote tumorigenesis. However, these cells also serve as tumor-suppressive cells by stimulating effector CD8+ T cells, supporting immune cell recruitment, and transitioning to Th1-lineage over time (224). Moreover, Th17 cells possess long-lived memory-like properties through the expression of stem-cell markers such as CCR7, LEF1, and TCF7 (225). Thus, the adoptive transfer of Th17 polarized CAR-T cells had been shown to produce superior tumor regression than Th1 cells in mice (225, 226). Follicular helper (Tfh) T cells characterized by IL21 secretion have been shown to contribute to antitumor responses by promoting the formation of intratumor follicular structures, which were positively associated with prognosis of cancer patients (227, 228).

Since the cytokine environment is pivotal in fate-determination of naive CD4 T cells, manipulation of CD4 T cells using cytokine signals has provided substantial promise in therapy. For example, Th17 polarized cells in cell culture with TGFβ, IL6, and anti-IFN-γ antibody (preventing Th1 differentiation) mediated effective tumor eradication and a survival advantage in a murine melanoma model (198). However, the lifetime of CD4 T cells generated is very short, and many other cytokines are necessary for their maintenance, which represents a significant barrier for use in clinical applications (229). Robust methods are still lacking for generating effector CD4 T subtypes with stem-like properties and longevity.

CYTOKINE RELEASE SYNDROME: EVERY COIN HAS TWO SIDES

As reviewed in previous sections, many therapies modulate cytokine and chemokine signaling to overcome the T-cell exclusion barriers in tumors (**Figure 2**). However, a danger of cytokine-based treatments is the severe toxicity from cytokine release syndrome (CRS). CRS is the over-activation of the immune system characterized by a flood of inflammatory cytokines, fever, and multiple organ dysfunction (230). CRS can happen after administration of therapeutic monoclonal antibodies or cytotoxic chemotherapies, such as the CD28 agonist TGN1412 (231), Rituximab and Obinutuzumab (targeting CD20) (232, 233), Dacetuzumab (targeting CD40) (234), Nivolumab (anti-PD1) (235), Oxaliplatin (236), and Lenalidomide (237).

Moreover, T-cell therapies, such as CAR-T and TCR-T cells, the bispecific T-cell (BiTE) single-chain antibody, and the dual-affinity re-targeting antibody, have produced the high CRS frequencies (238). CRS happens in nearly all CAR-T clinical trials, with presentations ranging from mild symptoms such as fever to life-threatening manifestations, including sepsis, thromboembolism, neurotoxicity, and multi-organ failure (230, 239). However, there is no conclusive evidence connecting the CRS severity to the immunotherapy response, and complete tumor remission can happen in patients without CRS (240).

The exposure of CAR-T cells to a tumor antigen can trigger CRS. The activation and proliferation of CAR-T cells release primary cytokines such as IL1, IFN-γ, and TNF, which induce the activation of other immune cells, such as macrophages, DCs, and monocytes (241). These cells then produce excessive amounts of secondary cytokines, such as IL6, IL10, and IL5 (242). Among these cytokines, IL6 is a crucial regulator of CRS and contributes to critical symptoms (243). The IL6/IL6R complex binds to the membrane-bound IL6ST (gp130) and activates a cascade of intracellular signaling, which results in severe CRS (244).

Several clinical factors are potentially predictive of the CRS severity. The first factor is the tumor burden. In many observations, the most severe CRS only occurs after the first

TABLE 6 | Negative signals of T-cell effector activity through differentiation.

Signal/Receptor	Producer/Target	Mechanisms	Therapy
(fn. 11) IL4/ IL4R+IL2RG	Th2, basophils, eosinophils, mast cells, NKT cells, Adipose tissue, cancer cells/Th1	(fn. 12) IL4 drives CD4+ T-cell polarization into the Th2 phenotype and suppresses IFNγ-producing Th1 cells (217).	MUC1 CAR T with a cytokine switch receptor of IL4 receptor extracellular domain fused to an IL7 intracellular signaling domain can proliferate and suppress tumor growth in mice breast cancer model (106).
TGFB1/TGFBR1,2,3	Leukocyte, fibroblast, tumor cell/ CD4 T cell	TGFβ promotes the conversion of effector T cells to Tregs (218).	

administered dose, and will not occur during the subsequent therapies, called the first-dose effect. It is believed that the first-dose effect is due to the high tumor antigen load at the initiation of treatment (245). The administered dose of an agent is another factor (246). The maturation of the immune system may be another factor for CRS because children are more likely to develop severe CRS following CD19 CAR-T-cell infusion in clinical trials. Also, the type of T-cell engaging agents affects the onset, duration, and severity of CRS (160). For example, first-generation of CAR T cells hardly triggered the CRS because of the lack of a costimulatory domain. Among the second-generation CAR-T cells (247), CARs with CD28 costimulation have a higher CRS rate than those containing a 41BB co-stimulation (248). Before CAR-T infusion, the lymphocyte depletion type also affected the risk, with a higher CRS incidence observed after fludarabine-based lymphodepletion (249).

The principle of CRS management is to prevent life-threatening toxicity and preserve the maximum antitumor immune responses. Low-grade CRS can be treated symptomatically with antipyretics and fluid therapy. As for severe CRS, in the BiTE blinatumomab context, some clinical trials advised the usage of corticosteroids to reduce the CRS incidence (250). Because clinicians can give BiTE repeatedly, immediate action can be taken upon CRS manifestation, even at the cost of lowering the antitumor response. However, unlike BiTE, the manufacture of CAR T cells allows only limited amounts for one-time administration. Therefore, corticosteroids should be avoided at the first-time treatment of CRS in patients receiving CAR T therapies, unless severe neurotoxicity has developed.

IL6 levels are significantly higher in the serum of patients with severe CRS after CAR-T treatment. IL6 and IL6 receptors are attractive targets for CRS treatment because IL6 is less critical for T-cell function than other inflammatory cytokines. The FDA approved Tocilizumab (monoclonal antibodies against IL6R) to treat severe CRS in patients who are at least two years old (251). For patients who are unresponsive to anti-IL6 or IL6R treatment, other ongoing clinical trials are evaluating T cell-depleting antibodies, such as Alemtuzumab, IL-1R-based inhibitors (Anakinra), and Ibrutinib (252, 253).

CONCLUSIONS AND FUTURE DIRECTIONS

The systematic categorization of cellular signaling mechanisms that modulate the T-cell infiltration, survival, and differentiation in tumors is the premise of overcoming the T-cell exclusion barriers. However, many cytokines have both positive and negative effects on the anticancer immune response depending on their different receptor usage, cellular context, and interactions with other signals. Meanwhile, cytokines promoting the T-cell functions may also induce the life-threatening cytokine release syndrome without clear indicators. Given a large number of signaling molecules and the intricate crosstalks among them, it remains a significant challenge for the field to gain a comprehensive view of the complicated immune ecosystem.

Leading-edge technologies such as single-cell omics and spatial genomics have enabled the profiling of the tumor microenvironment at high dimensions. Many of these new technologies, coupled with computational models, can reveal the cytokine and chemokine activities through the molecular status of their downstream signaling pathways. Meanwhile, the rapid development of automated technologies has enabled the large-scale screening of immunological assays in preclinical models. Catalyzed by recent technological advances, we foresee rapid knowledge growth on cytokines and chemokines.

Only two cytokines interferon-alpha and IL2 have been approved by the FDA for treatment of refractory melanoma and renal cell cancer, and are rarely used as monotherapy. Bottlenecks in the therapeutic application of cytokine therapies include the dose-limiting toxicity, the short half-life in the circulation, the low concentration at tumor sites when administered intravenously, and the unwanted recruitment of immune suppressive cells. Advancements in delivery technologies have shown encouraging safety and efficacy in preclinical models. For example, smart nanocarriers can respond to multiple stimuli in the blood circulation and tumor by changing their physical and chemical properties for precise and lasting cytokine release (254, 255).

T cells armed with stimulatory cytokines have great potential to remodel the suppressive tumor microenvironment. However, there remains a need to generate "smarter" T cells, given the multiple suppressive signals in tumors, the inter-and intra-tumor heterogeneity, and potential severe toxicities of current T cell-based therapies. Modern gene-editing technologies can encode immunomodulatory fusion proteins in T cells to rewire the inhibitory or death receptor signaling. Such engineered T cells should sense the host environment and react to different cues in a precise manner. The synthetic Notch receptors could serve as a versatile platform for engineered T cells. Activation of Notch and the intracellular transcriptional signal upon the customized antigen sensing should trigger production of a specific cytokine profile (256).

Given the complexity of tumor heterogeneity, it is unlikely that one therapeutic solution is sufficient to overcome the T-cell exclusion barriers in tumors. We foresee that future successful treatments will leverage rational combinations among different therapy modules after elucidating specific cytokine activities in patient samples through high-resolution genomic technologies.

AUTHOR'S NOTE

1. IL6 has a dual function in the tumor microenvironment. It has a dark face that acts on tumor cells through multiple intrinsically downstream mediators to support cancer cell proliferation, survival, and metastatic dissemination. IL6 also works on other cells within the tumor stroma, promoting angiogenesis and tumor evasion (17).

2. TNF plays a dual role in cancer immunity, TNF induced T-cell adhesion is ICAM1 and VCAM1 dependent, and most of the chemo-attracted cells are Tregs (257), Bregs (258, 259) and MDSC (259), which are negative modulators of the immune response. TNF also triggers the activation-induced cell death of

CD8 T lymphocytes (**Table 4**) and impairs tumor infiltration by CD8 T lymphocytes (**Table 2**).

3. CCL5 has a tumor-promoting role by inducing tumor cell proliferation (260), angiogenesis, and matrix metalloproteinases (47). It also suppresses the antitumor immune response by increasing the recruitment of TAM and Treg in tumors (63). Moreover, it stimulates the apoptosis of CD8 T cells (**Table 4**).

4. The IL35 receptor components vary by cell type. In T cells, IL-35 binds IL6ST (gp130) and IL12Rβ2. In B cells, IL35 signals through IL12Rβ2 and IL27RA (WSX-1) (5, 261).

5. CXCL8 has a tumor-supporting role by activating the epithelial-mesenchymal transition (262), promoting angiogenesis (262, 263), and stemness potential (264).

6. IL2 and IL15 share the β and γ components of their receptors and have similar functions on T cells, including stimulating proliferation of cytotoxic T lymphocytes. However, IL2 promotes terminal differentiation and elimination by AICD (265), but IL15 inhibits AICD and promotes the generation of long-lived stem memory T cells and maintains homeostatic proliferation (266). Other cytokines sharing the same γ-chain in their receptors (defined as the γc cytokine family) include IL4, IL7, IL9, and IL21 (150).

7. Reasons that prevent the extensive usage of IL2 for cancer therapy include short half-life *in vivo* (144), severe toxicity at therapeutic dosage (145), and propensity to promote Treg proliferation (146).

8. Treg-derived IL10 drives the exhaustion of CD8 T cells in tumors through the up-regulation of several inhibitory receptors (PD1, LAG3, and TIGIT) *via* a BLIMP1 dependent pathway (162). IL10 also inhibits the activation of CD8 T cells by decreasing antigen sensitivity (163).

9. NHS stands for the antibody NHS76 against DNA released by necrotic tumor cells.

10. Th17 is a double-edged sword in anticancer immunity. It may secrete high levels of the characteristic cytokine IL17 to stimulate angiogenesis and tumorigenesis. In contrast, Th17 may stimulate the effector CD8 T cells as a tumor-suppressive factor (224).

11. IL4 exerts controversial functions based on cancer types. In breast cancer, It promotes tumor growth by suppressing the effector function of Th1-polarized T cells (267). However, it drives the survival of B cell and T cells in other cancer types and promotes the long-term development of CD8 T memory cells (217).

12. Some studies demonstrated the antitumor activity of Th2 cells in collaboration with tumor-infiltrating granulocytes (268).

AUTHOR CONTRIBUTIONS

YZ and PJ outlined the review structure, finished the literature survey, and wrote the manuscript. X-yG participated in the discussion. All authors contributed to the article and approved the submitted version.

FUNDING

The work is sponsored by the NIH intramural research program and Bau Tsu Zung Bau Kwan Yeu Hing Research and Clinical Fellowship from the University of Hong Kong.

ACKNOWLEDGMENTS

We acknowledge the NIH Fellows Editorial Board for their language editing service. We also acknowledge suggestions from Dr. James Kochenderfer on our manuscript.

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

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frontiers in Immunology

REVIEW
published: 27 January 2021
doi: 10.3389/fimmu.2020.604967

Next Generation Imaging Techniques to Define Immune Topographies in Solid Tumors

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In recent years, cancer immunotherapy experienced remarkable developments and it is nowadays considered a promising therapeutic frontier against many types of cancer, especially hematological malignancies. However, in most types of solid tumors, immunotherapy efficacy is modest, partly because of the limited accessibility of lymphocytes to the tumor core. This immune exclusion is mediated by a variety of physical, functional and dynamic barriers, which play a role in shaping the immune infiltrate in the tumor microenvironment. At present there is no unified and integrated understanding about the role played by different postulated models of immune exclusion in human solid tumors. Systematically mapping immune landscapes or "topographies" in cancers of different histology is of pivotal importance to characterize spatial and temporal distribution of lymphocytes in the tumor microenvironment, providing insights into mechanisms of immune exclusion. Spatially mapping immune cells also provides quantitative information, which could be informative in clinical settings, for example for the discovery of new biomarkers that could guide the design of patient-specific immunotherapies. In this review, we aim to summarize current standard and next generation approaches to define Cancer Immune Topographies based on published studies and propose future perspectives.

Keywords: immune topography, solid tumors, immune exclusion, imaging techniques, deep learning, single-cell analysis

OPEN ACCESS

Edited by:
Carlo Bifulco,
Providence Portland Medical Center, United States

Reviewed by:
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Fudan University, China
Brian Piening,
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *10 September 2020*
Accepted: *03 December 2020*
Published: *27 January 2021*

Citation:
Pietrobon V, Cesano A, Marincola F and Kather JN (2021) Next Generation Imaging Techniques to Define Immune Topographies in Solid Tumors. Front. Immunol. 11:604967. doi: 10.3389/fimmu.2020.604967

INTRODUCTION TO CANCER IMMUNE TOPOGRAPHIES

Cancer Immunotherapy in Hematological and Solid Tumors

The first application of immunotherapy was reported in 1891, when Dr. William B. Coley saved a patient with inoperable multiple advanced tumors by infecting him with streptococcal bacteria. The immune reaction produced caused shrinkage of the malignant tumor. However, the advent of radiation and chemotherapy resulted in a dismissal of Coley's approach. This changed in the last decades leading in 2013 editors of the Science magazine to elect cancer immunotherapy as the "Breakthrough of the Year." In 2018, the Nobel Prize in physiology or medicine was awarded to two cancer immunotherapy researchers, J. P. Allison and T. Honjo, for their discovery of immune

checkpoints (1). Today, cancer immunotherapy is considered a promising therapeutic strategy against a variety of hematological and solid malignancies (2–4). Immune checkpoint inhibitors and chimeric antigen receptor (CAR) T-cells can induce durable remissions in many cancers and are clinically accepted as standard treatments for several cancers.

Significant clinical responses have been observed in hematological malignancies, using CAR-T-cells engineered to recognize CD19 molecules on B-cells (2, 5, 6). Treatments with checkpoint blocking antibodies have also been approved by the FDA for melanoma, lung cancer, breast cancer, kidney cancer and many other solid tumor types (7–15). Despite this progress, only a limited subset of patients responds favorably to the treatment and some tumors, such as prostate cancer and most gastrointestinal malignancies, have been proven to be particularly resistant to checkpoint inhibition, particularly when used as single agent (16–19). In general, solid tumors present various challenges to the applicability of immunotherapy, including the selection of the antigen to target, the infiltration of T-lymphocytes into the tumor core and the highly immunosuppressive tumor microenvironment (TME), which are all hallmarks of solid tumors (20). Solid tumors are heterogeneous ecosystems and they can contain different immunological niches in different regions of the same lesion. Systematic documentations of this phenomenon are scarce with the exception of a recent study which used computational image analysis inspired by geospatial data to quantify the heterogeneity of topographies in lung cancer (21). In some types of cancer, such as colorectal cancer (CRC), specific Immune phenotypes are linked to specific genotypes. For example, highly immune-infiltrated tumors are associated with hypermutation, which are mostly due to specific genetic features such as microsatellite instability or mutations in POLD1 or POLE genes in CRC (22).

Immune Topographies in Solid Tumors: Hot, Cold, and Immune Excluded Tumors

For decades, the gold standard method for diagnosing and subtyping almost any type of solid tumor has been visual examination of histopathology slides of tumor tissue. These slides are unspecifically stained with hematoxylin and eosin (H&E) which allows pathologists to discern cellular and subcellular structures. In spite of the technological progress in molecular biology assays, subjective evaluation of histopathology slides remains the backbone of solid tumor diagnostics. Although H&E staining dyes do not selectively bind to specific cell types, the visual characteristics of different cells allow a reproducible classification of cells into tumor cells, lymphoid immune cells, granulocytes, fibroblasts and other groups of cells in the tumor microenvironment. In particular, tumor-associated lymphocytes (including tumor-infiltrating and peritumoral lymphocytes) can be easily spotted due to their unique size, morphology and staining characteristics. Historically, the presence of such lymphocytes in or around tumor tissue has been regarded as an epiphenomenon of malignant tumor growth. However, mounting evidence supports the notion that the presence of these lymphocytes reflects an adaptive anti-tumor immune response by the host immune system and is a prognostic biomarker as well as a predictive biomarker of response to immunotherapy (23).

Systematic analyses of the distribution of lymphocytes in histopathology specimen have allowed to classify solid tumors according to three distinct Immune Topographies (24): a) Hot tumors, infiltrated by lymphocytes, i.e. lymphocytes are mixed with tumor cells in the tumor core (**Figure 1A**); b) Cold tumors, characterized by an absence of lymphocytes, i.e. almost no lymphocytes can be seen on histological slides (**Figure 1B**); c) Immune-excluded tumors characterized by an abundance of lymphocytes at the invasive edge of the tumor, but few to no lymphocytes in the tumor core (**Figure 1C**).

Hot tumors present a homogeneous infiltration of T lymphocytes together with the accumulation of pro-inflammatory cytokines, and usually respond better to immunotherapy (25). Immune excluded tumors embody a unique ecosystem, differing from hot tumors, as they display gradients of T-cell exclusion (26). Such gradients are specific to each tumor environment and not present in cold tumors, where T-cells are completely absent. This trichotomy can be detected across most solid tumors and is directly associated with clinical outcome and response to immunotherapy (24, 27–30).

However, few published studies have systematically quantified the trichotomous Immune Topography in solid tumors beyond histopathological description (24). At present, there is only a limited understanding about how cellular mechanisms of immune exclusion may relate to each other in shaping this peculiar phenotype in human cancers, either through converging biological pathways or from a causative standpoint. Compelling data collected through high-throughput analysis would shed light on the spatial and temporal dynamics in which such determinants are involved, allowing for the creation of a harmonized 'Theory of Everything' (31, 32). Moreover, mapping the spatial distribution of immune cells in solid tumors also provides quantitative information potentially informative in clinical settings, allowing for the design of combinatorial approaches aimed at improving immunotherapy efficacy.

Tertiary Lymphoid Structures and Other Second-Order Topographies

Hot, cold and immune-excluded tumors are among the most striking patterns that can be observed in histopathology images. However, in addition to this trichotomy, immune cells in cancer tissue can form additional patterns which have been quantified and linked to biologically and/or clinically meaningful endpoints. These "second-order" Immune Topographies do not rely on pre-defined compartments within the tumor and around the tumor. For example, one such pattern of lymphoid cells observable in histopathology images of cancer is a tertiary lymphoid structure (TLS, **Figure 1D**). These structures are composed of lymphocyte conglomerates organized to resemble lymph node germinal centers and can be observed outside of (33) or within (34) the tumor tissue. The presence and number of these TLS is positively correlated to survival (35) and immunotherapy response (36) in a number of cancer types.

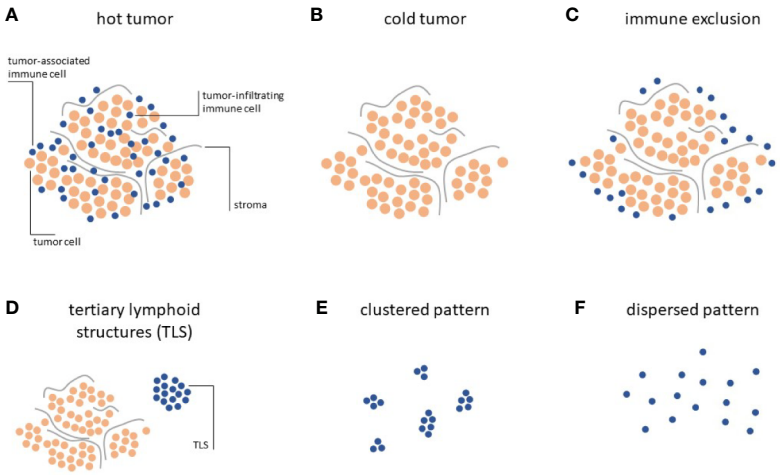


FIGURE 1 | Immune topographies of cancer. **(A–C)** First-order immune topographies. **(D–F)** Second-order immune topographies.

However, systematic analyses of association with TLS count and other types of Immune Topographies are still lacking. In summary, it is still not entirely clear how different Immune Topography patterns are correlated to one another and, collectively, to clinical outcome. Large-scale studies in thousands of patients treated with immunotherapy and annotated with clinical outcomes are needed to validate and reconcile these findings in the future. Another approach was recently described by Saltz and coworkers by an unbiased, computational approach to cluster tumors according to their spatial patterns of lymphocyte infiltration (37). In particular, this approach considered the notion of *clustered* (**Figure 1E**) and *dispersed* (**Figure 1F**) lymphocyte infiltration and the authors could demonstrate a link between the observed lymphocyte patterns and patient survival, the ultimate clinically relevant end point. However, this large-scale analysis was limited to a single multicenter dataset, the Cancer Genome Atlas (TCGA), which might suffer from batch effects and other sourcing bias (38).

In this review, we aim to summarize the current knowledge in standard and next generation techniques to define Cancer Immune Topographies, including the performed studies, outcomes and future perspectives.

BIOLOGICAL MECHANISMS OF IMMUNE TOPOGRAPHIES

Barriers to an Effective Immune Response in Solid Tumors

Determinants of immune exclusion can be classified into three groups: physical, functional or dynamic barriers. Physical barriers represent a category where T-cells do not come in direct contact with cancer cells, due to mechanical separations. Therefore, activation of the immune effector gene signature is not observed (30). However, T lymphocytes can also engage with cancer cells, but functional determinants block their migration, expansion, function and/or survival within the tumor core. Functional barriers consist of constitutive metabolic interactions among immune cells, cancer cells and cells in the TME. Finally, dynamic barriers include functional barriers, which may be induced only when a contact between T-cells and cancer cells occurs, preventing further infiltration, activation and/or survival of immune cells. Dynamic barriers may not be present in baseline conditions (39–41). Here, we will give a brief overview of these determinants, as it is beyond the scope of this review to describe them in more detail.

Physical Barriers

Physical barriers include the remodeling of the extracellular matrix (ECM), cancer cell coating factors and changes in vascular accessibility (**Table 1**). In tumor tissues, the most frequent alteration of the ECM includes increased collagen deposition and a rearrangement of its geometry; this leads to cancer-associated fibrosis and possibly to a physical barrier to T-cell penetration (115–120). A variety of chemokines are responsible for this process, which requires the activation of recruited and resident fibroblasts, myofibroblasts, cancer-associated fibroblasts (CAFs) and cancer-associated mesenchymal stem cells (42–44, 121–123). CAFs have also been shown to be responsible for the biosynthesis of CXCL12, which binds and shields cancer cells (64–66).

Another mechanism involved in the physical exclusion of T-cells may be related to tumor angiogenesis. As cellular proliferation outgrows blood supply, most solid tumors experience hypoxic conditions (124). In hypoxia, genes involved in angiogenesis are upregulated, including the vascular endothelial growth factor family (VEGF) (125, 126). Tumor angiogenesis often produces blood vessels with aberrant morphology and this could result in T-cell exclusion (87, 88, 127). Additionally, VEGF not only plays a prominent role in mediating T-cells infiltration into tumors, but also in regulating their function (59, 60, 92).

TABLE 1 | Determinants of immune exclusion.

		References
Mechanical barriers: physical impediments to a direct contact between T cells and cancer cells		
ECM remodeling	Cancer-associated fibrosis	(42–48)
	Epithelial-to mesenchymal transition	(49–55)
	Filaggrin, desmosomal proteins, endothelin B receptor	(30, 56–58)
Vascular accessibility	HIF-1 and HIF-2 driven angiogenesis	(59–63)
Cancer cell coating	CXCL12	(64–66)
Functional barriers: determinants limiting migration, function, and/or survival of T cells		
Metabolic alterations TME	Decrease in amino acids in the TME	(67–72)
	Warburg effect (increase in lactate)	(73–76)
	Increase in extracellular adenosine	(77–80)
	Increase in potassium	(62, 81, 82)
	Altered lipid metabolism	(83)
	Cyclooxygenase and prostaglandin metabolism	(84–86)
	Hypoxia	(87–91)
Soluble factors	VEGF-α	(59, 60, 92–94)
	Cytokines mediated immune suppressive mechanisms	(42, 43, 95–98)
Danger sensing molecules	TAM receptors	(99–102)
	don't eat me signals	(103–105)
	Tolerogenic cell death/absent immunogenic cell death	(106–108)
Tumor cell signaling	STAT-3, PI3K, NF-κB, Wnt/β-catenin, MAPK, p53	(109–114)
Dynamic barriers: barriers arising after interaction between T cells and cancer cells		
	Checkpoint/ligand interactions	(39–41)

Functional Barriers

Functional barriers consist in metabolic alterations of the TME, immune suppressive soluble factors, danger sensing molecules and tumor cell-intrinsic signaling (**Table 1**). Cancer cell metabolism often leads to the reshaping of TME conditions, depleting it of amino acids (i.e. glutamine), which are essential for proper T-cell function (67, 128–133). Moreover, TME often presents increased concentration of lactate, due to the shift toward glycolytic metabolism of cancer cells (Warburg effect) and increased concentration of ions and other immune suppressive components, such as extracellular adenosine (134–137). Therefore, low pH, low glucose and reduced amino acid presence in the TME collectively lead to T-cell dysfunction.

T-Cell Signaling

A complex signaling network is responsible for the impaired function of T-cells, leading to lymphocytes that are exhausted, anergic, senescent or presenting stem features. Stem-cell-like T-cells possess the ability to proliferate and persist, but they are unable to mature (81, 138). Recent evidence showed that an overabundance of potassium in the TME triggers suppression of T-cell effector function, while preserving stemness (138). This happens through metabolic remodeling as a result of caloric restriction and a T-cell starvation.

Interestingly T-cell stemness can also enhance the effectiveness of immunotherapy: the generation of CD19-specific CAR-modified $CD8^+$ memory stem cells led to long-lasting antitumor response and increased T-cell fitness, in a human acute lymphoblastic leukemia xenograft model (82, 139, 140).

A variety of chemokines have been implicated in the recruitment of T-cells into the tumor nest. In immune excluded tumors, it is possible that gradients of chemokines exist from the periphery to the center. However, additional repulsive mechanisms may limit the propulsion of T-cells, counterbalancing and overpowering attractive signals and reducing the chemo-attractive infiltration. In addition, stressed or dying cancer cells may inhibit proinflammatory signals, thereby affecting the efficiency of the immune response (**Table 1**) (141–143).

Dynamic Barriers in the Tumor Microenvironment

Finally, dynamic barriers represent a category of impediments absent in baseline conditions, but which arise as a consequence of the interaction between T-cells and cancer cells. This hints to a dynamic crosstalk between the two, at early stages. An example is the inducible activation of PDL-1 triggered by the production of IFN-γ by T-cells (**Table 1**) (39–41).

It is unclear if a predominant biology is responsible for most immune excluded cases or if an indiscriminate contribution of factors could better explain this complex phenomenon. Moreover, at present studies have not investigated if a correlation exists between immune exclusion mechanisms and tumor type or stage.

HOW TO QUANTIFY IMMUNE TOPOGRAPHIES

In Vivo and *Ex Vivo* Approaches

Over the years, studies have demonstrated the existence of a plethora of determinants playing a role in the immune excluded phenotype. Modern high-throughput techniques allow us to create pan-cancer Immune Topographies, characterizing spatial and temporal distribution of T-cells in the TME (24). Mapping *ex vivo* immune cells and correlating such distributions with determinants of immune exclusion and morphological parameters, would provide mechanistic insights into the dynamic organization of factors responsible for this phenomenon. It is

possible that specific determinants of immune exclusion could correlate with some tumor types or with the tumor stage, rather than appearing randomly and chaotically.

In vivo studies offer information to design effective personalized combinatorial immunotherapies and clinical monitoring. Finally, it may be possible to integrate the data obtained from *in vivo* and *ex vivo* techniques, for the different determinants of immune exclusion. Such a comprehensive analysis might lead to the understanding of a common biology at the basis of the immune excluded phenotype.

Histology Images

Histopathology images are a versatile and well established method to analyze the tumor microenvironment and Immune Topographies in solid tumors. Histological specimens are routinely generated from preclinical tumor models and are available for almost any patient with a solid tumor in the clinic. The standard staining method for histopathology slides is hematoxylin and eosin (H&E) which allows for a rough classification of cells in the TME. By visually looking at histopathology slides or digitized whole slide images, pathologists can quantify patterns of antitumor immune response.

Although hot, cold and immune-excluded Immune Topographies can be visually determined just by looking at H&E stained histopathology slides, two methods have enabled a more quantitative description of these topographies: Immunohistochemistry and computer-based analysis.

Immunohistochemistry and Multiplex Imaging Techniques

Immunohistochemistry (IHC) methods can be used to selectively label certain immune cell subtypes in histology images, allowing for a more nuanced definition of Immune Topographies. IHC uses antibodies which are directly or indirectly coupled to certain dyes, allowing it to highlight markers specific to certain cell types. For example, cytotoxic lymphocytes are defined by a presence of the CD8 protein on their cell membrane. IHC methods have recently inspired a whole range of more sophisticated multiplex methods, allowing to characterize the expression of multiple proteins in one image. Multiplex fluorescence imaging (144), multiplex brightfield imaging (145) are among the most widely used methods to deeply characterize tumor-associated immune cells in a spatially resolved way. However, these methods are much more expensive, time-consuming and complex than H&E staining and usually require access to specialized and costly equipment. Thus, the advantages of these deeper methods need to be balanced against the simplicity of classical H&E histopathology, which allows for a broader characterization of thousands of patient samples in larger cohorts. Accordingly, most studies which have analyzed H&E histopathology images include a much higher number of patients than studies adopting more specialized methods.

Hypoxia-Associated Proteins

As previously mentioned, hypoxia is a key player in the immune excluded phenotype. Hypoxia is responsible for a dramatic reshaping of cellular transcriptional programs, through the activity of specific transcription factors called Hypoxia Inducible Factor 1 and 2 (HIF-1 and HIF-2). These proteins are responsible for the upregulation of a subset of genes essential to ensure adaptation, survival and proliferation in hypoxic conditions (146–148). Common hypoxic markers used in IHC include: CAIX, VEGF-A, EPO, GLUT-1 and GLUT-3, osteopontin, BNIP3, PDK1, LDHA, and LOX (149–153). These proteins are transcriptionally induced by HIF-transcription factors. HIF-1 can also be directly assessed in IHC, but correct sample handling is essential. The half-life of HIF-1 at 20% oxygen is approximately 5 min while other markers (i.e. VEGF-A and CAIX) are more stable (147, 154–156). Therefore, it is crucial to select the most appropriate proteins to test, based on sample processing procedures. Exogenously administered compounds can also be used to detect hypoxic regions in IHC. A nitroimidazole molecule called pimonidazole and a pentafluorinated derivative of the 2-nitroimidazole etanidazole, called EF5, are the most widely used (157–159). These non-toxic compounds are administered from a few hours to 48 h prior to biopsy and immunochemical detection is then performed. Pimonidazole directly correlates with the severity of hypoxia, and IHC has been successfully used to assess tumor hypoxia in patients with cervical carcinoma, prostate cancer and head and neck carcinoma (150, 157, 160). EF5 is also routinely employed to detect gradients of hypoxia as shown in studies on patients with head and neck tumors and uterine cervix cancer (161, 162). EF5 has also been used to detect hypoxia in atherosclerotic plaques in mice and in xenograft models of human colorectal carcinoma and sarcoma (163, 164).

Digital Pathology Approaches

Digital pathology, i.e. computer-based processing of digitized histopathology slides, has been used to automatically detect and count tumor-associated immune cells. Such approaches can be used to automate detection of Immune Topographies and to establish quantitative thresholds for classification of a given sample in either class. In the early days of digital pathology, the sheer size of scanned whole slide images (WSI) has been an obstacle for many researchers to analyze such data. Nowadays, however, more widespread availability of digital slide scanners, cheaper storage media and the emergence of easily usable open source software such as QuPath (165) enables almost any researcher at academic institutions to use computer-based approaches for quantitative analysis of pathology slides. These quantitative analyses can be performed with H&E stained images, single-immunostained images or multiplex images. One way of quantifying hot-cold-excluded topographies by means of digital pathology is with the "Immunoscore" (166), which has been extensively validated in large-scale studies (28). While the original "Immunoscore" protocol relies on proprietary software marketed by a commercial entity, the underlying principle can be re-built based on publicly available information (167).

Deep Learning-Based Image Analysis

Medical image data, and particularly digitized histopathology slides contain a large amount of information which is not entirely

used. In particular, human observers, who visually analyze histopathology slides cannot objectively and quantitatively extract all relevant information. Deep learning is a method from the realm of artificial intelligence, which in recent years has revolutionized computer-based image analysis in non-medical and medical domains alike. Specifically, when applied to digital whole slide histology images, deep learning can extract biologically and clinically relevant information. In particular, immune-related features can be extracted from histopathology images. For example, gene expression signatures of cancer-infiltrating immune cells can be detected solely from H&E images in multiple types of solid tumors (168, 169).

Genome Sequencing Technologies

A comprehensive mechanistic insight regarding the correlation of functional, physical and dynamic barriers with morphological parameters in human cancer could be achieved thanks to last advances in high throughput analysis. These techniques are laying the foundation for a pan-cancer comprehension of the complexity of factors orchestrating the immune response in the TME. Recent studies profiled the TME of several cancer types including lung cancer, hepatocellular carcinoma, medulloblastoma, melanoma, head and neck cancer, pancreatic cancer and glioma (170–176).

Current developments in automated, multiplexed platforms to detect biomarkers and the increasing number of spatially resolved profiles of transcripts and metabolic products, could be important to provide an integrated landscape of molecular determinants driving the phenomenon of immune exclusion. Obtaining correlations between markers of different types of barriers, degree of immune infiltration/activity and tumor type/stage would allow us to investigate if one or multiple pathways are prevalent in human cancer or if these pathways just overlap indiscriminately. Such information, accumulated in a large cohort of human cancers, would be pivotal to improve diagnostic strategies and to predict the response to treatments.

Transcriptional profiling of "immune-mediated, tissue-specific destruction (TSD)" events led to the creation of an immune signature called immunologic constant of rejection (ICR) (31, 177–179). ICR is a 20-gene signature and characterizes a convergent pathway leading to TSD, also called immune rejection. Such signature can be observed in a variety of immune events: tumor regression, autoimmunity, clearance of pathogens and allograft rejection (179). ICR expression was correlated with a better prognosis in breast cancer patients and was validated as a prognostic predictive parameter in a pan-cancer cohort of patients treated with an anti-CTLA4 immune checkpoint inhibitor (109, 180, 181). The tumor inflammation signature (TIS) is considered as another immune predictive biomarker and it is an 18-gene signature (182, 183). TIS has been shown to be enriched in patients responding to anti-PD1 treatment and the expression patterns were conserved among tumor types (182).

Bioinformatics studies have been performed during the years, to investigate immune signatures in different types of tumors. These analyses rely on data from public cancer databases and provide a coarse evaluation of T lymphocytes functional status in bulk tumor samples. Computational deconvolution analysis on bulk RNA-seq data can be used to infer infiltrating cell types. Such analysis is limited by the existence of specific gene signatures, relative to cell types (184–187). However, no spatial or temporal resolution can be obtained from bulk bioinformatic studies. In order to achieve more detailed information about T-cell populations within the tumor mass and their functional state, single-cell techniques were developed and experienced tremendous progress in the past few years.

Single-Cell RNA Sequencing

Single-cell RNA sequencing (scRNA-seq) allows the investigation of the expression of hundreds of genes in a single experiment, enabling systematic identification of cell populations in a tissue. This technique provides insights into tumor heterogeneity and it has been used to assess both abundance and functional state of tumor associated cell types (188–193). ScRNA-seq has been increasingly employed due to a reduction in costs (sequencing and cell isolation) and improvement in throughput.

The most common scRNA-seq technologies rely on microfluidic devices which use patterned microwells for single-cell isolation or aqueous droplets in a continuous oil phase. Once isolated, cells are lysed and a whole transcriptome approach can be performed (i.e. Smart-seq2, MATQ-seq, SUPeR-seq) (194–196). Alternatively 3'-end or 5'-end sequencing technologies are available (i.e. Drop-seq, CEL-seq, Seq-Well, MARS-seq, Chromium, Quartz-seq, DroNC-seq, STRT-seq, etc.) (197–206). These two categories present different transcript coverage and each protocol has specific features, benefits and drawbacks.

Unfortunately, single-cell techniques require monodisperse cells, leading to loss of spatial information during cell isolation. Moreover, tumor solid biopsies could lead to biases and failure to identify the whole transcriptional profile or the truthful tumor-associated cell infiltration landscape. This can be due to the sampling location and to the fact that, only a small fraction of cells from the biopsy, can be sequenced (207–209). LCM microdissection is a method of sample dissection which consents the preservation of spatial information and partially rescues the technical limitation mentioned above. Cells from the region of interest are collected using laser pressure catapulting or through a near-IR laser, after being microscopically identified (210–212). Experimental or computational spatial reconstruction can also be obtained *via* immunohistochemistry, laser scanning microscopy, fluorescent *in situ* RNA sequencing (FISSEQ) or with anchor genes, through single molecule fluorescence *in situ* hybridization (smFISH) (213–217).

Single cell analysis allowed the investigation of the heterogeneity of tumor cells and tumor-infiltrating immune cells in breast cancer, clear cell renal cell carcinoma, colorectal, glioblastoma multiforme, melanoma, liver, ovary, non-small-cell lung carcinoma, nasopharyngeal cancer, squamous cell carcinoma of the head and neck and gastric cancer (168, 188–192, 218–222). The immune cells prevalently identified in most of these tumors were T lymphocytes; however myeloid cells, B cells and natural killer cells were also present at lower frequencies.

Immune cell subtypes were transcriptionally characterized, and their ontogeny was mapped, together with cell trafficking to different tissues or tumors (223). Clonotype expansion and migration was monitored by barcoding V(D)J recombination at the T-cell receptors (TCR) and B cell receptors (BCR) loci (224–226). V(D)J

recombination occurs during T and B cell maturation, resulting in the diverse repertoire of TCR and BCR present in the lymphocyte population. Gene expression signature of T-cell clusters reflects a specific functional status and such diversity is crucial in clinical settings, to predict immunotherapy response. Indeed, T-cell populations with lower exhaustion levels were associated with a better prognosis in a variety of different cancer types (227–229). A study on gastric cancer reported that the interferon regulatory factor-8 transcription factor (IRF-8) was downregulated in CD8+ tumor infiltrating lymphocytes (TILs), leading to an exhausted phenotype. Patients with lower IRF-8 levels in $CD8^+$ lymphocytes tended to have worse prognosis (230). A recent publication showed that, in early-stage triple-negative breast cancer, among $CD8^+$ TILs, tissue resident memory cells display high expression of cytotoxic molecules, inhibitory checkpoint and genes associated with proliferation. This study suggests that T-cell exhaustion is a gradual process (231, 232). In clear cell renal cell carcinoma, PD-1 was found widely expressed in both $CD8^+$ and $CD4^+$ T-cell populations, while other inhibitory molecules were present only in a subset of PD-1 positive T-cells (189). Studies on the functional state of T-cells were also performed in melanoma and non-small-cell lung carcinoma patients, allowing the characterization of dysfunctional T-cells in the TME (191, 233). Xiao et al. (222) developed a computational pipeline to investigate the metabolic landscape of the tumor, at single cell resolution. They analyzed metabolic gene expression profiles of more than 9000 single cells from melanoma and squamous cell carcinoma of the head and neck. Metabolic pathways in tumor cells were found to generally be more plastic and, interestingly, glycolysis and oxidative phosphorylation both correlated with hypoxia at the single cell level. Metabolic features of immune cells were also identified and found to be altered. Characterizing the metabolic landscape in the tumor core could provide insights into the organization and prevalence of functional barriers.

Interactions among cells play a central role in shaping the TME altering cell metabolism, immune response and creating barriers to lymphocytes infiltration or activity (234, 235). Despite the study of cell interactions using single cell approaches is at early stages, a new publicly available repository of curated receptors, ligands and their interactions is available. CellPhoneDB (www.cellphonedb.org) takes into account the subunit architecture of both ligands and receptors and, coupled to scRNA-seq data, is a powerful tool to infer cell-cell communication networks (236, 237). Recently, scRNA-seq coupled to CellPhoneDB has been used to reveal interactions between Th2 and mesenchymal cells, in asthmatic human donors (238). ProximID is another strategy to create a cellular network based on scRNA-seq data. ProximID can be used to discover new niches interactions in different tissues, *via* microdissection of small interacting cell clusters and inference of the cell types present in the dissected entity through scRNA-seq. Boisset et al. (239) used ProximID to study mouse bone marrow and found specific interactions between megakaryocytes and mature neutrophils and between plasma cells and myeloblasts and/or promyelocytes. Moreover, they identified stem cell interactions in small intestine crypts.

Single cell analysis is not confined to the investigation of the transcriptome and, recently, the combination of multiple measurements (DNA, RNA, proteins) has been suggested as a comprehensive strategy to understand the TME complexity (223, 240). Innovative techniques such as G&T-seq and DR-seq allow to sequence both DNA and RNA, from single cells (241, 242). Genomic DNA and full-length mRNA are captured and physically divided before amplification and library preparation. These techniques, despite allowing for the comparison of gene expression data and corresponding genomic data in the same cell, increase the risk of sample loss or contamination and present a moderate reduction in coverage distribution.

Another combination of multi-omics techniques, which provides information about the transcriptional status of cells, consists of coupling ATAC-seq with RNA-seq. ATAC-seq can be considered a technique to assess genome-wide chromatin accessibility (243–245) and it relies on a genetically engineered hyperactive Tn5 transposase (246). Such transposase allows fragmentation of chromatin and integration of NGS adapters into open chromatin regions (247, 248). ATAC-seq coupled with RNA-seq was used to identify potential gene regulatory regions in glucagon-secreting α-cells and insulin-secreting β-cells (249) and to unravel disruptions of transcriptional regulations and gene expression in lung cancers (250).

Quantification of proteins and mRNAs simultaneously in individual cells can be obtained through different methodologies: Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq), RNA Expression and Protein Sequencing (REAP-seq) and Antibody sequencing (Ab-seq) (251–254). The workflow includes the creation of a pool of barcoded antibodies againsT-cell surface proteins of interest. Then, cells bind to barcoded antibodies and are encapsulated within a droplet, as single cells. Finally, the scRNA-seq libraries are prepared and sequenced. Such an approach overcomes the lack of correlation that sometimes is found between mRNA and protein levels, providing a more accurate characterization of the cellular phenotype.

The integration of different layers of information could be pivotal to provide insights into signaling networks regulating the immune excluded phenotype. However, as mentioned before, a primary drawback of single cell techniques is the loss of spatial information, which occurs during sample processing. In order to create systematic Immune Topographies, it is of crucial importance to characterize spatial and temporal distribution of lymphocytes in the TME. Therefore, technologies permitting simultaneous transcriptional assessment and preservation of tumor morphology or restoration of spatial information are preferable.

Spatial Transcriptomic Methodologies

New spatial transcriptomic (ST) methodologies exploit spatially barcoded oligo-deoxythymidine microarrays, allowing for unbiased mapping of transcripts (**Figure 2**) (255). ST has been performed to investigate prostate cancer, gingival tissue, breast cancer, pancreatic ductal adenocarcinoma, melanoma, adult human heart tissue, mouse, human and mouse spinal cord tissue and mouse olfactory bulb (221, 256–262). ST does not

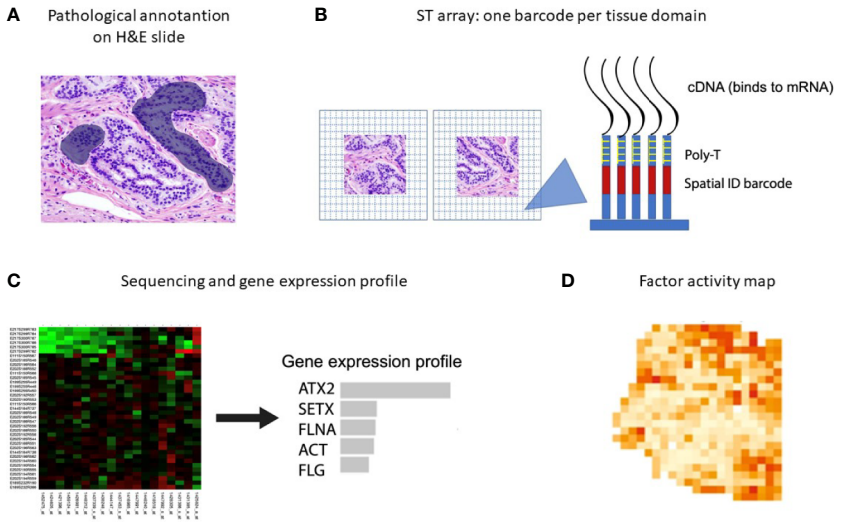


FIGURE 2 | Spatial transcriptomics workflow including the downstream analysis. **(A)** Histological tumor sections are annotated by a pathologist and sections of interest are stained with hematoxylin and eosin before permeabilization. **(B)** The sections are placed on glass slides containing RT-primers arrayed as spots that correspond to tissues domains. The RT-primers at each spot have a unique spatial ID barcode, which is sequenced along with the transcript to enable trace-back to a specific tissue domain. **(C, D)** After sequencing, gene expression profiles and factor activity maps are created.

provide a high resolution as each area resolves the transcriptome of 10-200 cells (~ 100 μm), depending on the tissue context.

Moncada et al. combined microarray-based ST with scRNA-Seq generated from the same sample, to identify enrichments of specific cell types and subpopulations across spatially-defined regions of pancreatic tumors (221). Berglund et al. assessed the transcriptomes of nearly 6,750 tissue regions through ST from a patient with prostate cancer. They extracted different expression profiles for stroma, immune cells, and cancer cells (**Figure 3**) (257). Another example is Thrane et al., who applied the ST technology to melanoma lymph node biopsies. The transcriptomes of over 2,200 tissue domains was sequenced, revealing a detailed landscape of melanoma metastases (259). Nanostring technologies recently developed a high-plex panel to be used with the GeoMx™ Digital Spatial Profiler (DSP). This panel includes more than 1,400 genes to spatial profile tumor and immune pathways, including checkpoint inhibitors, intrinsic cancer cell pathways and predictive markers (263).

ST protocols usually achieve a quite low resolution, however an implementation of this technique called Slide-seq can spatially resolve maps of histological sections at 10 μm resolution (264). Slide-seq substitutes the barcoded oligo-deoxythymidine with DNA-barcoded beads, harboring probes to trap the RNA. This technique was performed to map individual cell types, physically and functionally, in brain cryosections.

Other spatial techniques of interest include fluorescence *in situ* hybridization (FISH), NICHE-seq technology and spatially-resolved transcript amplicon readout mapping (STARmap). FISH allows to achieve a highly multiplexed single-molecule visualization of transcripts. In particular, multiplexed error-robust single-molecule fluorescent *in situ* hybridization (MERFISH) enables RNA imaging of individual cells using physically imprinted error-robust barcodes for individual RNA species. Subsequent rounds of imaging allow to measure these barcodes (265–269). Xia et al. (266) measured RNA species from ~10,000 genes in different subcellular compartments. He also observed transcriptionally distincT-cell states and revealed spatial patterning, in U-2 OS cultured cells.

NICHE-seq technology allowed isolation and sorting of immune cells from a visually selected niche in model animals, expressing a photoactivatable green fluorescent protein (215, 270). ScRNA-seq was performed on sorted cells. This technique preserves the cell states and allows the investigation of the TME influence on immune cells. NICHE-seq was performed to identify T and B cells in mouse lymph nodes and spleens, after virus infection (215). It also revealed niche-specific expression programs and changes in immune localization, in melanoma and naïve inguinal lymph nodes in mouse models (**Figure 4**). However, due to the two-photon laser scanning microscopy which is required to perform this technique, application of NICHE-seq is currently limited to preclinical research.

Three-dimensional distribution of transcripts in mouse model was achieved *via* STARmap. This technique integrates hydrogel-tissue chemistry, targeted signal amplification, and *in situ* sequencing (271, 272). STARmap was used to map 160 to 1,020 genes in 3D-intact tissue from brain mice. It successfully revealed molecularly defined gradient distribution and clustering of neuron subtypes (271).

Emerging Technologies for Multiplexed Molecular Profiling of the TME

The development of targeted therapies that may be efficacious in reprogramming the host immune response to recognize and eliminate tumor cells requires accurate identification of the

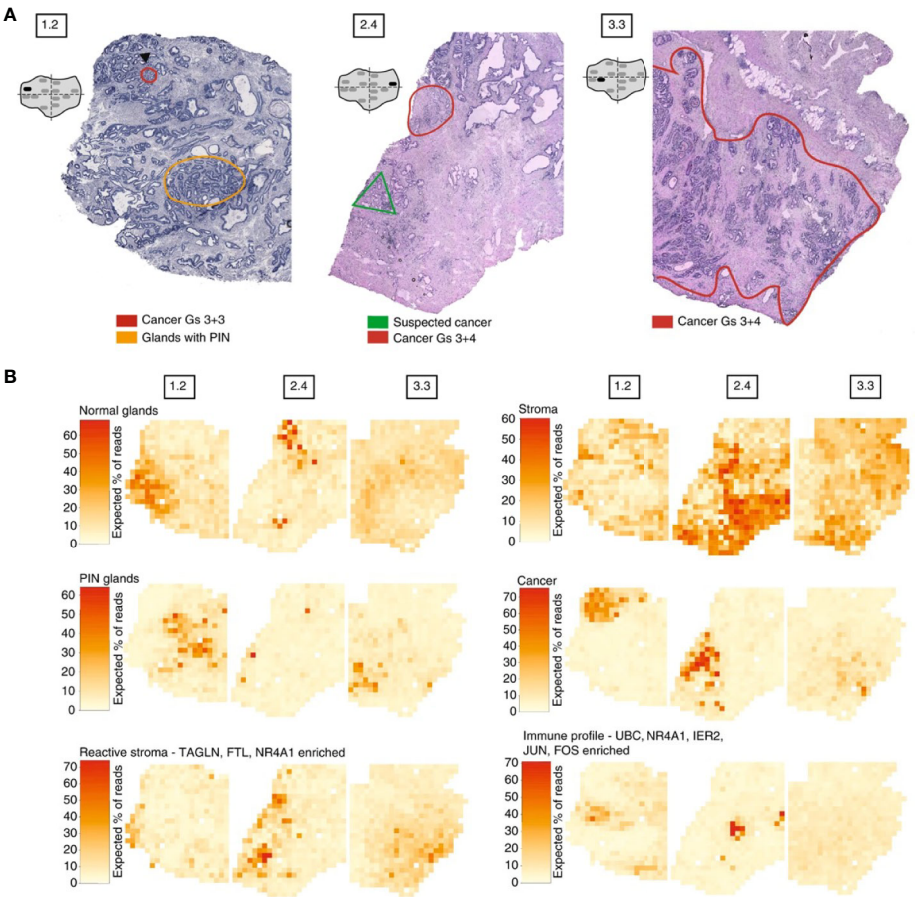


FIGURE 3 | Example of spatial transcriptomic analysis on three prostate cancer biopsies: histology and gene expression factors (257). **(A)** Annotated brightfield images of tissue sections of interest, stained with hematoxylin and eosin. **(B)** Factor activity maps for morphological features (normal glands, PIN glands, stroma and cancer cells) and for inflamed regions (reactive stroma, immune profile).

various inflammatory cells and the special relationships between them within the TME. While currently established techniques enable routinely interrogation for up to two protein markers and evaluate their expression by visual examination, there is a growing need to reliably query many more targets (including both proteins and mRNAs) simultaneously in a given tissue specimen, in order to more precisely characterize the TME within and between tumors. Three new technologies (i.e not based on IHC or IF platforms) aimed at achieving these goals, including multiplexed ion beam imaging (MIBI), codetection by indexing (CODEX) and digital special profiling (DSP) are discussed below.

Multiplexed Ion Beam Imaging (MIBI) is a new technology platform, based on the CYTOF technique that preceded it, with the capability to detect and visualize a large number of protein *in situ* using secondary mass spectrometry to image antibodies tagged with isotopically pure elemental reporters (273). In contrast to standard multiplexed IHC protocols, sample preparation involves one-step, rather than sequential, application of a cocktail of elementally tagged antibodies (274). Samples are subsequently subjected to a rasterized oxygen duoplasmatron primary ion beam which liberates the lanthanide adducts of the bound antibodies as secondary ions and recorded by a TOF-MS. For each physical pixel in the analyzed tissue a mass spectrum is recorded and reflects the abundance of the queried antigens in that location. Recent publications (275) showed that the high-parameter capability, sensitivity and resolution of MIBI are well suited to understanding the complex tumor immune landscape including the spatial relationships of immune and tumor cells and expression of immunoregulatory proteins.

Co-Detection by Indexing (CODEX) is another novel technology for highly multiparametric *in situ* analysis of protein expression using tissue sections. One of the benefits of this technique is its use of a standard fluorescence microscope rather than an ion bean coupled to a mass spectrometer. But unlike the other platforms, published reports involving CODEX have only utilized frozen tissue cut onto glass slides rather than formalin fixed, paraffin embedded (FFPE) tissue sections. Like

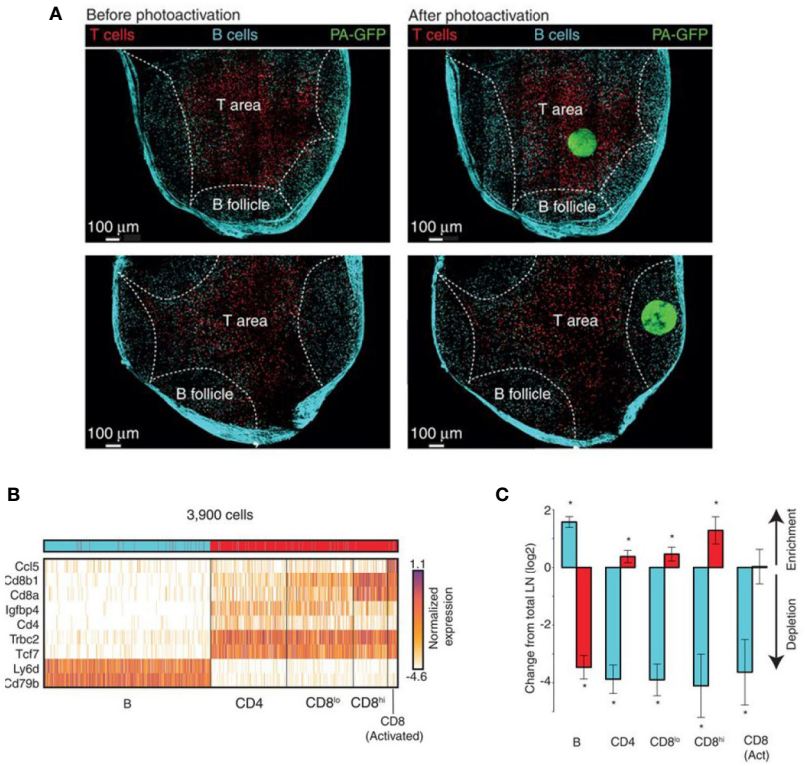


FIGURE 4 | Example of NICHE-seq, assessing the cellular composition of defined niches (215). **(A)** Two-photon laser scanning microscopy (TPLSM) images of naïve inguinal lymph nodes from PA-GFP host mice before and after photoactivation (green). In red, adoptively transferred cells and cyan marks the T-cell area and the B follicles, respectively. **(B)** Expression profile from photoactivated B follicles (cyan) or T-cell areas (red). **(C)** Relative enrichment of different T-cell types [(B), CD4, $CD8^{low}$, $CD8^{high}$ and CD8 Activated] in each subregion. *$p < 0.05$.

the other multiplexing methods, multiple antibodies are applied to a single tissue section for visualization simultaneously; however, the antibodies are tagged with unique DNA oligonucleotides, rather than fluorophores or rare metal elements and then crosslinked to their cellular targets. The process typically involves a single step of immunostaining with up to 40 antibodies each label with a distinct oligonucleotide tag. Visualization of a tissue-bound antibody requires specific PCR-based extension of each antibody bound oligo followed by annealing to a complementary strand of DNA coupled with specific fluorophore. This process is totally automated. However, since the analysis typically involves imaging two to five DNA tagged antibodies at the time a complete analysis of a single tissue requires approximately 30 h to image a 1 cm^2 at 400 nm resolution. At the end of the multicycle rendering protocol each group of antibodies is visualized at a known predefined cycle of the indexing protocol and the multiplexed image is reconstructed (276).

The above mentioned techniques all use antibody-based methods to detect antibody-protein complexes in a tissue section. By contrast, digital special profiling (DSP) is a technology platform which allows, in a non-destructive manner, to profile multiple proteins and RNA from a wide variety of samples types including FFPE tissue sections (263, 277). Briefly, the method uses antibodies or mRNA probes coupled with UV photocleavable oligo tags for the digital detection of specific proteins and transcripts, respectively. After probe hybridization to slide-mounted tissue, UV light exposure is used to liberate the oligo-tags within a small predefined region of interest (ROI) (278–280). The probes are then automatically collected and quantified on a standard nCounter systems (for up to 800-plex profiling or mRNAs or proteins) or sequenced on NGS platform (potentially for unlimited multiplexing) and counts are mapped back to the tissue location, thus producing a spatially resolved digital profile of analyte (protein or mRNA) abundance within each ROI (278–280). Since the UV light is projected into the sample using two digital micromirror devices containing one-million semi-conductor-based micromirrors, a complete flexibility in the pattern of light utilized for high-plex digital profiling of the tissue can be reached. This mechanism results in diverse, automatically configurable, ROI profiles including 1) tumor only; 2) tumor microenvironment only; 3) unique cell types and rare cell features; 4) spatial gradient around cell features; 5) simple hand-selected geometric areas or a combination of the above methods (277). Furthermore the technology does not destroy the sample thus allowing for multiple profiling cycles of the same tissue section or subsequent DNA sequencing of the same section.

Reports of application of this technology to immuno-oncology clinical trial samples are emerging (278, 279). Immuno-oncology clinical trial samples examined using DSP have already provided key insights into the mechanism of action of combination therapy in melanoma (278, 279). While such sophisticated approaches to tissue evaluation of biomarkers hold tremendous promise they are nonetheless in their infancy and therefore come with one or more caveats at this time including costs, lack of standardization across labs, time and labor intensive protocols and lack of widespread availability.

In Vivo Imaging and Functional Imaging

In vivo imaging of T-cell distribution could be a powerful strategy to provide dynamic and spatial information regarding immune exclusion in tumors, during preclinical studies and in a clinical setting. Non-invasive cell tracking would allow us to monitor and quantify cellular delivery and effectiveness of immunotherapeutic approaches. A robust technique would also allow the selection of an appropriate dosing regimen. Over the years, significant developments in imaging immune cells were made and a variety of techniques is currently available for preclinical or clinical use.

Optical detection includes fluorescence or bioluminescence imaging and is mainly performed in preclinical settings, due to its limited depth of penetration. However, numerous whole-body techniques are routinely used in health care and can also be a valid tool to monitor immune cell kinetics: positron emission tomography (PET), single photon emission computed tomography (SPECT), computed tomography (CT) and magnetic resonance imaging (MRI). These techniques require in-vitro or in-vivo labeling of T-cells (281). During in-vitro labeling, cells are harvested, processed, and then infused back into the model organism or patient. Labeling procedures can be classified into two types: direct and indirect (282). Direct labeling is easy to perform and radiotracers, MRI-based contrast agents or fluorophores are internalized by the immune cells. This technique does not allow long term monitoring of cells as mitotic events result in the dilution of the signal. Tracer uptake, retention capacity and changes in cellular features, due to the internalization of the probe, are further drawbacks of direct labeling (281, 283).

Indirect labeling requires genetic modification *via* stable transfection of cells with a reporter gene such as luciferases or fluorescent proteins, which do not require an additional tracer. Other reporter genes such as sodium iodide symporter (NIS), or herpes simplex virus–thymidine kinase (HSV-TK), require further probes for imaging. This approach is preferred for long term imaging because the reporter gene is inherited, but genetic manipulation raises safety concerns (284–287).

In vivo labeling occurs directly in the organism and requires the injection of radiolabeled antibodies into the body, to target immune cells. A two-step approach has also been developed, where bispecific antibodies, containing a binding domain for the epitope and one for the tracer, are injected into the organism. Labeled probes are then injected, in order to bind the previously administered antibody. This method allows the use of safer isotopes, with a faster radioactive decay (281, 282).

Optical Detection Techniques

Intravital microscopy includes a variety of approaches that allow one to distinguish individual cells from tissues and, therefore, investigate immune cell kinetics *in vivo* (288, 289). Optical probes allow for repeated scanning of tissues, providing a spatial and temporal dimension to cellular interactions. For example, intravital microscopy enabled visualization of the dynamic interactions between cancer cells and immune cells in the TME (290). It has also been used to decipher the behavior of B-cells and T-cells in germinal centers of lymph nodes (291).

Currently, the two main tools for intravital microscopy are the confocal microscope and the multiphoton microscope. Despite their potential for high-resolution and low phototoxicity, optical detection techniques are used exclusively in preclinical studies because of their low penetration depth (1–2 mm) and risk of photobleaching. Confocal microscopes have reduced costs but increased autofluorescence and scattering, therefore the imaging depth is in the range of 20 to 50 μm (289, 292). Tavri et al. (293) used fluorescence microscopy to track fluorophore-labeled, tumor-targeted natural killer cells to human prostate cancer xenografts.

In contrast, multilaser scanning microscopy uses tunable titanium-sapphire lasers that operate in the near infrared range (NIR), allowing for superior tissue penetration (200 – 300 μm). Increased imaging depths (500 μm) can also be obtained in brain and cleared tissues (294, 295). Two-photon laser scanning microscopy (TPLSM) requires simultaneous excitation by two photons with longer wavelengths than the emitted light. This particular type of excitation suppresses the background noise and reduces photobleaching. Moreover, using NIR for excitation also minimizes scattering in the tissue (296–298).

TPLSM was used to visualize the effects of anti-CD19 CAR-T treatment on intracranial primary central nervous system lymphoma (PCNSL), in the same animal over weeks (291). CAR-T-cells infiltrated the tumor inducing regression of PCNSL and increased long term survival. Multiphoton intravital microscopy was also used in lymph nodes, to show import dynamics of the nuclear factor of activated T-cells (NFAT) in the cells. A fluorescently labeled NFAT reporter was used in combination with the nuclear marker histone protein histone 2B (H2B) (299). Stoll et al. (300) developed a protocol for extended four-dimensional confocal imaging of T-cells and dendritic cells, reporting dynamic visualization of antigen-specific T-cells interacting with dendritic cells within intact explanted mouse lymph nodes. Two-photon laser microscopy was also performed to investigate the dynamic behavior of individual T-cells within intact lymph nodes (301, 302) (**Figure 5**). Finally, Bousso et al. (303), performed real-time analysis of the cellular contacts made by developing thymocytes undergoing positive selection in a three-dimensional thymic organ culture.

Bioluminescence (BLI) enables long-term cell tracking, through reporter enzymes such as firefly luciferase, renilla luciferase or bacterial luciferase (304, 305). BLI has higher sensitivity than fluorescence imaging due to the absence of autofluorescence generated by excitation light. BLI and

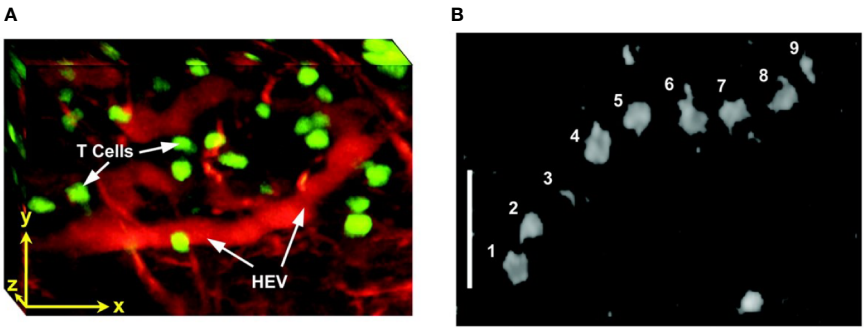


FIGURE 5 | Intravital two photon imaging of naïve T-cells in lymph nodes (302). **(A)** 3D reconstruction representing 85 x 120 x 75 µm of the T-cell area. Fluorescently labeled T-cells (green) are observed in the proximity of presumptive high endothelial venules (red), identified by i.v. injection of tetramethylrhodamine dextran. Scale bar 30 µm in all axes. **(B)** Video-rate imaging of a T-cell flowing in a small vessel within a T-cell region of the node. Image is a superposition of nine consecutive video frames and shows progression of a single labeled T-cell traveling at about 0.03 cm/s within a blood vessel. Scale bar 25 µm. *Copyright (2003) National Academy of Sciences, U.S.A.*

multilaser scanning microscopy were successfully adopted to investigate small populations of T-cells: less than 10.000 cells in live animals (306). Chewning et al. (307) created a novel transgenic mouse model for *in vivo* tracking of $CD4^+$ T-cells, using a human CD2 mini-gene to direct luciferase expression specifically to T-cell compartments. Kim et al., used BLI to show that tumor-specific T-cells upregulate IL-2 expression in hypoxic conditions in a model of human B cell lymphoma (308). BLI was also used to track migration of immune cells to sites of inflammation (306, 309).

Cerenkov luminescence imaging (CLI) is based on the detection of visible photons emitted by Cerenkov radiation. Cerenkov luminescence is emitted when a charged particle traverses a dielectric medium with a velocity greater than the phase velocity of light in the medium (310–312). CLI has been performed to optically monitor the biodistribution of ^{32}P-ATP labeled T lymphocytes, in small rodents, *in vivo* (313, 314). Results were comparable to those obtained with radioluminescence imaging and T-cell localization in the tumor mass was definitively confirmed by flow cytometry (313).

CLINICALLY APPLICABLE DETECTION TECHNIQUES

Digital Histopathology

Histopathology slides are available for almost any patient with a solid tumor, but immune topographies are currently not assessed in clinical routine. While subjective visual examination of tissue slides can be used to roughly quantify, computer-based approaches are ultimately much more scalable and objective. In several countries, digitization efforts for routine histopathology are underway (315). Once this digital infrastructure is established, development and refinement of histopathology-based Immune Topography biomarkers could be accelerated and in turn, clinical rollout of these biomarkers would be markedly facilitated.

Magnetic Resonance Imaging

MRI is a routinely used, non-invasive diagnostic technique that provides soft tissue contrast with high anatomical resolution. It is considered safer than PET, as it does not use ionizing radiation. Drawbacks of MRI include high instrumentation costs and relatively low sensitivity (316, 317).

MRI can complement PET imaging, co-registering soft-tissue anatomy and multimodal imaging for T-cell tracking is becoming more common. Multimodal imaging allows different imaging methods to be combined simultaneously, providing a multi-layered and complete information regarding the dynamics of immune cells (281, 318, 319).

Standard MRI is based on the detection of signals emitted by protons (^{1}H) that are part of the water present in human tissues. Due to the molecular composition of tissue, absorption of a specific electromagnetic impulse generates signals of different intensities. In addition to ^{1}H, MRI can be performed on other isotopes such as ^{31}P, ^{15}N, ^{13}C, ^{23}Na and ^{19}F (317, 320). In some cases these methods are considered less efficient because of the low abundance of these chemical elements *in vivo*, leading to poor signal intensity. ^{19}F MRI is gaining more interest as a tool to investigate cell behavior, driven by advances in MRI technology and scan protocols. Indeed, ^{19}F MRI provides images with high signal-to-noise ratio and current ^{1}H MRI instruments require minimal hardware upgrades to acquire ^{19}F-based images (321, 322). However, ^{19}F MRI has a detection limit of approximately 10^3 – 10^5 cells per voxel *in vivo* (323).

Magnetic nanoparticles (i.e. iron oxides, gadolinium and manganese chelates) can label cells by entering the cytoplasm, binding to the membrane or to reporter proteins. Labeled immune cells have been used to monitor cell interactions *in vivo* and to dissect immunological processes in deep tissue areas. One of the most extensively used nanoparticles in the study of T lymphocytes behavior is superparamagnetic iron oxide (SPIO) (324, 325). The long-term recruitment of cytotoxic T-cells to tumors was studied using a dextran coated SPIO particle,

derivatized with a peptide sequence from the HIV-tat protein (326). Wu et al. have developed negatively charged superparamagnetic iron oxide (PAsp-PCL/SPIO) nanoclusters to monitor the migration of dendritic cells into lymphoid tissues *in vivo* and correlated this with immunotherapy results in mice (327). Tremblay et al., used $CD8^+$ cytotoxic T-cells, regulatory T-cells and myeloid derived suppressor cells labeled with SPIO particles, to monitor the efficacy of DepoVax in mice implanted with HPV-based cervical cancer (328).

Cell labeling probes, based on perfluorocarbon nanoemulsions paired with ^{19}F MRI detection, have also been widely used to monitor immune cells. Fluorine-dense perfluorocarbon (PFC) nanoemulsions display hydrophobic and lipophobic characteristics and have been engineered to be endocytosed, even by non-phagocytic cells in culture (329). Commonly used PFC include perfluoropolyether (PFPE), perfluoro-15-crown-5-ether (PCE) and perfluorooctyl bromide (PFOB) (323, 330). Despite a lack of evidence supporting the exocytosis or degradation of PFCs, once internalized in the cells, mitotic events lead to the dilution of the signal limiting long- term studies. Nanoparticles are usually cleared by the reticuloendothelial system, in particular from the Kupffer cells in the liver (331, 332).

Chapelin et al. used ^{19}F MRI to monitor CAR-T biodistribution and immunotherapy efficacy on immunocompromised mice bearing subcutaneous human U87 glioblastomas (333). Another example includes the study performed by Gonzales et al., whereby T-cells were labeled with PFC *in vitro* and their distribution detected by ^{19}F MRI *in vivo*, in melanoma-bearing mice (334). A clinical trial was performed in 2014 to investigate the use of a PFC nanoemulsion in the detection of immunotherapeutic dendritic cells delivered to colorectal adenocarcinoma patients. Composite $^{19}F/^{1}H$ overlay images were created and showed that, despite the lack of treatment efficacy, ^{19}F MRI enabled visualization of injected cells in patients using a clinical scanner within acceptable scan times (335).

Immuno-PET

Immuno-PET is a sensitive and non-invasive method used to investigate immune cell interactions in clinical settings, allowing quantification of T-cell dynamics. Indeed, immuno-PET can quantify viability and retention of T-cells in the primary tumor mass and secondary lesions, which may provide insights into immunotherapy efficacy. Clinical imaging could be used to monitor steps of T-cell proliferation, trafficking and infiltration and give insights into mechanistic aspects of the process and effectiveness of induced T-cell response. Although PET and SPECT possess excellent signal-to-noise ratio and unlimited depth penetration, they provide limited anatomical information (336, 337).

Immuno-PET combines antibody specificity against immune cells with PET, which uses radioactive tracers to visualize human tissues. Antibodies recognizing specific features of immune cells are coupled with radioactive isotopes such as ^{11}C, ^{18}F, ^{68}Ga, ^{44}Sc, ^{64}Cu, ^{89}Zr, ^{124}I. Radionuclides need to be covalently bound to the antibody and remain kinetically and thermodynamically stable, in order to obtain good quality images. Therefore, their chemical properties and half-lives are fundamental parameters to consider when designing a study (338, 339).

Using monoclonal antibodies in immuno-PET produces images of optimal quality but, due to their size (~150 kDa), it can take up to a week to reach the imaging site after injection (**Figure 6**) (340, 341). Due to slower circulation and clearance times, radionuclides with longer half-lives are required for the labeling of monoclonal antibodies (^{89}Zr, ^{124}I). Although radionuclides can provide information over long periods of time, they constitute a biohazard as patients are exposed to higher radiation doses. Moreover, the size of monoclonal antibodies exceeds the clearance cut-off value of glomerular filtration (60 kDa), therefore their clearance occurs in the liver, thus precluding its imaging. Smaller molecules have been developed over the years (minibodies, diabodies, single-chain variable region fragments, nanobodies, affibodies), which still retain the specificity of the antibodies and have more desirable pharmacokinetic properties and deeper tissue penetration (**Figure 6**).

Adverse reactions to non-human antibodies are rare but they comport a safety risk for the patient, therefore it is pivotal to ensure that tracking antibodies have minimal pharmacological or toxicological effects. To minimize the risk of adverse reactions, antibodies of camelids, cartilaginous fish or human are often used (342, 343). Camelid antibodies are significantly smaller than standard antibodies and only consist of IgG heavy chains (344). Smaller antibodies can be conjugated with shorter-lived nuclides such as ^{18}F, ^{64}Cu, ^{44}Sc and ^{68}Ga. Antibody fragments contain only the targeting and binding components with their sizes ranging from 7 to 100 kDa. Another category includes affibodies, which are constituted of three alpha helices (~6–7 kDa) resulting in high contrast PET images that can be obtained within hours of their administration (339, 345, 346).

Diabodies labeled with ^{89}Zr or ^{64}Cu were used in specific targeting of $CD4^+$ and $CD8^+$ receptors resulting in targeting of T lymphocytes *in vivo* (347, 348). Tavare et al. showed that engineered anti-$CD8^+$ minibodies were applicable for immuno-PET imaging of endogenous $CD8^+$ T-cells in a murine model system (349). Nanobodies were also used to investigate the distribution of intratumoral $CD8^+$ T-cells and $CD11b^+$ myeloid cells in a colorectal mouse adenocarcinoma model. Response to anti-PD-1 treatment was assessed and showed the difference in $CD8^+$ and $CD11b^+$ cells infiltration, in responding and non responding tumors. Only the tumors that were completely infiltrated by $CD8^+$ T-cells went into full remission (350). Larimer et al., synthesized an anti-CD3 imaging agent labeled with ^{89}Zr, to predict tumor response to anti-CTLA-4 treatment in a murine tumor xenograft model of colon cancer. Higher presence of $CD3^+$ TILs, revealed by an increased uptake of the radiotracer, correlated with responsive tumors (351).

Reporter genes provide another strategy to target antibodies for immuno-PET. Reporter genes are transfected or transduced into cultured cells and they encode for a protein specifically targeted by the radiolabeled tracker. While genetic manipulation is considered a biohazard, modern gene-editing technologies have developed safe harbor locations and reduced the risk of

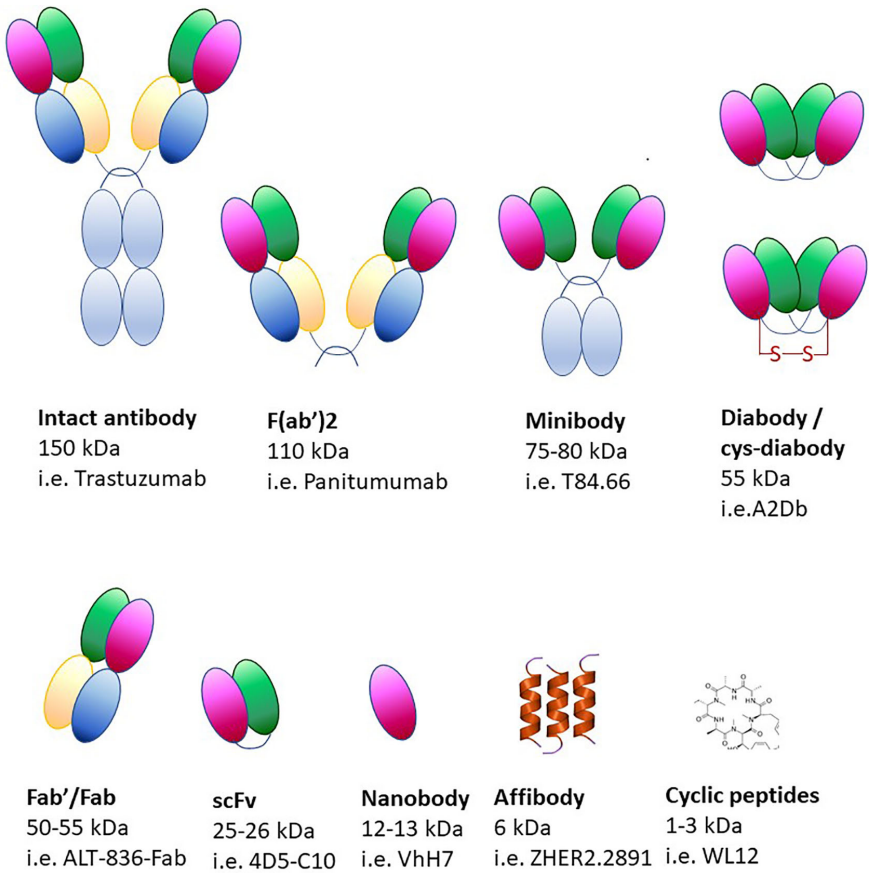


FIGURE 6 | Protein-based scaffolds for targeting cell antigens *in vivo*.

mutagenesis. Reporter genes have been used to monitor cell-based immunotherapies in preclinical and clinical studies (338, 352, 353). In a study with glioblastoma patients, cytotoxic T lymphocytes (CTLs) were engineered to express the herpes simplex virus type 1 thymidine kinase (HSV1-tk) alongside a glioblastoma-targeting interleukin-13 zetakine (353, 354). Immuno-PET coupled to MRI provided information regarding the locations of CTLs in the glioblastomas together with detailed anatomical context (353). The expression of a foreign protein can be recognized by a patient's immune system, causing adverse reactions. To avoid an immune response against the foreign reporter protein, endogenous human reporters have been designed. However, these probes trade off immunogenicity for reduced contrast. The sodium iodide symporter (NIS) is considered one of the most promising reporters for preclinical and clinical studies (284, 355, 356). Endogenous expression of NIS is confined primarily to salivary and lacrimal glands, lactating mammary glands, the thyroid and stomach. NIS probes have been used to monitor immune cells, viral vectors, oncolytic viruses, tumor cells and cellular therapies by PET and SPECT in both animal models and patients (284).

Antibodies engineered for use in immuno-PET can target a variety of epitopes on T lymphocytes. When naïve T-cells are activated, several cell surface markers are upregulated. However, expression of these markers does not imply, per se, cytotoxic effector functions. Metabolic activity is modified in active T-cells including glycolysis and the upregulation of nucleic acid metabolism. Therefore, specific enzymes involved in the metabolic pathways constitute good tracking candidates. Finally, the T-cell effector function can also be targeted (318, 338).

Surface markers used for activated T-cells include interleukin-2 receptor (IL-2R), OX40 (CD134), TCR complex and co-receptors CD3, CD4, and CD8. Activated T lymphocytes present high levels of IL-2R on their surface and several studies investigated the distribution of such immune cells *via* IL-2 labeling, with PET and SPECT.

For example, in primary melanoma [99mTc]Tc-HYNIC-IL-2 accumulation was observed at metastases. ^{18}F-labeled IL-2 was developed as a PET tracer and its uptake was shown to increase upon tumor treatment (357, 358). Clinical trial NCT02922283, completed in February 2020, aimed to study the biodistribution and kinetics of the tracer ^{18}F-FB-IL2 in patients with metastatic melanoma. Results have not yet been published. The activation marker OX40 has also been targeted and imaged with PET using a specific ^{64}Cu-conjugated murine antibody (359). Humanized

OX40 agonist monoclonal antibodies are currently being tested in early phase clinical trials.

T-cell receptors (TCR) present a constant membrane turnover that leads to internalization and accumulation of the anti-TCR probe. Studies have tracked human T-cells using ^{89}Zr labeled anti-mouse TCR. A highly sensitive imaging approach was proposed by Klar et al., targeting the TCR2.5D6 on T-cells, which recognize a peptide expressed on leukemia cells (360). T-cell co-receptor CD3 was targeted to monitor anti-CTLA-4 treatment in colon cancer xenograft mouse models. Anti-CD3 antibody labeled with ^{89}Zr was used to quantify T-cell infiltration revealing that tumor regression correlated with high levels of infiltration (351). Anti-CD4 and anti-CD8 labeled with ^{89}Zr or ^{64}Cu have been used to monitor T-cells in mice and human (361–364). Moreover, anti-CD8 minibodies are currently under investigation in clinical trials (NCT03107663, NCT03802123, NCT03610061).

Cell surface markers may present different expression patterns during the progression of the disease therefore, to obtain more comprehensive information, multiple markers should be tested concomitantly.

Metabolic changes during T lymphocyte activation can be monitored with labeled probes including amino acids, hormones, sugars or nucleosides (**Table 2**). As previously mentioned, activated T-cells switch to glycolytic metabolism and upregulate the intake of substrates.

Fluciclovine ^{18}F-FACBC, an analog of L-leucine, is a radiolabeled amino acid that is imported into activated T-cells due to upregulation of the amino acid transporter ASCT2 and LAT-1 (373–375, 400, 401). Labeled substrates for enzymes involved in the deoxyrubonucleoside salvage pathway have been developed such as ^{18}F-FAC, ^{18}F -CFA, ^{18}F-FLT, and ^{18}F-F-AraG (318).

^{18}F-FAC is a labeled deoxycytidine analog, with high-affinity for the enzyme deoxycytidine kinase (dCK). This tracer is being investigated in an early phase clinical trial (NCT03409419), that is recruiting patients with metastatic melanoma and who are undergoing TIM-3 targeted immunotherapy. ^{18}F-CFA is also a substrate for dCK and is studied as a potential cancer biomarker for treatment stratification and monitoring. ^{18}F-FLT is a thymidine analog that is trapped intracellularly due to its phosphorylation by thymidine kinase 1. It is used to monitor T-cell activation and cancer cell proliferation in medical practice (376, 379, 382, 383).

Arabinosyl guanine is a molecule with specific toxicity to T lymphoblastoid cells and T-cells. AraG prodrug has been used in patients with T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma (402). Engineered AraG leads to the development of a ^{18}F-F-AraG probe that is retained by primary T-cells and it is a substrate for deoxyguanosine kinase. Such a tracker could provide information about T-cell dynamics in the TME and other pathologies involving the immune system (337, 385). A clinical trial to assess ^{18}F-F-AraG biodistribution in cancer patients who are expected to undergo immunotherapy or radiation therapy is currently recruiting (NCT03142204). A recent study demonstrated that ^{18}F-F-AraG PET imaging could be used to report immune activation *in vivo*, in mice with rheumatoid arthritis (403).

Finally, the metabolic tracer ^{18}F-FDG measures the rate of glycolysis in active T lymphocytes, due to the upregulation of the glucose transporters GLUT isotypes. However, tumor cells also present increased rates of glycolysis, therefore imaging can lead to false positive signals (338, 387, 388). The increase in substrate uptake is similar between cancer cells and T-cells, therefore metabolic radiotracers often lead to difficulties in image interpretation.

In order to specifically monitor active T lymphocytes, probes targeting their effector functions have been developed (**Table 2**). For example, granzyme B, released by CD8$^+$ T-cells and natural killer cells, is considered one of the main mechanisms through which T-cells mediate cancer cell death (404). A recent study tested the probe [^{68}Ga]Ga-NOTA-GZP that targets murine or human granzyme expression. Imaging made it possible to differentiate responders from non-responders, within immunotherapy-treated mice (392). The human probe showed high specificity in human

TABLE 2 | Markers for T-cells imaging.

Category	Name	Reference and clinical trials
T cell surface	Interleukin-2 receptor alpha chain (CD25)	NCT02922283 (357, 358, 365–367)
	OX40 receptor (CD134)	NCT02318394 (359, 368)
	T cell receptor (TCR)	(361, 369, 370)
	CD3	(351, 371, 372)
	CD4	(362)
	CD8	NCT03107663, NCT03802123, NCT03610061 (347, 363, 364)
Metabolic pathways	L-leucine analogue	(373–375)
	^{18}F-FAC	(376–378)
	^{18}F -CFA	NCT03409419 (377, 379–381)
	^{18}F-FLT	(382–384)
	^{18}F-F-AraG	NCT03311672, NCT03142204, NCT03007719 (385, 386)
	^{18}F-FDG	(387–391)
Effector function	Granzyme B	(392–395)
	Interferon-gamma	(396)
	Checkpoint receptors	NCT03065764, NCT02760225, NCT03313323 (397–399)

samples, revealing a candidate predictive biomarker for cancer immunotherapy (392). Interferon-gamma (IFN-γ) is a pleiotropic molecule implicated in immune surveillance, playing a role in pro-apoptotic and antitumor mechanisms. However, evidence points to a protumorigenic role for IFN-γ in downregulating major histocompatibility complexes and upregulating checkpoint inhibitors. Although clinical trials assessing the efficacy of anti-cancer therapies based on IFN-γ reported limited success, IFN-γ-mediated response is still correlated with a positive patient prognosis (405). Gibson et al., used an ^{89}Zr-labeled anti-IFNγ probe to predict immunotherapy response after HER2/neu vaccination in mouse mammary tumors. Immuno-PET demonstrated that IFN-γ levels in situ, after vaccination, were inversely correlated with tumor growth rate (396). Therefore, targeting soluble cytokines by immuno-PET could be an interesting strategy to quantify immune response directly *in situ* and predict the response to immunotherapy.

OUTLOOK

A Comprehensive Biological Theory of Immune Phenotypes in Solid Tumors

A current major challenge in immunotherapy is the increase of its therapeutic potential in solid tumors. Preclinical research demonstrated the existence of a variety of determinants that play a role in shaping the TME, affecting immunotherapy response. The dynamics and distribution of these factors probably change during time and may also vary according to tumor type.

Further studies are required to understand the spatial and temporal distribution of mechanisms involved in the immune exclusion phenomenon and their interdence. Modern techniques allow high-throughput analysis of immune cell distribution *ex vivo* and *in vivo*. It would be interesting to correlate tumor stages with degree of immune infiltration and determinants of immune exclusion, in a pan-cancer investigation. This correlation would provide information on the mechanism(s) of immune exclusion, allowing to integrate the different determinants into a unified '*Theory of Everything*.' Moreover, a comprehensive analysis would also provide insights into approaches that should be adopted in order to improve the efficiency of immunotherapy and the rationale for innovative translational combinatorial treatments.

Clinical Translation of Immune Topographies

Ultimately, scientific insight into Immune Topographies in solid tumors could lead to a benefit of cancer patients. In particular, determining Immune Topographies at baseline (before starting a systemic treatment such as immunotherapy or chemotherapy) could inform physicians about the chances of treatment response. Thus, Immune Topographies could help to choose one of several available treatment options for a given patient. In addition, dynamically observing Immune Topographies during treatment might enable oncologists to adjust treatment accordingly. Compared to other biomarkers in oncology, Immune Topographies are intuitively understandable, linked to biological processes of demonstrated relevance and are comparatively easy to measure. However, clinical implementation will depend on larger-scale retrospective analysis and prospective clinical trials evaluating the utility of Immune Topographies for managing cancer treatment.

AUTHOR CONTRIBUTIONS

All authors contributed equally. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: VP and FM were employed by the company Refuge Biotechnologies. AC was employed by ESSA Pharmaceuticals.

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

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frontiers in Immunology

MINI REVIEW
published: 01 March 2021
doi: 10.3389/fimmu.2021.643291

Immunotherapy Goes Local: The Central Role of Lymph Nodes in Driving Tumor Infiltration and Efficacy

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Immune checkpoint blockade (ICB) has changed the therapeutic landscape of oncology but its impact is limited by primary or secondary resistance. ICB resistance has been related to a lack of T cells infiltrating into the tumor. Strategies to overcome this hurdle have so far focused on the tumor microenvironment, but have mostly overlooked the role of tumor-draining lymph nodes (TDLN). Whereas for CTLA-4 blockade TDLN have long since been implicated due to its perceived mechanism-of-action involving T cell priming, only recently has evidence been emerging showing TDLN to be vital for the efficacy of PD-1 blockade as well. TDLN are targeted by developing tumors to create an immune suppressed pre-metastatic niche which can lead to priming of dysfunctional antitumor T cells. In this review, we will discuss the evidence that therapeutic targeting of TDLN may ensure sufficient antitumor T cell activation and subsequent tumor infiltration to facilitate effective ICB. Indeed, waves of tumor-specific, proliferating stem cell-like, or progenitor exhausted T cells, either newly primed or reinvigorated in TDLN, are vital for PD-1 blockade efficacy. Both tumor-derived migratory dendritic cell (DC) subsets and DC subsets residing in TDLN, and an interplay between them, have been implicated in the induction of these T cells, their imprinting for homing and subsequent tumor control. We propose that therapeutic approaches, involving local delivery of immune modulatory agents for optimal access to TDLN, aimed at overcoming hampered DC activation, will enable ICB by promoting T cell recruitment to the tumor, both in early and in advanced stages of cancer.

Keywords: cancer, tumor draining lymph node, dendritic cell, immune check point, immune exclusion, t cell exhaustion, CTLA-4, PD-1

OPEN ACCESS

Edited by:
Sjoerd H. Van Der Burg, Leiden University, Netherlands

Reviewed by:
Praveen Bommareddy, Rutgers, The State University of New Jersey, United States
Howard L. Kaufman, Massachusetts General Court, United States
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *17 December 2020*
Accepted: *09 February 2021*
Published: *01 March 2021*

Citation:
van Pul KM, Fransen MF, van de Ven R and de Gruijl TD (2021) Immunotherapy Goes Local: The Central Role of Lymph Nodes in Driving Tumor Infiltration and Efficacy. Front. Immunol. 12:643291. doi: 10.3389/fimmu.2021.643291

INTRODUCTION

Over the past decade, it has become clear that for immune checkpoint blockade (ICB) to work, tumors need to contain sufficient numbers of infiltrating T cells (1, 2). Particularly in view of the perceived mechanism-of-action of PD-1 inhibitors this would make sense, since it is supposed to entail the release of cancer-imposed brakes from tumor-infiltrating cytotoxic effector T cells. An

intense research effort has therefore been ongoing to characterize the tumor microenvironment (TME) and find ways to ensure T-cell infiltration (3, 4). New insights point to the need for therapeutic targeting of tumor-draining lymph nodes (TDLN), rather than of the TME, to secure proper antitumor T-cell generation and at the same time brisk tumor infiltration (5–8). TDLN can either be more proximal or more distal from the tumor, but they are all part of the lymph catchment area of the tumor. As a result of this definition, non-TDLN may sometimes be more proximal to the tumor than TDLN, but due to the fact that tumor-derived factors will diffuse through the lymph basin, be less affected by the tumor, e.g., in terms of immune suppression (9). A growing number of studies are exploring the use of systemically administered immune checkpoint inhibitors (ICI) as neo-adjuvant therapy for patients in earlier (i.e., resectable) cancer stages (10–13). As in this setting both the primary tumor and TDLN are still in place (rather than surgically removed in the adjuvant setting) this approach will enable simultaneous immune modulation of the TME and of TDLN. As a result, these studies are generating a renewed interest in the contribution of TDLN to the efficacy of ICB. We and others have shown in pre-clinical models that TDLN play a pivotal role in PD-1/PD-L1 blocking antibody therapy, and that surgical resection of TDLN prior to treatment hampers therapeutic outcome (5, 14). In pre-clinical models, lymphatic drainage has also been shown to facilitate the priming of anti-tumor T-cell immunity (15, 16). Indeed, recent evidence points to the need for the recruitment to the tumor of newly primed and peripherally (e.g., in TDLN) expanded effector T cells to ensure efficacy of ICB (17). Clinical efficacy and durability of antitumor immunity appears to be associated with elevated frequencies of central-memory or early-effector T cells with the ability to home to lymph nodes (18–20). More in-depth knowledge on the exact nature of the T cells amenable to ICB and the underlying molecular mechanisms that control their activation, point to the importance of Dendritic Cells (DC) in driving waves of newly primed or reinvigorated early-effector T cells to facilitate effective ICB (7, 21–23). In this review we will discuss mechanisms underlying tumor-associated immune suppression of TDLN and how we can use this knowledge to devise new local intervention strategies aimed at harnessing TDLN to secure efficacy of cancer immunotherapy, both in early and in advanced stages of cancer development.

IMMUNE SUPPRESSION OF DENDRITIC CELLS IN TDLN: EARLY IMMUNE ESCAPE

TDLN represent the site where T cells will first be primed against tumor-associated (neo)antigens. In order to escape the immune response, it is vital for tumors to nip this induction of tumor reactive T cells in the bud. The more immunogenic the tumor, the more pressing this matter becomes. With a high mutational burden, melanoma is the most immunogenic tumor type identified to date (24). As tumors develop, the cellular content of their TDLN shifts (**Figure 1A**). In breast, melanoma and cervical TDLN shifts in CD4/CD8 T cell ratios and elevated Treg rates were observed prior to metastatic involvement, but even more pronounced after (28–30). As metastases in the TDLN grow, memory T cell rates grow and myeloid regulatory cells are recruited (29, 30). Already at early stages of melanoma development, the primary tumor exerts an immunosuppressive effect on its TDLN through the release of immune modulatory exosomes and soluble mediators, which can ultimately lead to a "tumor-supportive" microenvironment, i.e., the pre-metastatic niche (31). In the first-line draining TDLN, the so-called sentinel lymph node (SLN), we have found clear evidence of early suppression of DC (28, 29). DC subsets in TDLN encompass migratory conventional DC (cDC) subsets (marked by CD1a expression in human epithelium draining lymph nodes) as well as lymph node-resident cDC (LNR-cDC, marked by high CD11c levels, various CD1c, CD141, and CD14 expression patterns, and absence of CD1a) and plasmacytoid DC (pDC; $CD11c^{-}CD123^{hi}CD303^{+}$). Recent studies have shown the *in-vivo* exchange of antigens between migratory cDC and LNR-cDC and have demonstrated their concerted and coordinated activities to lead to optimal priming of an effective antitumor T-cell response (32–34). Whereas significantly lower levels of maturation and co-stimulatory markers were found in migratory cDC subsets already in Stage-2 melanoma, expression of these markers dropped profoundly in LNR-cDC only by Stage-3 (28). A significant negative correlation between the frequency and activation state of migratory cDC subsets in melanoma SLN and primary tumor size (Breslow thickness), suggested that the developing primary melanoma created a pre-metastatic niche in the TDLN by suppressing the migration of antigen-carrying cDC from the tumor to the TDLN. This early reduction in frequency of migratory cDC is consistent with observations made in murine models by Binnewies et al. (35) who reported that cDC2 migration from the tumor to TDLN was constrained by regulatory T cells (Tregs) in the tumor, resulting in suboptimal priming of Th cells and their failure to migrate to the tumor in sufficient numbers to support an anti-tumor response. In human melanoma SLN, metastasis size was inversely correlated with the frequency and activation state of LNR-cDC in SLN (28). Remarkably, whereas reduced frequencies of migratory cDC subsets was related to decreased local recurrence-free survival (RFS), reduced activation of LNR-cDC was related to decreased distant RFS (28). This suggests differential imprinting for homing properties of tumor-specific T cells by specific cDC subsets, and indicates an essential role for LNR-cDC in the induction of effective systemic antitumor immunity. In breast cancer SLN a similar progressive reduction in the activation state of LNR-cDC was observed, which was most pronounced upon metastatic involvement and then coincided with increased Treg rates, high co-expression levels of CTLA-4 and PD-1, and profoundly suppressed effector-T cell activity in the SLN (29). A possible role for LNR-cDC in keeping PD-1+ T cells in check was suggested by our finding that LNR-cDC in early-stage melanoma SLN expressed relatively higher PD-L1 levels as compared to CD80 (36). This indicates an inability of CD80 to keep PD-L1 from interacting with PD-1 on T cells through in-cis interactions (23, 37) and would fit with LNR-cDC subsets restraining antitumor T cells in early stages of cancer in a

PD-1 dependent manner as recently reported (22). Dammeijer et al. (7) showed an association between poor RFS with high frequencies of PD-1/PD-L1 interactions between T cells and cDCs in SLN from Stage-2 melanoma patients. In addition, we found a strong inverse correlation between the activation state of LNR-cDC and Treg rates in melanoma SLN (28). This increase of Tregs accompanying decreased LNR-cDC activation may be responsible for subsequent T-cell anergy induction and the conversion of Ag-specific naïve T cells into Tregs in TDLN, as described by Alonso et al. (38) in a lung adenocarcinoma mouse model. Polychromatic FACS analysis showed CLEC9A+ LNR-cDC to consist mostly of cDC2 expressing both CD1c and intermediate levels of CD141 and of a minority of CD141hi cDC1 (39, 40). Their superior cross-presentation and -priming ability and their apparent relationship to the generalized immune state of the SLN and distant RFS, make LNR-cDC attractive targets for early therapeutic intervention to curb metastatic spread and outgrowth (36).

THERAPEUTIC TARGETING OF EARLY-STAGE TDLN: "THINK GLOBAL, ACT LOCAL"

We have obtained clinical evidence that local administration (i.e., intradermal injection around the primary tumor excision site) of immune modulatory agents in early-stage melanoma, aimed at TDLN immune potentiation, leads to systemic antitumor immune activation and increased RFS. In two randomized Phase-II trials in clinical stage-1/2 melanoma patients, we have shown that this intradermal administration of one or two doses of the TLR9 agonist CPG7909 (CpG-B), with or without GM-CSF, in the week leading up to the SLN procedure, resulted in enhanced LNR-cDC activation and melanoma antigen-specific T-cell responses in both the SLN and in peripheral blood (39, 41–43). Clinical analysis of the 52 patients participating in these trials showed a significantly and profoundly lower number of tumor positive SLN and (at a median follow-up of 88.8 months) a significantly increased RFS in patients receiving CpG-B as compared to patients receiving a saline placebo (44). *In vitro*, cultures of single-cell suspensions of breast cancer SLN with CpG-B similarly showed enhanced LNR-cDC activation and increased expression of effector T-cell-recruiting chemokines and cytokines associated with a Th1 response (40). The addition of a JAK2/STAT3 inhibitor interfered with negative feedback loops activated by CpG-B, resulting in further enhanced DC activation, down-regulated Th2 rates, and constrained Treg expansion (40). Altogether, these observations are consistent with the reinvigoration and boosting of pre-existent but dysfunctional T cells in TDLN, through the activation of LNR-cDC, providing protection against metastatic spread and outgrowth (see **Figure 1**). This is consistent with findings from a previous study by Schietinger et al. (25) showing that *in-vivo* antigen-driven T-cell dysfunction in early developing tumors is reversible. These clinical studies have clearly demonstrated the systemic immune activating effects of locally administered immune modulatory agents, resulting in long-term protection against loco-regional as well as distant metastases. Moreover, they have delivered important proof-of-concept that in the absence of the primary tumor (but presence of TDLN), direct immune modulation of the TDLN can lead to effective systemic antitumor immunity.

OVERCOMING IMMUNE EXCLUSION BY TARGETING DC IN TDLN: REINVIGORATION OF EXHAUSTED T CELLS

While reinvigoration of suppressed T cells in early tumor stages may only require the "pushing of the gas pedal" by delivering DC-activating agents to TDLN, in more advanced stages simultaneous "lifting of the brakes" may be required by immune checkpoint blockade (**Figure 1B**). Recent insights hold that effective ICB would require the reinvigoration of so-called exhausted CD8+ T cells (7, 26, 45), which are regarded as a T-cell lineage that usually arises through chronic stimulation with high antigen doses (18). Exhausted T cells display loss of effector functions, accompanied by high expression levels of PD-1 in concert with multiple immune checkpoints. This exhausted phenotype has been linked to the activation of specific epigenetic regulatory programs (46). In cancer, exhausted T cells have been identified, which are replenished from a proliferative pool of so-called stem cell-like or progenitor-exhausted T cells: these progenitors are typified by intermediate surface levels of PD-1 and their expression of TCF-1 and SLAMF6. Recent findings show that in contrast to terminally exhausted T cells, these progenitor-exhausted T cells are still amenable to PD-1 blockade and as such may represent the prime targets for PD-(L)1 checkpoint inhibition (26). Other studies have pointed to CD8+ stem cell–like T cells or early-effector T cells as the foremost targets for effective PD-1 blockade (26, 45, 47–49). These populations, which may in part overlap, have been characterized as having a preserved capacity for proliferation and the ability to exert polyfunctional effector functions (26). Importantly, they are also commonly distinguished by their expression of CD28. Indeed, CD28 was shown to be required for effective PD-1 inhibition (48, 50). This is remarkable and points to the need for CD80/CD86 co-stimulation in addition to the "mere" interruption of PD-1 binding to its ligands PD-L1 and PD-L2 in order to unleash the full force of an antitumor effector T-cell response. This is in keeping with the observation of proliferative tumor-infiltrating CD8+ T cells upon clinical PD-1 blockade (1, 45). These proliferating T cells have a stem-cell phenotype and are found in niches with cDCs (51), which can provide CD80/CD86 co-stimulation. A recent study by Oh and colleagues showed that rather than tumor-expressed PD-L1, PD-L1 expression by infiltrating and cross-presenting DCs dictated PD-1 blockade efficacy (23). Similarly, Garris et al. (21) demonstrated that full-fledged activation of antitumor T cells by anti-PD-1 involved T-cell-DC crosstalk and was licensed by IFNγ and IL-12. This is all the more remarkable since macrophages by far outnumber DCs in tumors, and may be due to the fact that DCs, in contrast to tumor-associated

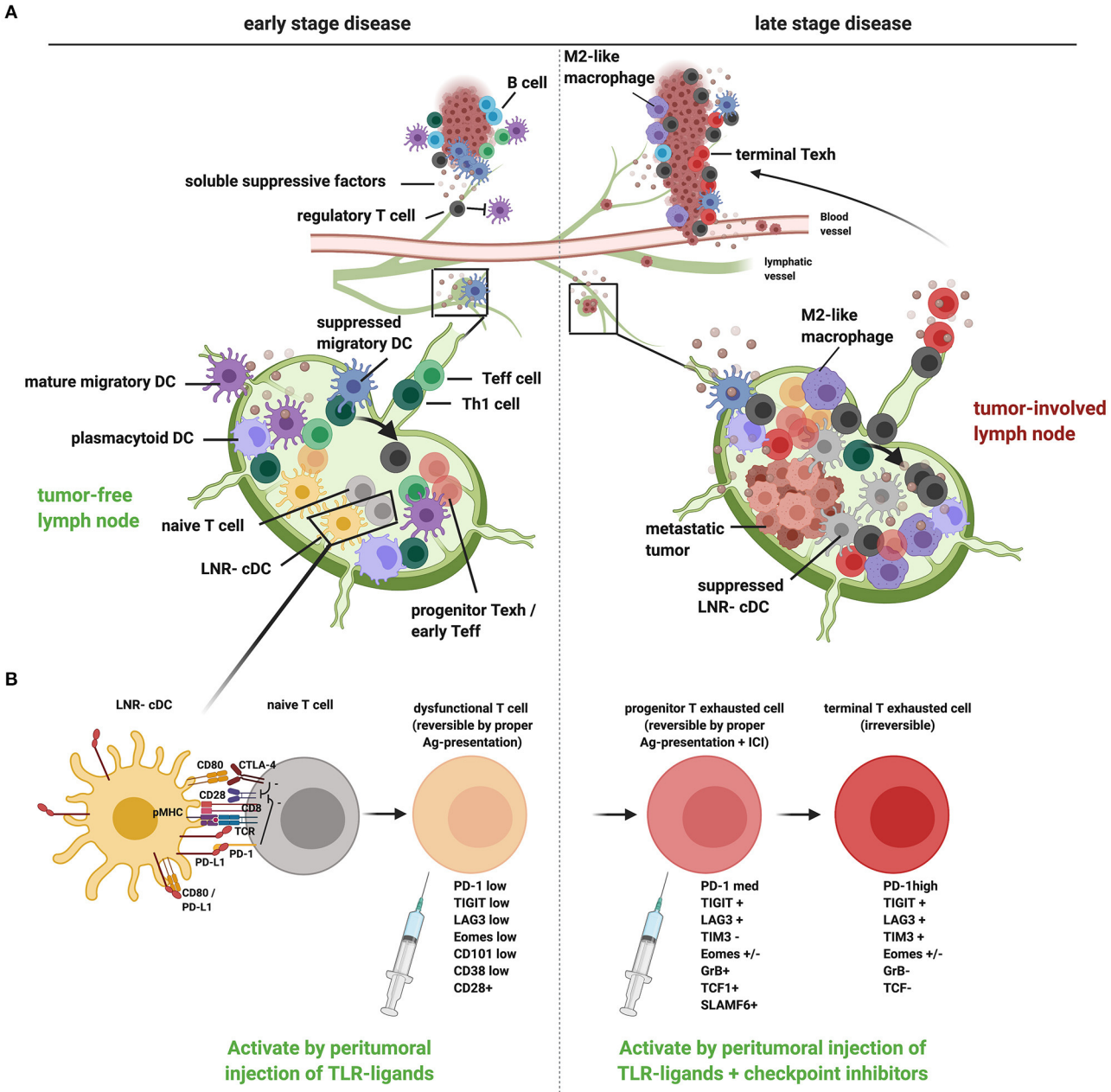


FIGURE 1 | A proposed model of tumor-induced immune suppression of draining lymph nodes and local therapeutic intervention opportunities to overcome T cell dysfunction and exclusion in early- and late-stage cancer development. **(A)** Schematic representation of how tumors, at early (left) and late stage (right), suppress the loco-regional immune response in the tumor as well as in the tumor-draining lymph nodes (TDLN). In early-stage disease, migratory DCs are hampered in their migration and activation [through release of suppressive factors in the tumor microenvironment (TME) and interactions with Tregs], resulting in suboptimal T cell priming and activation in the TDLN (generating dysfunctional T cells), conversion of Th cells to Tregs (see arrow), and reduced recruitment of Teff cells to the tumor. In late-stage disease, upon metastatic spread to the TDLN, LNR-cDC are profoundly suppressed, leading to the priming and expansion of dysfunctional progenitor exhausted T cells and Tregs in TDLN and poor recruitment of Teff to the tumor. Active suppression in the TME (with accumulating myeloid regulatory cells like M2 macrophages and a lack of mature DCs) contributes to the differentiation of terminally exhausted T cells and Treg recruitment with possible immune exclusion. **(B)** Suboptimal priming in the TDLN due to suppression of cDC, accompanied by excess PD-L1 surface expression, results in restrained T cell priming and deviated CD8+ T cell differentiation, marked by a reversibly dysfunctional state in early cancer development. Chronic high-dose (neo)-antigen stimulation in later stages of cancer development and progression will lead to the development of progenitor-exhausted and, ultimately, terminally exhausted T cells, marked by progressively higher PD-1 expression levels and the co-expression of other immune checkpoints, like LAG3, TIM3, and TIGIT. Typical markers for the different stages of dysfunction/exhaustion are listed (25–27). Whereas progenitor exhausted T cells can be rescued by immune checkpoint blockade, terminal exhaustion is an irreversible state due to epigenetic programming. Local immunotherapy, targeted at TDLN conditioning, can restore the anti-tumor T cell response by promoting DC activation (e.g., through local injection of TLR-L): in early cancer stages without tumor involvement of TDLN this may suffice to reverse T cell dysfunction and kick-start effective systemic antitumor immunity. In advanced cancer additional immune checkpoint blockade in the TDLN will enable reinvigoration of progenitor exhausted T cells, which can then home to the tumor and populate the TME, thus overcoming immune exclusion. This image was created using Biorender.com.

macrophages (TAMs), express CD80. CD80 interacts with PD-L1 in-cis (37), resulting in a block of PD-1 binding to PD-L1 with maintained CD80 co-stimulatory activity through interactions with CD28 on progenitor-exhausted, early-effector or stem-cell like T cells. Indeed, the importance of DCs for PD-1 inhibition efficacy *in vivo* was recently linked to the relative expression levels of PD-L1 and CD80, which were shown to dictate T-cell priming efficacy of DCs (22). This finding was confirmed by relatively high levels of PD-L1 on DC from patients with renal cell cancer, in line with their compromised T-cell induction ability (22). In particular tumor-infiltrating DCs expressing CCR7, indicative of their ability to migrate to TDLN, have been pinpointed as essential for effective PD-1 inhibition (33). Of note, increased PD-1/PD-L1 interactions in TDLN were identified as restraining antitumor T-cell immunity through increased PD-L1 levels on tumor-conditioned DCs (7); PD-L1 blockade resulted in DC-mediated expansion of progenitor-exhausted T cells, which, upon making their way to the tumor, could expand, and differentiate further to mediate antitumor effector functions. These observations provide a compelling argument for combining immune adjuvants, aimed at DC activation and T cell priming, with PD-1 blocking antibodies.

We propose that the lifting of immune suppressive barriers specifically in TDLN may increase the efficacy of ICB through facilitation of the priming and recruitment of new waves of tumor-specific T cells derived from progenitor-exhausted T cells. Indeed, our studies of local intradermal injections in patients with early-stage melanoma, where the primary tumor was removed but TDLN were still accessible to the locally injected immune stimulatory agents, have revealed the singular capacity of TDLN to prime and modulate the systemic antitumor T-cell response (39, 41–43). For CTLA-4 blockade this may entail both increased antitumor effector T-cell activation in the TDLN through CD28-mediated co-stimulation or Treg depletion or inhibition (52–54). In mouse models, we have demonstrated TDLN to also be vital in the efficacy of PD-1 blockade, regardless of local or systemic delivery of therapeutic antibodies (5). Egress of CD8+ effector T cells from TDLN proved vital for subsequent T-cell homing to the tumor and hence for PD-1 blockade efficacy. This finding echoes data we recently obtained from patients with cervical cancer (55): in patients with adenocarcinoma of the cervix there was an apparent accumulation of effector T cells in TDLN, coinciding with decreased frequencies of T cells infiltrating the primary tumor, indicative of faulty egress from the TDLN and homing to the tumor (55). These observations were related to a decreased cDC1-related transcriptional signature in the tumor and an increased Wnt/β-catenin response signature, similar to observations previously reported by Spranger and colleagues in melanoma, showing that β-catenin-mediated restriction in cDC1 recruitment to the tumor stood in the way of effective PD-1 blockade (56). The importance of T-cell trafficking from TDLN to tumor was further underscored by findings from Salmon et al. (8), showing that the absence of cDCs, presenting antigen in TDLN, resulted in a failure of CD8+ T cells to enter the tumor parenchyma after anti-PD-1 treatment, suggesting that increased T-cell infiltration was due to trafficking of T cells previously activated in TDLN. Thus, a picture is emerging of therapeutic PD-1 blockade involving the CD28-mediated expansion of tumor reactive T-cell clones by DCs in TDLN, rather than just the reversal of T-cell exhaustion in the TME. This is consistent with our own observation of superior effects of *in-vitro* PD-1 blockade on HPV16 E6-specific T-cell responses in cervical TDLN over tumors, which was related to the presence of CD8+FoxP3+ T cells with intermediate PD-1 expression levels (30), also previously described by others as prognostically favorable early effectors (18, 57). Such early-effector or progenitor-exhausted T cells can persist for long times in the TDLN in the absence of antigen, are polyfunctional, display a high proliferative capacity and share phenotypic traits with central-memory T cells (58). Upon PD-1 blockade they can efficiently home to the TME and there expand further and differentiate into effector T cells (6, 26). In keeping with this, Chow et al. showed that expression of CXCR3, required for tumor rejection after PD-1 blockade in the MC38 mouse model, was expressed at high levels by progenitor-exhausted or early-effector T cells, whereas it was hardly expressed by terminally exhausted T cells (59).

In conclusion, PD-1 blockade in TDLN can lead to efficient and DC-dependent tumor infiltration by reinvigorated progenitor-exhausted T cells, thus overcoming immune exclusion. In light of these observations, there is a clear rationale for intra- or even peri-tumoral delivery of ICI for optimal access to TDLN (see **Figure 1B**). Indeed, peritumoral administration ensures optimal access to the tumor's entire and exact catchment area and consequently the most efficient delivery to the greatest number of progenitor-exhausted T cells.

IN CONCLUSION: THE RISE OF LOCAL IMMUNOTHERAPY

Local administration of ICI has been described in pre-clinical models and tested in several types of cancer in clinical trials by us and others, with positive results (42, 59–70). We reported that peritumoral delivery of anti-CTLA-4 in mouse models resulted in an equally efficient antitumor response as observed after systemic administration, without the usually associated inflammatory side effects (65). A recent report from Francis et al. (6) elegantly showed in tumor models that intratumoral administration of CTLA-4 and/or PD-1 blocking antibodies ensured optimal access to TDLN (in contrast to systemic administration) and, moreover, that ipsilateral administration on a site different from the tumor but with lymph drainage to the same lymph node stations afforded equal tumor protection. This is in line with our own observations of the induction of systemic and protective anti-melanoma immunity in early-stage melanoma through local immune modulation of the SLN after surgical removal of the primary tumor, either by CpG-B (44) or by anti-CTLA-4 (71). Of note, systemic treatment with ICI, particularly in early stages of cancer, can result in unacceptably high toxicity. Local administration of lower doses may prove instrumental in limiting this toxicity, while maintaining efficacy by directly targeting the TME and, more importantly, TDLN. Both in breast cancer and

melanoma patients it has been well-established that completion lymphadenectomy in case of metastatic involvement of the SLN does not offer any prognostic benefits (72, 73). This notion, together with the recently developed insights that TDLN might be vital for ensuring effective anti-tumor immunity would argue for a neo-adjuvant ICB (or other immunomodulatory) strategy where the lymph nodes in the tumor draining basin are kept in place, possibly even in case of clinically detected lymph node involvement. Our approach of local administration of CpG-B to raise the DC activation state in TDLN and thereby systemic anti-melanoma T-cell immunity, might be used in concert with locally applied ICB to ensure DC-mediated T-cell activation in the TDLN, leading to systemic immunity, allowing new waves of T cells to be recruited to tumors. Indeed, recent reports have shown in patients with advanced melanoma that i.t. administration of CpG (likely ensuring optimal access to TDLN) can lead to increased T-cell infiltration (also of distant non-injected metastases) and even overcome prior resistance to PD-1 blockade (74). Oncolytic virus therapies, such as local treatment with the oncolytic Herpes Simplex virus Talimogene laherperepvec (T-VEC) are, similarly to local injection with TLR agonists, described as belonging to the category of so-called human intratumoral immunotherapy (HIT-IT). T-VEC is an engineered virus, that only replicates in tumor cells and induces secretion of the cytokine GM-CSF from its transgene. Oncolytic viruses can induce local and systemic anti-tumor immune responses through immunogenic cell death induction (75). The local release of GM-CSF results in recruitment and activation of DCs, but likely will also drains to nearby TDLN to activate lymph node resident (LNR)-DCs and promote T cell priming and activation. Moreover, the viral particles contain multiple TLR-ligands, which can directly activate DCs within the TME, but also, when produced and released by dying tumor cells [which in turn will also release damage-associated molecular patterns (DAMPs)], will drain, together with released DAMPs through the lymphatics and activate DCs in TDLN. The OPTiM phase 3 trial, that lead to the approval of T-VEC, compared local T-VEC treatment with systemic GM-CSF treatment and reported improved response rate and also showed signs of enhanced systemic immune responses by tumor regression in non-injected lesions (76). Moreover, enhanced immune cell infiltration upon local T-VEC treatment has been reported in non-injected lesions (77, 78). Combination treatment of local T-VEC with systemic anti-PD-1 therapy was shown to induce high response rates in metastatic melanoma patients (79). Altogether these observations clearly stress the importance of TDLN in immunotherapy efficacy and support the rationale for local delivery of ICI to ensure optimal access to, and modulation of, dysfunctional tumor-specific T cells "lying in wait" in the TDLN, which will subsequently provide systemic tumor control. These considerations have led to a remarkable surge in clinical studies exploring local or i.t. immunotherapy and the publication of a consensus statement on the standardization of terminology and methodologies used in their reporting (80). In time, increased knowledge on the role of TDLN in immunotherapy of cancer will lead to even more rational local therapy strategies in terms of dosing, timing in relation to surgery, and treatment combinations.

AUTHOR CONTRIBUTIONS

KP, MF, RV, and TG co-wrote the paper. All authors contributed to the article and approved the submitted version.

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

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frontiers in Immunology

ORIGINAL RESEARCH
published: 11 March 2021
doi: 10.3389/fimmu.2021.596646

Soluble Sema4D in Plasma of Head and Neck Squamous Cell Carcinoma Patients Is Associated With Underlying Non-Inflamed Tumor Profile

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OPEN ACCESS

Edited by:
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Reviewed by:
Rom Leidner, Earle A. Chiles Research Institute, United States
Rieneke Van De Ven, VU University Amsterdam, Netherlands

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *19 August 2020*
Accepted: *20 January 2021*
Published: *11 March 2021*

Citation:
Younis RH, Ghita I, Elnaggar M, Chaisuparat R, Theofilou VI, Dyalram D, Ord RA, Davila E, Tallon LJ, Papadimitriou JC, Webb TJ, Bentzen SM and Lubek JE (2021) Soluble Sema4D in Plasma of Head and Neck Squamous Cell Carcinoma Patients Is Associated With Underlying Non-Inflamed Tumor Profile. Front. Immunol. 12:596646. doi: 10.3389/fimmu.2021.596646

Semaphorin 4D (Sema4D) is a glycoprotein that is expressed by several tumors and immune cells. It can function as a membrane bound protein or as a cleaved soluble protein (sSema4D). We sought to investigate the translational potential of plasma sSema4D as an immune marker in plasma of patients with head and neck squamous cell carcinoma (HNSCC). Paired peripheral blood and tumor tissue samples of 104 patients with HNSCC were collected at the same time point to allow for real time analysis. Scoring of the histological inflammatory subtype (HIS) was carried out using Sema4D immunohistochemistry on the tumor tissue. sSema4D was detected in plasma using direct ELISA assay. Defining elevated sSema4D as values above the 95th percentile in healthy controls, our data showed that sSema4D levels in plasma were elevated in 25.0% (95% CI, 16.7–34.9%) of the patients with HNSCC and showed significant association with HIS immune excluded (HIS-IE) ($p = 0.007$), Sema4D^{+ve} tumor cells (TCs) ($p = 0.018$) and PD-L1^{+ve} immune cells (ICs) ($p = 0.038$). A multi-variable logistic regression analysis showed that HIS was significantly ($P = 0.004$) associated with elevated sSema4D, an association not explained by available patient-level factors. Using the IO-360 nanoString platform, differential gene expression (DGE) analysis of 10 HNSCC tumor tissues showed that patients with high sSema4D in plasma (HsS4D) clustered as IFN-γ negative tumor immune signature and were mostly HIS-IE. The IC type in the HsS4D paired tumor tissue

was predominantly myeloid, while the lymphoid compartment was higher in the low sSema4D (LsS4D). The Wnt signaling pathway was upregulated in the HsS4D group. Further analysis using the IO-360, 770 gene set, showed significant non-inflamed profile of the HsS4D tumors compared to the LsS4D. In conclusion, our data reveals an association between sSema4D and the histological inflammatory subtype.

Keywords: soluble, head and neck squamous cell carcinoma (HNSCC), Sema4D, immune excluded, real time, IFN-γ, biomarker, non-inflamed

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a devastating malignancy that occurs in close proximity to vital structures. A projection for the year 2020 estimated that 53,260 new cases, and 10,750 annual deaths of oral and pharyngeal SCC will occur in the US (1). Surgical excision remains the first line of treatment for oral cavity cancer. Depending on the disease presentation and pathological findings, other therapies are often required including radiotherapy as a single or adjuvant option, chemotherapy, targeted agents and immunotherapy as the most recent therapeutic advent (2). The overall 5 year survival rate is 65% with an average 6–10 month survival rate for platinum resistant patients (3). Several studies have described HNSCC as an immune suppressive tumor (4, 5). The recent advent of immunotherapy showed unprecedented improvement in overall response of advanced stage malignancy (2). Further understanding of HNSCC tumor inflammation can provide the basis for tumor stratification according to the underlying immune profile and hence may result in better treatment strategies and patient outcomes (6).

Immunoscore revolutionized our understanding of the histological patterns of HNSCC inflammation (7, 8). Initially referring to the inflamed tumor cores as hot and the non-inflamed as altered, the later descriptions included the immune excluded and the immune desert as the cold subtype (9, 10). The cold subtype showed poor prognosis with higher recurrence rate (9, 11). In addition, evidence-using the combination of inflammation and tumor mutational burden analysis revealed other intermediate subtypes that can include low mutational burden tumors that can be inflamed but with rich fibroblastic signature (12). In concordance, the inflamed tumor surrogate biomarker, programmed death ligand 1 (PD-L1) is used as a single analyte (13, 14) or the interferon gamma (IFN-γ) tumor immune signature as a multi-analyte, to represent T cell inflamed or non-inflamed tumors (15). IFN-γplays a key role in antitumor response. It is produced by activated T cells, as well as natural killer (NK) and NKT cells. The IFN-γ induced gene response includes pro-inflammatory, as well as feedback inhibitory signals, a feature that tumors take advantage of to progress and advance. An expanded and refined IFN-γimmune signature was initially generated using the nanostring nCounter IO-360 platform on melanoma samples, then tested on HNSCC and gastric cancer (15). The differential gene expression (DGE) of the IFN-γ signature was significantly discriminatory of the HNSCC response to anti-PD-1/PD-L1 standard immunotherapy. The final IFN-γ signature that further confirmed the significant correlation with HNSCC response to pembrolizumab from tumor samples obtained from KEYNOTE-012 was also validated on nine types of malignancies (13). It included 18 functional genes, that encompassed pro-inflammatory cytokines/chemokines (CXCR6, CCL5, CXCL9, STAT1, CMKLR1), T cell markers (CD8A, CD27,TIGIT), NK cell activity (NKG7, HLA-E), antigen presentation (HLA-DRB1, HLA-DQA1, PSMB10), and additional immunomodulatory factors (LAG3, IDO1, PDCD1LG2, CD274/PD-L1, CD276) (15). This immune signature was based on available tumor tissue biopsies. Tumor tissue represents an essential source of information that can be used in determining the course of treatment. Yet, in later stage malignancy, serial biopsies may not be feasible. Soluble immune biomarkers in the blood can potentially provide critical information at the initiation of immunotherapy or at any time point of the patient care in response to treatment (16–19). However, impact of soluble immune biomarkers in HNSCC is still limited, requiring further studies.

Semaphorin 4D (Sema4D; a.k.a. CD100) is an emerging immune biomarker that belongs to the fourth group of the Semaphorin family, which shares a conserved N-terminal domain called the 'sema' domain with highly conserved cysteine residues (20). Sema4D is expressed by almost all white blood cells of both lymphoid and myeloid origin (21–25). Interestingly, it can be expressed by activated T cells to bind its low affinity receptor CD72 on B cells or on antigen presenting cells, which results in the activation of humoral or T cell-mediated immunity, respectively (26, 27). Sema4D is a transmembrane glycoprotein that can function in the bound or soluble form. MT1-MMP and ADAM17 have been implicated in Sema4D proteolytic cleavage and shedding (28–30). The soluble form of Sema4D (sSema4D) inhibits myeloid cell spontaneous and cytokine-induced migration (22) (31). In the tumor microenvironment HNSCC-derived sSema4D was shown to induce immune suppression through upregulation of myeloid derived suppressor cells (MDSC) (5), as well as the increase of extracellular collagen deposition by fibroblasts (32). The expression of Sema4D has been described in tumor cells (TC) of several malignancies including HNSCC and by tumor infiltrating immune cells (IC), including tumor associated macrophages (TAMs) (32, 33). Sema4D also promotes tumor migration and invasion through Rho activation, microtubule organization, and epithelial mesenchymal transition (34). It has been associated with overall poor prognosis in sarcomas and cutaneous SCC (35, 36). Importantly, sSema4D is present in the

peripheral blood of HNSCC patients and other pathologic conditions including heart failure, autoimmunity and allergy (37–39). These findings reflect the significance of Sema4D as a soluble immune biomarker, and we sought to investigate its translational potential in HNSCC.

In the present work, we used paired tumor tissue and plasma samples obtained at the same time point to allow for real time analysis. The cohort was mainly immunotherapy naïve patients with HNSCC treated with surgical excision as the initial line of treatment. The current data suggests the potential of sSema4D as a soluble immune biomarker that can read non-inflamed tumor HIS and low IFN-γ immune signature in real time. It highlights Sema4D as a target for inhibition to be further investigated as a method to sensitize HNSCC to immunotherapy.

MATERIALS AND METHODS

Paired Tumor Tissue and Plasma of HNSCC Patients

Under an Institutional Review Board (IRB) approved protocol at the University of Maryland School of Medicine (UMSOM) (HP-00073603), paired blood samples and whole excision tumor tissue were collected prospectively at the day of surgery from 104 HNSCC upon informed patient consent. Our main inclusion criteria were primary tumors resected by surgery as the initial line of treatment. The blood was drawn pre-operatively prior to the planned surgical excision and processed to obtain plasma within 2 h of collection. Plasma from 51 patients with chronic pathological conditions served as controls. These included patients with autoimmune conditions (31 cases), allergy (10 cases) and osteoarthritis (10 cases) and 11 samples from healthy donors were collected retrospectively under an approved UMSOM IRB protocol HP-00074877. An additional 20 healthy donor plasma samples were purchased from Innovative Research (Novi, MI). All blood samples were processed to obtain plasma in sodium heparin tubes (BD Vacutainer glass tubes Medex Supply, NY; cat # 366480).

Sema4D ELISA

Sema4D concentration in the plasma was determined using direct ELISA as previously described (5). Briefly, Immulon 4 HBX microtiter plates (Thermo Scientific, Waltham, MA) were coated with undiluted plasma, washed with ELISA washing buffer, then incubated with anti-human CD100 antibody (clone: 133-1C6; Invitrogen, eBioscience, CA; cat # 14-1009-82) overnight, then followed by Goat anti-mouse IgM-Heavy chain, HRP conjugate secondary antibody (Invitrogen USA, IL; cat. # 62-6820), detection with TMB (Biolegend, CA; cat #421101) and Stop Solution (Biolegend, CA; cat #77316). The concentrations of Sema4D were calculated using the standard curve established using recombinant Sema4D (catalog no. 310- 29) (Peprotech, RockyHill, NJ). The detection limit was 3.1–1000 ng/ml. Plates were read at 450 nm wavelength using BioTek Epoch microplate spectrophotometer.

Immunohistochemistry

For Sema4D staining, the avidin–biotin complex (ABC) technique was used following Vectastain elite ABC kit (PK-6102, mouse IgG) (Vector Laboratories, CA). Briefly, FFPE tissue sections were deparaffinized, then rehydrated in graded ethanol, treated with Tris- EDTA buffer for antigen retrieval, and quenched in hydrogen peroxide to block endogenous peroxidase. Tissue sections were blocked with 2.5% normal plasma, incubated overnight at 4°C with anti- Sema4D antibody (clone 30/CD100; Catalog no. 610670) (BD Transduction Laboratories), followed by biotinylated secondary antibody (catalog no. BA-9200), then the ABC reagent. Primary antibody was omitted for negative control. Diaminobenzidine (SK-4105) was used as chromogen and counterstained with Mayer's hematoxylin (Sigma-Aldrich Corp.). PD-L1 staining (clone 28-8, catalog no. ab205921, Abcam) was used to stain the HNSCC sections according to Abcam IHC protocol. Universal HIER antigen retrieval reagent catalog # (ab208572), Rabbit specific IHC polymer detection kit HRP/DAB catalog # (ab209101) Amplifier and Detector, and DAB substrate kit catalog # (ab64238) were used from Abcam according to their PD-L1 IHC protocol.

The Sema4D and PD-L1 labeling index (LI) reflecting the intensity and extent of staining in the TC and the IC was defined semi-quantitatively using intensity and percentage of staining as (0, 1, 2, and 3). (0) was negative, (1) focal or diffuse weak staining, (2) focal strong positivity ≤25%, and (3) for diffuse strong positivity >25%. The combined positive score (CPS) was counted for both TC and IC positivity. The immunohistochemical (IHC) score standardization was carried by the surgical pathologists (JP, RC, RY), then the 104 HNSCC samples were scored by (RY and RC). Discordant scores were adjudicated by JP. Slides were scanned using Leica biosystem scanscope. Histological features included extent of peritumoral stromal fibrosis, the extent of inflammation taking into consideration the size of the tumor islands in relation to the number of immune (IC) infiltrate. The Histological inflammatory subtype (HIS) was scored according to Sema4D positive IC infiltrate into the tumor core; IC excluded by thin peritumoral fibrous rim or only at the tumor margin, or no IC in stroma, was carried using the *Aperio Imagescope.*

RNA Extraction and nSolver Analysis

For RNA extraction, three to five unstained, 5 mm thick FFPE tissue sections from 10 cases were used. Tumor tissue including 1–3 mm peritumoral stroma was mapped, manually micro-dissected by surgical oral pathologist (RY), and scrapped out the slides, guided by one H&E stained section of each tumor. RNA extraction using Rneasy FFPE kit (Qiagen, catalog no 73504) was carried out in the Genomic Core Facility, University of Maryland Baltimore. RNA quality control (QC) analysis was run on nanochip to ensure the required 200 nt in 50–300 ng with no prior amplification or enzymatic reaction. nCounter Human Pan Cancer IO-360 code set + panel standard cat # XT-CSPS-HIO360-12 and Master Kit-NAA-AKIT-012 platform were purchased from nanoString Technology (Seattle, WA). Hybridization was carried in a regular thermal cycler. The hybridized mix was purified using magnetic beads in the

nCounter machine in the institute of genome sciences (IGS) University of Maryland Baltimore. Data analysis was carried using IO-360 platform 770 genes code sets using the nSolver 4.0 software basic and advanced custom analysis.

Statistical Analysis

The distribution of Sema4D and other scale variables was compared between groups of patients using non-parametric statistics, the independent-samples Mann–Whitney U test in case of two groups and the independent-samples Kruskal–Wallis test in case of three or more groups. Associations between scale variables were quantified using Spearman's rank correlation coefficient, R_s, and tested against the null hypothesis of $R_s = 0$. Categorical variables were summarized as frequency distributions with indication of the relative frequency and its 95% confidence limits in parenthesis when relevant. All p-values are two-tailed; statistical significance was called for $p < 0.05$. A Bonferroni correction was applied to adjust for multiple comparisons, whenever relevant. Box-and-whisker plots were generated as a non-parametric representation of the distribution of a scale variable in a group; the sides of the box indicate the 1st and 3rd quartiles of the population distribution with a vertical bar indicating the median. The whiskers extend to 1.5 times the height of the box or, if no case has a value in that range, to the minimum or maximum values. In case of a normally distributed variable, approximately 95% or the data are expected to lie inside the whiskers. Outlying data outside this interval are marked with an asterisk. Binary logistic regression was used for multivariable analysis of patient- and disease-characteristics associated with having elevated sSema4D levels. Statistical analysis of patient engagement, and survey data was conducted using IBM® SPSS® Statistics for Windows, release 24.0.0 (IBM Corp., Armonk, N.Y., USA). nSolver 4.0 analysis uses the student T-test with statistical significance $p < 0.05$.

RESULTS

Histological Inflammatory Subtypes Scored Using Sema4D in HNSCC

Histological patterns of tumor inflammation were described using immunoscore of T cell infiltration in the tumor microenvironment (7, 11). Sema4D is an immune biomarker that may be informative of the global immune contexture and hence facilitates visualization of all leukocytes in the tumor core and peritumoral stroma (11, 21, 22, 25, 32). To describe the pattern of histological inflammatory subtypes (HIS) in the current HNSCC cohort, we performed IHC to assess Sema4D levels on 104 HNSCC that were treated with surgical excision as the initial line of treatment (**Figure 1**). We examined the Sema4D positive IC infiltrate in the invasive tumor front, peritumoral stroma, and tumor core. Eight cases of oral SCC were excluded due to lack of peritumoral stroma. The patients' demographics and tumor characteristics are described in **Table 1**. Sema4D showed moderate to strong membranous and cytoplasmic staining of the immune cells (IC). In the tumor cells (TC), Sema4D showed membranous and/or cytoplasmic staining that ranged from negative/weak to moderate/strong staining. The IC infiltration into the tumor islands was observed in 50 tumors (52%) that were scored as inflamed HIS (HIS-INF) (**Figures 2A, B**). IC infiltration mainly at the tumor invasive front or between the tumor islands, but excluded by a thin peri-tumoral fibro-myxoid/fibrous rim, was a discriminatory factor to score as immune excluded (HIS-IE). This was observed in 38 cases (40%) (**Figures 2C, D**). The stroma was almost deserted of IC infiltrate in eight cases (8%) and was scored as histologically immune deserted (HIS-ID) (**Figures 2E, F**).

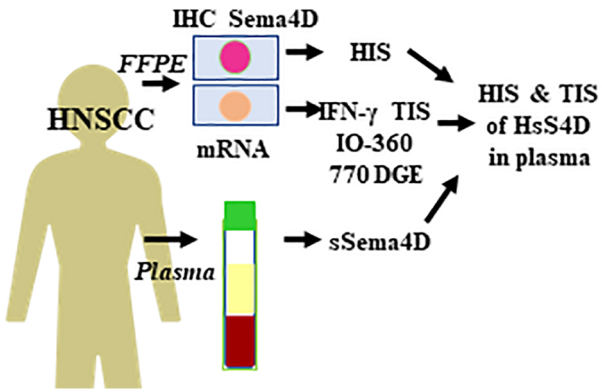


FIGURE 1 | Experimental outline and the workflow. Paired whole excisional tumor tissue and PB collected from 104 HNSCC patients at the same time point to allow for real time analysis of sSema4D in plasma in correlation to HIS and DGE. PB, peripheral blood; sSema4D, soluble Sema4D; IHC, immunohistochemistry; HIS, histological inflammatory subtype; TIS, IFN-γ tumor immune signature; DGE, differential gene expression; IO-360, Immuno-Oncologic 770 gene set. HsS4D, high soluble Sema4D in plasma.

The Sema4D HIS Validated Using IFN-γ Tumor Immune Signature

To validate the Sema4D HIS immune score, we tested representative samples using n Solver 4.0, principal component (PC) analysis. Indeed, the HIS-IE and HIS-INF segregate on PC.1 (**Figure 3A**). Then we examined the underlying immune transcriptional profile. We used the T cell inflamed immune signature, composed of active IFN-γ signaling, cytotoxic effector, and antigen presentation molecules, and T cell active cytokines that were previously sequentially validated to predict response to standard immunotherapy in HNSCC, as well as other tumor types (15). The refined six gene tumor immune signature (IDO1, CXCL10, CXCL9, HLA-DRA, STAT1, IFNG) was carried on a sample of cases representative of the Sema4D HIS subtypes using basic nSolver analysis (**Figure 3B**). The expanded IFN-γ16 gene signature (CD3D, IDO1,CD3E, CCL5,GZMK, CD2, HLA-DRA, CXCL13, IL2RG, NKG7, HLA-E, CXCR6, LAG3, CXCL10, STAT1, GZMB) was also run on individual samples and on grouped samples. Furthermore, the final validated T cell inflamed 18 gene signature (PSMB10, TIGIT, NKG7, PDCD1LG2, HLA-E, CCL5, CXCL9, STAT1, LAG3, IDO1,CD8A, CD27, CXCR6, HLA-DRB1, CMKLR1, HLA-DQA1, CD274, CD276) (15), was carried out on grouped samples representative of the three HIS subtypes

TABLE 1 | HNSCC Patients demographics and correlation with sSema4D in blood.

HNSCC	N (%)104 (100%)	Correlation to sSema4D in plasma p- value
Age, median (IQR)	67 (60, 73)	0.32
Gender		
Females	44 (42%)	0.17
Males	60 (58%)	
Race		
African American	7 (7%)	0.021[$]
Caucasian	88 (84%)	(–) 0.008[ϕ]
Hispanic	2 (2%)	
Asians	7 (7%)	
Location		
Oral and mobile Tongue	97 (93%)	0.15
Oropharynx	7 (7%)	
Stage[#]		
CIS	3 (2%)	0.29
I	35 (34%)	
II	11 (10%)	
III	10 (9%)	
IV	45 (45%)	
PATH grade		
CIS/Sup inv	7 (7%)	0.34
well	47 (46%)	
mod	39 (38%)	
poorly	10 (9%)	
HPV		
Yes	4 (4%)	0.046
No	100 (96%)	
Smoking		
Yes	43 (41%)	0.103
No	61(59%)	
Alcohol		0.70
Yes	19 (18%)	
No	85 (82%)	
History of dysplasia or HNSCC		
No history	57 (63%	0.01[‡]
History of Dysplasia	5 (6%)	
History of HNSCC	28 (31%)	

Decimals are rounded to the nearest whole number. [#]AJCC 8th edition staging system. Only significant p values for the HNSCC racial groups are mentioned. Caucasian versus African American[$] and Caucasian versus Asian [ϕ]. HPV +ve: one oropharyngeal and three oral SCC. [‡]HNSCC patients with history of dysplasia showed less level of sSema4D in plasma compared to other HNSCC groups.

(**Figure 3C**). Interestingly, Sema4D HIS-INF tumors showed a positive IFN-γ six gene signature, and the HIS-IE and HIS-ID were negative for the IFN-γ six gene signature (**Figure 3B**). The same distribution was observed using the expanded 16 gene (**Supplemental Figure 1**) and the final validated IFN-γ 18 gene signature (**Figure 3C**) (15).

Sema4D in Peripheral Blood of HNSCC Patients

High levels of sSema4D (HsS4D) have been described in chronic inflammatory conditions like osteoarthritis (OA), rheumatoid arthritis (RA), other autoimmune conditions (AI), and allergic reactions; like asthma (A) (38, 39). sSema4D was also previously described in plasma of HNSCC (5). To investigate the potential of sSema4D in peripheral blood as a soluble immune biomarker that can read the level of inflammation in HNSCC patients, we analyzed the level of sSema4D in the plasma of the 104 HNSCC patients compared to the control groups of healthy donors, AI, A, and OA patients (**Supplementary Table 1**). Our data showed that there was no statistically significant difference between sSema4D levels among the AI/A/OA conditions (p = 0.07). The level of sSema4D was highest within the Collagenous AI (Col AI) group (p = 0.011), and in the RA group compared to other Col AI diseases (p = 0.012) (**Supplementary Figure 2**).

sSema4D levels were significantly higher in the AI/A/OA group compared to HNSCC (p = 0.003, adj p = 0.009) and healthy donors (p < 0.001, adj p= 0.001). There was no statistically significant difference observed between sSema4D level in healthy donors and HNSCC (p = 0.051, adjust p = 0.152). However, 75% of HNSCC cases had higher sSema4D levels in plasma than the median of the healthy donors (above 83 ng/ml). There was no statistically significant difference observed between sSema4D levels in plasma and other clinical or demographic characteristics including smoking or alcohol drinking (**Table 1**). The HPV +ve cases and various racial groups were limited in number, which prevented conclusive results related to these variables in the current HNSCC cohort.

sSema4D in Plasma Reads the Underlying HIS and Immuno-Oncologic Signature in HNSCC

To investigate the potential of sSema4D as a soluble immune biomarker that can read the underlying tumor inflammatory stromal subtype, we analyzed the level of sSema4D in plasma with the paired tumor HIS subtype. The paired plasma and tumor tissue were collected at the same time point to allow for real time analysis (**Figure 1**). We defined a patient as having elevated levels of sSema4D (HsS4D) if the value exceeded 155 ng/ml, the 95th percentile of the levels measured in healthy donors. Using this definition, 25.0% (95% CI, 16.7–34.9%) of patients with HNSCC presented with elevated Sema4D (**Figure 4A**) (**Supplementary Table 2**). Our data showed a statistically significant association between HIS and HsS4D in plasma, P = 0.007. This is driven by HIS-IE, where the proportion of cases with HsS4D is 42% with exact binomial confidence limits (26%, 59%) as compared with HIS-INF where 14% (6%, 27%) presented with HsS4D (**Figure 4B**, **Table 2**).

The tumor was scored for the Sema4D and PD-L1 in IC and TC. Interestingly, sSema4D levels in plasma correlated significantly with Sema4D^{+ve} TC (p = 0.018) (**Figure 4C**) and PD-L1^{+ve} IC (p = 0.038). Furthermore, Sema4D $^{+ve}$ TC also correlated with PD-L1^{+ve} IC (p = 0.031). There was no statistically significant correlation observed between sSema4D and Sema4D in IC, PD-L1 in TC, nor PD-L1 or Sema4D CPS (**Supplementary Table 3**).

We performed a multivariable logistic regression analysis to identify patient-level factors associated with elevated HsS4D in patients with HNSCC. Patient-level factors tested in the model were age, gender, race, stage of disease, lympho-vascular invasion, smoking history, alcohol use, HIS, PD-L1 in IC, and PD-L1 in TC. Among these, HIS was highly statistically significant in the final model, P = 0.0014 (Likelihood ratio test)

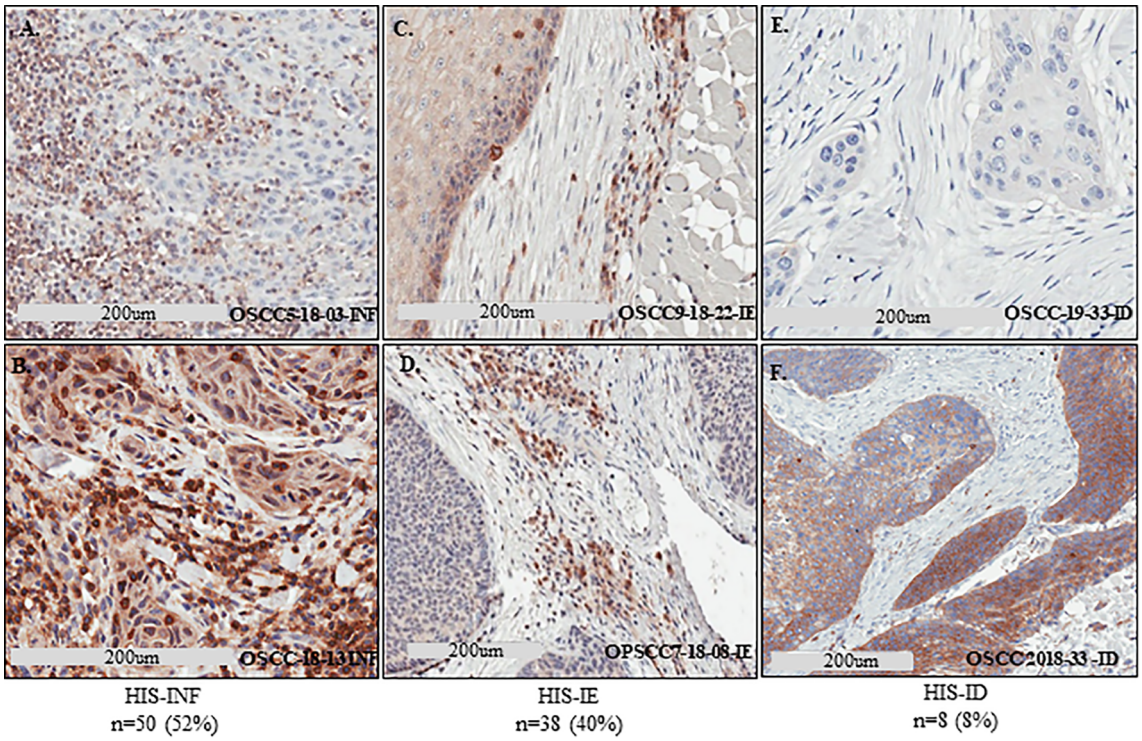


FIGURE 2 | Defining the HIS subtypes in HNSCC using Sema4D. IHC stain of Sema4D in HNSCC tissue. **(A, B).** HIS-INF OSCC tumors demonstrating IC infiltrate into the core of the tumor islands. **(C, D).** HIS-IE in OSCC **(C)** and base of tongue SCC **(D)**, shows IC excluded from tumor island by a rim of PTSF **(C)** or FMX rim **(D)**. **(E, F).** HIS-ID in OSCC showing cold non-inflamed fibrotic dense stroma deserted of IC. IHC, immunohistochemistry; HIS, histological inflammatory stroma subtype; INF, inflamed; IE, immune excluded; ID, immune desert; FMX, fibromyxoid; PTSF, peri-tumoral stromal fibrosis.

in a model adjusting for PD-L1 in TC (P = 0.035). The *post-hoc* test showed that most of the contrast related to HIS was between HIS-IE and HIS-INF, with an odds ratio for presenting with elevated sSema4D of 6.3 with 95% CI (2.2, 18.4), P = 0.0007 for patients with HIS-IE tumors. This analysis suggests that the information on HIS conveyed by Sema4D is independent of PD-L1.

We further analyzed the final 18 gene IFN-γ signature in a sample of 10 cases in relation to the level of sSema4D in plasma, using basic nSolver 4.0 analysis. The 10 cases were selected to include replicates of the HIS patterns (INF *versus* IE &/or ID), and Sema4D +ve and −ve tumor cells. They were also selected to include replicates of Sema4D/PD-L1 co-positive tumor cells *versus* Sema4D-ve/PD-L1+ve tumor cells, as previously characterized (32) (**Supplementary Figure 3**). The reference cutoff value >155 ng/ml for high sSema4D (HsS4D) in plasma was used. Four cases that were scored as HIS-IE or HIS-ID revealed HsS4D in plasma and clustered as negative IFN-γ immune signature. On the other hand, two cases of HIS-INF were LsS4D in plasma and clustered as positive IFN-γ signature. The remaining four cases represented a gray zone of IFN-γ expression, two of which were HIS-IE with a group of downregulated IFN-γ genes, and HsS4D in plasma that clustered more towards the positive IFN-γ signature. One case of HIS-IE with considerable number of downregulated IFN-γ genes was LsS4D in blood and clustered towards IFN-γ positive. One case of the HIS-INF, with IFN-γ positive signature was HsS4D. Interestingly, this case had a group of downregulated IFN-γ genes (**Supplementary Figure 3**).

We then investigated the underlying immune-oncologic profile of the HsS4D compared to the LsS4D. We used the IO-360, 770 genes representative of the oncogenic pathways, tumor inflammation and tumor microenvironment. RNA was extracted from mapped FFPE tumor tissue. Using the sSema4D a predictor variable, with a customized, advanced nSolver analysis, differential gene expression of ~40 genes in LsS4D *versus* HsS4D was observed (**Figure 5**). SPP1, ULBP2, COL11A1, and MMP7 were significantly upregulated in HsS4D compared to the LsS4D. The T cell inflamed tumor biomarker CD274 (PD-L1) was significantly upregulated in the LsS4D in addition to BATF3, CD247 (CD3ζ), CD80 among others (**Figure 5**). The distribution of the IC type demonstrated total tumor infiltrating leukocytes (TILs) to predominate in LsS4D tumors (**Figure 6A**). Analysis of the IC type relative to total TILs, showed Tregs to be the most numerous followed by B cells, exhausted T cells, then cytotoxic T cells in the LsS4D. Cytotoxic T cells were low in the LsS4D but were still higher than in HsS4D samples. Interestingly, the HsS4D had more mast cells, macrophages, and neutrophils in the TILs (**Figure 6A**). A trend plot of the immune-oncologic signaling pathways showed that the highest was the lymphoid

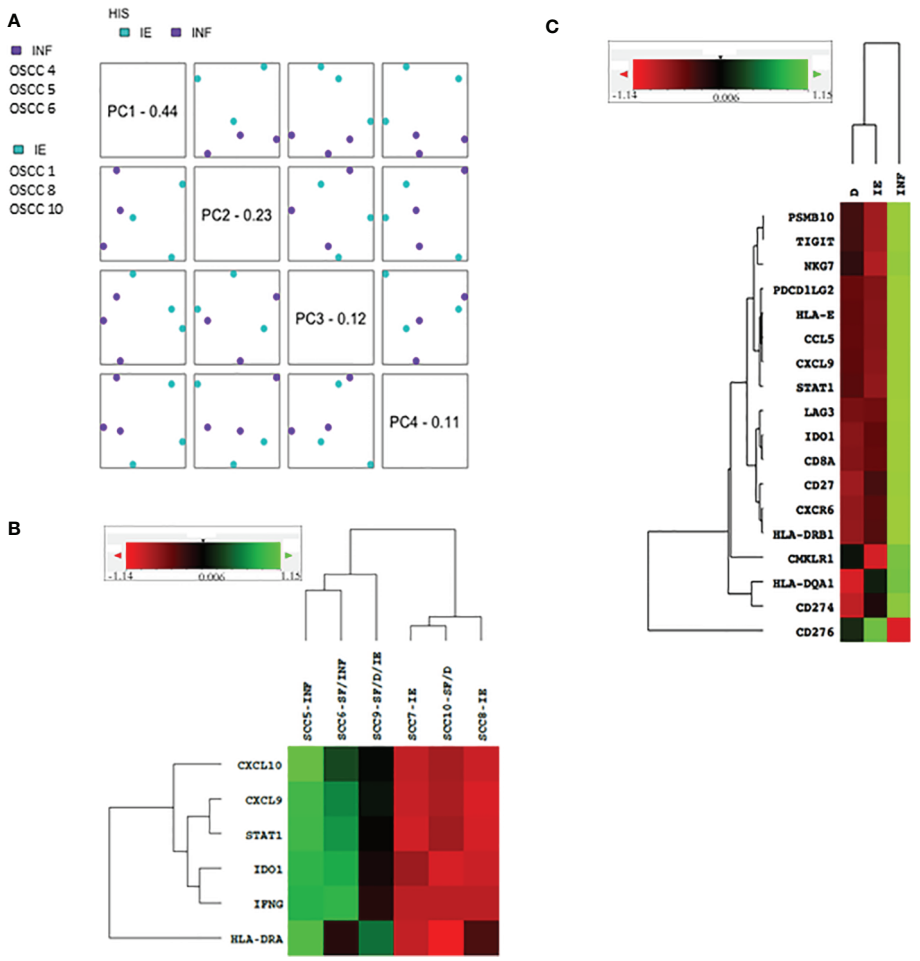


FIGURE 3 | Validation of the Sema4D HIS subtypes using PCA and IFN-γ signature on nSolver 4.0 analysis. **(A)** PCA analysis of the HIS-IE and HIS-INF. **(B)** Heat map of the refined six IFN-γ-signature in representative HIS tumors. **(C)** Heat map illustrating final IFN-γ18 gene signature between grouped cases of the three HIS subtypes (SCC 5&6 (INF), 7&8 (IE), 9&10 (ID). INF, inflamed; IE, immune excluded; ID, immune deserted; SF, stromal fibrosis. PCA, Principal component analysis. Green is positive; read is negative.

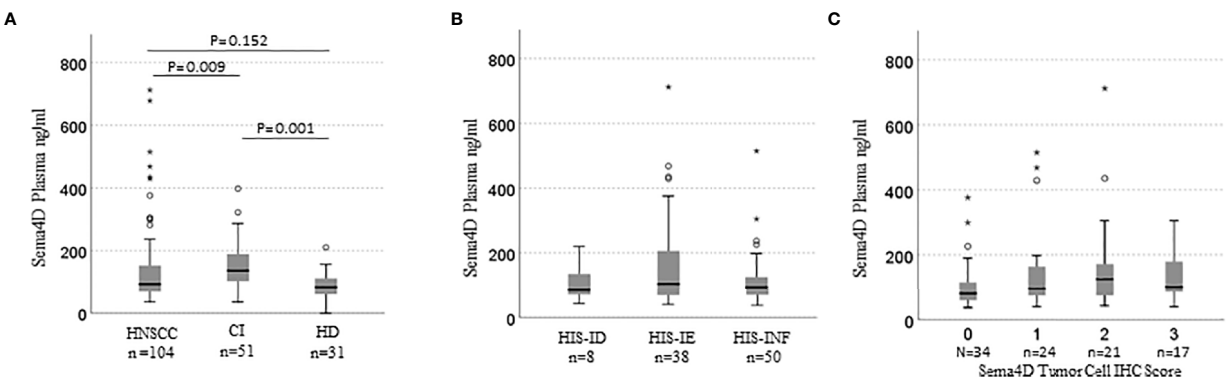


FIGURE 4 | sSema4D in plasma correlates with HIS-IE and Sema4D +ve TC in real time. Box and whisker plots illustrating independent samples Kruskal–Wallis test of **(A)** sSema4D level in plasma in HNSCC, CI (AI/A/OA) and HD. **(B)** The sSema4D in relation to the HIS subtypes (INF, IE, ID). **(C)** sSema4D levels correlate with Sema4D in TC. TC, tumor cell; HIS, histological inflammatory subtype; INF, inflamed; IE, immune excluded; ID, immune deserted. HD, healthy donors; CI, chronic inflammation; AI/A/OA, autoimmune; allergy and osteoarthritis. The circles and asteriks represent samples with higher level brackets.

TABLE 2 | HsS4D in plasma is associated with HIS-IE.

		Plasma		Total	p-value
		LsS4D	*HsS4D		
HIS	ID	7	1	8	
		87.5%	12.5%	100.0%	
	IE	22	16	38	0.007
		57.9%	42.1%	100.0%	
	INF	43	7	50	
		86.0%	14.0%	100.0%	
	Total	72	24	96	
		75.0%	25.0%	100.0%	

*HsS4D defined as sSema4D in plasma >155 ng/ml, LsS4D, Low sSema4D; ID, immune deserted; IE, immune excluded, INF, Inflamed.

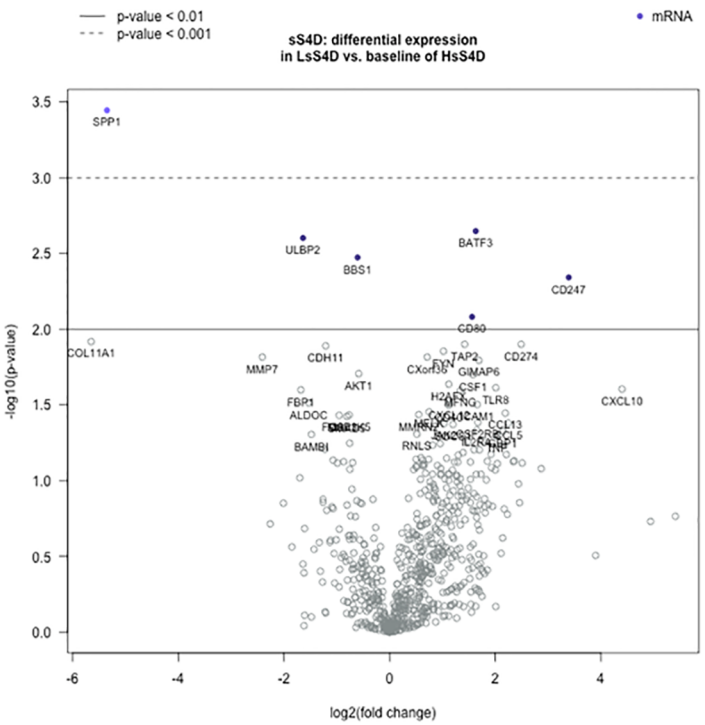
component, followed by costimulatory signaling, cytokines/chemokines, immune cell adhesion and migration pathways. IFN-γ, antigen presentation, and cytotoxicity pathways were also elevated. JAK-STAT and NF kappa B pathways were also among the upregulated pathways in the LsS4D compared to the HsS4D. In the HsS4D the Hypoxia and the Wnt signaling pathways were the most upregulated, followed by the Hedgehog pathway, metabolic stress, and TGF-β1 signaling (**Figure 6B**). Taken together, these findings suggest that HsS4D in plasma reads the underlying non-inflamed tumor, hypoxic and metabolically stressed microenvironment.

This biological association between Sema4D and HIS-IE raises the question whether Sema4D is of potential use as a predictive biomarker for the underlying tumor inflammatory stromal subtype? To this end, we performed a ROC curve analysis. The area under the ROC curve for Sema4D as a predictor of HIS-IE was 0.59 with 95% CI (0.46, 0.71). This was not statistically significantly different from the null hypothesis of AUC = 0.5, P = 0.17. The sensitivity of elevated HsS4D for predicting HIS-IE was 42% with 95% CI (26%, 59%), and the specificity was 86% with 95% CI (74%, 73%) (**Figure 7**).

DISCUSSION

Here we used Sema4D staining of the immune cells in the tumor microenvironment to score the three patterns of histological inflammatory subtype (INF, IE, ID) (9, 10). It is worth mentioning that scoring of tumor inflammation using Sema4D has not been previously reported. Interestingly, the percentage of INF/immune high *versus* IE/immune low using Sema4D scoring in the current cohort (**Figure 2**) is in concordance with the previously reported immunoscore using CD3/CD8 in HNSCC (7, 40, 41).

Biologically, our findings imply that the HsS4D in HNSCC can modulate a fibrotic type of tumor stromal inflammation similar to the chronic collagenous inflammation in RA and SLE.



mRNA	Log2 fold change	P-value
SPP1	-5.35	0.000
BATF3	1.63	0.002
ULBP2	-1.64	0.003
BBS1	-0.607	0.003
CD247	3.39	0.005
CD80	1.56	0.008
COL11A1	-5.65	0.012
TAP2	1.42	0.013
CD274	2.49	0.013
CDH11	-1.21	0.013
FYN	1.02	0.014
MMP7	-2.41	0.015
CXorf36	0.711	0.015
GIMAP6	1.69	0.016
AKT1	-0.585	0.020
CSF1	1.58	0.020
H2AFX	1.12	0.023
TLR8	2.01	0.024
CXCL10	4.4	0.025
FBP1	-1.68	0.025

FIGURE 5 | Immuno-oncologic analysis of HNSCC tumor tissue using sS4D as a predictor variable. DGE Volcano plot, presenting linear regression of 770 genes using IO-360, in LsS4D versus baseline of HsS4D. Table presents the top 20 significant DGE genes. Duplicates of LsS4D with IFN-γ+ve (SCC05, SCC06), were compared to HsS4D with IFN-γ–ve (SCC08 & SCC10) and HsS4D IFN-γ+ve (SCC01 and SCC04) (guided by **Supplemental Figure 3**). DGE, differential gene expression; LsS4D, low level of sSema4D in plasma; HsS4D, high level of sSema4D in plasma. P < 0.05 is significant.

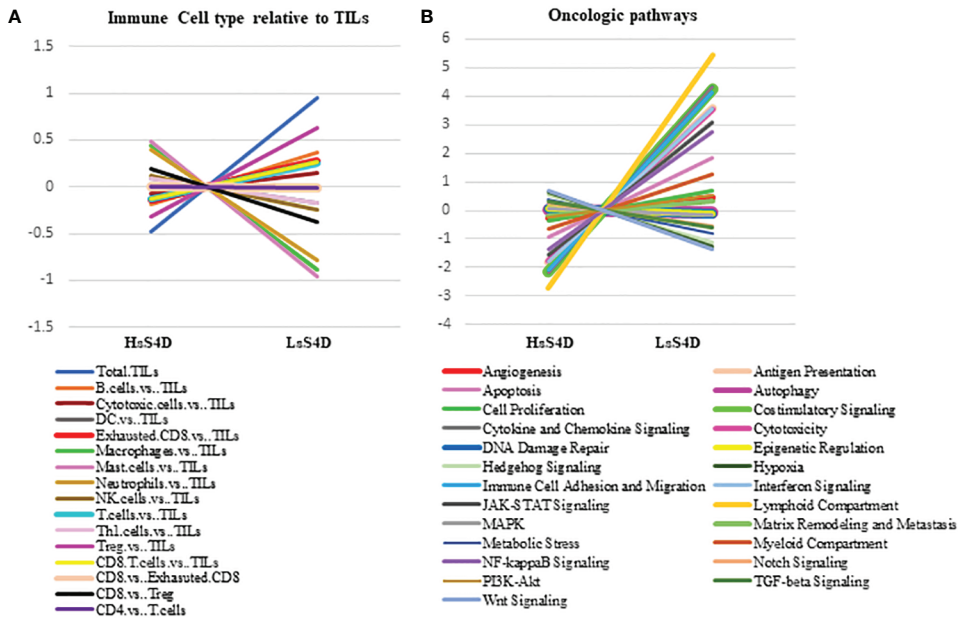


FIGURE 6 | Immune cell type and Immuno-oncologic pathways analysis *versus* sSema4D level in plasma. **(A)** Trend plot of immune cell type relative to total TILs in LsS4D *versus* HsS4D. **(B)** Trend plot analysis of immune-oncologic signaling pathways in LsS4D *versus* HsS4D. The same samples used for **Figure 5** were used. TILs, tumor infiltrating leukocytes; LsS4D, low level of sSema4D in plasma; HsS4D, high level of sSema4D in plasma.

It can promote immune mediated reactions in cancer patients characterized by a stromal phenotype that acts as an immune exclusion barrier.

The current work demonstrates that a statistically significant association between HIS and HsS4D in plasma, was driven by HIS-IE (P = 0.007). The nanoString analysis of a sample of the HsS4D-paired tissues was mostly IFN-γ signature negative, high in the myeloid component, in SPP1, and COLL11A gene expression, Wnt, hypoxia and TGF-β signaling (**Figures 5** and **6**). Interestingly, a recent study based on TCGA and GEO data set analysis in HNSCC showed that COL11A1, TGF-β and SPP1 were among the highest scored and selected hub genes. High SPP1 and high TGF-β expressing HNSCC were associated with a lower overall survival rate than the low expressing tumors (42).

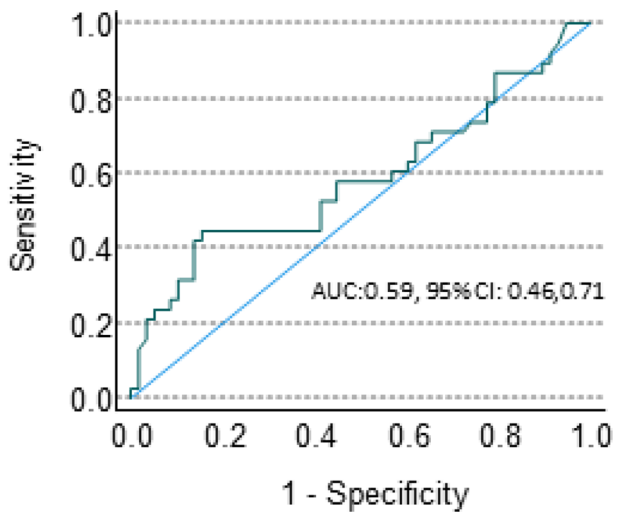


FIGURE 7 | sSema4D ELISA blood assay in HNSCC. ROC curve of the specificity and sensitivity of the HsS4D as a predictive marker of HIS-IE.

LsS4D patients had more HIS-INF tumor pairs that were positive for IFN-γ signatures with high lymphoid compartments and costimulatory signals. The Tregs predominated the lymphoid compartment in the LsS4D paired tissue. Interestingly, high Tregs in the tumor invasive margin and core were reported to correlate with better survival in HNSCC independent of HPV status (43). In addition, a recent study showed that the proximity of Tregs to CD3/CD8 cells can be a more precise estimate for overall survival of patients with HNSCC rather than summative assessment (8).

Previous studies showed that inhibition of Sema4D in an *in vivo* tumor model facilitated IC infiltration into the tumors and decreased the MDSC component (44, 45). Humanized anti-Sema4D antibody is currently under investigation in cancer and autoimmune neurogenic disorders. It is well tolerated, and the immune cell levels at baseline and progression-free survival were consistent with an immune-mediated mechanism of action (46, 47). Future studies to investigate the level of sSemaD in plasma in response to Sema4D inhibitory antibody and in combination with standard immunotherapy would be informative. The current work suggests that Sema4D level in the blood should be maintained within the healthy donor range to keep the physiologic homeostasis that Sema4D regulates in the immune and the nervous system (20, 25).

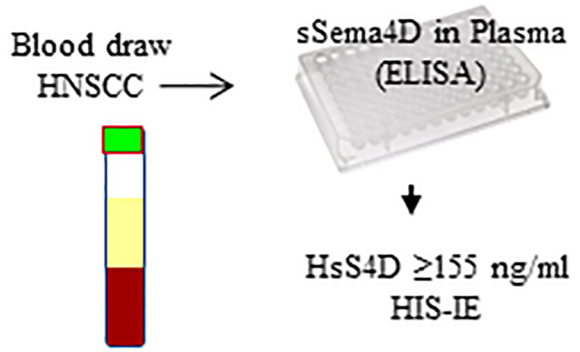


FIGURE 8 | Diagram of the sSema4D ELISA blood assay in HNSCC. HsS4D, High soluble Sema4D; HIS-IE, Histological immune excluded phenotype.

Our current cohort is mainly HPV-ve HNSCC of the oral and mobile tongue (**Table 1**), treated with surgery as the initial line of treatment. It is mainly immunotherapy naïve and accordingly provides basal level of the immune biomarker sSema4D in peripheral blood. Although the high level of sSema4D in plasma of AI/A/OA is a limitation to the current technology, none of the patients included in this study had a medical history of any of these conditions. The level of sSema4D in HPV +ve patients, oropharyngeal SCC, as well as patients with previous chemo or radiotherapy, and terminal stage malignancy has yet to be investigated.

Our data suggest that the Sema4D +ve TC can be the source of the sSema4D in blood (**Figures 4C**, **Table 2**), but does not rule out the production of sSema4D in blood by activated circulating immune cells. Intriguingly, cancer patients can demonstrate both pro-inflammatory and anti-inflammatory response, as the patient's immunity at the tumor tissue level and in the circulation is altered at different stages of tumor development. Further investigations to demonstrate the direct evidence of the source of HsS4D in plasma of HNSCC patients and its correlation with disease progression and patient survival are warranted. This can include future comparative analysis of pre-operative *versus* post-operative levels of sSema4D in plasma of HNSCC.

The current work suggests that HNSCC with elevated sSema4D could be a distinct phenotype. The observed associations raise the hypothesis that changes in sSema4D can monitor the underlying dynamics of tumor and stromal inflammation in real time. This would be the topic of a subsequent, larger study (**Figure 8**). Characterization of the differential immune oncologic profile and the histological pattern of inflammation in relation to the soluble immune biomarker Sema4D can provide a translational aspect that can further enhance our understanding and stratification of HNSCC patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE163131.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Maryland Baltimore Institutional review board, Human Research Protection Program. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RY generated the research hypothesis, the experimental design, carried the tumor tissue mapping for RNA extraction, carried the Sema4D immunoscore and the IHC scoring, oversaw the ELISA assay, the RNA extraction, carried the basic and custom analysis on nSolver, and wrote the manuscript. IG ran all the ELISA, for the HNSCC and control plasma samples, and contributed to the immunoscore of the Sema4D IHC. ME and RC ran all the PD-L1 IHC and contributed to the scoring. ME scanned all the Sema4D and PD-L1 slides using aperio scanscope for digital analysis. VT assisted in the IHC. JP contributed to the immunoscore. DD, RO, and JL contributed to the HNSCC clinical diagnosis and patient selection. JL collected all relevant clinical, pathological, and demographic parameters of the patients. TW contributed to the direct ELISA assay design. ED and LT contributed to the data analysis. SB carried out the statistical analysis of the Sema4D level in plasma in correlation to all clinical, demographics, and histological findings and generated the graphs. All authors contributed to the article and approved the submitted version.

FUNDING

The current work was funded by the Maryland Technology Development Corporation (TEDCO) MII Phase I (PID 0719-012, UMB-Younis). The Institute for Clinical and Translational Research, University of Maryland Baltimore (150-Younis-ICTR), Angel research fund to the Department of Oncology and Diagnostic Sciences, UMSOD.

ACKNOWLEDGMENTS

The authors would like to acknowledge the faculty and research coordination staff in the Department of Oral and Maxillofacial Surgery in the School of Dentistry, University of Maryland Baltimore. The core facility services at the Institute for Genome Sciences, the Pathology Biorepository shared services, Biostatistics and Bioinformatics shared services, the Genomics Core facility, at

the University of Maryland Baltimore. Finally, we would like to acknowledge the staff at the human research protections comprehensive program, the institutional review board at UMB, and all HNSCC patients who consented to the current study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.596646/full#supplementary-material

Supplementary Figure 1 | Heat map analysis of the HIS subtypes using expanded IFN-γ signature. **(A)** Heat map illustrating individual tumors using IFN-γ expanded 16 genes signature. **(B)** Heat map of expanded IFN-γ16 genes signature between grouped cases of the three HIS subtypes (SCC 5&6 (INF), 7&8 (IE), 9&10 (ID). INF, inflamed; IE, immune excluded; ID, immune deserted.

Supplementary Figure 2 | sSema4D in plasma of AI/A/OA. Box and whisker plot Independent-Samples Kruskal–Wallis test illustrating **(A)** sSema4D level in plasma of AI/A/CI. **(B)** sSema4D in RA *versus* other CoI AI conditions. AI, autoimmune diseases; A, Asthma; CI, chronic inflammation (osteoarthritis); other CoI AI, other collagenous AI; HD, healthy donors.

Supplementary Figure 3 | HsS4D in plasma of HNSCC is associated with negative IFN-γ signature. Tumor tissue heat map for the IFN-γ 18 gene immune signature with corresponding HIS scoring and sSema4D levels in plasma. The 10 samples were mainly selected based on the HIS, and Sema4D in TC. HIS, histological inflammatory subtype; INF, inflamed; IE, immune excluded; ID, desert; HsS4D, high sSema4D in plasma; LsS4D, Low sSema4D in plasma; TC, tumor cell; Green, positive; Red, negative.

Supplementary Table 1 | Descriptive analysis of the AI/A/CI controls for sSema4D in plasma. Collagenous AI conditions: RA, L, SC. Non-collagenous AI conditions: MS, Crohn's, Sarcoidosis and MG. Three allergy patients had osteoarthritis and one RA had osteoarthritis.

Supplementary Table 2 | Descriptive analysis of age, race, and sSema4D in plasma of HNSCC patients, AI/A/OA and HD.

Supplementary Table 3 | Sema4D and PD-L1 in tumor cell and immune cell in relation to sSema4D in plasma. Decimals are rounded to the nearest whole. 0; negative, 1; weak, 2; positive, 3; strongly positive. Extent of INF; extent of inflammatory cell present independent of the pattern (INF or IE).

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Conflict of Interest: TW is CEO of WebbCures, LLC, co-founder of Screen Therapeutics and serves as an advisor for Immunaccel Labs, LLC.

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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frontiers in Immunology

REVIEW
published: 13 April 2021
doi: 10.3389/fimmu.2021.654877

The Cancer Cell Dissemination Machinery as an Immunosuppressive Niche: A New Obstacle Towards the Era of Cancer Immunotherapy

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OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 17 January 2021
Accepted: 23 March 2021
Published: 13 April 2021

Citation:
Asiry S, Kim G, Filippou PS, Sanchez LR, Entenberg D, Marks DK, Oktay MH and Karagiannis GS (2021) The Cancer Cell Dissemination Machinery as an Immunosuppressive Niche: A New Obstacle Towards the Era of Cancer Immunotherapy. Front. Immunol. 12:654877. doi: 10.3389/fimmu.2021.654877

Although cancer immunotherapy has resulted in unpreceded survival benefits to subsets of oncology patients, accumulating evidence from preclinical animal models suggests that the immunosuppressive tumor microenvironment remains a detrimental factor limiting benefit for many patient subgroups. Recent efforts on lymphocyte-mediated immunotherapies are primarily focused on eliminating cancer foci at primary and metastatic sites, but few studies have investigated the impact of these therapies on the highly complex process of cancer cell dissemination. The metastatic cascade involves the directional streaming of invasive/migratory tumor cells toward specialized blood vessel intravasation gateways, called TMEM doorways, to the peripheral circulation. Importantly, this process occurs under the auspices of a specialized tumor microenvironment, herewith referred to as "Dissemination Trajectory", which is supported by an ample array of tumor-associated macrophages (TAMs), skewed towards an M2-like polarization spectrum, and which is also vital for providing microenvironmental cues for cancer cell invasion, migration and stemness. Based on pre-existing evidence from preclinical animal models, this article outlines the hypothesis that dissemination trajectories do not only support the metastatic cascade, but also embody immunosuppressive niches, capable of providing transient and localized immunosubversion cues to the migratory/invasive cancer cell subpopulation while in the act of departing from a primary tumor. So long as these dissemination trajectories function as "immune deserts", the migratory tumor cell subpopulation remains efficient in evading immunological destruction and seeding metastatic sites, despite administration of cancer immunotherapy and/or other cytotoxic treatments. A deeper understanding of the molecular and cellular composition, as well as the signaling circuitries governing the function of these

dissemination trajectories will further our overall understanding on TAM-mediated immunosuppression and will be paramount for the development of new therapeutic strategies for the advancement of optimal cancer chemotherapies, immunotherapies, and targeted therapies.

Keywords: cancer immunotherapy, tumor microenvironment, endothelial anergy, lymphocyte exclusion, lymphocyte exhaustion, metastasis, macrophages, T cells

INTRODUCTION

Molecular investigations of the intricate and reciprocal interactions between tumor and immune cells have been at the frontier of cancer research in the past decade, a trend that will likely continue given the recent development of highly effective cancer immunotherapies (1–7). In general, antitumor immunity is strongly reliant on the trafficking of $CD8^+$ T cells in both primary and metastatic tumor microenvironments (TMEs) and can be characterized as a highly dynamic and tightly regulated process (8–10). There is abundant preclinical and clinical evidence that the presence of tumor-infiltrating lymphocytes (TILs) correlates with favorable clinical outcomes (11–17), but contradictory results have also been reported (18, 19). Moreover, recent studies have demonstrated that the spatial distribution patterns of TILs within the tumor microenvironment may play an even more drastic role in determining the prognostic outcome, than the density of TILs alone (20–22). In yet other studies, the co-assessment of immune cell signatures related to specific functional status (or subtypes) of TILs may be critical for a more accurate assessment of prognostic outcomes (23–30). These observations collectively suggest that T cell trafficking into the TME is one of the critical aspects of antitumor immunity. The overall immune landscape in the TME is therefore a key determinant for the efficiency of $CD8^+$ T cell-mediated antitumor immunity in either natural, induced or engineered immune responses.

The intricate relationship between immune and cancer cells in the context of tumor development and progression has long been recognized (31). Since the initial proposal of the cancer immunosurveillance theory (32, 33), numerous immunotherapies have been developed including monoclonal antibodies (34, 35), chimeric antigen receptor (CAR) T cells (36–38), and tumor vaccines (39, 40). Despite the success, which is primarily seen in hematological malignancies, such as in leukemia and lymphoma (41, 42), the efficacy of these treatment modalities has been less dramatic in solid tumors, such as in breast, colorectal, and prostate cancers (31, 43). The lack of promising outcomes in these solid tumor types is likely a multifactorial and cumulative result arising not only from intrinsic defects of antitumor immunity, but also from the intricate relationships among tumor cells, immune cells, and their surrounding microenvironment, which can obfuscate these antitumoral immune responses (44–46). Although the majority of these mechanisms will not be detailed as they are beyond the scope of the current perspective, here we focus on the emerging roles of the tumor-infiltrating myeloid cell population in limiting antitumor $CD8^+$ T cell responses.

A plethora of terminally differentiated myeloid cells and/or their immature counterparts, including monocytes, macrophages, neutrophils, and myeloid derived suppressor cells (MDSCs) among others, have been identified in the tumor stroma, whereby they conspire with tumor cells to promote the acquisition of metastatic hallmarks (47–52). In this heterogeneous landscape, a flurry of proangiogenic and proinflammatory cytokines (VEGF, IL6, etc.) rising from hypoxic and acidic microenvironments instigate myeloid cell infiltration and activation (53–60). There is now ample evidence that this myeloid cell-dominated milieu constitutes a rather inhospitable and antagonistic microenvironment for T cell trafficking and further promotes T cell exhaustion and deactivation (61–68). As such, the latest advances in immunotherapy have been directed at overcoming the immunosuppressive mechanisms within the tumor microenvironment, with a special focus on counteracting the function of protumoral myeloid cell populations (31).

It is undeniable that modern immunotherapies, including immune checkpoint blockade (anti-PDL1, anti-CTLA4, etc.) and adoptive transfer of genetically engineered T cells to express a receptor that is specific for a tumor antigen have revolutionized cancer treatment (41, 69–72). However, most such studies have primarily evaluated cancer cell growth and proliferation endpoints, such as primary and metastatic tumor burden, to document their efficiency as potential anticancer treatment modalities. The degree to which natural or engineered antitumor immunity can successfully target the highly invasive and migratory tumor cell subpopulation is poorly understood. As seen by the high recurrence rates in many solid malignancies, invasive/migratory tumor cells can evade the cytotoxic effects of chemotherapy, radiotherapy, and other treatments, as well as escape immunological detection and destruction (47, 73, 74). Cancer cell dissemination is regulated by a specialized network and subsets of myeloid cells, which form dedicated niches for the nurturing of migratory/invasive cancer cells (47, 50, 75–77). In this perspective, we propose that certain myeloid cell subsets, particularly perivascular M2-like macrophages, are contextually associated with cancer cell dissemination trajectories, offering a localized immunosuppressive niche to the metastasizing tumor cell population, while in the act of active dissemination. We conclude that thorough understanding of these immunosuppressive mechanisms in the tumor microenvironment at the molecular level will lead to more effective therapeutic targeting of cancer metastasis and will possibly improve the outcome of modern immunotherapies.

THE *CANCER CELL DISSEMINATION TRAJECTORY*: TUMOR MICROENVIRONMENT THAT REGULATES THE INITIAL STEPS OF THE METASTATIC CASCADE

From earliest portrayals to more recent representations, two generic components have been distinguished as integral parts of the metastatic cascade, a cancer cell dissemination step and a cancer cell growth/proliferation step at the metastatic site, the latter also known as colonization step (78–84). Both these steps are regulated and may even be reinforced by a diverse array of biological programs in the tumor microenvironment, including epithelial-to-mesenchymal transition (EMT), invasion/migration, chemotaxis, and dormancy (85–92), among others. Recent advances in the underlying mechanisms of cancer cell dissemination have indicated that cancer cells that have undergone EMT, and thus have lost epithelial polarity and gained mesenchymal properties, participate in a reciprocal juxtacrine-paracrine signaling loop with tumor-associated macrophages (TAMs), eventually leading them to the underlying vasculature for subsequent intravasation. Cancer cell intravasation, however, does not occur along the entirety of the cancer-associated endothelium, but is rather restricted to specialized intravasation sites, known as Tumor MicroEnvironment of Metastasis (TMEM) doorways. In this section, we will briefly discuss the factors that underlie the spatial and functional relationship between the disseminating tumor cell subpopulation and the TMEM doorways, a critical ingredient that regulates the initial steps of the metastatic cascade in primary tumors.

Cancer cell intravasation doorways, also known as TMEM doorways, constitute intratumoral niches characterized by the physical juxtaposition of a tumor expressing high levels of the actin-regulatory protein Mammalian enabled (MENA), a perivascular macrophage and an endothelial cell, and represent an independent prognostic indicator of metastatic risk in breast cancer patients (93–96). Perivascular macrophages residing in TMEM doorways express the tyrosine kinase receptor TIE2, thus assuming an M2-like polarization status and tumor-promoting effects. Under the tight regulation of TIE2 signaling, TMEM macrophages secrete large amounts of vascular endothelial growth factor (VEGF), which in turn, functions in a paracrine fashion on the TMEM endothelial cell to promote the reversible breakdown of endothelial cell-to-cell adhesions, localized vasculature opening, and the subsequent intravasation of invasive/migratory tumor cells from the immediate area surrounding the TMEM doorway. Despite that the precise role of the TMEM tumor cell in the TMEM triad has not yet been clearly elucidated, high-resolution microscopy has suggested the presence of invadopodia stemming from TMEM tumor cells and extending in between the underlying vasculature (73). Thus, the current understanding is that TMEM tumor cells pinpoint the breaching point of the endothelial wall following TIE2-dependent TMEM doorway activation.

Formation of active TMEM doorways has not only been observed in primary tumors, but also in their respective loco-regional and distant metastatic sites, such as in the lymph nodes and lungs, respectively (75, 93, 97–99). Indeed, prior work has documented that established lymph node metastases attract $TIE2^+$ macrophages in the perivascular niche, which in turn assemble TMEM doorways *de novo* (99). More importantly however, photoconversion experiments that can specifically label tumor cells in metastatic lymph nodes and observe their behavior in real time have indicated that cancer cells within metastatic foci are capable of utilizing TMEM doorways to re-disseminate to tertiary metastatic sites, such as to the lungs (99). Overall, these studies support TMEM doorway-mediated cancer cell dissemination as a universal mechanism of cancer cell dissemination at all stages of cancer progression.

Within the constantly evolving landscape of tumor cell heterogeneity, it is crucial to appreciate that not all tumor cells are equally capable of cancer cell dissemination *via* TMEM doorways. Rather, only a small subset of tumor cells in primary tumors is co-opted to utilize TMEM doorways for intravasation in the peripheral circulation. Expression profiling studies have specifically identified this subset as overexpressing an alternatively spliced isoform of the actin-regulatory protein Mammalian enabled (MENA), called $MENA^{INV}$, and having concurrently lost expression of the antimetastatic and cell cohesion-promoting alternatively spliced isoform MENA11a (100–104). MENA is one of the key members of the Ena/VASP family of proteins, involved in regulation of cell movement, shape and adhesion (105), mainly through regulating actin filament polymerization and rate of filament elongation during the formation of cellular protrusions (106, 107). Cancer cells that overexpress $MENA^{INV}$ are characterized by formation of extracellular matrix-degrading cellular protrusions, called invadopodia, by increased sensitivity to chemotactic factors in the tumor microenvironment such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF), which both facilitate cancer cell invasion and migration (75, 98, 107–114). It is therefore not surprising that $MENA^{INV}$-expressing tumor cells are preferentially co-opted for TMEM-mediated cancer cell intravasation. It should be noted that $MENA^{INV}$-expressing cancer cells also share markers and phenotypic characteristics that indicate they have undergone epithelial-to-mesenchymal transition (EMT), which is a crucial landmark of metastatic dissemination (85, 115–118). However, in the current perspective we will primarily refer to the migratory/invasive tumor cell compartment as the $MENA^{INV+}$ cancer cell subpopulation, given that prior studies have suggested that MENA isoform switching is crucial for the establishment of metastatic disease (103, 104, 106).

Multiphoton intravital imaging studies in live mice have suggested that $MENA^{INV}$-expressing tumor cells migrate along collagen fibers with partnering TAMs in the tumor microenvironment. A well-described, reciprocal paracrine loop between the two cell types, involving colony stimulating factor-1 (CSF1) secretion from the tumor cell and epidermal growth factor (EGF) secretion from the macrophage, leads to the chemotactic attraction of one cell towards the other, coupling them in sequence in a unique migratory pattern called "cancer cell streaming" (104, 119, 120). The specific targeting of either of

these factors, either pharmacologically or *via* genetic engineering, is sufficient by itself to disrupt cancer cell streaming and suppress cancer cell dissemination (104, 121–123). Eventually, MENA^{INV+} tumor cells reach down to the perivascular niche, whereby they utilize pre-existing TMEM doorways to intravasate into the blood vessel (104, 114, 124, 125). Chemotactic factors, such as hepatocyte growth factor (HGF) and stromal derived factor-1 (SDF1), either secreted by the cancer endothelium itself or by cells associated with the endothelium (e.g. TMEM macrophages), are responsible for the directed migration of the entire cancer cell "streams" towards the TMEM doorway (126, 127).

A few studies have previously investigated the mechanisms *via* which MENA^{INV} expression is induced in the migratory/invasive cancer cell subset. Although the exact mechanism has not been deciphered at the molecular level, there is strong indication that TAMs streaming with tumor cells are crucial for MENA^{INV} induction in the latter. Specifically, *in vitro* co-culture experiments have indicated an up to 50-fold increase in MENA^{INV} expression when tumor cells were co-cultured with macrophages, and this phenotype was demonstrated to be contact-dependent, suggesting that juxtacrine signaling loop may also be elicited during the cancer cell streaming behavior (124). In support to these observations, the *in vivo* depletion or suppression of macrophage differentiation has shown a significant reduction of cancer cell dissemination (128).

In this perspective, the described MENA^{INV+} migratory/invasive cancer cell population partnered with intratumoral TAMs during streaming, along with corresponding TMEM doorways used during the intravasation process, will be collectively referred to as the "*Dissemination Trajectory*" (**Figure 1**). Indeed, it is expected that different signaling pathways, cytokine/chemokine profiles, and metabolic patterns will characterize the dissemination trajectories versus the more proliferative compartments of solid tumors. Here, we explore dissemination trajectories as immunosuppressive landscapes, in an effort to explain current translational and clinical observations on why natural or engineered antitumor immunity is not efficient in preventing the metastatic cascade, albeit demonstrating promising results in eliminating tumor growth potential.

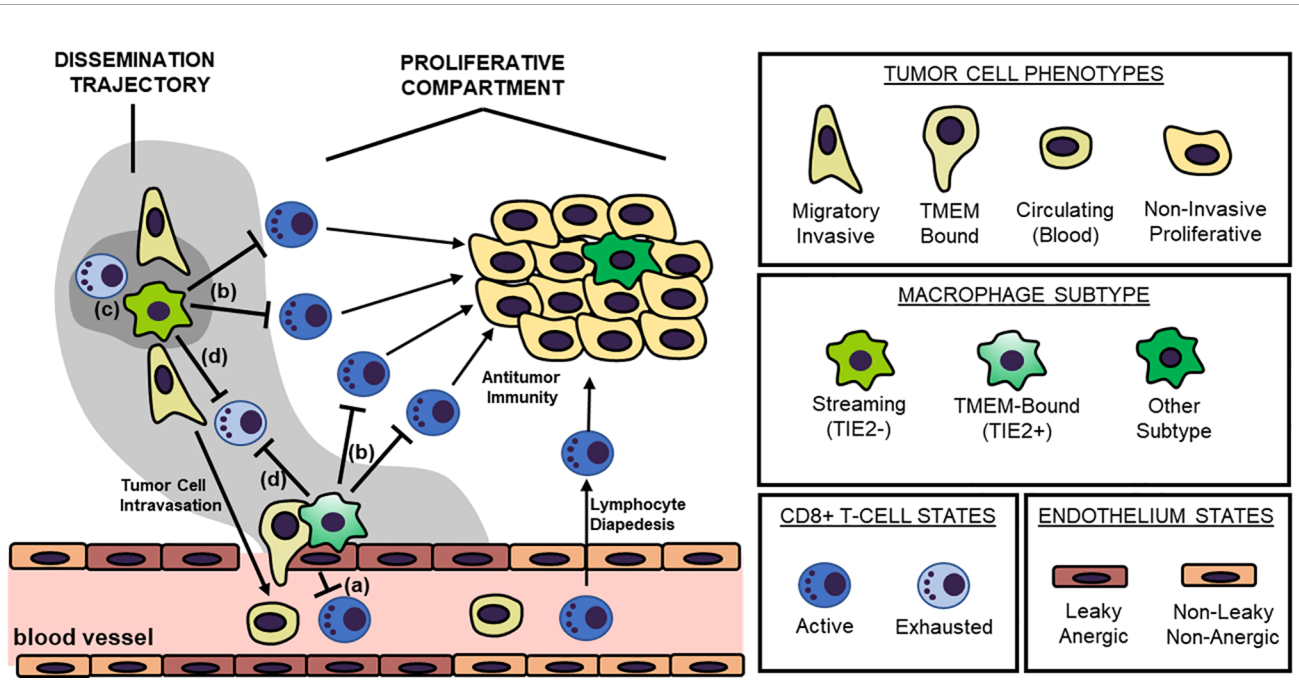


FIGURE 1 | The "Dissemination Trajectory" Working Model of Metastatic Dissemination. Two major cellular prerequisites are necessary for cancer cell dissemination: a TMEM doorway and a highly invasive, highly migratory cancer cell subsets streaming toward TMEM doorways. TMEM doorways are composed of three cell types, a TIE2^{+} macrophage, an endothelial cell and a tumor cell forming an invadopod in the vasculature, and signaling conversation among these three cells results in localized vascular opening to facilitate transendothelial migration of the highly invasive, highly migratory cancer cell subset. The highly invasive and migratory cancer cell subsets participate in a reciprocal paracrine and juxtacrine signaling loop with intratumoral macrophages that do not express TIE2, resulting in the increased induction of the actin-regulatory protein MENA^{INV}. Eventually, these interactions result in the so called "streaming migration", which is directed toward TMEM doorways, and MENA^{INV}-facilitated transendothelial migration and metastatic dissemination. TMEM doorways and their streaming MENA^{INV+} cancer cell subsets are herewith referred to as "dissemination trajectories". These specialized microenvironments are distinguishable from other tumor compartments with rapidly dividing tumor cells that do not share similar molecular pathways, here described as "proliferative compartments". Four layers of immunosuppressive mechanisms dominate within the dissemination trajectories, that result in the development of immune deserts further facilitating the process of metastatic dissemination. These mechanisms postulate that: (a) the TMEM endothelium is anergic, thus not allowing for T cell diapedesis; (b) dissemination trajectories do not support cytokine/chemokine matching for allowing T cell chemotaxis; (c) dissemination trajectories have a unique metabolic landscape that is refractory for T cell chemotaxis and/or function; and finally (d) dissemination trajectories are characterized by the induction of immune checkpoint signaling, that promoted exhaustion of T cells. Overall, tumor-associated macrophages (TAMs) within these dissemination trajectories play the pivotal role in regulating all four layers of immunosuppression, although secondary mechanisms have also been identified.

THE CANCER CELL DISSEMINATION TRAJECTORY AS AN IMMUNOSUPPRESSIVE NICHE

A substantial amount of preclinical and clinical studies has indicated that tumor-associated myeloid cells, predominantly tumor-associated macrophages (TAMs), neutrophils, and myeloid-derived suppressor cells (MDSCs), sustain an immunosuppressive tumor microenvironment, which is particularly refractory to both T cell trafficking and antitumor T cell functions (4, 129–134). Extensive research in this field has additionally concluded that the specific targeting and/or elimination of this myeloid-driven immunosuppressive program can render the natural, induced, and engineered immunological responses against tumors more concrete and effective (135–137). In line with the above, here we first provide proof-of-principle evidence of this notion using the Mouse Mammary Tumor Virus Polyoma Middle-T antigen (MMTV-PyMT) mouse model of breast carcinoma, which successfully recapitulates human breast cancer progression (138). During the natural progression of MMTV-PyMT carcinomas, T cells are spatially restricted to the peritumoral stromal sheaths and are visually excluded from multicellular tumor cell cohorts (**Figure 2A**), insinuating structural and/or functional impediments of intratumoral T cell trafficking. However, upon the pharmacological depletion of TAMs *via* the administration of clodronate liposomes, intratumoral T cell trafficking is clearly improved (**Figure 2B**), pinpointing TAMs as the responsible structural and functional impediments to T cell trafficking. It has been previously shown that immune cells can excessively infiltrate primary tumors as a result of a cytokine surge, induced by cytotoxic factors, such as chemotherapy treatment (47, 73, 74, 139). Indeed, administration of paclitaxel, a taxane-based chemotherapy known to inflict prometastatic modifications as a consequence of a cytokine surge (139–144), results in a dramatic increase of TILs, which are otherwise restricted to the peritumoral stromal sheaths (**Figure 2C**). This distribution pattern appears to be the consequence of immunosuppressive TAMs, because clodronate-mediated depletion of TAMs in the chemotherapy setting facilitates the intratumoral trafficking of T cells that have responded to the chemotherapy-driven cytokine surge (**Figure 2D**). Of note, similar observations by other groups have corroborated our findings using a diverse array of macrophage suppression or

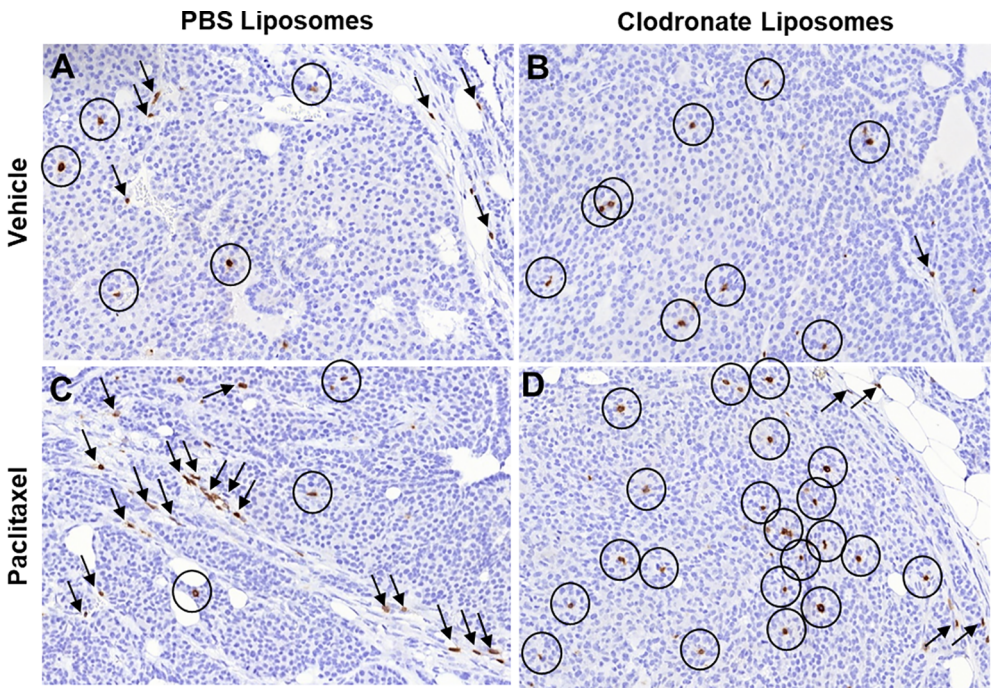


FIGURE 2 | Immunohistochemical indication of how different pharmacologic modifications of the immunosuppressive tumor microenvironment may affect T cell trafficking into tumors. **(A–D)** Immunohistochemistry for T cell specific marker CD3 in tumor sections from mouse mammary tumor virus – polyoma middle T antigen (MMTV-PyMT) mice, developing spontaneous breast carcinomas. The images are high power fields (x40), representative from a total of three mice in each experimental condition. Circles, $CD3^+$ T cells infiltrating the tumor nests; Arrows, $CD3^+$ T cells infiltrating the tumor stroma. Notice the significant changes in intratumoral versus stromal T cell infiltration upon different treatments that modify the immunosuppressive microenvironment.) In breast carcinoma, T cells are found in both tumor cell nests and the tumor stroma **(A)**. Upon macrophage depletion with clodronate liposomes, most T cells can leave the stroma and penetrate the tumor cell nests **(B)**. However, treatment with cytotoxic chemotherapy is known to induce lymphocyte infiltration and significantly larger number of T cells is found compared to the vehicle **(C)**. Notably however, most of these T cells are restricted in the tumor stroma, as chemotherapy attracts immunosuppressive myeloid cells at the same time, resulting in lymphocyte exclusion **(C)**. If such immunosuppressive myeloid cells are depleted through clodronate liposomes in chemotherapy-treated tumors, the increased influx of T cells is now relocated in the tumor nests **(D)**. *Immunohistochemistry was performed in archival tissue from experiments originally conducted in the manuscript by Karagiannis et al.* (139)*, in which ethical approval for the use of the experimental mice was also obtained* (139).

re-polarization strategies (77, 145, 146). In conclusion, these experimental data along with accompanied literature collectively demarcate the detrimental impact of TAMs in T cell trafficking and distribution in primary tumors.

As described above, specialized tumor microenvironments within primary carcinomas comprising of TMEM doorways and their associated prometastatic $MENA^{INV+}$ cancer cell compartments, herewith defined as *dissemination trajectories*, are both structurally and functionally supported by distinct TAM subsets (47). Given the experimental and literature evidence on the immunosuppressive properties of TAMs described above, here, we surmise that dissemination trajectories signify immunosuppressive niches, reminiscent of *immune deserts*. The term "immune dessert" is used here as an interchangeable term for collectively describing tumor microenvironments with immune excluded and immune desert phenotypes, as defined in multiple prior studies (147–150). It is hereby suggested that at least four distinct mechanisms may contribute to the function of dissemination trajectories as immune deserts in the primary tumor microenvironment: First, dissemination trajectories are sites of endothelial anergy; Second, they represent sites of lymphocyte exclusion; Third, they represent sites of metabolic reprograming, refractory to anti-tumor lymphocyte functions; Fourth, they constitute sites of lymphocyte exhaustion. Collectively, the aforementioned immunosuppressive mechanisms (**Figure 1**) significantly undermine the capacity of the tumor-infiltrating lymphocytes for targeting the disseminating cancer cell population, thus allowing for a narrow, but solid window of opportunity for the successful execution of the initial steps of the metastatic cascade.

(a) Dissemination Trajectories as Beacons of Endothelial Anergy

Lymphocyte migration needs to be precisely coordinated to contribute to effective T cell trafficking in both physiological and neoplastic contexts. This process can be summarized into selectin-dependent leukocyte rolling, chemokine-driven integrin activation, integrin-dependent leukocyte tethering in the vascular wall, and leukocyte diapedesis (8, 9, 151–153). It should be mentioned that this process is primarily mediated by lymphocyte-endothelial cell interactions, and as a consequence, the integrity and functionality of the endothelium in either a physiological or neoplastic context, could have a dramatic effect on T cell trafficking. Under the control of growth factors and abnormal contextual signals, the tumor (neo)vasculature often displays a high angiogenic potential coupled to irregular distribution, enlarged vessels, excessive branching morphology, microhemorrhaging, and disturbed blood flow, when compared to traditional blood vessel architecture and physiology (154–158). Another decisive factor contributing to tumor endothelium instability, and consequently to defective T cell trafficking into the tumor tissue, is the failure to support endothelial integrity and functions *via* adequate mural cell (e.g. pericyte) coverage (159–162). One could intuitively, but erroneously, assume that high endothelium instability/permeability should render immune cell trafficking much easier. However, circulating lymphocytes require specialized molecular signatures (e.g. selectin, integrin and chemokine profiles) in tissue endothelial barriers to help with their homing into tissues (163–167). These molecular signatures, which are magnanimously present in High Endothelial Venules (HEVs) of various lymphoid organs for example (168–171), are characteristically disrupted or absent in tumor endothelia, rendering them "leakier" and insensitive to pro-inflammatory signals (172). This phenotype, known as "endothelial anergy", is characterized by impaired adherence of effector T cells to the endothelial cells and their subsequent extravasation to the tumor microenvironment (9, 173).

Although macrophages provide essential trophic factors to facilitate generation and retention of pericytes in certain developmental contexts (174, 175), at least one study has previously indicated that TMEM doorways are devoid of $NG1^+$ pericyte coverage (128), signifying one potential signature of endothelial anergy at TMEM doorways. In yet other studies, it has been shown that M2-polarized TAMs may in contrast support macrophage-pericyte interactions in the tumor microenvironment, but such interactions lead to enhanced neovascularization and tumor progression (176–179), again flagging the immediate surroundings of M2-like TAMs as potential niches of endothelial anergy.

For a long time, it has been theorized that the tumor vasculature is under constant and simultaneous control of proangiogenic and antiangiogenic factors, with vascular endothelial growth factor-A (VEGFA) representing a well-known paradigm of angiogenesis inducers (155, 180–186). However, prior evidence also suggests that different blood vessel subtypes in the tumor microenvironment do not all respond homogeneously to anti-VEGF treatment (187), inferring the presence of contextual factors promoting heterogeneity in VEGFA expression and activity. Indeed, the increase of VEGF around TMEM doorways may be the cause of the overall heterogeneity of VEGF expression around blood vessels in tumors. Under the transcriptional control of the Ang2-Tie2 signaling axis, $TIE2^+$ TMEM macrophages can locally release large quantities of proangiogenic factors, most prominently VEGFA, which is critical for both eliciting an angiogenesis program and sustaining TMEM function and TMEM-mediated cancer cell dissemination (128, 188–190). Importantly, VEGFA regulates blood vessel wall permeability *via* a variety of mechanisms, for instance *via* increasing endothelial cell fenestration at lower concentrations, or *via* breaking down and dissolving the endothelial cell adherens and tight junctions at higher concentrations (191–197). The latter is especially critical in the process of metastasis because it provides an effective paracellular passageway for the disseminating cancer cell subpopulation into the blood circulation (128). Indeed, the conditional ablation of the VEGFA gene *via* targeted expression of Cre recombinase under the control of the macrophage-specific promoter that regulates transcription of the colony stimulated factor-1 receptor (CSF1R) in a mouse model of breast carcinoma results in successful assembly of TMEM-doorways, which are otherwise entirely incapable of breaking down endothelial junctions and facilitating cancer cell transendothelial migration and intravasation (128). Overall, these data suggest that TMEM

doorways within the dissemination trajectories reflect to TMEs with high VEGFA expression and activity, suggesting that they function as candidate beacons of endothelial anergy within the tumor microenvironment.

TMEM doorways are functionally regulated by M2-like TAMs, which represent a prominent source of angiogenic molecules in the perivascular niche (47, 51, 75, 189, 190, 198–201). Besides the well documented VEGFA, TAMs release a plethora of other proangiogenic factors, such as tumor necrosis factor-α, basic fibroblast growth factor, thymidine phosphorylase, urokinase-type plasminogen activator, adrenomedullin, and semaphorin-4D (47, 189, 190, 201–205). These proangiogenic factors are known to downregulate the expression of adhesion molecules (ICAMs, VCAMs, and selectins), which are actively involved in lymphocyte trafficking, thus resulting in endothelial anergy and lymphocyte tolerance (9, 172).

In summary (**Figure 3A**), TMEM doorways likely serve as proponent components of endothelial anergy in the tumor microenvironment, subduing intratumoral recruitment of $CD8^+$ T cells. Possible contributors of endothelial anergy at TMEM doorways are the reported defects in pericyte coverage, as well as the localized, high concentration of VEGFA and other proangiogenic molecules secreted by the TMEM macrophage. These mechanisms may together prompt a highly permeable vasculature at TMEM doorways, which is otherwise unable to support T cell trafficking due to the lack of characteristic molecular signatures for lymphocyte diapedesis.

(b) Dissemination Trajectories as Crossroads for Lymphocyte Exclusion

Among the critical mechanisms leading to inadequate T cell trafficking into solid tumors are those culminating in mismatching between bioavailable chemokines in the tumor microenvironment and chemokine receptors expressed on the surface of cytotoxic T cells (206). The disruption of the immunosuppressive chemokine/cytokine network either pharmacologically or *via* genetic manipulations in animal models can therefore reliably covert the tumor microenvironment into a receptive niche for T cell trafficking and further sensitize tumors to immunotherapy (207). The dissemination trajectories are functionally and contextually associated with distinct macrophage subtypes, which represent a prominent source of immunosuppressive cytokines and chemokines in the tumor microenvironment (47, 73–75, 206). As mentioned, perivascular TMEM doorway macrophages, express high levels of the tyrosine kinase receptor TIE2 (also known as CD202b), and the mannose receptor MRC1 (also known as CD206), suggesting that they are skewed towards an M2 (or M2-like) phenotype according to the traditional macrophage polarization spectrum (47, 119, 208, 209). In this perspective, we support the working model that M2-like macrophages within dissemination trajectories represent the major orchestrators of chemokine/chemokine receptor mismatching that leads to inadequate $CD8^+$ T cell trafficking (206).

Peripheral monocytes are usually recruited within tumors *via* the CCL2/CCR2 chemokine pathway and transdifferentiate into M2-like macrophages under the regulation of the CSF1/CSF1R pathway (210). It is now strongly documented that CSF1-dependent macrophage polarization into M2-like phenotype leads to the acquisition of an immunosuppressive macrophage subtype, characterized by T cell exclusion (145, 206, 211–213). Indeed, the depletion of tumor-associated macrophages *via* inhibiting either CSF1/CSF1R or CCL2/CCR2, are both capable of overcoming T cell exclusion within tumors (145, 214). There is sufficient evidence that M2-like macrophage functions are antagonistic to Th1 immunological responses, which would theoretically favor antitumoral immunity. Specifically, M2-like macrophages may suppress the interferon-gamma (IFN)-mediated responses that culminate in the induction of CXCL9 and CXCL10 chemokines, which, in turn, are able to attract $CXCR3^+CD8^+$ memory T cells (215). The critical association between CXCR3-binding ligands CXCL9/10 and $CD8^+$ T cell trafficking has been well documented (216–220). Although the dominance of M2-like macrophages within the dissemination trajectories can by itself account for the suppression of such favorable Th1 immunological responses, several macrophage-independent mechanisms of Th1 suppression have also been reported in this context. For example, certain tumors (e.g. ovarian carcinomas) can use epigenetic mechanisms to silence the expression of CXCL9 and CXCL10. Moreover, nitrosylation by reactive oxygen species (ROS) in the tumor microenvironment may result in altered proteolytic processing of CXCL11, another chemoattractant of CD8+ T cells (221), which incapacitates its binding-induced signaling (222).

It has been demonstrated that once homed in tumors under the control of CCL2/CCR2 and CSF1/CSF1R pathways, M2-like macrophages begin to also express the chemokine receptor CXCR4, possibly under the control of the pleiotropic cytokine TGFβ (210). The *de novo* expression of CXCR4 may force prometastatic macrophages into a unidirectional migration toward the perivascular niche where CXCL12, the chemokine ligand of CXCR4 is abundantly expressed, and where they eventually assemble TMEM doorways (210). Although many sources of TGFβ within the tumor microenvironment have been reported (223–225), human monocytes and macrophages can also activate TGFβ *via* the expression of integrin αvβ (226). TGFβ has been previously documented as among the strongest immunosuppressive cytokines, capable of excluding T cells from human and murine tumors (227, 228). These observations collectively suggest that TGFβ expression within dissemination trajectories represent a critical mechanism of lymphocyte exclusion as a result of cytokine/cytokine receptor mismatching.

As mentioned, $CXCR4^+$ macrophages within dissemination trajectories can chemotactically respond to the presence of the CXCL12 ligand at the perivascular niche (210). Prior evidence suggests that mesenchymal stromal cells, such as cancer-associated fibroblasts (CAFs) and possibly mural cells coating the blood vasculature, serve as the primary source of CXCL12 production and secretion (229–234). In a recent model of cancer cell dissemination, the chemotactic migration of $CXCR4^+$ macrophages with their partnering $MENA^{INV+}$ tumor cells, has been rendered as the possible driving force for the observed streaming migratory behavior within dissemination

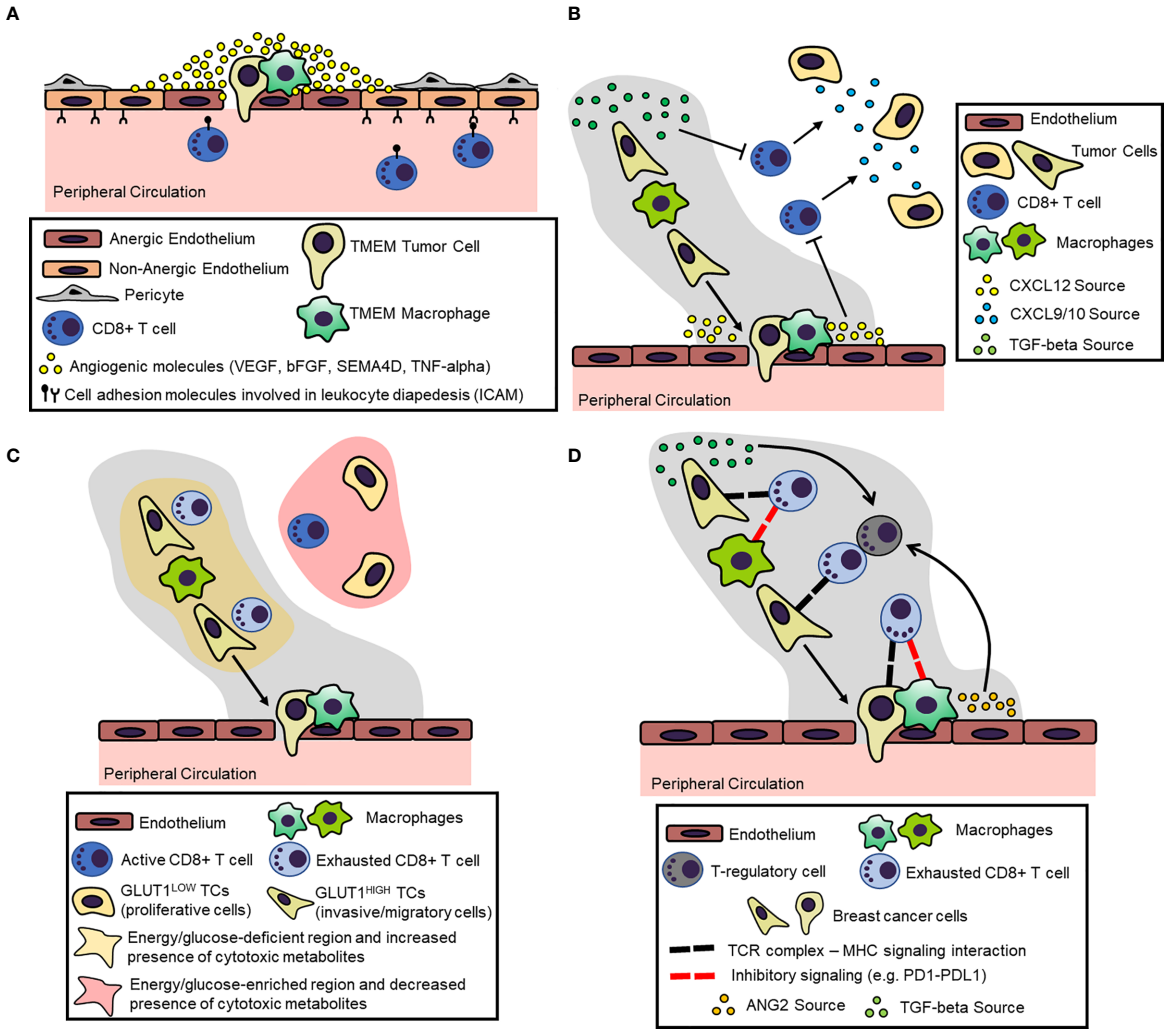


FIGURE 3 | Proposed Mechanisms for the Induction and Maintenance of an Immunosuppressive Microenvironment within the Dissemination Trajectory. **(A)** Dissemination trajectories as beacons of endothelial anergy. Perivascular (TMEM doorway) macrophages secrete a number of proangiogenic factors (e.g. VEGFA) in the peri-TMEM area, which downregulate cell adhesion molecules in endothelial cells critical for lymphocyte diapedesis, thus resulting in "locally" anergic endothelium. **(B)** Dissemination trajectories as crossroads for T cell exclusion. Cytokine/cytokine receptor mismatching mechanisms within the dissemination trajectories result in the exclusion of T cells. For example, prometastatic macrophages suppress the expression CXCL9/10 within the dissemination trajectories, which function as the primary chemoattractants for T cells. Instead, dissemination trajectories are characterized by the expression of other cytokines/chemokines, like TGF-beta and CXCL12, which act as repellents for T cells. **(C)** Dissemination trajectories as primers for metabolic burdening of T cells. Highly migratory tumor cells within the dissemination trajectories tend to upregulate glucose transporters (e.g., GLUT1), which on one hand reduces the bioavailable energy resources (i.e., glucose), while on the other hand, may produce metabolites. This metabolic landscape is burdensome for immune cells, resulting in T cell exclusion and exhaustion. **(D)** Dissemination trajectories as checkpoints for T cell exhaustion. Chronic TCR signaling within the dissemination trajectory along with overexpression of inhibitory ligands (e.g., PDL1) by the prometastatic macrophages may result in T-regulatory (Treg) cell expansion and CD8+ T cell inactivation/exhaustion.

trajectories (210). Moreover, a concrete body of evidence supports that intratumoral distribution of CXCL12 inversely correlates with the presence of T cells (235), although it is not yet clear whether the CXCL12/CXCR4 pathway can directly suppress T cell trafficking into CXCL12-enriched microenvironments (230, 236). Indeed, pharmacological inhibition of the CXCL12/CXCR4 pathway alleviates the tumor microenvironment from the lymphocyte exclusion phenotype (236). On one hand, CXCL12 appears to be a critical chemokine for cancer cell dissemination (231, 233, 237, 238); still, it may comprise a major chemokine/chemokine receptor mismatching mechanism for the trafficking of T cells into dissemination trajectories.

In summary (**Figure 3B**), the immunosuppressive M2-like macrophages may orchestrate the expansion of a cytokine/chemokine network, which excludes T cells from the dissemination trajectories. Foremost, M2-like macrophages seem to directly suppress the expression of the CXCR3-binding ligands CXCL9 and CXCL10, which are the primary chemokine

attractants for $CD8^+$ T cells. Moreover, the reciprocal interactions among disparate cells within dissemination trajectories seem to be highly dependent on the induction and contextual expression of several cytokines and chemokines, including but not limited to CXCL12 and TGFβ, which may disrupt lymphocyte trafficking and exclude $CD8^+$ T cells from the landscape.

(c) Dissemination Trajectories as Primers for Metabolic Burdening of Lymphocytes

In general, sugars, amino acids, and fatty acids are the major fuel sources utilized by eukaryotic cells, but rapidly proliferating tumor cells tend to exhaust them, thus subjecting both tumor and immune cells to nutrient-deficient microenvironments and imposing considerable bioenergetic constraints on their functions (76, 151, 239–242). Cancer cells tend to upregulate the expression of glucose transporters, such as GLUT1 (243, 244), amino acid transporters, such as ASCT2 and LAT1 (245–247), and fatty acid elongation enzymes, such as FAS (248–250), to facilitate their adaptation to energy-deficient microenvironments. This metabolic reprogramming does not only limit nutrient availability for cytotoxic $CD8^+$ T cells, but may also generate metabolic byproducts that may overwhelm T cell function, survival, and expansion (151). In this chapter, we briefly explore certain mechanisms, *via* which the metabolic landscape within dissemination trajectories may interfere with lymphocyte trafficking and function.

Foremost, the metabolic machinery of $MENA^{INV+}$ tumor cells within dissemination trajectories remains poorly understood. However, it is generally known that fatty acids are primarily required by rapidly dividing tumor cells to form new plasma membrane lipid bilayers, thus explaining why most tumors overexpress FAS and malignant transformation depends on lipogenesis (251). However, neither migratory tumor cells nor effector T cells seem to heavily depend on fatty acid oxidation, although the development of antitumor memory T cells is affected (252–254), suggesting that such pathways may not be as immunocompromising within the dissemination trajectories. On the other hand, there are certain lines of evidence suggesting that the highly migratory/invasive cells that have undergone EMT tend to express high levels of the glucose transporter GLUT1, which partially supports high energy demands for the active process of invasion and migration (255, 256). Accordingly, it has been shown that proteolytic modifications of the extracellular matrix by highly migratory cells *per se* can also promote GLUT1 expression and aerobic glycolysis (257). Concomitantly, GLUT1 overexpression has been associated with low T cell trafficking in renal cell and squamous cell carcinomas (258, 259), suggesting that dissemination trajectories could potentially limit both T cell trafficking and their functional capacity in a GLUT1-dependent manner.

The metabolic landscape within dissemination trajectories may also impair T cell functions through generation of immunosuppressive metabolites and byproducts, not only *via* the direct competition for energy resource availability. For example, indoleamine 2,3-dioxygenase (IDO), an enzyme that converts tryptophan into kyunerines (260), is a well-established suppressor of $CD8^+$ T cell infiltration into tumors and most of the associated antitumor T cell responses (132, 261). In addition, diminished tryptophan deposits in IDO^{High} tumor microenvironments can prevent T cell proliferation, while kyunerines can promote T cell death and interference with TCR signaling (132, 261, 262). Despite that dendritic cells have been identified as major inducers of IDO within the immune microenvironment (261), TAMs can also participate in IDO-mediated tryptophan metabolism under certain contexts (263–265), suggesting that dissemination trajectories may be characterized by the accumulation of immunosuppressive metabolites.

In summary (**Figure 3C**), dissemination trajectories are associated with a metabolic landscape that results in diminished T cell trafficking into tumors and associated antitumor T cell functions. On one side, highly migratory tumor cells within the dissemination trajectories may successfully outcompete TILs for the scant availability of energy resources, such as glucose, because they tend to upregulate corresponding transporters (e.g., GLUT1). On the other hand, TAMs within the dissemination trajectories may be engaged in metabolic pathways that not only deplete essential elements (e.g., tryptophan), but also produce immunosuppressive metabolites along the process (e.g., IDO-induced kyunerines).

(d) Dissemination Trajectories as Checkpoints for Lymphocyte Exhaustion

In recent years, it has been suggested that effector T cells ($CD4^+$ and $CD8^+$), which infiltrate tumors tend to exhibit impaired functional and proliferating capacity, characterized by progressive loss of their ability to produce their characteristic effector cytokines (i.e., TNF-α, IFN-γ, IL-2) and lyse tumor cells, a state described as *lymphocyte exhaustion* (45, 151, 207, 266–269). The existence of this particular phenotype is further corroborated through experimental evidence showing that certain cancer immunotherapies, such as those that specifically target immune checkpoint pathways, may alleviate T cell exhaustion, and restore the ability to eradicate cancer cells (270). In this section, we propose that dissemination trajectories rich in M2-like immunosuppressive macrophages can yield a contextual milieu that promotes T cell exhaustion, potentially accounting for the lack of treatment response seen in many patients following checkpoint therapies.

Similar to the case of chronic viral infections, the most prominent hallmark of T cell exhaustion in the tumor microenvironment is the co-expression of a wide range of immune checkpoint receptors by the T cells (271, 272). These inhibitory receptors primarily include programmed cell death protein 1 (PD1), lymphocyte activation gene 3 protein (LAG3), T-cell immunoglobulin domain and mucin domain protein 3 (TIM3), cytotoxic T lymphocyte antigen-4 (CTLA4), band T lymphocyte attenuator (BTLA) and T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) (273). Although it is beyond the scope of the current perspective to delineate the detailed biology of these immune checkpoint pathways, it should be mentioned that intracellular

signaling *via* these receptors in T cells can generally lead to functional deficiencies characteristic of the lymphocyte exhaustion phenotype (274–279). However, in a certain context, $PD1^{+}TIM3^{+}$ tumor-infiltrating T cells were functional despite the co-expression of both immune checkpoint receptors, suggesting that certain competitive intracellular pathways to unruly T cell exhaustion may also exist (280). It has been generally known that TAMs are prominent inducers of T cell exhaustion in the tumor microenvironment through interference with immune checkpoint control. For example, TAMs from renal cell carcinoma patients induce the skewing of autologous blood derived $CD4^{+}$ T cells towards an exhausted phenotype, with decreased production of effector cytokines and enhanced expression of PD1 and TIM3 (281). Of all immune checkpoint pathways mentioned above, the prominent expression of PDL1, a ligand for PD1, and B7-H4, a ligand for CTLA4, are perhaps the most well-known immunosuppressive mechanisms leading to macrophage-driven T cell exhaustion (282–286). Of particular interest is the fact that ligands for immune checkpoint receptors are mostly expressed by M2-like macrophages, which are also integral components of TMEM doorways, providing another attractive theory for immune evasion by the migratory/invasive cancer cell subpopulation within the dissemination trajectories.

Prior research has suggested that chronic T cell receptor (TCR) signaling in functional T cells can normally lead to elevated expression of inhibitory receptors, such as PD1, TIGIT and CTLA4 (271, 287). This observation further postulates that increased expression of these inhibitory receptors in TILs may accordingly be the result of chronic exposure to neoantigens and/or persisting tumor antigens (151, 268). However, the expression of inhibitory receptors in TILs is markedly higher compared to those in functional T cell states, suggesting that other factors, possibly microenvironmental ones, may be responsible for increased immune checkpoint control and lymphocyte exhaustion (151). In accordance with these observations, prior experimental evidence has demonstrated that certain cytokines, often expressed in the tumor microenvironment (tumor cells, cancer-associated fibroblasts, immune cells, adipocytes), such as angiopoietin-2 (ANG2), interleukin-10 (IL10), and transforming growth factor-β (TGFβ), are sufficient for T cell exhaustion and suppression of anticancer immunity (288–291). Although, this cytokine network leads to lymphocyte exhaustion through a variety of mechanistic pathways, both direct and indirect *via* the expansion of $CD4^{+}CD25^{high}FOXP3^{+}$ T-regulatory (Treg) cells, have been suggested (270, 272, 273, 292). Certain of these cytokines, especially TGFβ, have been discussed in prior chapters with regards to their functional relevance within dissemination trajectories. Others, like ANG2, are also critical for cancer cell dissemination, as ANG2-dependent activation of TIE2 receptor in the TMEM macrophage leads to the localized production and secretion of VEGF, which in turn, is critical for TMEM-associated vascular opening and the transendothelial migration of $MENA^{INV+}$ tumor cells (75, 98). Therefore, it seems that dissemination trajectories are enriched in cytokines that not only promote lymphocyte exclusion, but also lymphocyte exhaustion.

In summary (**Figure 3D**), T cells that are not excluded from and manage to eventually infiltrate dissemination trajectories have acquired an "exhausted" phenotype rendering them unable to produce effector cytokines and successfully target tumor cells. This phenotype is regulated by an abnormally high expression of immune checkpoint receptors, such as PD1, CTLA4 and TIM3, at their surface. Among other cells, M2-like immunosuppressive TAMs within dissemination trajectories express a spectrum of corresponding ligands for these inhibitory receptors, thus offering immunosuppressive "sanctuaries" around the exhausted $CD8^{+}$ T cells. Furthermore, the cytokine network within the dissemination trajectory, including primarily TGFβ and ANG2, among other factors, serves as a critical driver of Treg expansion and inhibitory receptor overexpression, thus maintaining and perpetuating the dysfunctional T cell states.

CONCLUSIONS AND FUTURE PERSPECTIVES

In recent years, the molecular/cellular investigation of the immune tumor microenvironment and the comprehensive studying of the immunosuppressive mechanisms harbored therein have been at the frontier of cancer research, as an attempt to improve the already promising landscape of cancer immunotherapy (1, 86, 132, 135, 151). In this regard, we offer a fresh perspective on the topic by distinguishing disparate sets of immunosuppressive mechanisms in different tumor microenvironments. In particular, here we focused on analyzing multiple layers of immunosuppression, which involve mechanisms preventing T cell trafficking and mechanisms promoting T cell exhaustion within the specialized microenvironments dedicated to cancer cell dissemination (i.e., dissemination trajectories). This unique distinction serves a dual purpose: First, it offers an attractive explanation on why most immunotherapies do not target the migratory/invasive tumor cell subpopulation but instead are primarily restricted in promoting antitumor immunity within the more proliferative - less migratory tumor compartment. Second, it provides a rational framework on thinking the diverse immunosuppressive mechanisms as a multilayered obstacle against antitumor immunity, clearly suggesting that we should focus on targeting the immunosuppressive "network" rather than a "pathway" to be able to either restore the natural or orchestrate an engineered antitumor immunity. This perspective certainly does not aim at understating the importance of studying mechanisms of T cell exclusion and exhaustion in the proliferative and/or the cancer stem cell niches of the tumor microenvironment, given that targeting these microenvironments is also critical for establishing efficient anticancer immunity. However, this perspective aims at drawing significant attention to the frequently neglected concept of cancer cell dissemination, which may lead to a significant burden of dormant tumor cells in the distant metastatic sites, which may eventually grow into overt once they have found a way to avoid immunological detection and

acquired resistance to immunotherapy or other therapeutic modalities (293). Therefore, the rational targeting of immunosuppressive mechanisms within the dissemination trajectories would serve as a promising *antimetastatic therapy*, given that its purpose would be to improve T cell trafficking and to alleviate T cell exhaustion, thus rallying an immunological attack against the migratory/invasive cancer cell population while in the act of departure from the primary tumor.

In pursuit of understanding the escape of migratory/invasive ($MENA^{INV+}$) cells from antitumor immunity, here, we propose a unified model with at least four distinct layers of immunosuppression. Foremost, we propose that endothelial anergy and cytokine/cytokine receptor mismatching mechanisms do not allow for robust T cell trafficking within dissemination trajectories, and, in case that these mechanisms are somehow breached, alternative mechanisms promoting T cell exhaustion from either metabolic burdening or immune checkpoint control may become dominant (**Figure 1**). It should be noted that all these individual mechanisms are strictly context-dependent and may occur simultaneously within dissemination trajectories, not in tandem. As a consequence, therapeutic targeting of these mechanisms for purposes of improving cancer chemotherapy and/or immunotherapy should consider all the aforementioned categories of immunosuppression, because counteracting a single one would likely be inadequate. Fortunately, therapeutic strategies that target each individual immunosuppressive layer in our model (**Figure 1**) are in development. Therefore, the greatest challenge for the next decade will fall back to eliciting the most appropriate combinations to successfully cripple the immunosuppressive niche within the tumor microenvironment, including within the dissemination trajectory. For example, prior reported antiangiogenic approaches aimed at promoting blood vessel normalization were shown to concurrently disrupt endothelial anergy, resulting in (re)sensitizing tumor blood vessels to lymphocyte diapedesis and improved T cell trafficking (294). Furthermore, immune checkpoint receptor/ligand blockade (primarily of CTLA4, PD1, and PDL1) with monoclonal antibodies has emerged as a successful therapy against intratumoral T cell exhaustion in human patients (1, 69, 291, 295, 296). Combining such antiangiogenic therapies with immune checkpoint blockade could represent the most attractive strategy to counteract immunosuppression and render cancer immunotherapy more successful (297).

Most conclusions regarding the immunosuppressive cues described in this review article have risen from literature evidence on the immunosuppressive properties of M2-like macrophages in general. The most critical aspect of the working model of spatial immunosuppression (**Figure 3**) is the contextual positioning of M2-like TAMs within the dissemination trajectories, either those represent "streaming" or "TMEM-doorway" macrophages. Therefore, the immunological properties of all the distinct tumor compartments are attributed to the topographical enrichment of M2-like macrophages within the dissemination trajectories rather than to unique or specific M2-like macrophage phenotypes.

As mentioned earlier, TMEM doorways are also formed in diverse metastatic sites, such as in the lungs and lymph nodes, and such *de novo* dissemination machineries may participate in the re-dissemination of cancer cells to tertiary sites, accelerating metastatic burden (97, 99). Indeed, analysis of TMEM doorways in secondary/metastatic sites suggests that their ensuing biology can mimic to great extent the biological programming of cancer cell dissemination observed in primary tumors (97, 99). It would therefore be interesting to investigate in the future if identical or similar immunosuppressive cues are recapitulated in the metastatic microenvironments that assemble "re-dissemination machineries".

The deeper we delve into the complex circuitries involving immune cells and their associated cytokine/chemokine signatures in the tumor microenvironment, the necessity for more sophisticated technologies to study the processes they are involved with, will constantly emerge. Indeed, conclusions from many studies included in this perspective would be impossible to be drawn in the absence of high-throughput technologies for multiplex imaging and/or single cell expression profiling. In addition, high-resolution imaging (e.g., intravital fluorescence microscopy and planar bioluminescence imaging) has yielded important spatiotemporal data at single cell resolution, furthering our understanding on the immunological pathways supporting the active process of cancer cell dissemination (298–300). To complement the aforementioned efforts, such emerging technologies will additionally provide feasible tools for analyzing mutation antigen profiles, gene signatures and epigenetic modifications of both tumor and immune cells, the breadth of antibody responses, as well as the magnitude, homing capacity, cytotoxic function, and T cell receptor (TCR) repertoires of tumor-infiltrating lymphocytes. Overall, we anticipate that new technologies in this intriguing field of research will bring us a step closer to achieving personalized medicine and more promising immunotherapies.

In brief, here we describe an alternative perspective that tumor microenvironments dedicated to cancer cell dissemination may elicit strong immunosuppressive cues that prevent T cell trafficking and promote T cell exhaustion, processes that undeniably facilitate the initial steps of the metastatic cascade. Interestingly, these mechanisms are primarily orchestrated by certain well-recognized subsets of tumor-promoting TAMs (e.g., $TIE2^{+}$ TAMs), and their corresponding cytokine/chemokine network deployed around the cancer cell dissemination machinery. This working model of compartmentalized "immunosubversion" provides the groundwork for future studies on alleviating the immunosuppressive milieu for more optimal cancer immunotherapies.

AUTHOR CONTRIBUTIONS

SA, GKi, PF, DE, DM, MO, and GKa: wrote the manuscript. LS and GKa: produced data and designed **Figure 2**. GKa: designed illustrations for **Figures 1** and **3**. All authors contributed to the article and approved the submitted version.

FUNDING

This article is supported by the following resources: NIH K99 CA237851; T32 CA200561; DOH01-ROWLEY-2019-00037; S10 OD019961 for the use of the Perkin Elmer Scanner; Gruss-Lipper Biophotonics Center and its Integrated Imaging Program at the Albert Einstein College of Medicine (AECOM); Jane A. and Myles P. Dempsey.

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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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frontiers in Oncology

REVIEW
published: 22 April 2021
doi: 10.3389/fonc.2021.653625

The Dynamic Entropy of Tumor Immune Infiltrates: The Impact of Recirculation, Antigen-Specific Interactions, and Retention on T Cells in Tumors

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Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: 14 January 2021
Accepted: 06 April 2021
Published: 22 April 2021

Citation:
Blair TC, Alice AF, Zebertavage L, Crittenden MR and Gough MJ (2021) The Dynamic Entropy of Tumor Immune Infiltrates: The Impact of Recirculation, Antigen-Specific Interactions, and Retention on T Cells in Tumors. Front. Oncol. 11:653625. doi: 10.3389/fonc.2021.653625

Analysis of tumor infiltration using conventional methods reveals a snapshot view of lymphocyte interactions with the tumor environment. However, lymphocytes have the unique capacity for continued recirculation, exploring varied tissues for the presence of cognate antigens according to inflammatory triggers and chemokine gradients. We discuss the role of the inflammatory and cellular makeup of the tumor environment, as well as antigen expressed by cancer cells or cross-presented by stromal antigen presenting cells, on recirculation kinetics of T cells. We aim to discuss how current cancer therapies may manipulate lymphocyte recirculation versus retention to impact lymphocyte exclusion in the tumor.

Keywords: T cell, recirculation, tumor, clonality analysis, retention, recruitment

INTRODUCTION

The evolutionary development of an adaptive immune system requires different systems to localize responses. An adaptive immune response against a specific virus can result in viral antigen-specific B cells and T cells that remain throughout life (1). The progressive accumulation of such responses over the lifetime of an individual would make it impractical to have all of the different types of antigen-specific cells located at all possible sites of infection. Rather than distribute all immune cells uniformly, the mammalian immune system uses a system of sensors and rapid responses to mobilize responses to a location. Compared to a fully distributed immune system this will result in a delay, but allows a more flexible system.

To achieve this the mammalian immune system employs selective sensors, which provide an initial indication of the type of immune response required, to mobilize suitable cells. For example, intracellular sensors for nucleic acids that might detect a viral infection, such as TLR3, RIG-I-like receptors and cGAS/STING can generate a different pattern of cytokine responses to extracellular sensors for bacterial components such as TLR2 and TLR4 (2, 3). Moreover, the various sensors are not uniformly shared across innate immune cell types, so that specific immune cells can specialize in

detecting certain infections (3, 4). In this way the immune response is pre-warned of the nature of the infection, and the appropriate adaptive response that is needed.

Among the cells that participate in the adaptive immune response, T cells have a particular limitation in that they must be in physical proximity to a target cell expressing antigens in order to exert their effect. B cells can retreat to stable niches in the bone marrow and secrete antibody for the duration of the animal's lifespan (1). This isn't possible for T cells. To overcome this limitation, the immune system exploits a mechanism to move antigens to T cells, and T cells to antigen (5). Naïve T cells do not travel to tissue sites and so cannot scan for their cognate antigen at the site of infection. Instead, dendritic cells are able to carry antigen from tissues to draining lymph nodes where they are able to present the antigen to naïve T cells and initiate antigen-specific immune responses. Mathematical modeling suggests direct lymph node entry of T cells is low in the absence of inflammation (6). For naïve T cells, entry is classically dictated by CCR7, which also directs T cells to the vicinity of dendritic cells within the T cell zone of the lymph node (7). However, inflammation in the upstream site can lead to remodeling of the lymph node to increase infiltration of naïve T cells and recruitment of all T cells to the node (7, 8). Once in the lymph node, T cells that continue to receive chemokine signals, or are held in place *via* cognate interaction and retention signals such as CD69, will remain in the lymph node. However, there is an ongoing pull *via* S1PR1 on the T cells and S1P in the lymphatics that results in exit of T cells that fail to meet their cognate ligand or have disengaged from antigen presenting cells (9). This ongoing pressure to leave ensures continued recirculation of T cells in search of cognate ligands.

Once they are antigen experienced, T cells are subsequently able to travel through the blood to tissues due to a range of changes including altered selectin expression (7) and explore local MHC for their cognate antigens. Importantly, a recirculation system exists to return these T cells through the draining lymphatics and back into blood circulation (5, 7). Without such a system of recirculation, antigen-experienced cells would be 'lost' to the tissues resulting in a progressive loss of antigen-experienced cells from the circulation. This is the critical feature that provides our circulating, distributed form of adaptive immune system. While the principle of recirculation is well known and a fundamental of basic immunology, what is often unappreciated is the rate of recirculation. In studies performed over 50 years ago it was demonstrated that the total blood pool of lymphocytes can be refreshed 11 times per day based purely on the output from the thoracic duct (10). The drug FTY720, which prevents lymphocyte egress from lymph nodes by blocking Sphingosine-1-phosphate receptor 1 (S1PR1), can result in complete loss of thoracic duct lymphocytes within 4 hours (11) and 90% loss of peripheral blood lymphocytes in 3-24 hours (12), demonstrating an extremely high recirculation rate. While FTY720 treatment rapidly removes lymphocytes from the blood it has a lesser effect on the tissues, and these data have allowed investigators to calculate transit times of approximately 18 hours through tissue parenchyma (13). Thus, T cells spend their least amount of time in the peripheral blood, with estimates ranging from 1-2 circulations through the heart, which corresponds to timescales of minutes in the bloodstream of mice (6, 14). By contrast lymph node transit even in the absence of cognate antigen can take approximately 10 hours (6, 15). In models of long-term memory to viral infection, it was found that less than 4% of the virus-specific T cell memory population was present in the peripheral blood at any one time (16). Thus, T cells spend the majority of their time in tissues or secondary lymphoid organs scanning for cognate antigens.

As with all rules, there are exceptions. Recently, resident memory T cells (Trm) have been described that remain in peripheral sites long term and provide rapid local antigen-specific responses (7, 16, 17). The majority of experiments that identified these cells were performed in very clean laboratory settings where the mice had an extremely limited history of infection. This makes it difficult to assess how diverse the Trm pool is in peripheral tissues, since there will clearly be a 'space' constraint in supporting a fully diverse T cell population at all peripheral sites. Human neonates have increased populations of naïve T cells compared to adult humans, and antigen-experienced populations are less frequent in neonatal tissue where there is likely a limited experience of antigen (18, 19). Therefore, with antigenic experience, peripheral niches are populated with antigen-experienced cells. By analyzing wild and pet-shop mice, Beura et al. demonstrated a dramatically higher population of memory T cells populating peripheral tissues of wild mice compared to laboratory mice (20). In normal human pancreas tissue, Trm were found to express similar defining markers such as CD69 and CD103, but were phenotypically distinct from jejunal Trm (21). This included decreased expression of a range of inflammatory markers (21), which may relate to the lower ongoing exposure to infectious agents in the pancreas. As would be expected given the potential for differing local antigen exposure, Trm clones in the pancreas and jejunum also had limited overlap (21). In agreement with this, Trm in normal lung but not other sites demonstrated reactivity to influenza antigens (22); however, influenza-specific Trm in the lung share clonotypes with non-Trm memory subtypes in the lung (23). These data demonstrate that while Trm provide local recognition, their function is reinforced by recirculating populations. Difficulties in comparing clonotypes is highlighted by Schoettler et al, who demonstrated using lung samples that only approximately 5% of more than 100,000 TCR clones were found in more than one tissue or patient sample and only TCRs expressed by CD4 T cells were identified as shared across multiple memory populations in both the lung and lung-draining lymph nodes (24). It remains unclear quite how comprehensively protective the Trm cell response can be given the limited size of the Trm niche in any one place, compared to the diversity of the combined repertoire that is recirculating or resident elsewhere at any moment. It is possible that the Trm niche reflects recent antigen exposures, and pre-existing cells are displaced to new recruits. In this way the peripheral resident population will proportionally represent frequent infectious agents, including non-pathogenic

colonizing organisms and this resident response will therefore be of most use for rapid responses to these most frequently exposed agents. This is supported by data in lung infection, where repeated antigen exposure ensures a durable lung Trm population (25). In this, way, earlier exposures to a common organism may come to dominate the local response. By monitoring the response to viral infections containing model antigens, Muschaweckh et al. demonstrated a selective preference for T cells specific for the model antigen Ovalbumin to form Trm as compared to T cells specific for the viral antigen $B8R_{20}$ (26). However, if the tissue was first allowed to form Trm specific for the viral antigen $B8R_{20}$ through prior infection, then $B8R_{20}$ -specific T cells dominated the Trm niche even after challenge with a $B8R_{20}$ and Ova-expressing virus (26). These data suggest that Trm formation is affected by immunodominance (discussed more later), and that existing Trm populations can outcompete simultaneous incoming new responses to retain their place in the tissue niche. Importantly, T cells specific for the same antigen can be found as Trm and as classical circulating memory populations (27), so even if Trm that recognize a specific infection are lost from the tissue niche through progressive rounds of infection with other agents, the circulating memory can remain.

The principle of recirculation is also essential to overcome the fact that T cell recruitment to tissue sites is not antigen specific. Recruitment to peripheral sites is dependent on inflammatory patterns. Inflammation in the tissue site generates cytokines such as TNFa, which activates endothelial cells to express adhesion molecules such as ICAM1 [reviewed in (28, 29)]. This is critical to initiate the process of tissue entry by lymphocytes by allowing rolling along the endothelial surface of the blood vessel lumen. In addition, local inflammation results in chemokine secretion, and chemokine binding to receptors on rolling cells permits changes in adhesion to tight binding, and eventually diapedesis through the endothelia and into the tissue (28). This means that an infection that results in a local inflammatory response triggered *via* infection sensors will non-specifically recruit any T cells expressing selectins and appropriate chemokine receptors, regardless of TCR specificity. Since the recruited T cells need both the correct chemokine receptors and activation-regulated adhesion molecules to permit diapedesis through the vasculature into the tissue, there will be selection for activated T cells (30). In animal models, there is likely only one major ongoing infection at any one time, so the majority of the emerging activated cells are likely specific for the infection (20). However, in a human there are likely multiple ongoing infections simultaneously occurring at different sites, therefore T cells specific for an ongoing flu infection may also be recruited to the site of an infected splinter, and vice versa. Recirculation permits non-specific cells to leave the tissue and be available for recruitment again. This can also result in dominance of a highly inflamed tissue. For example, a lung infection can recruit T cells specific for other pathogens to the lung as part of the local inflammatory response (31). Similarly, in tumors, T cell recruitment is not antigen-directed but instead attracts all T cells with appropriate activation markers (32). To return these cells to the general circulation, and ensure they are available to respond to their cognate targets should they return, requires efficient recirculation.

Using tools such as Kaede mice, where cells can be photoconverted at a specific site then followed for their movement (33), it is clear that not all T cells are equivalent in their recirculation dynamics. By labeling the tumor then analyzing the tumor draining lymph node, within 1 day of tumor labeling the majority of emigrated cells are dendritic cells and T cells (34). Lymph nodes draining tumors exhibited a much higher overall number of recirculating cells than normal skin (34), suggesting a high rate of immunosurveillance in tumors, despite tumor progression. Though the number of T cells in the lymph node that had been in the tumor at conversion peaked at 1 day following conversion, tumor-originating cells were still in the lymph node at day 3 (34). It is not clear whether this is continued emigration or retention of these cells in the lymph node, but the tumor was still observed to hold a large proportion of the converted T cells at day 3 after conversion (34). As would be expected based on the requirements for initial tumor infiltration, the majority of cells recirculating to the draining lymph node are enriched for effector and central memory phenotypes. Using the Kaede system to convert skin resident T cells in infectious models, Park et al. demonstrated that Trm in the skin remain in place following viral rechallenge, and do not recirculate *via* the draining lymph node (35). However, circulating virus-specific T cells are recruited to the skin site, and themselves become Trm following infection (35). In tumor models, by day 3 following conversion of Kaede cells in the tumor, some of the converted T cells are detectable in the lymph nodes draining an identical tumor at a distant site but the converted cells are poorly detectable in distant lymph nodes that do not drain tumors (34). These data suggest that by this time point dissemination through the peripheral blood has occurred and antigen-mediated retention has allowed accumulation of tumor-specific populations in distant lymph nodes. Thus, even following exit from tumors, recirculation and accumulation at sites of distant antigen are rapid *in vivo*.

In this review we will discuss how the principle of T cell recirculation impacts lymphocyte exclusion in the tumor environment. In addition, we will explore the effect of therapy on lymphocyte numbers in the tumor, with a focus on the differing effects on recruitment versus retention. In response to cognate antigens T cells can also proliferate locally, which will also result in T cell accumulation at the tumor. In our review we will not discriminate the mechanisms that result in lymphocyte arrest versus proliferation in response to cognate antigen. Together these are grouped as retention mechanisms, rather than recruitment mechanisms. Different treatments may differently affect recruitment versus retention of lymphocytes, and this may play a role in their successes and failures.

Lymphocyte Recirculation Kinetics

Most assessments of tumor infiltration view only a single snapshot in time within the tumor. Although such assessments may show a high degree of tumor infiltration by T cells, it may also represent a tumor with a high throughput of T cells entering

and leaving the tumor, so actually representing a high rate of surveillance (**Figure 1A**). If we consider the lymphocytes in a tumor over time, we can anticipate that some continue to recirculate, while others remain (**Figure 1B**). Assuming constant inflammatory conditions there is little reason for the overall number of cells to change over time, but when comparing the specificity of the T cells present at any two timepoints we could expect completely different T cells are infiltrating the tumor. With inflammatory flux, the numbers may go up or down as recruitment changes, but their overall time spent in the tumor may be unaffected if their retention is unchanged. Tools such as TCRSeq allow us to sequence the TCR of T cells and examine their diversity (36, 37). Expansions of specific T cells as clonal populations are detectable as repeated TCR sequences with increased frequency and using this technique to examine tumors shows that there are measurable clonal expansions in tumors. If we were to use TCRSeq to compare TCR clones in the tumor over time, we would anticipate an overall change in TCR clonotypes according to the degree of recirculation. Among the population that is retained over time, we would anticipate enrichment for properties of resident cells, such as Trm, or ongoing antigen engagement that results in prolonged adhesive interactions with their targets that impacts retention. This is supported by data from patient tumors, demonstrating that tumor reactive CD8 T cells in tumors express markers associated with tissue residency (38, 39). Multiple additional markers that may define the Trm phenotype have been described (40); however, experimental limitations means that residency has only been proven in murine systems. Nevertheless, ongoing studies in human tissues have identified shared features of cells expressing the canonical CD103/CD69 signature in humans (40). Bystander viral-specific CD8 T cells in tumors can also express these Trm markers (39), therefore CD39, a marker of chronic T cell activation is useful in distinguishing between the bystander and tumor-reactive T cell populations (38, 39). Duhen et al. used the combination of CD39 and the Trm marker CD103 to enrich for tumor-reactive CD8 T cells, and compared these cells in the tumor to those from the blood and lymph nodes (38). The $CD103^{+}CD39^{+}$ (double positive – DP) CD8 T cells in the tumor were shown to have clear enrichment for specific clonotypes. Duhen et al. demonstrated that the greatest TCR diversity was found in CD8 T cells in the blood, and the lowest TCR diversity

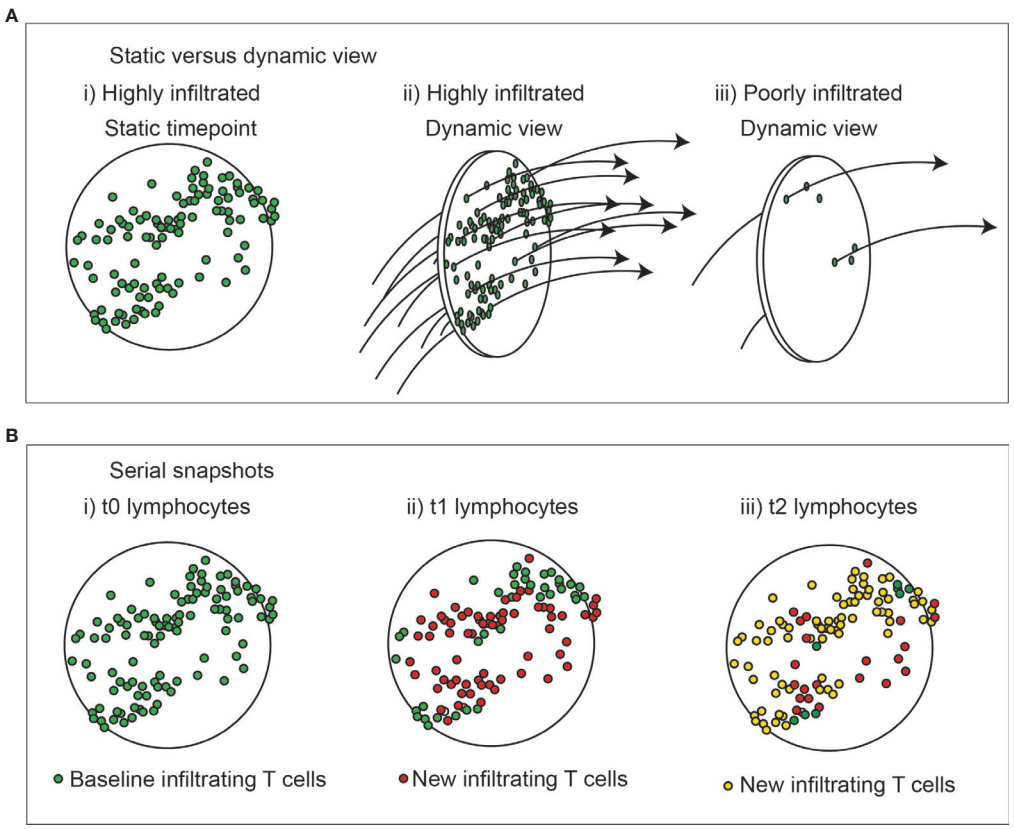


FIGURE 1 | Dynamic view of cell infiltration in tumors. **(A)** A highly infiltrated tumor may also represent a high rate of throughput of immune cells. i) a static view shows large numbers of T cells in the tumor. ii) a dynamic view shows a high rate of surveillance and recirculation. Iii) a dynamic view of a poorly infiltrated tumor shows a low rate of surveillance and recirculation. Understanding the kinetic will help understand the rate of accrual versus accumulation in the environment. **(B)** Change in lymphocytes in tumors over time with unchanged overall infiltration. i) Lymphocytes present at baseline are green. ii) Those newly present at the second timepoint are red, and iii) newly present at the third timepoint are yellow. At each timepoint not all cells are replaced, and those exhibiting prolonged interactions in the tumor are more likely to have engaged their cognate antigen.

was found in the DP CD8 T cells in the tumor (38). DP CD8 T cells did not significantly share TCR sequences with CD8 T cells in the tumor draining lymph node or the peripheral blood, while $CD103^{-}CD39^{-}$ (double negative – DN) CD8 T cells did share TCR sequences with circulating cells (38), suggesting that the DP CD8 T cells are selectively retained within the tumor and the DN CD8 T cells are recirculating. This also means that examining any two different time-points might show a similar proportion of clonally expanded cells, but the non-specific clones would change over time and the cells that are present at both time points would be expected to be enriched for tumor antigen-specific cells. This proposition appears to be supported by current data. Using a combination of scRNASeq and TCRSeq Yost et al. demonstrated clonal expansions of CD8 T cells in tumors were enriched for exhaustion markers (41), and that these same cells also exhibited evidence of tumor reactivity based on expression of CD39 and CD103. Importantly, they found little overlap in TCR clonotypes between the exhausted population and CD8 T cells with effector phenotypes, suggesting that these cells have distinct specificities (41).

While we assume that recirculation will result in selective replacement of only non-specific cells, and that while the overall diversity of the infiltrate might remain consistent, there will be some notable caveats. We all have clonally expanded T cells specific for common viruses meaning that our circulating T cell pool is not uniformly distributed among possible TCR sequences. These clonally expanded T cells would also be present in the tumor through non-specific recruitment, but these would not be expected to be selectively retained. However, any chronically active T cell populations, such as those for CMV or EBV might be enriched in the activated T cell pool that is recruited to tumors. Thus, not all clonally expanded cells in tumors can be predicted to be tumor specific. Scheper et al. cloned the TCR from tumor infiltrating T cells and evaluated their specificity for autologous cancer cells. They found that only a small proportion – from 1-10% of T cells in the tumor were specific for cancer cells, and that in some examples T cells specific for EBV were three times more frequent in the tumor (42). As many as 3% of CD8 T cells infiltrating tumors have been shown to be specific for a CMV epitope (39). This number likely varies significantly between tumors, according to the degree of non-specific recruitment and the antigen-specific retention. For example, in the above paper, the authors analyzed a melanoma specimen where 90% of the tumor-infiltrating T cells expressed PD1, and 50-80% of all T cells were estimated to be tumor specific (42). Shitaoka et al. demonstrated that $CD8^{+}CD137^{+}$ cells represented 10-70% of CD8 T cells infiltrating human tumors, and a large proportion of these were clonally expanded (43). In murine tumors $CD8^{+}CD137^{+}$ cells represented up to 5% of tumor-infiltrating CD8 T cells, and TCRs from clonally expanded $CD8^{+}CD137^{+}$ cells were mostly tumor-reactive (43), suggesting that this population also enriches for tumor-reactive cells. Tumor-specific T cells generated *ex vivo* and adoptively transferred into an animal circulate widely, with no particular selectivity for a tumor expressing the cognate antigen (44). However, functional activation was limited to the site of antigen. These data suggest that non-tumor specific clonally expanded populations would be expected in the tumor, and tumor-specific clonally-expanded populations would be expected elsewhere. In this way clonal expansion may be an insufficient measure of specificity for antigens at that site. This is supported by current data. Penter et al. explored clonal expansions in colorectal tumors as well as uninvolved sites, and found similar rates of clonal expansions inside and outside the tumor (45), indicating that clonal enrichment is not unique to tumors. However, while on aggregate uninvolved and tumor regions had similar expansions, the data suggest that individual patients may exhibit enrichment for clonal expansions in the tumor. The tumor-associated clones exhibited increased expression of activation and exhaustion markers such as PD1, which are not seen in non-tumor clones (45), suggesting they are chronically recognizing antigen while in the tumor. Clonally expanded populations in the peripheral blood were stable over time (45), likely representing circulating memory populations specific for common targets such as EBV and CMV, and importantly a dominant CMV-specific clone was shown to be expanded in the blood, tumor, and uninvolved tissue site, demonstrating that these cells recirculate widely. In addition, clones that were highly expanded in the tumor were also detectable in the uninvolved tissue site (45), suggesting that tumor-specific cells may also be recirculating or can take up residency elsewhere. Analysis of lung tumors versus distant normal lung tissue demonstrated that highly expanded clones were more frequent in normal tissue than the tumor and the T cell in the tumor had greater TCR diversity (46), suggesting that the tumor recruits more non-specific T cells compared to normal tissue. Interestingly, in this and other studies there are data suggesting that a lower clonality and an increased T cell diversity in tumors is associated with worse outcome to conventional therapies and immunotherapies (46, 47). This would fit with clonal populations representing accumulated tumor-specific T cells among a background of diverse non-specific T cells. In this way, overall infiltrate is less informative than infiltrates of specific T cells.

For these reasons, a critical measure of tumor specificity or selectivity may be serial assessment. However, such analyses are rare. In part this is due to the clinical scenarios, since most analyses are performed on single biopsies or a tumor resection specimen. Yost et al. used bulk TCRSeq to compare T cell clonality in untreated tumors at two timepoints and found no significant changes in the TCR sequences present in clonally expanded populations over time (41). This was in contrast to tumors sampled before and after PD1 blockade, which resulted in an influx of new expanded clones (41). Later we will explore the effects of therapy on recirculation kinetics, but it is reasonable that at baseline the T cell infiltration of any tumor will be directly related to recruitment and retention, and be generally split into rapidly recirculating non-specific cells and selective retention of antigen-specific cells. Thus, increasing recruitment *via* inflammation and chemokines has the potential to increase the diversity of T cells in the tumor, but this occurs without any selectivity for tumor-specific cells (**Figure 2A**). As we will discuss

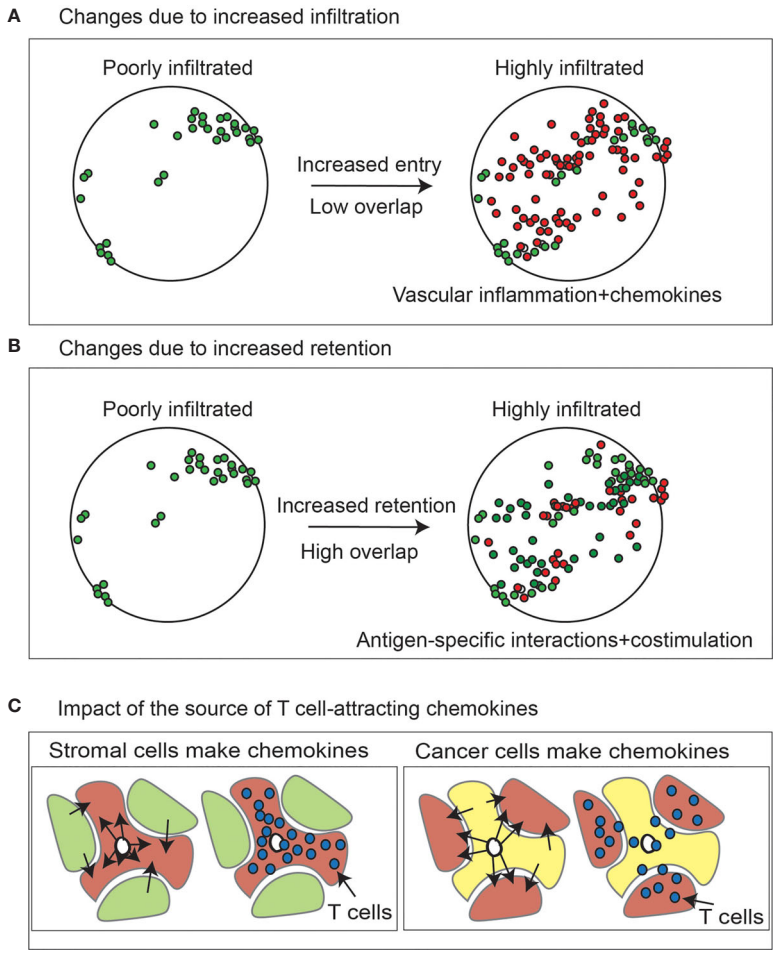


FIGURE 2 | Change in lymphocytes in tumors due to increase infiltration versus increased retention. **(A)** Effect of increasing lymphocyte infiltration *via* chemokines and inflammatory changes in the tumor vasculature on T cell diversity in the tumor. Lymphocytes present at baseline are green. Those newly present at the second timepoint are red. **(B)** As with **(A)**, but the effect of increasing T cell retention *via* increased antigen-specific interactions *via* altered antigen presentation or costimulation. **(C)** Higher expression of chemokines that can attract activated T cells in cancer cells will cause proportional enrichment of immune cells in the vicinity of cancer cell nests. By contrast higher expression of the same chemokines in the tumor stroma may cause their enrichment in the stroma but exclusion from the cancer cell nests, limiting their cytotoxic potential.

later, recruitment is not selective for tumor-specific T cells, and will attract any cells expressing the appropriate chemokine receptors, whether tumor-specific or CMV-specific. By contrast increasing antigen presentation and altering recognition thresholds using costimulatory agonists or coinhibitory blockade has the potential to increase clonality by increasing retention of tumor-specific cells (**Figure 2B**).

Intravital 2 photon microscopy has helped understand the dynamics of T cells in tumors. Using cancer cells expressing ovalbumin as a model antigen to view tumor-specific responses of OT1 TCR transgenic T cells, Breart et al. demonstrated that in a highly responsive model, transferred T cells were first visualized in the vicinity of vascular entry points and rapidly spread throughout the tumor resulting in cure (48). Salmon et al. demonstrated higher motility of T cells in the tumor stroma, and their comparative exclusion from tumor nests (49). Motility was highest in the immediate perivascular location, with fibers of the extracellular matrix serving to prevent direct T cell interactions with cancer cells (49). Initial estimates suggested that tumor-specific T cells and cancer cells required a 6hr duration of interaction to result in cancer cell death (48). This timing is interesting since only a proportion of T cells exhibit a longer duration interaction with cancer cells in tumors, with the majority exhibiting short-term interactions with multiple cells despite having specificity for tumor-associated antigens (50). Random migration of the T cells occurs prior to stable interaction with the target, and the kinetics of T cell movement differs depending on the presence of the cognate antigen (50). While T cells pause when meeting cancer cells that they can recognize, in models where the T cells are known to be responsible for curing tumors, they resume their motility once they successfully kill their targets (51). Interestingly in these models, T cells could be seen actively moving along the exterior of blood vessels in the periphery of the tumor, which would

represent a stromal location, and they adopted a non-motile appearance once detached from the blood vessel (51). T cells only entered deeper regions of the tumor when cognate antigen was present, and these changes took more time and followed the kinetic of successful tumor elimination at the periphery. This potentially relates to inflammatory changes in the vicinity of T cells actively engaging antigen that are propagated to neighboring regions that were formerly poorly inflamed and thus poorly infiltrated, or due to the T cells being restrained in the periphery until cancer cells were eliminated and they could resume their motility.

Studies using pertussis toxin demonstrate that chemokine signals are necessary for T cell motility within the tumor stroma (49), and cancer cells engineered to express chemokines can increase recruitment of T cells into the stroma (52), and on into cancer nests (49). In such a scenario, the ability of newly recruited T cells to meet their cognate antigen presented on cancer cells will be highly dependent on local chemokine signals that recruit T cells out of the stroma and towards cancer cell nests (**Figure 2C**). At the same time, inflamed lymphatic endothelial cells may secrete their own chemokines (53) and direct T cells out of the stroma to recirculation. The pre-existing Trm populations resident within cancer cell nests have both a phenotype that encourages adhesion to the cancer cells (38, 54) and lack SIP1R resulting in limited capacity for lymphatic traffic (55). Thus, once past the tumor stroma circumstances favor tumor residency versus recirculation of tumor antigen specific T cells.

Chemokine Modification of Tumors to Increase Recruitment

To understand recruitment and retention it is useful to take the example of two different tumors, one highly infiltrated and another poorly infiltrated with T cells. At baseline, we have no information as to whether the tumors are highly infiltrated due to an increased recruitment of T cells to the tumor, or increased retention of T cells within the tumor (**Figure 2**). If recruitment is the key criteria, then manipulating inflammatory signals within the tumor will influence infiltration. Over the last few decades, we and others have explored modification of cancer cells with cytokines and chemokines to increase immune cell infiltrates into tumors. Engineering tumors to express chemokines that attract T cells results in increased T cell infiltration and increased tumor immunogenicity (56–58). These data suggest that T cell recruitment to tumors is limited by suboptimal chemokine expression. Analysis of tumors with high versus low T cell infiltrates demonstrated that expression of a panel of 12 chemokine genes could predict increased T cell infiltration into tumors (59, 60). However, in view of the recirculation behavior of T cells, how will increasing chemokine levels change anti-tumor immunity? As discussed above, increased inflammation in the tumor or increases in chemokine expression will lead to a non-specific influx of T cells into these inflammatory areas, where the majority of cells are not tumor-specific. Among these recruited T cells there may also be populations of unconventional T cells, for example gamma delta T cells. Such cells can share recruitment mechanisms, and while they may not recognize tumor antigens, they may respond to the altered metabolic status in the tumor environment (61).

The fact that tumors may progress despite the presence of targetable neoantigens can in part be explained by immunological ignorance (62). T cells specific for cancer cells can recirculate through the tumor without impacting the tumor growth, unless given some external stimulus. This can be due to limited ongoing cross presentation (63, 64), or limited direct presentation due to low expression of the cognate antigen or low levels of antigen processing and presentation in the cancer cells (65, 66). In some circumstances these limitations can be overcome through increased antigen release from the cancer cells (67, 68). However, T cell ignorance of tumors can also be impacted by a poor rate of recirculation through tumors as well as their limited exposure to tumor-associated antigens. In addition, whether T cells are ignorant of the tumor or are actively recirculating through the tumor, these T cells will still face the broad range of immune suppression mechanisms that operate in the tumor environment. These issues are well reviewed (69–71), and such immune suppression may be the dominant pathway regulating adaptive immune control of growing tumors.

If chemokine levels in a tumor alter over time, the proportion of newly recruited cells that are tumor-specific before and after chemokine expression would be anticipated to remain identical, though their absolute numbers would be expected to change. However, increasing chemokine levels may increase the rate of recirculation, and therefore increase the likelihood that a tumor-specific T cell can meet it's cognate ligand. In this way, increased T cell recruitment may result in an increased proportion of tumor-specific T cells in the tumor through a more efficient screening of the recirculating T cell repertoire. For this to impact tumor control, entry to the tumor must have been the limiting factor preventing tumor-specific T cells from exerting their function. This is plausible, since as described above, low chemokine expressing tumors and low T cell infiltrated tumors have worse outcome than their matched counterparts (72–76). CXCR6 has been shown to play a role in the recruitment of Trm to tissue sites and their retention in tissues *via* its ligand CXCL16 (77). However, this chemokine receptor and ligand are not tissue specific. Up to 20% of all peripheral blood $CD8^+$ T cells express CXCR6 in cancer patients (78) and healthy donors, and CXCR6-mediated recruitment occurs in multiple healthy tissues including the lung and liver (77, 79). The percentage of CXCR6+ cells in the peripheral blood is much lower in mice with no history of infection, but is upregulated following infection (80). Therefore, CXCR6-expressing T cells may represent the diverse array of immune responses occurring in humans. Consistent with this, from 20-60% of EBV-specific T cells circulating in patients express CXCR6, and CXCR6 can be rapidly upregulated on antigen rechallenge (78). Importantly, CXCL16, the ligand for CXCR6, is upregulated in normal tissues following infection (79, 81) so all CXCR6-expressing T cells may be recruited to the site along with specific T cells. CXCR6 is enriched on cells that infiltrate tumors, as are other chemokines associated with activated T cells, such as

CCR5 (78). In particular, CXCR6 is particularly associated with a Trm population (77, 82, 83). Given the low recirculation of Trm phenotype cells and the high proportion of circulating CD8 T cells that express CXCR6, it is unlikely that CXCR6 is a specific means to recruit Trm. Alternatively, since CXCR6 is an activation marker on T cells, it is potentially a marker of T cells that have received additional cognate stimulation in the tumor environment, and may serve to retain antigen specific cells where both CXCL16 and the cognate antigen co-exist. In this way chemokine receptors that are induced by activation are likely to be enriched on the antigen-specific populations in the tumor environment, akin to the activation markers CD69 and CD39. It may also be important that CXCL16 is an unusual chemokine in that it is membrane bound, until cleaved by proteases that are regulated under inflammatory conditions and during cancer treatment (84–87). Therefore, CXCR6 may generate a retentive niche for antigen-reactive cells in close contact with epithelial cells, or recruitment from systemic circulation under inflammatory conditions.

Where chemokine expression is already high, or T cell infiltration is high, it would seem that recruitment is not a limiting factor in tumor control. It is logical that a tumor that has abundant T cells yet continues to grow may be more impacted by other issues (88). It is possible that these infiltrating T cells are in an unsuitable location, are suppressed, are unable to engage with antigen presented by cancer cells, or are simply not specific for the cancer cells. In addition, as will be discussed below, chemokine expression by the cancer cells might additionally impact the distribution of T cells within the tumor environment, encouraging T cell migration through the stroma and to cancer cells nests. For this reason, experiments that evaluate the role of chemokine expression on immune responses artificially alter the biology of the system. When cancer cells are engineered to express chemokines, as we have used in the past (52, 56), the chemotactic gradient will peak around the cancer cells, so recruitment of immune cells will be to the cancer cell nests. By contrast increased chemokine expression by cells of the tumor stroma may lead to a non-productive accumulation of T cells in the stroma without impacting their contact with cancer cells. Non-cancer cells of the tumor stroma are critical sources of chemokines in tumors, and altering the recruitment of T cells into versus out of the stroma can have therapeutic consequences that do not relate to the overall chemokine production in the broader tumor environment (**Figure 2C**). A heightened inflammatory environment that is restricted to the stroma may therefore negatively impact functional tumor control. Moreover, the infiltrating non-specific T cells may limit the ability of the specific cells to establish a niche and engage cognate antigen. This phenomenon is evident in a model of diabetes, where increased infiltration of non-specific T cells into the islet actually reduced the ability of islet-specific T cells to cause autoimmune diabetes (89). In this model, there is a threshold number of islet specific T cells that are necessary to bring about diabetes (90); however these islet-specific T cells represent a small proportion of the T cells infiltrating the islet (90). By providing large numbers of T cells of irrelevant specificity through a vaccination approach, the islet-specific population in the islet were less activated and less effective (89). This data is likely very impactful to cancer in patients, where ongoing irrelevant immune responses are likely present to a much greater degree than are present in murine models in clean animal facilities (91), and any attempt to increase recruitment to the tumor will occur regardless of specificity. In this scenario, the mechanisms impacting retention and activation may be more critical than recruitment to improve anti-tumor immunity.

Role of Antigen in Tissue Retention

Advances in genomic analysis of tumors and bioinformatic models to identify tumor mutations has resulted in a dramatic increase in the discovery of patient-specific neoantigens (92). The number of these antigens per patient vary considerably, but thus far most patients tested have been found to have targetable neoantigens and T cells that can recognize them (93–95). However, when considering the additional restriction based on MHC-binding of any neoantigens, it is theoretically possible that a poorly mutated tumor could have no targetable tumor neoantigens. This may be a stronger possibility in pediatric malignancies, where highly penetrant driver translocations/mutations can result in tumorigenesis with few additional passenger mutations (96–98). In these cases, recruited T cells would have no interaction with cognate targets, and would freely recirculate with no possible retention of specific cells. However, since recruitment is not antigen specific, it is possible that such a tumor could still have T cell infiltrates. Spranger et al. examined the neoantigen profile of tumors that were highly or poorly infiltrated with T cells, and found no correlation between the number of antigenic targets and the numbers of T cells infiltrating the tumor (99). These data suggest that T cell infiltration is unrelated to antigen density. However, in addition to neoantigens, there are an array of tumor-associated antigens (100) and in some cases viral antigens that can be effective targets for T cells. For example, in a recent clinical study in head and neck cancer, $CD8^+$ T cells in the tumor did not make measurable responses to any of the mutated neoantigens that were present in the cancer cells (101). However, the T cells made strong responses to the E6 and E7 proteins from human papilloma virus. Notably, these responses were exclusively found in the $CD103^+CD39^+$ population of resident CD8 T cells (101), demonstrating that these cells are not restricted to mutated neoantigen reactivity. In B16 tumors in murine models, approximately half of the clonally expanded T cells in the tumor were reactive to the unmodified gp70 epitope that is shared in many murine tumors (43). Thus, mutated neoantigens may not be essential for adaptive immune control of tumors.

In interpreting antigen density we must be cautious not to assume that we will make T cells specific for all potential targets. In infectious disease models, despite a wide range of potential antigenic targets the immune response generally focuses on a small number of antigens. This is known as immunodominance and occurs in antibody and T cell responses (102–104). This suggests that once a tumor passes some antigenic threshold, immunodominant antigens may focus the immune response

around a restricted set of T cells, and additional antigens may not impact the T cell response. Immunodominance is a potential problem in the immune response to infectious agents and to cancer, since the high antigen specificity can generate selective pressure that can permit outgrowth of variants. This has been observed as antigenic drift in infection (102) and immunoediting in tumors (105). Thus, despite large numbers of potential neoantigen targets in patient tumors, commonly only a very small number of tumor-specific clones can be cultured, often as low as 1-2 clones per tumor (92, 95). While this may be a technical issue relating to T cell expansion from tumor tissue given their suppressed status, if immunodominance limits the number of responses per patient it may provide an alternative explanation for the disconnect between the number of neoantigens and the degree of T cell infiltrate. However, this is also an opportunity for therapy, since we have the potential to introduce additional T cell responses capable of contributing to tumor control. Linnette et al. identified neoantigens present in tumors using genomic sequencing, and identified T cell specific for these tumor neoantigens using peptide stimulation and a DC vaccination approach in patients (106). Prior to vaccination, T cells specific for tumor antigens were below detection limits in the peripheral blood, but T cells specific for neoantigens could be expanded from blood and tumor *ex vivo*, with more of them found in the tumor (106). Vaccination resulted in expansions in neoantigen-specific T cells in the peripheral blood, but importantly, these experiments allowed the investigators to identify neoantigen-specific TCR sequences. TCR sequencing of the tumors demonstrated that the majority of these tumor-specific TCR sequences were absent from the tumor prior to vaccination, and even where present only a proportion of the potential specificities were detectable (106). Thus, patient tumors exhibit only a small proportion of the potential reactivity to unique neoantigens, supporting both some degree of immune ignorance and some degree of immunodominance in tumors. Kalaora et al. comprehensively characterized potential neoantigen targets in melanoma patients and corresponding TCR sequences in T cells expanded *in vitro* from tumors (107). Importantly, while there was significant variability between patients, distinct metastases within an individual patient overlapped in both neoantigen targets and TCR sequences present. In one example, these experiments demonstrated that 11 TCR sequences accounted for 90-99% of the tumor specificity (107). Zhang et al. demonstrated that the degree of clonality in tumors was positively correlated to the overall mutational burden (108). Interestingly, this paper also demonstrated a negative correlation between clonality and the percent tumor in the specimen (108), suggesting that an increased stromal component results in a decreased clonality likely due to increased infiltration of non-specific T cells in the stroma. Recent studies have demonstrated that a 'mutator phenotype' associated with loss of mismatch repair pathways is a stronger predictor of outcome than quantity of mutations (109, 110). Such tumors have increased T cell infiltrates counterbalanced by local immune suppression, including increased PDL1 expression (109, 111–113). However, it is unclear whether the mutations in these tumors dictates this infiltration phenotype. Tumors with the mutator phenotype have a more rapid tumorigenesis (114) and these tumors are highly inflamed before they have a high mutational burden (115). It is plausible that the mutator phenotype regulates immune activation through multiple mechanisms of which increased mutational frequency is only one (116).

One significant feature of tumors that is different from acute models of infectious disease is that the antigen target is chronically present in tumors. In most acute infectious disease models, antigen is present for only a few days to weeks in the infection site, and cross-presented antigen has a similarly short half-life. Following elimination of the pathogen, antigen is lost from the environment and as discussed above, the antigen-specific T cells remain as both tissue infiltrating resident memory T cells and circulating conventional memory T cells (17). Broadly, once established these are thought to be non-overlapping populations in the absence of further antigen exposure. Trm show little propensity to reenter circulation, and if they do, they have no directed pressure to establish themselves in other sites. This has best been shown in experiments where two mice – one antigen experienced and the other naïve – are surgically connected so that they share blood circulation. In this setting, only the antigen-experienced animal retains local Trm-mediated responses to rechallenge with the infectious agent (25, 117, 118). In the absence of further stimulation, Trm may traffic as far as the tissue draining lymph node, in a slower event over the course of weeks to months following their initial local antigen exposure (119). These cells retain residency features in the lymph node, and this mechanism can ensure locoregional memory within both the tissue site and the draining lymphatics to provide rapid response to infection (119). The literature is divided on whether these cells can re-enter circulation on rechallenge (27, 35), with some studies showing antigen challenge causes only local proliferation (35), other studies showing that rechallenge with a local infectious agent can cause the Trm to proliferate locally, enter the draining lymphatics and form conventional circulating memory populations (27). These data are impactful for understanding the Trm population in tumors. While cells with a Trm phenotype in tumors do not share TCR sequences with T cells in the draining lymphatics or the peripheral blood (38), it is possible that appropriate activation of these cells can cause recirculation. Similarly, it has been shown that circulating memory cells can become Trm following a subsequent local antigen challenge (120). Thus, in tumors where antigen is chronic, there may be a greater potential for turnover between resident and circulating tumor-specific T cells even though it is difficult to measure this with only steady state data. Yet, as discussed earlier, cells with Trm phenotypes in the tumor have unique clonotypes that are not readily detectable in the tumor-draining lymph node or peripheral circulation (38). These data suggest that despite chronic antigen presence, tumor-associated Trm are not measurably recirculating. As will be discussed later, Trm in the tumor express a range of exhaustion markers (38, 121) that are not typically observed on Trm in post-infection normal tissue.

It is possible that by the time cancers become clinically evident, the reactivation potential of the tumor-infiltrating Trm is greatly reduced, and the cells that remain in the tumor have achieved a degree of balance between the antigen-presenting capacity of the cancer cells and their activation state.

Stromal Versus Cancer Distribution of T Cells

The tumor is not a homogenous structure, and there are microenvironments within the broader tumor environment. One of the more critical distinctions is between the nests of cancer cells and the tumor stroma (**Figure 3**). The extent of tumor stroma varies considerably between individuals and between tumor pathologies. In addition, tumors can incorporate tertiary lymphoid structures, which are lymphocyte aggregates with varying levels of organization that can be found in cancer, and are similarly found in other scenarios where chronic inflammation disrupts the tissue architecture (122). In pancreatic cancer, the presence of these structures does not correlate with the overall tumor mutational burden (123), though the lymphoid structures are more likely where strong MHC-binding neoantigens are present. Tumors with tertiary lymphoid structures are likely to have more T cells infiltrating the tumor, but these T cells are less enriched for $CD103^+$ cells (123), suggesting that the formation of tertiary lymphoid aggregates versus cancer-associated Trm occur through distinct mechanisms. Notably, the 12 chemokine gene signature used to predict T cell infiltration in tumors also predicts the presence of tertiary lymphoid structures in tumors (59, 60). However, it remains unclear whether there is direct movement between the lymphoid structures and the vicinity of the cancer cells. B cells in these structures can recognize tumor-associated antigens (124), so it is reasonable to infer that there are CD4 T cells with similar specificities in the lymphoid structures. However, thus far there is no direct evidence the tertiary lymphoid structures of tumors are significant sources of the tumor-specific effector CD8 T cells

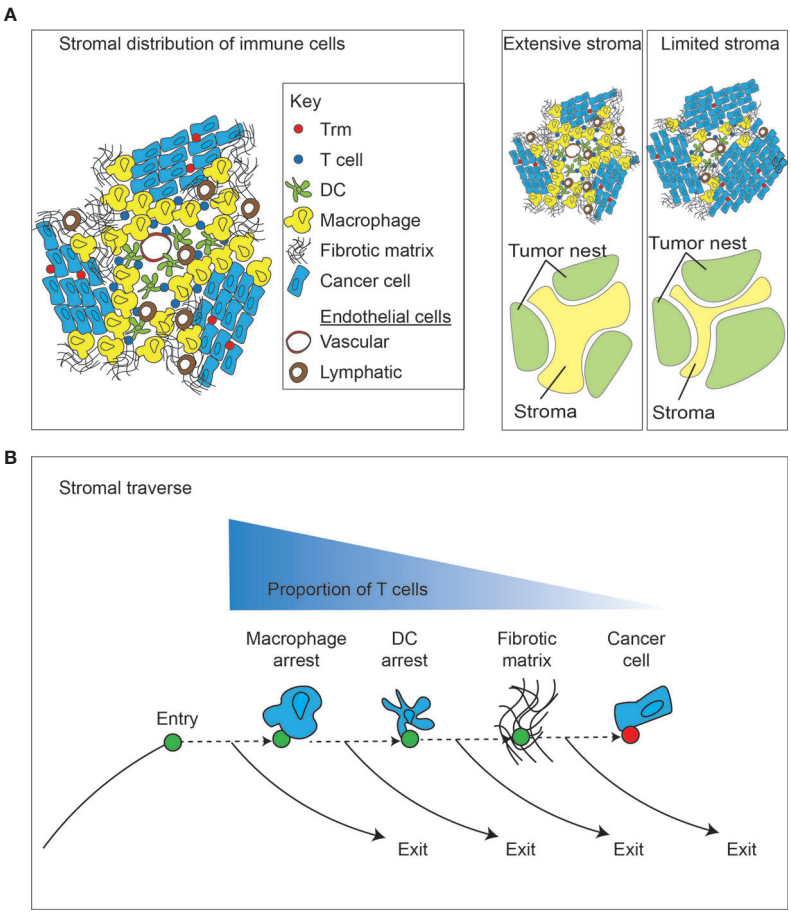


FIGURE 3 | Impact of the tumor stroma and the stromal traverse on T cell dynamics. **(A)** The tumor is a non-homogenous structure that can be generally split into tumor and stroma. A diverse population of immune cells enrich in stroma away from direct cancer cell contact, and closer to vascular points of entry and lymphovascular points of exit. Different tumors can vary widely in the extent of tumor stroma. capacity for direct cancer cell cytotoxicity. **(B)** Following entry of lymphocytes into the tumor stroma *via* the vasculature, there are multiple stromal barriers that can cause T cell arrest and provide opportunities for a dominance of exit signals for continued recirculation through efferent lymphovasculature, rather than continuing through the stroma to meet cancer cells. More extensive stroma may increase the duration of traverse and decrease the likelihood of T cells meeting cancer cells.

that participate in tumor control. Pancreatic cancer is a commonly mentioned example where it is often possible to detect extensive desmoplastic stroma punctuated with relatively small nests of cancer cells (**Figure 3**). The stroma can incorporate a diverse set of non-cancer cells and plays an important role in tumor growth and immune regulation. T cells are not uniformly distributed between the stroma and cancer cell nests – they are generally enriched in the stroma (49, 125, 126). Importantly, T cell subtypes are differentially distributed between stroma and cancer cell nests. For example, in breast cancer it has been demonstrated that cells with the Trm phenotype are enriched in cancer cell nests rather than the tumor stroma (126). By contrast, stem-like CD8 T cells expressing the Tcf1 marker were shown to be enriched in the immediate perivascular region (127). These stem-like cells have been shown to co-localize with APC in their stromal niche (128), suggesting that their interactions are more impacted by cross-presentation than by direct presentation by cancer cells. Considering features regulating recirculation, it is important to know that lymphatic density is highest in the tumor stroma surrounding cancer cell nests, driven by both cancer cell and stromal cell factors that guide lymphangiogenesis [reviewed in (129)]. Similarly, vascular endothelia are a defining feature of the tumor stroma, and the proximity of these entry and exit vessels and their separation from cancer cells means that recirculation can occur without T cells having an opportunity to directly contact the cancer cells.

The role of lymphatic endothelial cells in tumor immunity is multifaceted (129), but includes direct negative regulation of T cell activation as a result of inflammatory feedback (130). Since lymphatic endothelial cells mediate T cell exit from tissues, loss of lymphatic endothelial cells would be expected to decrease the ability of T cells to leave the tissue site. If ingress into the tumor is sustained and exit decreased, this should result in T cell accumulation. However, since as discussed above, rapidly recirculating T cells are more likely to be non-specific, this may not impact outcomes. Alternatively, since lymphatic endothelial cells can suppress T cells (130), more effective local immune responses might also be expected if there are fewer lymphatic endothelial cells in the tumor. Interestingly, contrary to these expectations tumors implanted into mice that lack functional lymphatic endothelial structures have fewer T cells infiltrating the tumor and reduced overall inflammation in the tumor (131). One possible explanation for this data is that impaired recirculation also results in impaired initial anti-tumor immunity, which will skew these results. As discussed earlier, to initiate new immune response dendritic cells must travel from the antigen site to draining lymph nodes *via* lymphatics to meet and stimulate naïve T cells. Loss of lymphatics might also mean loss of this initial anti-tumor immunity. Consistent with this, in viral models a lack of lymphatic endothelial structures results in impaired local control because of impaired initial immune activation in the draining lymph nodes (132). This initial immunity to tumor implantation is dependent on cross-presenting cDC1 and requires CD40 to generate both CD4 and CD8 T cell responses to tumor-associated antigens in the draining lymph node (133, 134). Using adoptive transfer to overcome this initial limitation in T cell responses, transferred tumor antigen-specific T cells have been shown to improve tumor control where tumors lack lymphatic endothelial cells (131), and pre-existing virus-specific T cells were equivalently capable of controlling a viral infection in the absence of lymphatic endothelial structures (132). These data suggest that lymphatic endothelial cells are required to prime new immune responses, but do not impair or suppress tumor immunity by existing tumor-specific T cells. Interestingly, increasing lymphangiogenesis in tumors increased their responsiveness to immunotherapies, and is associated with changes in the T cell populations that were recruited into the tumor immune environment (135). It is difficult to isolate the exact contribution of lymphatic structures due to the myriad of mechanisms by which they can interact with immune cells and the tumor stroma. However, given their role as an immunoregulatory component of the tumor stroma, the lymphovascular cells have a significant capacity to regulate the tumor immune environment and T cell recirculation (28).

The extracellular matrix represents an additional important limiting factor in T cell motility within the tumor. As discussed above, Salmon et al. demonstrated high T cell motility in the tumor stroma, and their comparative exclusion from tumor nests (49). Fibers of the extracellular matrix were shown to prevent direct T cell interactions with cancer cells (49). In such a setting the newly infiltrated T cells find it difficult to physically interact with the cognate antigen, but have an easy path to draining lymphatics which may comparatively promote their exit. For these newly-entered T cells the antigen presenting cells within the tumor stroma may play a significant role. Macrophages are prevalent in tumor stroma and are important in driving neoangiogenesis, lymphangiogenesis, and matrix remodeling for cancer cells growth (71, 129). Therefore, these cells are located amidst the key features regulating recirculation. Using live cell imaging, Peranzoni et al. demonstrated that tumor macrophages formed stable interactions with infiltrating CD8 T cells in the tumor stroma which reduced T cell motility (125). Depletion of macrophages using CSF1R inhibition restored T cell mobility and increased direct T cell interaction with cancer cells (125). Thus, macrophages in the tumor stroma may limit T cell mobility and thus limit functional interactions with cancer cells. Importantly, while macrophages can take up tumor antigens and present them to CD4 T cells *via* MHCII, they cannot cross-present antigen to CD8 T cells *via* MHCI. Dendritic cells are present in dramatically reduced proportions compared to other myeloid populations in the tumor stroma (136, 137), but they have the unique capacity to cross-present cell-associated antigens to infiltrating CD8 T cells (137, 138). Using cancer cells expressing fluorescent proteins, Englehardt et al. identified that dendritic cells closest to the cancer cells had the most phagocytosed cancer-cell material (139). They demonstrated that T cells closest to cancer cells exhibited reduced motility *in vivo*, and also that the T cells exhibited a more prolonged interaction with the dendritic cells that were close to the cancer cells compared to those dendritic cells that were further

away and that had less phagocytosed cancer cell material (139). These data support the role for stromal dendritic cells in cross-presenting tumor associated antigens and this may increase T cell retention even where the T cells do not directly contact cancer cells. Interestingly, these dendritic cells were poorly able to support T cell proliferation unless first treated with innate adjuvants (139), suggesting that they maintain an immature phenotype and are unable to provide adequate co-stimulatory signals and cytokines to support T cell proliferation or effector function. These data suggest that those APC closest to the cancer cells have more abundant antigen for cross-presentation, but in progressively growing tumors these cells cannot sufficiently activate anti-tumor immunity for tumor control. Thus, they increase retention of tumor-specific clones, but do not necessarily result in tumor elimination. Tumor antigen-specific T cells have been described as being trapped in a dendritic cell network, restricting their access to cancer cells (140). This closely matches the observed state in snapshot views of tumors, where tumor-specific T cell clones are enriched but in poorly functional states permitting progressive tumor growth. Consistent with this, under steady state conditions tumor dendritic cells often have impaired functionality (136, 141). Dendritic cells in tumors can become poorly functional early in tumorigenesis (142, 143), and tumors with poorly functional dendritic cells are also poorly responsive to conventional therapies (136). These data indicate that tumor-infiltrating T cells must pass both physical barriers and intercepting APC that may drive local tolerance before they can even directly access cancer cells (**Figure 3B**). The role of intratumoral dendritic cells in regulating T cell control of tumors remains controversial, in part due to difficulties in distinguishing myeloid subtypes. While cross-presenting dendritic cells are required to initiate immune responses to tumor-associated antigens (133), they can be dispensable for tumor control by adoptively transferred T cells (144). This continued exit of matured and maturing dendritic cells makes it difficult to interpret the biology of dendritic cells in tumors at steady state, since by definition, the tumor resident dendritic cells should be immature since the mature cells have exited. However, features of the tumor environment that keep dendritic cells in the environment without permitting their maturation have the potential to generate a tolerogenic APC barrier. A wide range of immune interventions aim to provide signals that can drive dendritic cells maturation (145, 146), and many of these have shown efficacy in preclinical settings.

Impact of Cancer Cell Antigen Presentation on Recirculation

Antigen presentation is a regulated process and can be dynamically upregulated in response to stimuli. For both mice and humans, non-MHC genes that are integral to antigen presentation on MHC-I are also contained within the Mhc region, including the genes for tapasin, TAP1, TAP2, and LMP7; however, in both humans and mice the gene for β2m is located on a separate chromosome. Expression of classical MHC-I elements is mediated by three major regulatory elements: enhancer A, IFN-stimulated response element (ISRE), and the SXY module. Within MHC-I promoters, enhancer A elements are bound by NF-κB/rel family members and ISRE elements are bound by interferon regulatory factors (IRFs) including IRF1; these transcription factors notably mediate transcription of MHC-I proteins downstream of IFNγ and TNFα stimulation (147–149). The SXY module, comprised of S/W, X1, X2 and Y boxes, binds a number of nuclear factors including RFX (comprised of RFX5, RFXAP, and RFXANK/B), CREB/ATF, and NF-Y, which require a transcriptional regulator, either the Class I Transactivator (CITA, or NLRC5) or the Class II Transactivator (CIITA), to coordinate enhanceosome assembly (147, 150, 151). NLRC5 is a dominant regulator of MHC-I in most cells (152), and expression of NLRC5 can be induced by IFNγ (153).

Downregulation of antigen presentation on MHC-I is a common immune evasion mechanism employed by tumors (154). While decreased antigen presentation is a common feature of cancer, total loss of MHC-I expression (for example *via* biallelic loss of B2M) is less common (155), likely due to selective pressure from natural killer cells whose cytotoxic function is inhibited by the presence of MHC-I. Antigen presentation on MHC molecules can be reduced without total ablation by epigenetic suppression or genetic loss of factors regulating MHC-I expression (e.g. NLRC5) (156–158), downregulation of molecules involved in peptide loading onto MHC-I (159–161), loss of specific HLA alleles (162), or suppression of cytokine-activated pathways for augmenting MHC-I expression (e.g. loss of IFNγR/IFNAR or downstream JAK/STAT molecules) (163–165).

Conventional cancer therapies have the potential to regulate MHC expression by cancer cells. Reits et al. demonstrated that MHC-I expression is increased after radiation due to increased availability of intracellular peptides available for loading (166). Cancer cell irradiation can also activate the cGAS/STING pathway, triggering extracellular release of type I IFN (167). Ligation of IFNAR with type I IFN triggers downstream signaling *via* STAT1, STAT2 and IRF9 and leads to transcription of interferon-stimulated genes including MHC-I related proteins (168, 169); resulting in IFN-dependent upregulation of MHC-I by cancer cells (170). Similar mechanisms have been proposed for chemotherapy-induced activation of the STING-IFN pathway (171, 172). In addition, radiation therapy can upregulate NLRC5 independently of STING and IFN activation (173), potentially *via* distinct DNA-damage detection mechanisms. This suggests that cancer cell MHC expression can be regulated *via* an array of conventional approaches to alter lymphocyte dynamics in the tumor environment.

Significantly, alteration of cognate MHC-peptide expression on target cell surfaces can affect the magnitude and efficacy of CD8+ T cell responses (174). Differential responses of CD8+ T cells to varying MHC-peptide concentration have been observed, where increased epitope density corresponds with greater responsiveness to IL-2, enhanced proliferation and increased cytotoxic function including cytokine production (174–176). This phenomenon is better understood in naïve T cells, where

high levels of antigen presentation in combination with costimulation and integrin stabilization are required to generate a stable immunological synapse and to cross an activation threshold of TCR signaling (174, 177). In activated T cells, MHC-peptide:TCR interactions at the synapse are much shorter: a single MHC-peptide complex can serially engage with rapidly internalizing TCRs and a CD8+ T cell can exert cytotoxic functions after engaging with as few as 1-3 MHC-peptide complexes per target cell (178, 179). It is clear that higher concentrations of MHC-peptide can engage more TCRs and it has been proposed that serial engagement of the TCR allows increased stability and enhanced signaling within the TCR/MHC-peptide/CD8 molecular complex (176). Functionally, downregulation of MHC-I induced by viral infection can significantly attenuate the ability of CD8+ T cells to kill infected targets (180). Interestingly, expression of the early activation marker CD69 appears to be independent of epitope density (181), and similarly CD69 and PD1 can be induced in the tumor environment independent of cognate antigen (182). Different densities of MHC-peptide can activate different thresholds in T cells for expression of early activation markers, cytolytic degranulation, versus cytotoxic cytokine release (183) (**Figure 4**). As discussed earlier, we have not explicitly examined the effect of local proliferation on T cell accumulation in tumors, instead grouping that as a retention mechanism. However, the degree of antigen presentation directly impacts the threshold for T cell proliferation in addition to cytotoxic activity. Since antigen presentation below a threshold can eliminate T cell responses, total loss of MHC-I is not necessary for resistance to T cells, and can result in various stages of activation without functional cancer cell cytotoxicity. This can explain why baseline tumor downregulation of antigen presentation *via* MHC-I results in checkpoint blockade resistance in human patients (164, 184), and presents a role for conventional therapies to increase T cell recognition and killing of cancer cells. In addition, antigen transfer that occurs as a result of cancer cell death driven by chemotherapy been shown to increase T cell infiltration into tumors, and increase T cell interactions with dendritic cells in the tumor (140). Similar mechanisms of antigen transfer to antigen presenting cells occur following radiation therapy (66), and have the potential to further manipulate the kinetics of lymphocyte movement through tumors by altering thresholds for cross-presentation in addition to direct presentation. Finally, T cell interactions that result in successful signalling and cytokine production can result in IFNg secretion and consequently increased antigen presentation in the vicinity of the T cell. While this may be tempered by simultaneous upregulation of negative regulation *via* PDL1, an initial cognate interaction by T cells can start a positive feedback loop in the microenvironment that can permit the T cell to pass critical activation thresholds that can result in cytotoxicity. For these reasons, manipulating the threshold for T cell activation in the tumor environment has the potential to dramatically alter tumor control.

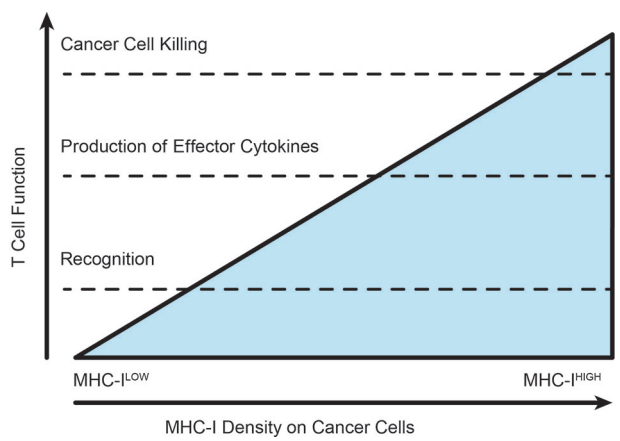


FIGURE 4 | Varying thresholds for T cell function according to extent of TCR-MHC interactions. As the extent of cognate antigen increases on a target, whether by improved antigen expression or increased antigen processing and presentation, the intensity of T cell recognition allows the T cell to pass through various thresholds of response. The position of these thresholds can be altered by checkpoint blockade or costimulatory agonists. T cells in established tumors are typified by phenotypic modification without successful cytotoxic elimination of the cancer cells.

Effect of Checkpoint Regulators on Retention

As described above, while T cell entry into tumor is antigen-independent, retention in the tumor results from antigen-specific interactions with antigen-presenting cells or the cancer cells themselves. Immunotherapy has the potential to increase T cell surveillance of tumors through either mechanism – by increasing the recruitment or increasing their retention. However, to focus our efforts on antigen-specific cells it may be more useful to understand the mechanisms that increase the retention of this subpopulation in the tumor. As discussed earlier, we consider local proliferation to be a feature of retention, resulting in increased numbers of antigen-specific T cells in the site without changes in recruitment. In addition to the direct TCR interaction with cognate MHC-peptide on target cells, there are an array of costimulatory and coinhibitory signals that regulate that interaction (185). These are the targets of most of the immunotherapies currently being tested in clinical studies, and may function by regulating antigen-specific T cell retention and access to cancer cells, as well their function in the tumor, including local proliferation. The effect of checkpoint inhibition on TCR diversity and clonality has recently been reviewed (186), and this is an area of rapid research advancement. For example, Zhang et al. demonstrated that patients exhibiting a major pathological response to PD1 inhibition showed increased sharing of highly expanded clones between the tumor, peripheral blood, and non-tumor tissue (108). These data suggest that PD1 signals ordinarily limit T cell recirculation, or that the poorly responsive tumors in particular have limited active recirculation. Using live cell imaging it was shown that PD1 blockade increased the duration of T cell interactions with cancer cells (125), slowing their overall motility in the tumor environment. However, recent evidence suggests that PD1 blockade may increase the infiltration

and function of a new clonally expanded population in the tumor, rather than restoring function of the existing exhausted cells (41). Single cell RNASeq plus TCR clonotype analysis of basal cell carcinoma before and after anti-PD1 therapy identified that T cells with shared TCR clones also tended to share a phenotype and that this phenotype was preserved following treatment (41), with exhausted clones remaining exhausted. Two thirds of the clones that expanded on treatment were new to the tumor, and included newly exhausted clones that made up the majority of exhausted cells in the treated tumor (41). Interestingly, this same precursor-like population is responsible for the proliferative expansion in T cells following PD1 blockade in viral models (187), suggesting that in both cases the response is dependent on expansion, recruitment and accumulation of new cells independent of the pre-existing clonally enriched, but exhausted T cells. Thus, in both viral infection models and tumor models, it has become clear that PD1 blockade is unable to restore the function of exhausted T cells and drive their conversion into memory and effector populations (188, 189). Rather, a distinct population current described as 'progenitor exhausted cells' that do not express the antigen-recognition and exhaustion marker CD39 expand following PD1 blockade and are more capable of generating effector function and tumor control (189). Siddiqui et al. demonstrated that these Tcf1+ progenitor-like cells were responsible for the majority of the proliferation following anti-PD1 therapy, and this expansion and tumor control could occur while new recruitment from the periphery was blocked using FTY720 (127). These data suggest that the progenitor-like clones may already be present in the tumor, and the apparent recruitment may be due to the frequency of these cells passing detection thresholds rather than recruitment from elsewhere. Since patients that have a pre-existing clonally expanded population of T cells expressing exhaustion markers is associated with improved outcome following PD1 blockade (47, 190), this suggests two major possibilities. Firstly, that the pre-existing population impacts the subsequent responses even if they are not the proliferative cells. Secondly, that the clonally expanded population in the tumor is evidence of a permissive environment in the cancer, which as discussed above might include effective antigen presentation and recruitment of cells into cancer cell nests to increase accumulation of tumor-specific clones. While it is currently difficult to break down these features, it is notable that clonal expansions of T cells following PD1 blockade occur in neighboring tissues as well as in the tumor (191). This suggests that the biology is not unique to the tumor but to applies to recirculating cells through other tissue sites.

Anti-CTLA4 therapy has been shown to increase T cell diversity in the peripheral blood of cancer patients (192, 193), though this was not necessarily reflected in positive phenotypic changes in the tumor (192). This data is consistent with expansion in functional specificities in the peripheral blood following anti-CTLA4 therapy (194), but little evidence of correlation between clonotypic changes in the peripheral blood versus the tumor following anti-CTLA4 therapy (195). Interestingly, in 4T1 mammary cancer models, anti-CTLA4 treatment increased motility of T cells in the tumor (196). Since this experimental design did not discriminate antigen-specific T cells it is possible that this increased motility reflects an increase in diverse T cell populations that are not tumor specific and so have increased overall motility in the tumor environment. Notably, adding radiation therapy to anti-CTLA4 therapy resulted in decreased overall motility in this model, and the change could be blocked with anti-MHCI antibodies, suggesting that cognate interactions are increased by the combination (196). TCRSeq in the same model shows that the combination of radiation and anti-CTLA4 increases the clonality of T cells in the tumor, and the proportion of antigen-specific T cells in the tumor, but no change in the distribution of TCR clones within the antigen specific population (197). This suggests that the tumor likely experiences increased infiltrates of clonal non-specific cells following anti-CTLA4 therapy. In patients, both anti-CTLA4 therapy and anti-PD1 therapy similarly increased T cell clonality in the tumor (190), but the extent of clonal expansion in the tumor pre-treatment was only predictive of outcome following anti-PD1 therapy (190). These data suggest the two agents have very different effects on T cell dynamics. By correlating patient responses to therapy with T cell populations in the blood, Wei et al. demonstrated that different T cell subpopulations in the peripheral blood correlated with response to single versus dual agent therapy (198). However, the frequency of these populations in the blood did not correlate well with their proportions in the tumor. Lau et al. demonstrated that combined PDL1 and CTLA4 blockade resulted in increased overall numbers of antigen-experienced T cells in murine tumors, and that these cells were more heterogeneously distributed than in untreated tumors (199). As observed in other models and discussed above, in these untreated murine tumor models T cells exhibited greater motility in the tumor periphery than in the tumor core (199). Following PDL1 blockade alone or combined PDL1 and CTLA4 blockade T cell motility was decreased in all regions of the tumor and overall infiltration was increased (199), consistent with increased cognate interactions in the tumor environment.

In contrast to checkpoint inhibitors like anti-PD1, costimulatory agonists like anti-OX40 (CD134) and anti-41BB (CD137) provide additional signals to T cells to overcome limited TCR stimulation, and can dramatically expand new populations of antigen-specific cells (200–202). This may occur because their antigen-specific interaction was below the necessary activation threshold as a lower affinity/avidity interaction, or because limited adjuvant signals have generated APC that are cross-presenting antigen but are not adequately providing costimulatory signals (203). In both cases, costimulation can generate a broader pool of antigen specific cells and also improve the quality of T cells as measured by memory formation and effector function (200–203). In the tumor, administration of anti-OX40 has been shown to increase the clonality of T cells in both the tumor and the spleen, suggesting that only some populations are being expanded by the therapy (204). Consistent with tolerogenic hypotheses limiting T cell responses in the tumor, T cells required anti-OX40 agonism to generate functional TCR signals in the tumor environment (182). In addition, higher affinity T cells in the tumor are more likely to express the

costimulatory target OX40 (182), and the effects of anti-OX40 therapy was more pronounced on this tumor-infiltrating T cell population than those in the draining lymph nodes (182). These data suggest that OX40 costimulation specifically targets the existing antigen-specific T cell population that infiltrate but fail to cure tumors. Interestingly, the combination of anti-OX40 therapy and anti-PD1 therapy also enriches for T cells that receive high affinity TCR signals (204), as measured by activation of a Nur77-GFP reporter system (205). Thus, anti-OX40 therapy has been shown to remodel the tumor immune environment *via* activation of existing CD8 T cells that were previously functionally limited by the tumor immune environment (206, 207). To understand the response to agonist antibodies to 4-1BB, Weigelin et al. performed intravital imaging of tumors expressing ovalbumin as a model antigen to view tumor-specific responses of OT1 TCR transgenic T cells (208). As discussed above in other tumor models, these experiments demonstrated that OT1 T cells moved at slower speeds when tumors expressed their cognate antigen (208). The addition of agonist antibodies to 4-1BB (CD137) slowed the transit of the tumor-specific T cells, and increased the dwell time of T cells with target cells (208). These data demonstrate that as with PD1 blockade, costimulation can increase retention of T cells in the tumor resulting in T cell accumulation.

The consequence of manipulating T cell interactions with their cognate targets *via* checkpoint blockade and costimulatory agents can therefore be viewed through overlapping mechanisms. Firstly, new cells that previously were able to stably interact with their target can be incorporated into the anti-tumor immune response by decreasing the activation threshold of the T cells. This may occur *via* removal of negative regulation in T cell activation (209), or through provision of costimulatory support (210). Again, these data are consistent with ongoing immune surveillance of tumors by T cells as part of baseline recirculation. Those clones that are newly able to interact with cancer cells, can arrest and accumulate when checkpoints or costimulation are regulated. Secondly, there is clonal expansion. While the data is limited at present, costimulatory agents appear to expand existing populations that had been limited in their function in the tumor environment (204), while checkpoint inhibition through anti-PD1 appears to permit a new population of T cells to participate in the anti-tumor immune response (41). As the data improves, we will obtain a better picture of how these therapies impact recirculation kinetics versus local function, and how these features explain their effects in tumors.

Conclusions

As we have discussed, lymphocyte exclusion from the tumor environment predominantly revolves around recruitment and recirculation kinetics, but within those contexts the retention of antigen specific T cells and their ability to meet cancer cells are key. A tumor with a high throughput of T cells through high recruitment and high recirculation is very *dynamic*. Such a tumor has a great deal of potential for T cell-mediated control of cancer cells should cells of the appropriate specificity exist. When faced with a tumor that is not very dynamic, meaning that infiltration is poor, it will likely also lack the inflammatory signals that recruit and mature dendritic cells, so such a tumor may fail to generate cells specific for tumor antigens and also not recruit any that are expanded or provided through immunotherapy.

However, there is little reason to assume that a high recruitment tumor has a higher proportion of tumor antigen specific cells. A random assortment of T cells being recruited to the tumor stroma would not be expected to impact tumor growth and progression, so a high degree of *entropy* would have no advantage even in a highly infiltrated tumor. Moreover, cells that recirculate through an inflamed tumor stroma may minimally pass out of the stroma and meet the cancer cells to permit antigen-specific destruction. If T cells can be recruited to cancer cell nests, it may not be necessary to have a high recirculation rate to eventually result in an accumulation of cancer-specific cells amongst cancer cells. A wide array of data discussed above suggests that this is the most important feature – high pre-existing clonality, high Trm infiltrates, T cells infiltrated into tumor nests. That is, a low entropy tumor.

Currently, therapies that assist the tumor specific T cells complete their tasks, such as anti-PD1 are the most effective immunotherapy agents in the clinic. As discussed above, recent data suggests that PD1 blockade functions to recruit a new population to participate in tumor control rather than convert the function of terminally exhausted cells. In turn, this suggests that an ability to direct these new T cells to the tumor is essential for responses. Therapies that will help expand the existing population of tumor-specific T cells, such as anti-OX40 and anti-41BB have not yet shown sufficient promise for clinical approval despite their preclinical power. Understanding the critical issues of recruitment and retention of tumor-specific T cells to the tumor, as well as mechanisms that allow us to initiate new anti-tumor immune responses where they are currently lacking, will be key to success. To do this we will need to look carefully so that we can discriminate these responses from the constant recirculation of irrelevant T cells, and understand how these might interact to regulate site specific immune responses, to control the dynamic entropy of tumor lymphocytes.

AUTHOR CONTRIBUTIONS

TB, writing, editing. AA, writing, editing. LZ, writing, editing. MC, writing, editing. MG, writing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NCI R01CA182311, R01CA244142, and R01CA208644.

ACKNOWLEDGMENTS

The decision to frame the title and conclusions in terms of dynamic entropy is courtesy of xkcd (https://xkcd.com/2318/).

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Conflict of Interest: MG and MC receive research funding from Bristol Myers-Squibb, Jounce, and Mavupharma that is unrelated to the content of this manuscript.

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

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frontiers in Immunology

REVIEW
published: 23 April 2021
doi: 10.3389/fimmu.2021.643771

Macrophage Biology and Mechanisms of Immune Suppression in Breast Cancer

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Macrophages are crucial innate immune cells that maintain tissue homeostasis and defend against pathogens; however, their infiltration into tumors has been associated with adverse outcomes. Tumor-associated macrophages (TAMs) represent a significant component of the inflammatory infiltrate in breast tumors, and extensive infiltration of TAMs has been linked to poor prognosis in breast cancer. Here, we detail how TAMs impede a productive tumor immunity cycle by limiting antigen presentation and reducing activation of cytotoxic T lymphocytes (CTLs) while simultaneously supporting tumor cell survival, angiogenesis, and metastasis. There is an urgent need to overcome TAM-mediated immune suppression for durable anti-tumor immunity in breast cancer. To date, failure to fully characterize TAM biology and classify multiple subsets has hindered advancement in therapeutic targeting. In this regard, the complexity of TAMs has recently taken center stage owing to their subset diversity and tightly regulated molecular and metabolic phenotypes. In this review, we reveal major gaps in our knowledge of the functional and phenotypic characterization of TAM subsets associated with breast cancer, before and after treatment. Future work to characterize TAM subsets, location, and crosstalk with neighboring cells will be critical to counteract TAM pro-tumor functions and to identify novel TAM-modulating strategies and combinations that are likely to enhance current therapies and overcome chemo- and immuno-therapy resistance.

Keywords: breast cancer, tumor associated macrophages, immune suppression, T cell inhibition, cancer immunity

OPEN ACCESS

Edited by:
Carlo Bifulco, Providence Portland Medical Center, United States

Reviewed by:
Mario Leonardo Squadrito, Vita-Salute San Raffaele University, Italy
Jennifer Richer, University of Colorado, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *18 December 2020*
Accepted: *17 February 2021*
Published: *23 April 2021*

Citation:
Mehta AK, Kadel S, Townsend MG, Oliwa M and Guerriero JL (2021) Macrophage Biology and Mechanisms of Immune Suppression in Breast Cancer. Front. Immunol. 12:643771. doi: 10.3389/fimmu.2021.643771

INTRODUCTION

Macrophages are innate immune cells and play a myriad of important roles such as host defense, tissue homeostasis, and modulating inflammatory responses (1, 2). To perform these functions, immature macrophages with high plasticity respond to microenvironmental cues, causing them to adopt a spectrum of effector function, among which M1-like and M2-like represent extreme polarization states (3–5). Classically activated M1 macrophages exhibit pro-inflammatory behavior by migrating to inflamed tissues, targeting pathogens with the production of reactive oxygen species (ROS), and having high antigen-expressing potential (6–8). Due to their inflammatory behavior, anti-tumor macrophages are commonly called M1 macrophages. These macrophages can be potent effector cells that kill tumor cells and can recruit cytotoxic T lymphocytes (CTLs) to activate adaptive immune responses. On the opposite side of the macrophage polarization spectrum, alternatively activated M2 macrophages secrete anti-inflammatory cytokines to induce immune tolerance and attract T regulatory cells (Tregs) and Th2 T cell subsets capable of protective

type 2 responses but devoid of cytotoxic functions. M2 macrophages facilitate canonical tissue repair functions and in cancer are regarded as pro-tumor where they promote tissue remodeling and repair, stimulate angiogenesis with vascular endothelial growth factor (VEGF), and encourage tissue growth with transforming growth factor beta (TGF-β) (9). Therefore, for simplicity, tumor-associated macrophages (TAMs) have been described as either M1-like (anti-tumor) or M2-like (pro-tumor), but it should be recognized that the M1/M2 dichotomy represents idealized polarization states (**Figure 1**), while in nature, there exists a broad spectrum of macrophage phenotypes, which will be discussed in detail below.

TUMOR INFILTRATING MACROPHAGES IN BREAST TUMORS

Recruitment of Monocytes and Macrophages to Breast Tumors

TAMs represent a significant component of the inflammatory infiltrate in breast tumors (10, 11). Tumor-derived growth factors such as chemokines and cytokines facilitate recruitment of monocytes and macrophages into tumors (**Figure 2**, step 1) (12). One of the best-characterized cytokines responsible for recruiting TAMs into the tumor is chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1). CCL2 is expressed by both stromal cells and tumor cells (13) and is associated with poor prognosis in breast cancer (14, 15). Through recruitment of CCR2-expressing monocytes, CCL2 has been shown to promote pulmonary metastasis in mouse models of breast cancer (16). Activation of the CCL2–CCR2 axis promotes CCL3 production from macrophages, enhancing metastatic seeding of breast cancer cells (17). CCL5, also known as Regulated upon Activation, Normal T Cell Expressed and Secreted (RANTES), is another well-known factor that recruits TAMs to the breast tumor. CCL5 is expressed by malignant epithelial cells in breast carcinoma and is associated with advanced disease progression (18–20). Macrophages express high levels of its receptor (CCR5) and respond to CCL5 produced by tumor cells by infiltrating to the TME (21, 22). Importantly, CCL5 has been reported to alter the functionality of TAMs toward a tumor-promoting phenotype in colorectal cancer (23).

Several other factors produced by tumor cells help recruit macrophages. Colony-stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor (M-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are other tumor-derived factors produced by breast cancer cells (12, 24, 25). CSF-1 expression is associated with poor prognosis and increased infiltration of CSF-1 receptor (CSF-1R)-expressing macrophages in mouse models of breast cancer (26, 27). Tissue factors important for angiogenesis, such as vascular permeability factor (VPF), are also known to attract monocytes to tumors (28). In line with this, increased VEGF expression in tumor cells correlates with macrophage infiltration in human breast cancer (29). Hypoxia is another well-described factor that alters the activation and accumulation of macrophages in hypoxic regions and prevents migration out of these regions (30–32). Recruitment of TAMs into hypoxic niches occurs in a hypoxia-induced Semaphorin 3A (Sema3A)-dependent manner through VEGF-R1 phosphorylation in a mouse model of breast cancer (33). VEGF signaling and hypoxia also play a crucial role in angiogenesis, an important hallmark of cancer, providing links between angiogenesis and TAMs (34).

To further add complexity to TAMs, there are at least three lineages of macrophages that arise at different stages of development and persist into adulthood (35, 36). Each tissue in the body is composed of 5–20% tissue-resident macrophages, which are yolk sac-derived and seeded during embryogenesis. During homeostatic adaptations, such as tumorigenesis, macrophages of different phenotypes can be recruited from the monocyte reservoirs of blood, spleen, and bone marrow (37) and from resident progenitors or through local proliferation (38, 39). One report documented the loss of resident macrophages and a concomitant increase in monocyte-derived TAMs in a breast cancer model (40). Tissue macrophages have vastly different transcriptional profiles between tissues, suggesting that the macrophages at metastatic sites may differ from the primary tumor site and may need to be targeted differently (36). It is currently unknown how the ontogeny of TAMs influences primary and metastatic breast cancer and the extent to which TAM origin is important for clinical outcome.

Differentiation and Maturation of TAMs in Breast Tumors

TAM differentiation and polarization is regulated by the tumor microenvironment (TME) and results in a heterogeneous population of cells, but the subset diversity is only just beginning to be understood. Tumors are generally poorly vascularized and lack nutrients, causing recruited monocytes to differentiate into mature wound-repairing macrophages. To an infiltrating monocyte, the tumor is a "wound" that needs repair, and therefore, TAMs polarize to an M2-like phenotype driven by CSF-1, IL-3, IL-4, IL-10, and TGF-β (**Figure 2**, step 2) (41, 42). TAMs subsequently show pro-tumor functions by promoting tumor cell survival, proliferation, angiogenesis, and dissemination (42–47). Whether or not differentiated macrophages are able to undergo M1/M2 repolarization after adopting a mature phenotype *in vivo* is still under debate, or if instead new monocytes recruited to the TME acquire their phenotype and remain in that state (41, 48–50). At least e*x vivo* M1-like human macrophages can be repolarized to M2-like macrophages upon exposure to M2 cytokines (51) and that M2-like macrophages are reprogrammed to express M1-like genes following exposure to TLR ligands or IFN-γ (52, 53).

PRO-TUMOR FUNCTIONS OF MACROPHAGES IN BREAST TUMORS

Promotion of Tumorigenesis

The contribution of TAMs to cancer progression and outcomes is multifaceted due to their wide spectrum of phenotypes. *In vitro* and *in vivo* studies have shown that macrophages are involved in a feedback loop between tumor cells at

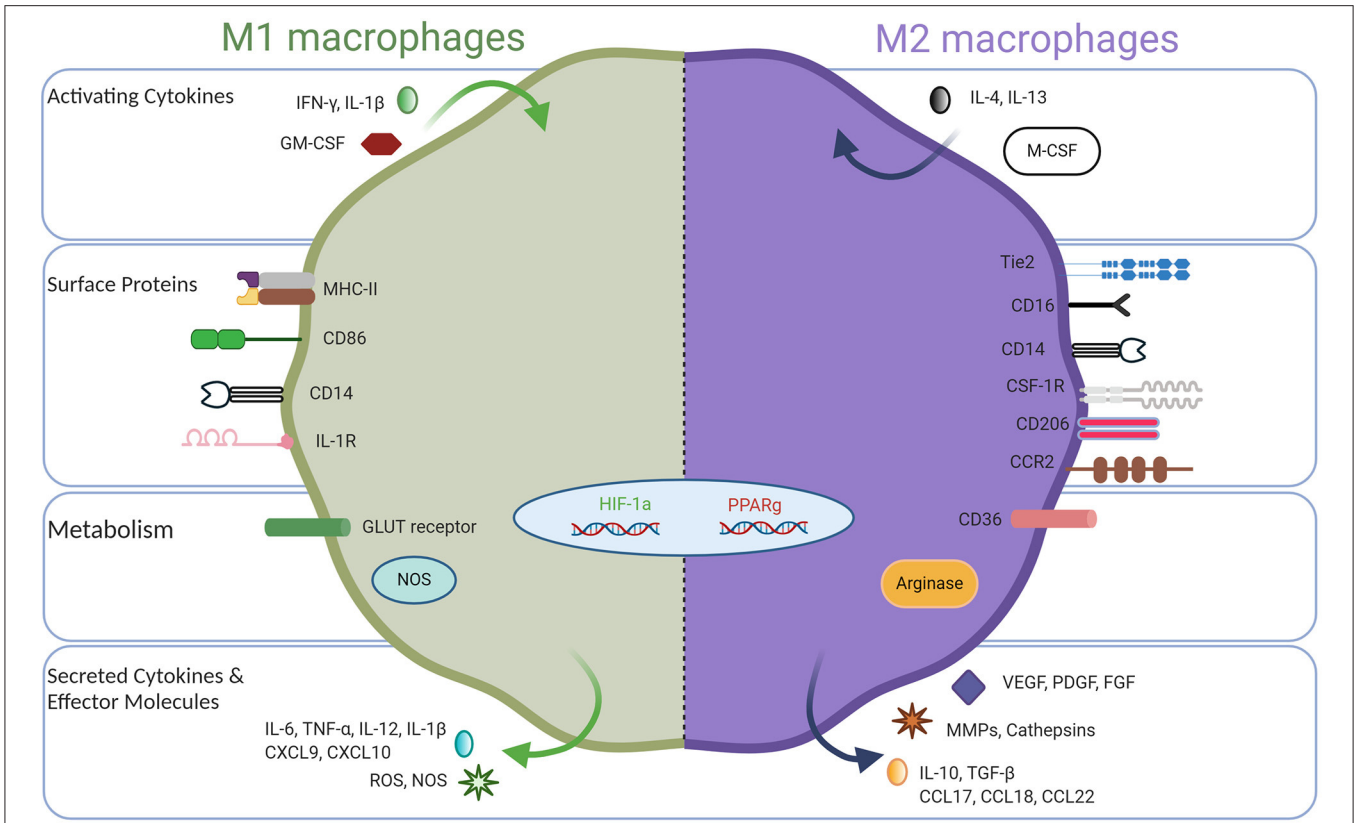


FIGURE 1 | Opposing phenotypes of M1-like and M2-like tumor-associated macrophages.

invasive fronts of breast tumors, where macrophages provide factors that enhance tumorigenesis. TAMs can directly promote the proliferation of tumor cells by secreting growth factors and inflammatory mediators such as CCL2, IL-1α, IL-6, and TNF-α (**Figure 2**, step 3) (54). Importantly, TNF-α secreted by TAMs leads to the activation of NF-κB in tumor cells, preventing tumor cell death and enhancing tumor cell invasion (55). In that regard, tumor cells recruit and activate M2-like macrophages, which then produce M2-related cytokines, such as CCL18, which causes breast cancer cells to elongate, lose contact inhibition, and increase vimentin expression. In a humanized murine model, anti-GM-CSF and anti-CCL18 both reversed the epithelial–mesenchymal transition (EMT) state of cancer cells, inhibiting metastasis. This concept was further confirmed with human breast tumors, revealing that high GM-CSF expression significantly corresponds to CCL18-expressing macrophages, cancer cell EMT and metastasis, and poor clinical outcome (56).

Facilitation of Angiogenesis

Angiogenesis is a complex, multistep process of forming new vasculature that facilitates tumor growth and progression (31). Macrophages are a major contributor of angiogenesis through the secretion of angiogenic cytokines (**Figure 2**, step 4) (57). TAMs secrete a key angiogenic cytokine, VEGF-A, directly, or indirectly by secreting matrix metalloproteinase 9 (MMP9) that activates latent forms of VEGF-A (29, 58, 59). TAMs also produce other pro-angiogenic factors from the fibroblast growth family that includes TGF-α, TGF-β, EGF, and PDGF (12, 60). Further supporting the role of macrophages in tumor angiogenesis, studies have shown that increased infiltration of TAMs promote angiogenesis whereas TAM depletion inhibits the angiogenic switch (61, 62). TAMs also contribute to angiogenesis by producing CCL18, which promotes angiogenesis both *in vitro* and *in vivo* (63). In addition to angiogenesis, CCL18 production from TAMs has also been associated with tumor invasiveness and metastasis in breast cancer (64). Furthermore, a subset of angiopoietin receptor Tie2-expressing monocytes, also known as TEMs, promote tumor angiogenesis (65, 66). In murine models, TEMs were initially identified as leukocytes expressing Tie2, CD11b, and CD45 in the peripheral blood and Tie2, CD11b, and Sca-1 in mammary tumors (65, 67). Additionally, TEMs also show a unique surface marker profile consisting of Tlr4, Mrc1, Il4ra, and CD163, which differentiate them from TAMs in murine mammary tumors (68). In humans, TEMs express CD45, CD11b, CD11c, CCR5, CD33, M-CSF-1R, and CD13 but lack expression of CD62L and CCR2 in circulating blood. Interestingly, TEMs from tumors of primary invasive breast carcinoma patients display different markers associated with antigen presentation, such as HLA-DR, CD80, CD86, and CD1a in addition to CD14, Tie-2, and VEGFR-1, which suggests their role in tumor-specific immune responses (69, 70).

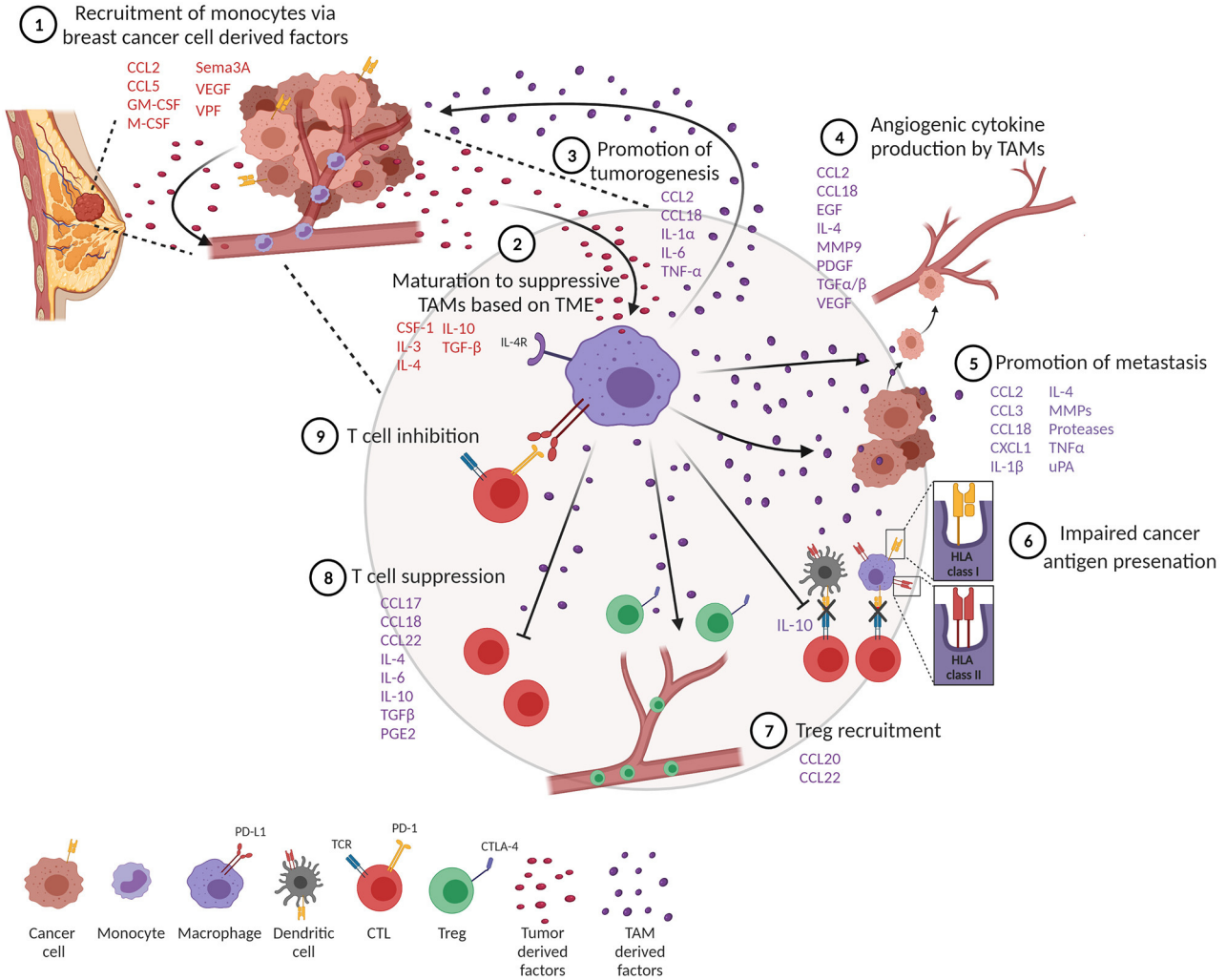


FIGURE 2 | Tumor-associated macrophages (TAMs) impede a productive anti-tumor immune response. (1) Monocytes are recruited to the tumor through tumor-derived factors. (2) One of the tumor monocytes mature to suppressive TAMs. (3) TAMs produce factors that promote tumor cell proliferation and survival. (4) TAMs promote angiogenesis and metastasis (5). (6) TAMs impair productive antigen presentation by dendritic cells and themselves downregulate MHC class I and II molecules. TAMs inhibit T cell function through recruitment of T regulatory cells (Tregs) (7) and suppression (8) and upregulation of co-inhibitory molecules (9).

TEMs are also capable of secreting large amounts of IL-10 and VEGF, promoting a pro-angiogenic and immunosuppressive environment that inhibits tumor-specific T cell responses in breast tumors (70).

Promotion of Metastasis

TAMs play a major role in tumor progression and metastasis by producing various matrix proteolytic enzymes including matrix metalloproteases (MMPs) and urokinase (uPA; **Figure 2**, step 5) (71, 72). This allows for the local invasion and attachment of cancer cells to other adjacent epithelial cells and the extracellular matrix (ECM) through the formation of adherens junctions. Next, the multistep process of invasion-metastasis occurs with intravasation of cancer cells to blood and lymphatic vessels, escape of cancer cells to distant tissues, and, finally, the growth of small lesions in tumors (34, 73). Direct visualization of macrophages using intravital multiphoton imaging showed that perivascular macrophages are important for the intravasation of cancer cells in the mammary tumor (74). In line with this, the genetic ablation of CSF-1 reduced macrophage density and slowed tumor progression and metastasis in the MMTV-PyMT model of breast cancer (75). CXCL1 secretion from TAMs is known to promote breast cancer migration and epithelial–mesenchymal transition in both mouse and human breast cells (76). Furthermore, a study showed that macrophages were required for breast tumor metastases, and Ets-2 transcription factor in TAMs was important for promoting angiogenesis and growth of both primary tumors and lung metastases (77). In another study, macrophage depletion using clodronate-encapsulated liposomes in an ovarian tumor reduced metastasis

in ovarian cancer (78). In the MMTV-PyMT mouse model of breast cancer, TAMs show high levels of cathepsin protease activity, which promotes tumor invasion and angiogenesis (79). It is also reported that IL-4 released from tumor cells and T cells induces cathepsin protease activity in TAMs to promote tumor invasion (79).

Importantly, tissue-resident macrophages at metastatic sites may vary greatly. For example, hypoxic primary breast tumor environments release lysyl oxidase (LOX), triggering NFACTc1-driven osteoclastogenesis, which increases bone resorption and thereby creates a metastatic niche for circulating tumor cells. Bisphosphonate treatment inhibits bone osteoclasts, which could be a potential combination therapeutic for patients with high-LOX primary breast tumors (80). The ability for therapeutics to reach sites of metastasis should also be considered when targeting TAMs. For example, in a murine breast cancer model, local pulmonary administration of CSF-1R inhibition allowed the drug to overcome lung physiological barriers of administration better than oral administration, significantly enhancing the M1/M2 ratio at a much lower dose. Therefore, orally administered medications may not effectively reach the lung-resident macrophages due to mucous layers, enzyme degradation, or mucociliary clearance; therefore, locally administered routes specific to lung metastatic sites should be considered (81).

Non-productive Antigen Presentation

Detrimental to the TME, protumor TAMs can become less efficient antigen-presenting cells, subsequently causing these cells to produce lower levels of pro-inflammatory cytokines and become less tumoricidal or able to activate T cells (82–84). TAMs that express pro-tumor features such as the IL-4R and arginase activity express low levels of MHCII (85). Subsets of TAMs characterized by lower expression of MHCII have been shown to be less effective in antigen presentation (85–87). In general, macrophages are less efficient in processing internalized antigens compared to conventional DCs, and macrophages in tumors appear to be greatly restricted in their antigen-presenting capacity (85, 86). Importantly, IL-10 derived from TAMs can also inhibit DC antigen presentation, further dampening tumor immunity (**Figure 2**, step 6) (88).

Suppression of T Cell Function

TAMs release anti-inflammatory cytokines that promote an immune-suppressive tumor microenvironment through the recruitment of Tregs, which suppresses effector T cell activation (**Figure 2**, step 7). For example, CCL22 and CCL20 secreted by TAMs induce an immunosuppressive microenvironment by recruitment of Tregs in ovarian and colorectal cancer (89, 90). In addition, TAM-derived chemokines CCL17, CCL18, CCL22, IL-4, IL-10, TGF-β, and prostaglandin-E2 (PGE2) have all been well-characterized in their ability to suppress T cell functions (**Figure 2**, step 8) (91–97). As a third way to inhibit T cell function, blocking B7-H4 on macrophages improves macrophage-mediated T cell activation (98), and at the heart of T cell activation as it relates to immunotherapy, TAMs induce immunosuppression through the expression of ligands that bind to PD-1 and CTLA-4 molecules on T cells, which inhibits T cell activation (**Figure 2**, step 9) (99).

MACROPHAGE METABOLISM AS A BARRIER TO ANTI-TUMOR IMMUNE RESPONSES

Macrophage metabolism is a critical feature of the macrophage phenotype. Previous work has revealed that M2-like macrophages have a distinct metabolic profile compared to M1-like macrophages (100). *In vitro*, the metabolic profile of macrophages activated with IL-4 (M2-like stimuli) is distinct from those activated with lipopolysaccharide (LPS) + IFN-γ (M1-like stimuli) (100). Studies have revealed that IL-4-stimulated macrophages depend on fatty acid oxidation (FAO) known as β-oxidation (101) and glutamine metabolism for the production of key metabolic intermediates such as α-ketoglutarate and succinate (100), to fuel the oxidative tricarboxylic acid cycle (TCA) cycle. IL-4 activates glutamine catabolism in macrophages, and interestingly, glutamine deprivation or inhibition of N-glycosylation decreased M2 polarization and production of the chemokine CCL22 (100). However, macrophages stimulated with LPS and IFN-γ utilize glycolysis and fatty acid synthesis (FAS) (102). Some early work has revealed that enhanced uptake of glucose through the induction of glucose transporter 1 (GLUT1) is a key feature of LPS-activated macrophages (103). Furthermore, A[1,2-^{13}C2] glucose tracer-based metabolomics approach, coupled with mass isotopomer distribution analysis of the newly formed metabolites, revealed that stimulated macrophages are highly glycolytic cells. The expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2), which regulates fructose-2,6-bisphosphate concentration and the glycolytic flux, was found to be the key molecular switch between the two phenotypic states and was independent of hypoxia-inducible factor 1α (HIF-1α) activation (104).

Metabolic regulation is a major difference between the extreme states of macrophage polarization and related to their downstream effector function. M1 macrophages preferentially depend on glycolysis, whereas M2 macrophages facilitates ATP production through the oxidative tricarboxylic acid cycle (TCA) coupled with oxidative phosphorylation (OXPHOS) (105–107). Previous work shows that M1 macrophages heavily depend on glycolysis to fulfill their energy demands; however, glycolysis only generates two ATP molecules per unit of glucose whereas the TCA cycle and OXPHOS generates 36 ATP molecules (108). It is speculated that M1 macrophages produce enough energy and intermediate metabolites for quick execution of effector functions like cytokine production and microbial killing (109, 110). Indeed, the metabolic differences between M1 and M2 macrophages are critical for their effector function. M1 and M2 macrophages display major differences in L-arginine metabolism via nitric oxide synthase (NOS) or arginase, respectively, which ultimately impact their functional outcome. NOS2 is activated through proinflammatory cytokines such as IFNγ, TNFα, IL-1β, and LPS (111) and has been well-characterized to metabolize

L-arginine to L-citrulline and cytotoxic nitric oxide (NO; a pro-inflammatory mediator responsible for anti-tumor immune response) (112, 113). The expression of arginase is induced by Th2 cytokines such as IL-4 and IL-13 that activate the signal transducer and activator of transcription 6 (STAT6), which acts with STAT3 and CCAAT/enhancer binding protein β (C/EBPβ) to bind to the arginase 1 enhancer locus. Additionally, GM-CSF, TGFβ, prostaglandin E2 (PGE2), cyclic adenosine monophosphate (cAMP), and TLR agonists can induce the expression of arginase 1 (111). In M2 macrophages, arginase catalyzes L-arginine to urea and L-ornithine (113). In M2 (wound repairing) macrophages, ornithine production promotes cell proliferation and repairs tissue damage through generation of polyamines and collagen, which are important for wound repair, but highly immunosuppressive in the TME (114–117) and involved in mediating TME remodeling (118). Recent studies have challenged this dichotomy of macrophage metabolism. A transcriptomic and metabolomics-based analysis of macrophages stimulated either with LPS or Pam3CysSK4 [P3C; a toll-like receptor (TLR) 2 agonist] revealed divergent metabolic responses such as TCA, OXPHOS, and lipid metabolism pathways between both stimuli, which regulated cytokine production and phagocytosis. Unlike LPS, the TLR2 ligand P3C induced a complex metabolic rewiring by upregulating both glycolysis and OXPHOS (119).

The Role of the TME on Mediating TAM Metabolism

The intricate communication between TAMs and the TME has been well-studied (36, 120–123), and the metabolic interactions between TAMs and cancer cells have been recently reviewed (124). There are growing efforts to understand the crosstalk between metabolic pathways, their metabolites, and the intracellular signaling in macrophages that in turn affects the epigenetic and transcriptional profile, which ultimately controls macrophage plasticity (100, 105, 125–127). One recent study showed that lactic acid production from tumor cells was an essential component in driving the M2-like polarization of TAMs by upregulating ARG1 and vascular endothelial growth factor A (VEGFA), with the effect of lactic acid being dependent on HIF-1α (128). Another study showed that lactate released from cancer cells acts as ligand for G protein-coupled receptor 132 (Gpr132, a pH sensor) that converts macrophages to an immunosuppressive M2-like phenotype. Importantly, genetic deletion of Gpr132 impaired M2-like macrophage phenotype with reduced tumor burden and metastasis in a preclinical model of breast cancer (129). In line with this, in areas of hypoxia, TAMs upregulate HIF-1α, which enables migration and survival (130), suggesting a feedback loop and signaling between tumor cells and TAMs that regulate their metabolic phenotype. Tumor-derived lactic acid has also been shown to induce the activation of the angiopoietin receptors (Tie1 and Tie2) and AXL receptors on TAMs. These receptors have been associated with pro-angiogenic and immunosuppressive phenotypes (131, 132). Similarly, macrophages treated with conditioned media from thyroid cancer cells underwent functional reprogramming. This effect was partly mediated by tumor cell-derived lactate that induced upregulation of cytokine production through an AKT1/mTOR-dependent increase in aerobic glycolysis. Immunohistochemistry (IHC) analysis confirmed the increase in glycolytic enzymes and lactate receptor in TAMs from human tumors (133).

Tumor cell-derived long-chain fatty acids including oleic acid have been shown to reprogram mitochondrial respiration in TAMs and subsequently induce polarization to an immune-suppressive phenotype through activation of the mTORC signaling pathway (134). In another study, tumor cell-derived succinate resulted in TAM polarization toward a pro-tumor phenotype through a succinate receptor (SUCNR1) and was dependent on the PI3K/HIF1α signaling pathway that resulted in enhanced metastasis (135). Similarly, prostaglandin E2 (PGE2), a prostanoid lipid synthesized by tumor cells, has been reported to polarize TAMs toward a pro-tumor phenotype through the cyclic AMP-responsive element binding (CREB) pathway (136, 137). These studies indicate that there is an intricate relationship between the factors secreted by the TME that dictates the outcome of macrophage phenotype and function. Additional studies to evaluate the differences of how the TME at different metastatic sites regulates TAM metabolism are warranted. An in-depth understanding of the crosstalk between TAMs and TME will be critical to design therapeutic strategies to target TAMs at both the primary and metastatic sites.

CLINICAL SIGNIFICANCE OF TAMS IN BREAST TUMORS

TAM infiltration is associated with poor prognosis, in a variety of cancers, including breast cancer (61, 92, 138, 139). This finding has been corroborated in a meta-analysis where TAM density significantly correlated with poor survival in patients with breast cancer (140). In an analysis of 11,000 breast tumors, the immune cell type that correlated most significantly with poor clinical outcome in estrogen receptor-positive (ER+) breast tumors was the presence of TAMs (141, 142). Clinically, the presence of TAMs is associated with metastasis (44) and poor survival (45–47) and has been shown to induce endocrine therapy resistance in ER+ breast cancer cells *in vitro* and *in vivo* through NF-κB- and IL-6-dependent signaling pathways (143). Importantly, a higher fraction of M1-like TAMs in ER+ breast cancer correlated with a higher pathological complete response (pCR) rate as well as prolonged disease-free survival (DFS) and overall survival (OS) (142). In a cohort of 40 HER2+ breast cancer patients who received trastuzumab, an anti-HER2 therapy, a high number of M1-like macrophages (iNOS+) were significantly associated with improved survival whereas high expression of M2-like macrophages (CD163+) were associated with worse prognosis (144). Additionally, high numbers of iNOS+ M1-like macrophages combined with high numbers of CD8+ T cells were significantly associated with improved survival, and this combined marker predicted a patient's ability to remain progression-free without trastuzumab after initially responding to therapy (144). This is in line with other reports demonstrating low TAM and high CD8+ T cell populations are associated with

better recurrence-free survival in patients with invasive breast cancer (145).

Relative Numbers of Macrophages Across Breast Tumor Subsets

Evidence for differential TAM regulation between breast tumor subsets comes from ER+ and triple-negative breast cancer (TNBC) cell lines co-cultured with macrophages. Tumor-induced phenotypic drift toward M2-like phenotype has been shown in differentiating bone marrow-derived macrophages (BMDMs) in breast cancer cell-conditioned media (146). Additionally, macrophages exposed to a TNBC cell line upregulated CCL2, reinforcing the concept that macrophage infiltration is a vicious feedback loop. In contrast, ER+ breast cancer cells co-cultured with macrophages were more broadly pro-inflammatory, secreting CXCL10, IL-2RA, and IL-3 (147). Furthermore, macrophage infiltration by breast cancer subtype has been explored using gene expression data with adjacent normal tissue as a baseline, which reported lower macrophage scores in ER+ tumors, especially ER+/HER2− subsets, in comparison to ER− tumors (148). A recent study examined immune-rich ER+ and immune-rich TNBC tumors based on microarray expression scores and compared relative fractions of immune cells. Although TNBC had a higher overall macrophage count, ER+ tumors had a higher relative percentage of M2-like macrophages and upregulation of TGF-β expression, which are both indicators of poor prognosis (149). TGF-β and IL-4 have been shown to upregulate YKL-39, a chitinase-like protein secreted by TAMs, which facilitates monocyte recruitment and angiogenesis. Importantly, YKL-39 expression levels were six times higher in patients with metastases than without metastases after neoadjuvant chemotherapy (NAC), independent of tumor stage or grade (150). YKL-39 has also been shown to be elevated in estrogen receptor 1 (ESR1) mutant metastatic breast cancer (151) and has been shown to be regulated by the androgen receptor in prostate cancer (152). These differences highlight unique targeted therapies, such as targeting the TGF-β pathway or reprogramming M2-like macrophages into M1-like macrophages, as having high potential, especially in ER-positive breast cancer.

Immunohistochemistry (IHC) has revealed that TNBC has significantly more tumor-infiltrating T cells and macrophages compared to non-TNBCs (153). To further understand the extent of macrophage infiltration in TNBC, immune infiltrates in 41 TNBC tumors were characterized using multiplexed ion beam imaging by time of flight (MIBI-TOF) (154). The fraction of infiltrating immune cells ranged widely from 1 to 91% of cells in the analysis fields selected from each tumor. On average, macrophages were among the most abundant immune cell in the tumor region, accounting for 25% of the immune population. Other immune populations also significantly contributed to the immune landscape, including the following: $CD8^+$ (19%), $CD4^+$ (15%), regulatory T cell (1%), B-cells (11%), and NK cells (<1%). However, these numbers varied greatly between patients. In fact, macrophages comprised as high as 100% of the immune population in tumors with low immune infiltration or as low as 5% in more immunologically abundant TNBC tumors. Our group has recently investigated macrophage abundance in TNBC using multiplex cyclic immunofluorescence (CyCIF) (155–158). We also found that TAM infiltration can vary greatly between tumors (**Figure 3**). In addition, we recently reported that TNBC harboring mutations in the breast cancer gene 1 (BRCA1) has a nearly 10-fold increase of TAMs compared to *BRCA1*-wildtype TNBC (159). This work highlights that tumor cell intrinsic mutations, such as BRCA mutations, may play a key role in regulating the TME. Indeed, the current and other studies have shown that BRCA-deficient cancer cells have an increase in cytosolic DNA leading to STING pathway activation and secretion of type 1 interferon-related cytokines such as CXCL9 and CXCL10 that can recruit immune cells, including macrophages, to the tumor (160, 161). Another work has confirmed an increase in CD68+ macrophages in TNBC compared to other subsets, and interestingly, breast tumors with high frequency of c-Myb-positive cells, identified through mRNA levels in breast cancer patients from public datasets, were correlated with a lower density of CD68+ macrophages, which was also found within the molecular subtypes (162).

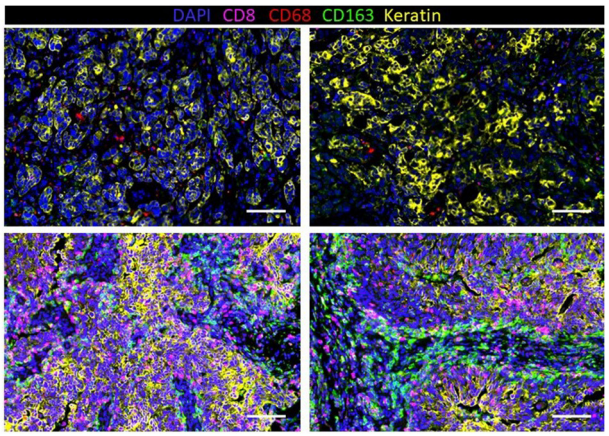


FIGURE 3 | Infiltration of macrophages (CD68 and CD163) and T cells (CD8) in human triple-negative breast cancer (TNBC) with a low density of immune cells (top row) and high density (bottom row).

Relative Numbers of Macrophages in Breast Cancer Progression

Not only does macrophage abundance vary between breast cancer subtypes but also with the stage of cancer progression. In preclinical models, macrophages have been shown to orchestrate early breast cancer dissemination and metastasis in a mouse model of HER2+ breast cancer, where CCL2 produced by both cancer cells and myeloid cells recruited CD206+/Tie2+ macrophages to propagate the disease (163). Similar results were seen in the MMTV-PyMT murine model of luminal B breast cancer, where blocking CCL2 prevented recruitment of TAMs to breast tumors, reduced metastasis, and prolonged the survival of mice (16). Other preclinical studies have shown a significant

correlation between CSF-1 and breast cancer metastasis using the MMTV-PyMT model (26).

Compared to normal breast tissue, macrophage numbers are significantly higher in ductal carcinoma *in situ* (DCIS) and remain higher through progression to invasive ductal carcinoma (IDC) (164). TAMs from human breast cancer have been shown to have a distinct transcriptomic signature from macrophages in healthy breast tissue, which is enriched in more aggressive forms of breast cancer (165). Additionally, CD68+ macrophages (166, 167), especially those defined by authors as M2 macrophages (CD68+PCNA+), have been shown to be elevated in high-grade compared to low-grade DCIS (167). Recent work by Gil Del Alcazar et al. compared immune cell infiltration in HER2+ and TNBC as a function of disease progression by comparing DCIS to IDCs (168). IDC had higher numbers of macrophages compared to DCIS. Interestingly, in DCIS, higher fractions of CD8+ T cells were associated with a significantly higher frequency of macrophages. Gene expression profiling revealed that Th1 and Th2 cells were enriched in HER2+ IDCs, while TNBC IDCs had enrichment of Th17 cells and T regulatory cells (Tregs). For TNBC tumors, but not HER2+ tumors, the transition from DCIS to IDCs correlated with an increased number of TILs, but fewer were in the activated state, indicative of T cell exhaustion in advanced stages of TNBC. Macrophages were not examined in this context, indicating that further characterization is needed to fully understand how macrophages participate in different stages of cancer progression.

Location of Macrophages in Breast Tumors

In addition to breast cancer subtype and disease state, the location of macrophages within the TME may be a predictor of their function and correlate with clinical outcome. However, the field has not yet come to a consensus on the extent of region-specific TAM behavior as a prognostic factor. Generally, macrophages in the stroma are associated with angiogenesis, immunosuppression, and cancer cell migration. In contrast, macrophages in the cancer nest tend to be more heterogeneous across cancer types and are correlated with reduced overall survival (OS) and recurrence-free survival (RFS) in breast cancer (169). Gwak et al. found stromal, intratumoral, and total TAMs to all have similar prognostic value, while Merdeck et al. reported stromal TAMs, but not tumor nest TAMs, are significantly correlated with tumor size and high tumor grade (45, 170). Recently, CD68 and CD163 were evaluated in both tumor nests and tumor stroma in 60 specimens of invasive breast cancer. High numbers of CD68+ TAMs in the tumor stroma were significantly associated with larger tumor size and positive nodal metastasis, whereas high numbers of CD163+ TAMs in the tumor stroma were significantly associated with positive vascular invasion, nodal metastasis, and molecular subtypes (171). Macrophage behavior may be modulated by breast cancer subtype and TME location, which in turn may regulate tumor behavior with stimulatory and inhibitory signals. For example, in HER2+ and basal-like subtypes, macrophages concentrate in invasive fronts and use TGF-β signaling to thicken the extracellular matrix (ECM), contributing to breast tumor metastasis (172). Therefore, not only are macrophages shaped by their location within the TME but they also reciprocally influence the surrounding TME composition. The significance of histologic localization of TAMs and the degree of TAM infiltration add additional layers of complexity to targeting TAMs in breast tumors for anti-cancer therapy.

Macrophage Characterization After Therapy

The characterization of TAMs before and after therapy may provide insight to how TAMs may change with treatment and play a role in drug resistance and metastasis. Increases in macrophages have been reported after chemotherapy in both preclinical and clinical settings (145). In a primarily ER+ patient cohort, neoadjuvant chemotherapy increased the percentage of CSF-1R+ macrophages (173). In line with these findings, Waks et al. examined changes in TAM populations after neoadjuvant chemotherapy in ER+ breast cancer, reporting an influx of CD68 macrophages with a larger proportion associating with an M2-like phenotype (174). These findings are in line with preclinical studies showing chemotherapy significantly increased F4/80+ macrophages in chemotherapy-sensitive tumors, but not chemotherapy-resistant tumors, indicating that macrophages are recruited to the site of chemotherapy-induced apoptosis and play a role in drug response (175). These changes in the TME could be implicated in mechanisms of chemotherapy resistance.

MACROPHAGES AS A BARRIER TO CHEMOTHERAPY AND IMMUNOTHERAPY

While the heterogeneity of TAM populations is still being deconvoluted, clinical evidence suggests that macrophages can contribute to shortcomings of chemo- and hyphenate immunotherapy. High TAM density has been shown to be an independent poor prognostic marker in breast cancer patients, especially for HR-positive breast cancer (170). Importantly, macrophages have been shown to contribute to reduced efficacy of chemotherapy. Shree et al. discovered that *in vitro* cathepsin-expressing BMDMs shield mammary cancer cells from paclitaxel-induced cell death. They further demonstrated that tumors from MMTV-PyMT mice treated with paclitaxel had increased TAM infiltration, and cathepsin inhibition in combination with paclitaxel increased long-term survival (176). Similarly, tamoxifen-resistant ER-positive and HER2-negative clinical samples had a higher density of CD163+ macrophage populations and increased expression of EGFR than tamoxifen-sensitive samples, which positively correlated with tumor size and metastasis (177). Furthermore, macrophages can disrupt T cell infiltration, which immunotherapies rely on to mediate efficacy. For example, in lung cancer patients, macrophage exclusion of CD8+ T cells from tumor nests correlated with poor response to anti-PD-1 therapy. When macrophages were depleted with anti-CSF-1R therapy, CD8 T cells successfully infiltrated the tumor to interact with malignant cells and delay tumor progression (178). Also, importantly, in preclinical breast cancer models, when macrophages are depleted using anti-CSF-1R therapy (145, 178) or their phenotype was converted to an anti-tumor phenotype

(179), anti-PD-1 therapy induced potent anti-tumor immunity. This highlights the deleterious effects of TAMs in tumors and the importance of targeting both innate and adaptive immune cells to achieve the full potential of immunotherapy and a durable anti-cancer immune response.

Targeting TAMs for Anti-Cancer Therapy

Both preclinical and clinical strategies to target the tumor-promoting functions of TAMs in cancer are being developed. These approaches have been reviewed in great detail and include inhibiting the recruitment of macrophages to tumors by blocking the CCL2–CCR2 or CCR5–CCL5 axes, depleting TAMs by blocking CSF-1 or CSF-1R; blocking macrophage "checkpoint inhibitors" such as CD47/SIRP1α, PD-1/PD-L1, MHCI/LILRB1, and CD24/Siglec-10; and suppressing macrophages' pro-tumor activity (inhibition of TGF-β or VEGF) (36, 180–184). Depletion or inhibition of macrophages using CCL2, CSF-1, and CSF-1R inhibitors has been shown to be effective against both mouse and human tumors (16, 36, 145, 185). Importantly, a recent study showed that CCL2 inhibition as a monotherapy led to more metastasis when the therapy was discontinued, which was driven in an IL-6- and VEGF-dependent manner (186). This study challenges the use of CCL2 as a monotherapy and highlights the need to understand the tumor microenvironment composition for successful anti-metastatic therapy.

CSF-1R is a promising target to address TAMs therapeutically, as high expression of CSF-1 or CSF-1R predicts cancer progression and mortality (187). Blockade of CSF-1R has been shown to decrease TAM infiltration, which subsequently results in the increase in CD8+ T cells and improves response to chemotherapy (95, 145). In a phase Ib study with advanced solid tumors, the combination of pexidartinib, a CSF-1R inhibitor, and paclitaxel was well-tolerated, and the combination showed reduced macrophage infiltration in the tumor microenvironment (188). However, in another phase I a/b study, emactuzumab, a monoclonal antibody against CSF-1R, showed reduction in immunosuppressive TAMs but did not demonstrate clinical benefit alone or in combination with paclitaxel (189). These studies suggest that a careful evaluation of the TME is important before deciding which patients would best benefit from CSF-1R therapy. Other caveats to anti-CSF-1R therapies include reports showing that inhibition of CSF-1R signaling can promote breast cancer metastasis (190).

To enhance anti-CSF-1R therapies, combining anti-CSF-1R with complementary chemotherapy and agents that enhance T cell function may markedly improve outcomes. In that regard, a recent study showed that addition of a CD40 agonist before anti-CSF-1R therapy induced a short-lived hyperactivated macrophage state that was enough to generate an effective T cell response in ICB-resistant tumors (191). Additionally, we have recently shown that CSF-1R inhibition leads to a significant reduction in TAMs and when combined with PARP inhibitor therapy results in an increase in overall survival, with some mice experiencing tumor-free survival for at least 1 year (159). Studies with CSF-1R signaling antagonists, combined with the drug paclitaxel or carboplatin, showed enhanced tumor control and reduced metastasis in preclinical models of breast cancer. Importantly, blockade of CSF-1 signaling also enhanced anti-tumor immunity and cytotoxic T cell infiltration to chemotherapy (145). The blockade of the CCR5–CCL5 axis, which decreased macrophage infiltration in tumors, is another exciting therapeutic target with ongoing clinical trials for breast cancer (192).

An alternative strategy is to convert pro-tumor TAMs to an anti-tumor phenotype. CD40 agonists (193), PI3Kγ inhibitors (194), CD47 inhibitors (195), and a class IIa HDAC inhibitor (179) have all been shown to reduce primary and metastatic murine breast tumors (179) and have emerged as novel modalities to convert TAMs to anti-tumor macrophages. In addition, other strategies have been shown to convert TAMs to an M1 phenotype and include Bruton's tyrosine kinase (BTK) inhibitors (196), TLR agonists (197), STAT3 inhibitors (198), IL-1Ra inhibitors (199), and LILRB2 inhibitors (200) Taken together, strategies to deplete or inhibit suppressive TAM functions or activate anti-tumor TAMs combined with chemotherapy and/or immunotherapy may have a great potential for the treatment of breast cancer patients. However, while many of these compounds in preclinical and clinical development are now filling our toolbox with TAM-targeting strategies, it will likely be necessary to further elucidate the complexity of TAM subsets including their ontogeny and phenotype for successful therapeutic targeting (**Figure 4**).

Therapeutic Targeting of TAM Metabolism for Anti-Cancer Therapy

The metabolic programming of TAMs is complex, and the underlying molecular mechanisms and crosstalk between tumor cells and stroma remain to be characterized. An in-depth analysis of these metabolic circuits may facilitate better appreciation for the functional fates of macrophages, including their pro- vs. anti-tumor phenotype. This important information would further support the clinical application of targeting TAM metabolism for anti-cancer therapy. There is some insight of the potential of this strategy from several recent publications that utilized other immune cell types including Tregs. Recently, Tregs were shown to activate the sterol regulatory element-binding protein 1 (SREBP1)-mediated fatty acid synthesis pathway in TAMs. SREBP1 induced M2-TAM metabolic fitness, mitochondrial integrity, and survival (201). Pharmacological inhibition of *de novo* fatty acid synthesis using a SREBP1 inhibitor, fatostatin, showed anti-tumor immunity when combined with ICB (anti-PD-1) in a B16 melanoma preclinical tumor model (201). Our group recently reported that PARP inhibition directly modulated macrophage metabolism by shunting glycolysis and inducing a dependence on lipid metabolism, which generated an immunosuppressive TME by inhibiting T cell function and thereby contributed to PARP inhibitor resistance (159). The use of fatostatin in combination with PARP inhibition and macrophage modulation significantly enhanced the overall survival of mice bearing brca1-deficeint TNBC (159). In line with our findings, inhibition of PARP induced upregulation of lipogenic genes by modulating the transcription factor specificity protein 1 (Sp1), which leads to the accumulation of lipid droplets

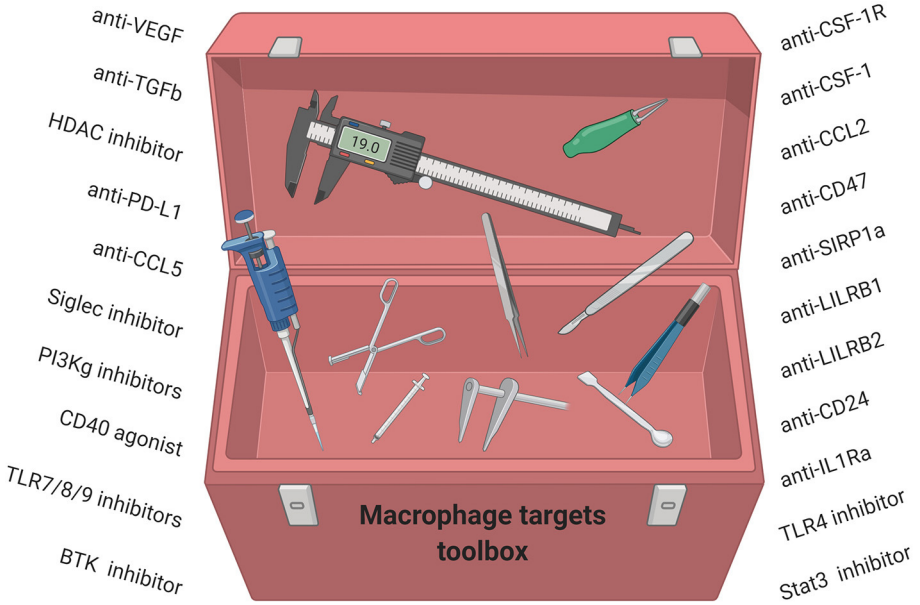


FIGURE 4 | Macrophage-targeting strategies for anti-cancer therapy have started to fill our toolbox. We now need to understand how these compounds work, which subsets of TAMs they modulate, and which breast cancer patients will benefit.

in the liver (202). Similarly, a study suggested that genetic deletion as well as pharmacologic inhibition of PARP induced the expression of ATP-binding cassette transporters (ABCA1) and cholesterol efflux in macrophages (203). These studies highlight the role of both Tregs and PARP inhibitors in regulating macrophage lipid metabolism. Further molecular understanding on the mechanisms of how PARP inhibitors regulate TAM metabolism would provide future opportunity for promising therapeutic strategies.

In a preclinical syngeneic model of pancreatic ductal adenocarcinoma (PDAC), a TLR9 agonist, CpG oligodeoxynucleotide, induced a metabolic state that required fatty acid oxidation and shunting of TCA intermediates for *de novo* lipid biosynthesis. This shift in central carbon metabolism activated highly phagocytic macrophage that could overcome the CD47 "don't-eat-me" signals on tumor cells to mediate an antitumor response (204). Macrophages cultured with PDAC-conditioned media compared to normal pancreatic cells had higher levels of vascular network formation, enhanced metastatic potential, increased levels of EMT, and a pronounced glycolytic signature. Inhibiting hexokinase II (HK2) with 2-deoxyglucose (2DG) inhibited glycolysis and reversed the pro-tumor TAM phenotype, highlighting the therapeutic potential of modulating TAM metabolism for anti-cancer therapy (205). Molecular metabolic control of TAMs has been demonstrated *in vitro* by inhibiting glutamine synthetase (GS). In human monocytes, GS expression activates an M2-like phenotype, which is reversed through pharmacological inhibition of GS by methionine sulfoximine (MSO). Inhibition of GS resulted in production of succinate, a critical regulator of the pro-inflammatory response, and enhanced glucose flux through glycolysis. Importantly, in *ex vivo* studies, GS restored T cell recruitment. *In vivo*, genetic deletion of macrophage-specific GS reduced metastasis in a preclinical mouse model of lung cancer (206). Taken together, precisely targeting the metabolic rewiring of TAMs may re-educate their phenotype and may overcome TAM-associated immunosuppression.

BEYOND M1 AND M2 PHENOTYPES AND NEXT STEPS

To date, characterizing the diversity of macrophage subsets has been difficult, as researchers have relied on a limited number of macrophage markers, and gene expression profiling has been done in bulk tissue or total macrophage populations, which preclude detection of unique subsets. Although gene expression data is high throughput, it inherently lacks the ability to determine spatial relationships, precise cellular function, or biochemical analysis at a single-cell level. Another consideration for leukocyte-containing samples is the canonical expression of RNase, which could potentially degrade RNA transcripts and interfere with single-cell analysis sample quality. Likewise, protein analysis should be used to support gene expression data, but is comparably tedious and low yield. The intracellular glycoprotein CD68 is widely used in clinical studies as a TAM marker, but it also detects other cell types such as some lymphoid and non-hematopoietic cells (207, 208) and does not identify TAM phenotype or functional status. The scavenger receptor CD163 has also been used to identify some TAM populations and has been shown to associate with early recurrence and reduced survival in breast cancer patients (209). CD68 and

CD163 have been broadly used to show that high TAM density correlates with a worse clinical outcome in breast cancer but do not predict their functional phenotype (122, 140, 210). As high-resolution techniques uncover the heterogeneity and plasticity of macrophage populations, researchers will need to look beyond the binary M1/M2 nomenclature and incorporate an extensive panel of markers in their analysis. Indeed, TAMs have high plasticity and can express both M1-like and M2-like phenotypes simultaneously, creating a need for more nuanced categorizations of macrophages (169). Multiplex IF such as MIBI-TOF and CyCIF have the unique advantage of analyzing a relatively large number of proteins, while maintaining spatial context of the tumor. These technologies will be invaluable in the next era of TAM studies, in which distinct phenotypes will need to be pursued.

Immune-focused mass cytometry has shed light on the diversity of TAMs in breast cancer, where in a study of 144 breast cancer patients, 19 distinct subsets of myeloid cells were identified, which clustered into five broader categories (211). These clusters included CD14-expressing monocytes, early immigrant macrophages, tissue-resident macrophages, TAMs, and myeloid-derived suppressor cells, with each group containing several additional subsets of myeloid cells. The distribution of the 19 clusters was distinct regarding the location within the tumor microenvironment and the subtype of breast cancer. For example, ER– tumors had more PD-L1+ TAMs than ER+ tumors, while luminal B tumors contained a higher proportion of PD-L1+ TAM subsets than luminal A. Although the sample set was predominantly from ER+ tumors, the results expose high-resolution categorizations of macrophages that could shed light on current therapeutic barriers. This work highlights the complexity of macrophages and tumors and is the right step forward; however, it is yet to be understood what the functional significance of these subsets are for clinical outcomes (211). Assays that test the functional ability of macrophages are warranted including high-throughput efferocytosis assays or gene signatures that may predict enhanced efferocytosis. The abovementioned studies have contributed to understanding the immune profile related to macrophages in breast cancer subtypes using bulk and single-cell RNA-sequencing, single- and dual-color IHC, multiplex spatial analysis such as MIBI-TOF and CyCIF, as well as flow cytometry, which compile data with different perspectives and limitations. As researchers continue to assess the breast TME, it is critical to acknowledge the strengths and limitations of different methods regarding their resolution, accuracy, and ability to represent tumor heterogeneity.

TAMs play a major role in tumor progression and metastasis and promote a highly suppressive TME that may limit breast cancer therapy. Therefore, modulating TAMs in combination with chemo- and/or hyphenated immuno-therapy will be critical to achieve maximum tumor reduction and elimination. Thus, it is imperative to characterize TAM subsets and their location. In addition, differences among breast tumor subtypes and how TAMs change after therapy will be important to characterize, along with differences in tissue-resident macrophages at metastatic sites. To develop effective macrophage-targeting therapies for the treatment of breast cancer, it is critical to have a precise understanding of the unique macrophage populations in each subset of breast cancer, as well as their location within different regions of the tumor. In addition, when considering the variety of influences that macrophages have on the TME, it is apparent that the surrounding TME will also need to be considered. Therefore, it is crucial to assess the number of infiltrating macrophages, their phenotype and function, as well as their spatial location to other cells in the TME. Expanding beyond M1 and M2 macrophage nomenclature will enhance the field of cancer immunology by providing better understanding of the tumor environment for rationale design of immunotherapy strategies including future development of macrophage-targeting therapies (**Figure 4**).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

JG was supported by the Dana-Farber/Harvard Cancer Center (DF/HCC) Specialized Program of Research Excellence (SPORE) in Breast Cancer P50 CA1685404 Career Enhancement Award, The Susan G. Komen Foundation Career Catalyst Award CCR18547597, The Terri Brodeur Breast Cancer Foundation, and The Ludwig Center at Harvard.

ACKNOWLEDGMENTS

Images were created with BioRender.com.

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Conflict of Interest: JG is a consultant for Glaxo-Smith Kline (GSK), Codagenix, Verseau, Kymera, and Array BioPharma and receives sponsored research support from GSK, Array BioPharma, and Eli Lilly.

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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frontiers in Immunology

REVIEW
published: 26 April 2021
doi: 10.3389/fimmu.2021.642958

Tumor-Intrinsic Mechanisms Regulating Immune Exclusion in Liver Cancers

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Representing the fourth leading cause of cancer-related mortality worldwide, liver cancers constitute a major global health concern. Hepatocellular carcinoma (HCC), the most frequent type of liver cancer, is associated with dismal survival outcomes and has traditionally had few treatment options available. In fact, up until 2017, treatment options for advanced HCC were restricted to broad acting tyrosine kinase inhibitors, including Sorafenib, which has been the standard of care for over a decade. Since 2017, a multitude of mono- and combination immunotherapies that include pembrolizumab, nivolumab, ipilumumab, atezolizumab, and bevacizumab have been FDA-approved for the treatment of advanced HCC with unprecedented response rates ranging from 20 to 30% of patients. However, this also means that ∼70% of patients do not respond to this treatment and currently very little is known regarding mechanisms of action of these immunotherapies as well as predictors of response to facilitate patient stratification. With the recent success of immunotherapies in HCC, there is a pressing need to understand mechanisms of tumor immune evasion and resistance to these immunotherapies in order to identify biomarkers of resistance or response. This will enable better patient stratification as well as the rational design of combination immunotherapies to restore sensitivity in resistant patients. The aim of this review is to summarize the current knowledge to date of tumor-intrinsic mechanisms of immune escape in liver cancer, specifically in the context of HCC.

Keywords: hepatocellular carcinoma, immune escape, immune exclusion, immunotherapies, CTNNB1

OPEN ACCESS

Edited by:
Jason J. Luke, University of Pittsburgh Medical Center, United States

Reviewed by:
David James Pinato, Imperial College London, United Kingdom
Hongbin Wang, California Northstate University, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *17 December 2020*
Accepted: *24 February 2021*
Published: *26 April 2021*

Citation:
Lindblad KE, Ruiz de Galarreta M and Lujambio A (2021) Tumor-Intrinsic Mechanisms Regulating Immune Exclusion in Liver Cancers. Front. Immunol. 12:642958. doi: 10.3389/fimmu.2021.642958

INTRODUCTION

Liver Cancer and Hepatocellular Carcinoma

Liver cancers represent the fourth leading cause of cancer-related mortality worldwide, with estimates from the World Health Organization predicting over 1 million deaths in 2030 (1). As the second-most lethal malignancy behind pancreatic cancer and harboring a 5-year survival rate of 18%, liver cancers represent a major global health concern (1). The two most frequent forms of liver cancer are hepatocellular carcinomas (HCCs) and cholangiocarcinoma, which represent 80–90% and 6–15% of all primary liver cancers, respectively (2). While patients diagnosed with early-stage HCC may be eligible for potentially curative surgical resection, most patients are diagnosed with recurrent or advanced stage disease (1). Until recently, treatment options for advanced HCC were restricted to tyrosine kinase inhibitors (TKIs) that confer limited survival benefits (3–6). Sorafenib, a TKI, has been the standard of care for advanced HCC for over a decade (3) but confers a survival

benefit of merely 2.8 months over placebo. More recently, additional TKIs have been approved as first or second-line treatment for advanced HCC patients including regorafenib (4), cabozantinib (5), and ramucirumab (6). However, since 2017, two immune checkpoint inhibitors targeting the programmed cell death-1 (PD-1) pathway, pembrolizumab and nivolumab, the latter alone or in combination with the monoclonal antibody targeting cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4) have been FDA-approved as second-line treatment for advanced HCC (7–9). Most recently, the combination of monoclonal antibodies atezolizumab (anti-programmed death ligand-1; anti-PD-L1) and bevacizumab (anti-vascular endothelial growth factor; anti-VEGF) has shown, for the first time in any HCC clinical trial, superiority over sorafenib and is now FDA-approved as first-line treatment for advanced HCC (10). With objective response rates of around 30%, these immunotherapies have demonstrated unprecedented results in the treatment of advanced HCC (7–10). However, this also means around 70% of patients are insensitive to this treatment, making it imperative to understand mechanisms of immune escape in liver cancers in order to design novel combination therapies that restore sensitivity in these immunotherapy-resistant patients as well as identify biomarkers of resistance or response to improve patient selection.

Immunoediting and Immune Escape

To understand the notion of immune escape in cancer, it is important to first understand the concept of cancer immunoediting as well as the cancer immunity cycle. Immunoediting describes the process by which the immune system protects the host from cancers (i.e., immune surveillance); however, in doing so the immune system also places evolutionary pressure on malignant cells causing them to undergo immunogenic sculpting that enables disease progression (i.e., immune escape) (11, 12). Immunoediting proceeds through three phases: elimination, equilibrium, and escape (11). During the elimination phase, transformed cells that have escaped normal cell-intrinsic apoptotic/senescence checkpoints are recognized and killed by cells of the innate and adaptive immune systems (11). In the equilibrium stage, tumor subclones that survived the elimination phase (e.g., through the acquisition of additional genetic alterations that promote immune suppression) begin to expand (11). However, the overall net growth of the tumor is still being prevented primarily by the adaptive immune system, which maintains tumor cells in a state of functional dormancy (11, 12). Over time, the evolutionary pressure placed on the developing tumor by the immune system, coupled with the genetic instability associated with rapidly dividing malignant cells, promotes the selection and expansion of tumor subclones that have acquired alterations that suppress host immune responses and tumor cell destruction (11, 12). In this final stage of escape, tumor outgrowth is no longer restricted or blocked by the host immune responses and these tumor subclones emerge to cause clinically apparent disease (11, 12). While the immune system is capable of recognizing and killing malignant cells and constraining tumor growth, this theory of cancer immunoediting describes the process by which the same mechanism also promotes the emergence of malignant subclones that have undergone immunogenic sculpting to evade detection and destruction.

In the cancer immunity cycle (13) (**Figure 1**), certain somatic mutations in tumor cells result in the production of a modified protein product (neoantigen), which has the potential to be recognized by the host's immune system as foreign. Additionally, cancer-specific antigens resulting from expression of viral genes or aberrant expression can also be recognized by the immune system. These antigens can be released into the tumor microenvironment and sampled by dendritic cells, which travel to secondary lymphoid organs where they prime tumor antigen-specific adaptive (T and B lymphocyte-mediated) immune responses (13). Primed antigen-specific cytotoxic CD8+ T cells subsequently traffic to and lyse tumor cells that are presenting tumor-specific antigens through the Major Histocompatibility Complex (MHC) class I molecules, which results in the release of more tumor-associated antigens into the microenvironment (13) (**Figure 1**). The cancer immunity cycle represents the adaptive arm of the immune surveillance cancer immunoediting phase. However, innate immune cells, such as natural killer cells and γδT cells, also participate in immune surveillance by these cells (14–16). Tumors can escape immune surveillance through a variety of strategies, such as the acquisition of genetic alterations that perturb the aforementioned processes.

TUMOR-INTRINSIC MECHANISMS OF IMMUNE ESCAPE: GENETIC ALTERATIONS IN LIVER CANCER

Known tumor-intrinsic mechanisms that induce immune escape in the context of liver cancer are limited; however, a few studies have demonstrated that mutations affecting the WNT/ß-catenin pathway, which affects ∼27–37% of human HCC patients, promote immune escape in HCC. One group published correlative data on human HCC samples suggesting that ß-catenin activation may promote immune escape (17). Here, Sia et al. analyzed gene expression profiles from 956 HCC patient samples and virtuallyand, using a non-negative matrix factorization algorithm, separated the gene expression profiles from tumor, stromal, and immune cell compartments. Expression patterns were correlated to immune cell infiltration by pathology and immunohistochemical analysis. Then, using data from The Cancer Genome Atlas (TCGA), these immune cell gene expression profiles were correlated with chromosomal aberrations and mutations. Through this analysis, they found that around 25% of HCCs displayed high expression of inflammatory markers (termed "immune class"), either indicative of an adaptive T cell immune response or an immune exhausted phenotype. However, they also found that tumors with a "*CTNNB1*-mutation gene signature" were specifically excluded from the "immune class." This CTNNB1 class also showed lower T cell enrichment scores and downregulation of CCL-4. Previously, using an autochthonous mouse model of melanoma, Spranger et al. demonstrated that activation of the ß-catenin pathway led to impaired T cell priming through repression

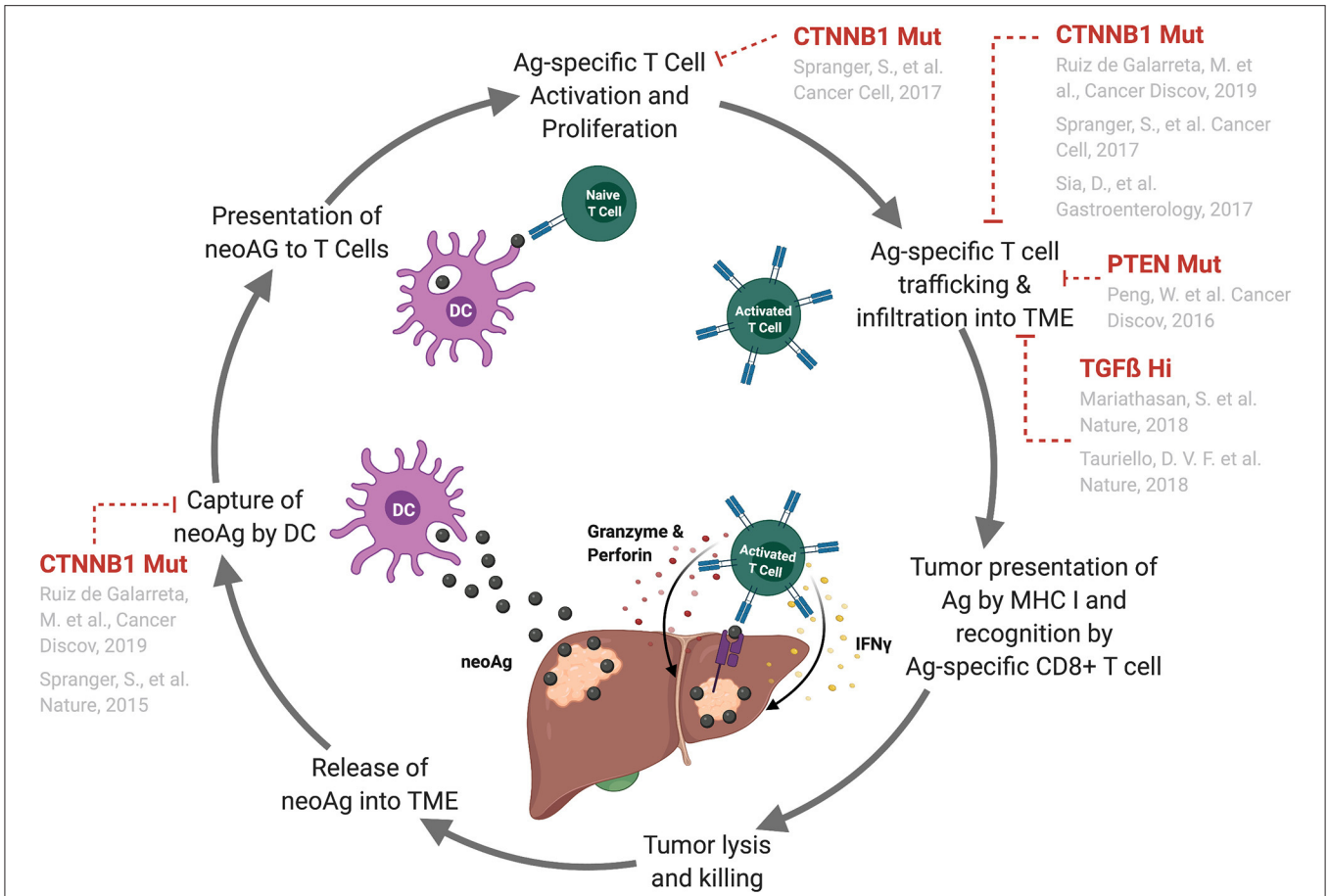


FIGURE 1 | Genetic alterations that perturb the cancer–immunity cycle and lead to tumor immune escape. Depiction of the cancer-immunity cycle (13). Tumor cells release antigens into the tumor microenvironment where they are sampled by circulating antigen presenting cells (APCs), such as dendritic cells. These APCs traffic to the lymphoid organs where they present antigens to T cells leading to T cell priming and activation. These activated T cells traffic back to the tumor site where they infiltrate, recognize, and kill tumor cells expressing their cognate antigen. Known mechanisms of immune escape in HCC and other tumor types that perturb specific points in this process are indicated. Abbreviations are as follows: neoAg, neoantigen; TME, tumor microenvironment; DC, dendritic cell; Ag, antigen.

of CCL-4-mediated dendritic cell recruitment to the tumor microenvironment and, subsequently, led to resistance to anti-PD-L1 and anti-CTLA-4 immunotherapy (18). However, the resistance to immunotherapy here could be due to the lack of endogenously activated T cell responses and thus no baseline infiltration into the tumors. A subsequent study by Spranger et al. used adoptive T cell transfer to address this and found that still, adoptively transferred effector T cells failed to traffic to the tumor site and this was due to the absence of CXCL9/10 production from CD103+ dendritic cells (those with the ability to cross-present extracellular antigen to CD8+ T cells) (19).

More recently, using a murine model of HCC based on hydrodynamic tail vein injection of genetic elements *in vivo,* our laboratory demonstrated that activating mutations in *CTNNB1* lead to immune escape in HCC (1). We generated two models with *MYC* overexpression and knockdown of *TP53*; one version that is non-immunogenic (*MYC-luc;sg-p53*) and one that is immunogenic due to expression of 3 model antigens (*MYC-lucOS;sg-p53*). The *MYC-lucOS;sg-p53* model had significantly better overall survival and decreased tumor burden compared with *MYC-luc;sg-p53* mice and this was found to be due to CD8+ T cell-mediated tumor clearance. We then generated two additional models overexpressing an activating form of ß-catenin (encoded by *CTNNB1*): non-immunogenic *MYC-luc;CTNNB1* and immunogenic *MYC-lucOS;CTNNB1*. There was no difference in survival or tumor burden between the non-immunogenic and immunogenic mice expressing an activating form of ß-catenin, which suggests that ß-catenin induces immune escape in the context of antigen expression. We found that this was due to diminished CCL5 expression, which in turn impaired dendritic cell recruitment to the tumor microenvironment and thus led to ineffective CD8+ T cell tumor clearance (1). Furthermore, overexpression of CCL5 restored dendritic cell infiltration into the tumors leading to active immunesurveillance and restored survival in the *MYC-lucOS;CTNNB1* mice. Finally, using TCGA data, Luke et al. showed across multiple tumor types, including HCC, an inverse correlation between a T-cell inflamed gene signature and

ß-catenin pathway activation (20). ß-catenin pathway activation was inferred through somatic mutations in pathway signaling elements, pathway prediction from RNA-sequencing data, as well as ß-catenin protein levels (20).

Correlative data from human HCC patients as well as mechanistic studies in mouse models of HCC are highly suggestive that tumor-intrinsic activating mutations in the WNT/ß-catenin pathway promote immune escape and resistance to immunotherapy in HCC. More specifically, these studies suggest the mechanism of immune escape is through defective recruitment of dendritic cells to the tumor microenvironment leading to inferior anti-tumor T cell responses (**Figure 1**). While mutations affecting the WNT/β-catenin pathway account for a large proportion of human HCC cases (27% to 37%), this disease is highly heterogenous with complex genetic etiology underlying each case. It is unlikely that the mutations affecting the WNT/β-catenin pathway are the sole genetic alterations that perturb effective anti-tumor CD8+ T cell responses and promote immune escape and resistance to immunotherapies in HCC. As immunotherapies have only recently demonstrated success in the treatment of HCC, to date, the roles of additional genetic alterations in promoting immune escape and response or resistance to immunotherapies in the context of HCC have not been well elucidated. In other solid tumor types, for example non-small-cell lung cancer, tumor mutational burden and microsatellite instability/mismatch repair deficiency have been implicated as good predictors of response to immune checkpoint therapies - the idea being that these tumors have a higher probability of expressing immunogenic neoantigens capable of eliciting anti-tumor immune responses (21–24). Currently, there is little evidence suggesting a prominent role of tumor mutational burden and microsatellite instability/mismatch repair deficiency as biomarkers of HCC responsiveness to immunotherapies. Two recent studies suggest that these features are, in fact, infrequent in HCC and poor predictors of response to immunotherapy in HCC (25, 26). As a relatively new field, more studies with larger cohorts of patients are needed to investigate the role of tumor mutational burden and microsatellite instability/mismatch repair deficiency as biomarkers of immunotherapy responsiveness in HCC. However, there are a few other genetic alterations with known relevance to human HCCs that have been shown to mediate immune escape and resistance to immunotherapies in other tumor types.

TUMOR-INTRINSIC MECHANISMS OF IMMUNE ESCAPE: GENETIC ALTERATIONS IN OTHER TUMOR TYPES

While studies investigating tumor-intrinsic mechanisms of immune escape involving acquisition of genetic alterations in liver cancers, and HCC in particular, are scarce, there are further examples published in the context of other solid tumors that may have relevance in HCC. One study by Peng et al. demonstrated that PTEN loss leads to decreased T cell trafficking to tumors and impaired T cell-mediated tumor killing in a murine model of melanoma (27) (**Figure 1**). Specifically, PTEN loss induced upregulation of CCL2 and VEGF expression and inhibited tumor cell autophagy (27). In melanoma patients, PTEN loss was associated with lower T cell infiltration in tumors and poorer response to anti-PD-1 immunotherapy (27). Though nothing has been published to date implicating PTEN in promoting immune escape in HCC, PTEN is altered in 7% of human HCC patients (28) making this pathway an appealing option for targeted therapies; however, future studies are needed to demonstrate whether or not tumor-intrinsic loss of PTEN leads to immune escape in HCC.

Another example of a tumor intrinsic mechanism of immune escape in cancer is overexpression of the Notch signaling pathway. Shen et al. demonstrated in a murine model of spontaneous mammary carcinoma that Notch overexpression leads to upregulation of proinflammatory cytokines, IL-1ß and CCL2, which in turn promote the recruitment of tumor-associated macrophages (29). Further, in breast cancer patients, expression data revealed a correlation between Notch activation, IL-1ß/CCL2 expression, and macrophage infiltration (29). It is possible that these findings hold true in the context of liver cancer, as NOTCH2 is amplified in 10% of HCC patients (28); however, again, further studies are needed to demonstrate a role for tumor-intrinsic Notch signaling in promoting immune escape in the context of liver cancer.

Finally, two studies have demonstrated a role for TGFß overexpression in inducing immune escape in solid malignancies. First, Mariathasan et al. showed TGFß expression from fibroblasts leads to T cell exclusion within the peritumoral stroma and subsequent resistance to anti-PD-1 immunotherapy in urothelial cancer (30). Additionally, Tauriello et al. demonstrated that stromal cell-derived TGFß overexpression induces T cell exclusion as well as prevents acquisition of Th1 effector phenotype and resistance to anti-PD-1 immunotherapy in colon cancer (31) (**Figure 1**). While TGFß production from stromal cells might not be considered "tumor-intrinsic," this immune escape mechanism may be important in the context of HCC as TGFß is overexpressed in 28% of human HCCs (28). Further, TGFß has been identified in multiple HCC classification systems based on expression data from HCC patients (17, 32).

TUMOR-INTRINSIC MECHANISMS OF IMMUNE ESCAPE: OTHER EXAMPLES IN LIVER CANCER

Beyond acquisition of genetic alterations that induce immune escape, there have been other tumor-intrinsic mechanisms of immune escape described in liver cancers. For example, two tumor-derived non-coding RNA molecules have been implicated in such mechanisms. Yang et al. reported that the pseudogene or long non-coding RNA RP11-424C20.2 as well as it's parental gene, UHRF1, are upregulated in HCCs and promote immune escape, in part, through the IFNgamma-mediated CTLA-4 and PD-L1 pathways (33). Similarly, Liu et al. provided a mechanism by which endoplasmic reticulum stress in HCC leads to the release of exosomes containing the microRNA miR-23a-3p,

which promotes immune escape through PTEN inhibition and subsequent upregulation of PD-L1 in macrophages (34). They showed that expression of proteins related to ER-stress were positively correlated with CD68+ macrophage recruitment and PD-L1 expression in HCC tissues (34). Furthermore, co-culture of macrophages stimulated with these exosomes and T cells led to a decrease in CD8+ T cells and IL-2 production as well as an increase in apoptosis in T cells (34). Finally, they found that miR-23a-3p levels in HCCs negatively correlated with overall survival (34). Another example of tumor-intrinsic immune escape described in HCC involves epithelial-to-mesenchymal-transition (EMT). A study by Shrestha et al. investigated the association between EMT and induction of immune checkpoint expression in HCC (35). TNFalpha induced EMT in Hep3B and PLC/PRF/5 cells and led to the upregulation of PD-L1, PD-L2, CD73, and B7-H3, whereas reversal of EMT (MET) led to suppression of these markers (35). In a cohort of 422 HCC patients, they demonstrated that high expression of TNFalpha and PD-L1 is associated with poor overall survival and expression of TNFalpha and PD-L2 was associated with increased HCC recurrence (35).

Additional examples of tumor-intrinsic mechanisms of immune escape described in liver cancer involve overexpression of secreted immunomodulatory molecules. For example, Chan et al. provided a mechanism by which IL-6-activated JAK1 phosphorylates PD-L1, which then results in PD-L1 glycosylation that maintains PD-L1 stability (36). Combination of IL-6 and TIM-3 antibody blockade resulted in synergistic T cell-mediated tumor killing *in vivo* (36). Further, they identified a positive correlation between IL-6 and PD-L1 expression in HCC patients, making this a potentially relevant and targetable mechanism in HCC (36). Another study by Li et al. detected indoleamine 2,3-dioxygenase 1 (IDO1) expression in tumor cells in 109/112 HCC patients analyzed and this expression was associated with CD8+ T cell infiltration (37). They also showed that IDO1 expression is significantly correlated with IFNgamma and CD8a transcripts in HCC and this is associated with better overall as well as disease-free survival (37). Additionally, Zhu et al. demonstrated that tumor cell-intrinsic osteopontin correlates with PD-L1 expression and tumor-associated macrophage infiltration in tumor tissues from HCC patients (38). Mechanistically, they showed that oseopontin promotes chemotactic migration of macrophages and PD-L1 expression in HCC through activation of CSF1R pathway (38). *In vivo*, dual blockage of PD-L1 and CSF1R resulted in enhanced anti-tumor immune responses and resulted in improved survival in mice with high expression of osteopontin (38). This was attributed to increased CD8+ T cell infiltration, reduced tumor-associated macrophages, as well as polarization of Th1 responses (38).

In addition to tumor-derived secreted molecules, overexpression of other molecules as well as surface receptors on tumor cells have been implicated in promoting immune escape in liver cancer. For example, Qiu et al. suggested a role for Annexin A2 in promoting immune escape in HCC by leading to an increase in regulatory T cells and expression of inhibitory molecules as well as a decrease in natural killer cells and dendritic cells (39). In another study, Zhou et al. demonstrated that tumor cell-intrinsic TLR9 activation negatively regulates PARP1 expression, promoting STAT3 phosphorylation, and leading to increased transcription of PD-L1 (40). They also show that TLR9 is positively correlated with increased STAT3 phosphorylation and PD-L1 expression while negatively associated with PARP1 expression in HCC patients (40). Finally, they demonstrated that combination therapy with TLR9 agonist and anti-PD-1 or anti-PD-L1 therapy inhibited HCC growth *in vivo* (40). Another example involved overexpression of decoy receptor 3 (DcR3) in HCC mediated by the TGFß-Smad-Sp1 pathway. Overexpression of DcR3 promotes Th2 and regulatory T cell while inhibiting Th1 differentiation and knockdown of DcR3 restored CD4+ T cell immunity (41). Another study by Ren et al. provided a mechanism by which CD147 expression on HCC tumor cells promotes immune escape through binding secreted cyclophilin A (42). This subsequently led to tumor cell proliferation through ERK1/2 pathway activation and knockdown of CD147 Hepa1-6 cells led to increased T cell chemotaxis (42).

Other studies have also demonstrated expression of immune checkpoint inhibitors on liver cancer cells, which attenuate anti-tumor immune responses. In this regard, Li et al. defined 5 subtypes of stage I/II HCCs based on gene expression profiles from TCGA, gene expression omnibus, and the International Cancer Genome Consortium that each differ in immune profile and clinical responses (43). For example, subtype C4 was associated with upregulation of immune profiles as well as expression of immune checkpoint inhibitors (e.g., PDCD1, CD274, CTLA4, etc.) whereas subtype C5 was associated with downregulation of the same immune profiles (43). Similarly, Zhou et al. characterized tumor-infiltrating lymphocytes from HCC patients who underwent surgical resection and found higher expression of PD-1, TIM3, LAG3, and CTLA4 on CD8+ and CD4+ T cells isolated from tumor tissue compared with control tissue or blood (44). They also found expression of PD-1, TIM3, and LAG3 was higher on tumor-specific CD8+ T cells compared with other CD8+ T cells (44). Tumor infiltrating lymphocytes with expression of these checkpoint inhibitors had higher expression of activation markers, but similar or lower levels of granzyme B expression compared to tumor infiltrating lymphocytes not expressing these checkpoints (44). Blocking antibodies against these checkpoints resulted in increased proliferation of CD8+ and CD4+ tumor infiltrating lymphocytes and cytokine production in response to stimulation (44). Another interesting study by Li et al. set out to investigate the mechanism of resistance of HCCs to MET inhibitors (45). They found that MET inhibitors promote immune escape through stabilization of PD-L1 and decreased anti-tumor T cell inactivation (45).

MECHANISMS OF IMMUNE ESCAPE: IMMUNE INFILTRATES IN LIVER CANCER

In addition to tumor-intrinsic mechanisms of immune escape, some studies in liver cancer have described immune escape due

to perturbations within the tumor-immune microenvironment. First, Dong *et al.* demonstrated in a cohort of 15 patients with multifocal HCC^{+} that those arising from intrahepatic metastasis vs. multicentric occurrence had a unique tumor-immune microenvironment (46). Specifically, those with multicentric occurrence had higher expression of immune checkpoint inhibitors and higher levels of immunoediting while those with intrahepatic metastasis had less T cell and M2-like macrophage infiltration (46). Another study found an association between M1-like macrophage infiltration and PD-L1 expression in HCC and further demonstrated that M1-conditioned media from THP-1 cells induced expression of PD-L1 in HCC cells (47). In this study, they identified IL-1ß to be the major driver of PD-L1 expression through transcription factors p65 and IRF1 (47). Additionally, Liu et al. found a role for CCL15-mediated recruitment of CCR1+CD14+ monocytes in promoting tumor invasion and metastasis and these tumor-derived monocytes also expressed high levels of immunosuppressive molecules including PD-L1, B7-H3, and TIM3 (48). Moreover, CCR1+CD14+ monocytes positively correlated with CCL15 expression and predicted survival in HCC patients (48). Aside from macrophages and monocytes, Ye et al. found that HCC patients show higher TIM-1+ regulatory B cell infiltration within tumors compared to peri-tumoral sites, and that these cells express IL-10 and promote CD8+ T cell suppression (49). Mechanistically, this was shown to be due to HMGB1 from tumor-derived exosomes, which lead to activation of B cells and expansion of TIM-1+ regulatory B cells through TLR2/4 and MAPK pathways (49). The accumulation of TIM-1+ regulatory B cells was associated with advanced stage HCC and was associated with reduced survival and predicted early recurrence of disease (49). Another interesting study by Kang et al. compared conventional HCCs (cHCCs) with HCCs containing immune cell stroma (isHCCs) and found that isHCCs had higher Epstein-Barr virus (EBV)-positivity in CD20+ tumor infiltrating lymphocytes (50). isHCCs also had higher CD8+ T cell infiltration, PD-L1 and PD-1 expression in tumor infiltrating lymphocytes, PD-L1 expression in tumors, and association with a favorable recurrence-free survival (50). However, paradoxically, a subgroup of isHCCs with high EBV-positivity in tumor-infiltrating lymphocytes demonstrated poorer recurrence free and overall survival as well as higher enrichment scores for CD8+ T cell exhaustion (50). Furthermore, *CTNNB1* mutations were not identified in isHCCs, whereas 24.1% of cHCCs harbored such mutations (50). Interestingly, viral infections such as hepatitis B precede many cases of liver cancer and these viral infections can lead to expression of unique viral antigens. However, there are also tumor antigens that are produced due to mutations generated throughout the process of tumorigenesis. A study conducted by Bubie et al. provided strong evidence that tumor neoepitopes are more immunogenic than viral epitopes in hepatitis B virally infected liver cancer and that this could potentially drive immune response in this context (51).

CONCLUSIONS

As an immune privileged site, the liver can tolerate the introduction of innocuous antigens without mounting an immune response (52). This is necessary as the hepatic portal system brings blood through the portal vein and hepatic arteries. The portal vasculature supplies blood from the gastrointestinal tract, spleen, and associated organs whereas the hepatic arteries bring oxygenated blood from the aorta. Though immune privileged, the liver is enriched in immune cells. The liver has the largest reservoir in the body of tissue-resident macrophages, which are called Kupffer cells (53). Additionally, the liver contains resident γδT cells, natural killer cells, B cells, and other antigen presenting cells (54, 55). More comprehensive reviews on liver immunology have been conducted (53–55). Furthermore, underlying liver diseases (e.g., hepatitis viral infections, alcohol abuse, or non-alcoholic fatty liver disease) occur in the majority of patients with HCCs (1), meaning most cases of HCCs arise in the context of chronic inflammation. Thus, tumor immunology in the context of liver cancer is likely a critical factor in disease initiation and progression. This is further supported by the recent unprecedented success of immunotherapies in the treatment of advanced HCC. However, there is still very little known regarding tumor-intrinsic mechanisms of modulating immune responses specifically in the context of liver cancer, but also in most tumor types in general. As a very heterogeneous disease, this is an exciting area of study in HCC and lends the opportunity to design personalized combination immunotherapies for patients with advanced HCC that are rationally designed based on unique genetic alterations and the mechanisms by which these genetic alterations induce immune escape. However, much more mechanistic work in this regard needs to be conducted.

AUTHOR CONTRIBUTIONS

KL drafted the manuscript. KL, MR, and AL reviewed and revised the manuscript. KL and AL conceived the review. All authors contributed to the article and approved the submitted version.

FUNDING

KL was supported by NIH 5 T32 AI78892-12; MRG was supported by Damon Runyon-Rachleff Innovation Award (DR52-18). AL was supported by Damon Runyon-Rachleff Innovation Award (DR52-18), Pfizer Emerging Science Fund, NIH/NCI R37 Merit Award (R37CA230636), and NIH/NCI R01 (R01 CA251155-01).

ACKNOWLEDGMENTS

Figure created with BioRender.com.

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Conflict of Interest: Research grants from Pfizer and Genentech for unrelated projects AL.

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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frontiers in Immunology

ORIGINAL RESEARCH
published: 07 May 2021
doi: 10.3389/fimmu.2021.637146

TIGIT and PD-1 Immune Checkpoint Pathways Are Associated With Patient Outcome and Anti-Tumor Immunity in Glioblastoma

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Glioblastoma (GBM) remains an aggressive brain tumor with a high rate of mortality. Immune checkpoint (IC) molecules are expressed on tumor infiltrating lymphocytes (TILs) and promote T cell exhaustion upon binding to IC ligands expressed by the tumor cells. Interfering with IC pathways with immunotherapy has promoted reactivation of anti-tumor immunity and led to success in several malignancies. However, IC inhibitors have achieved limited success in GBM patients, suggesting that other checkpoint molecules may be involved with suppressing TIL responses. Numerous IC pathways have been described, with current testing of inhibitors underway in multiple clinical trials. Identification of the most promising checkpoint pathways may be useful to guide the future trials for GBM. Here, we analyzed the The Cancer Genome Atlas (TCGA) transcriptomic database and identified PD1 and TIGIT as top putative targets for GBM immunotherapy. Additionally, dual blockade of PD1 and TIGIT improved survival and augmented $CD8^+$ TIL accumulation and functions in a murine GBM model compared with either single agent alone. Furthermore, we demonstrated that this combination immunotherapy affected granulocytic/polymorphonuclear (PMN) myeloid derived suppressor cells (MDSCs) but not monocytic (Mo) MDSCs in in our murine gliomas. Importantly, we showed that suppressive myeloid cells express PD1, PD-L1, and TIGIT-ligands in human GBM tissue, and demonstrated that antigen specific T cell proliferation that is inhibited by immunosuppressive myeloid cells can be restored by TIGIT/PD1 blockade. Our data provide new insights into mechanisms of GBM αPD1/αTIGIT immunotherapy.

Keywords: glioblastoma, immunotherapy, PD1, TIGIT, MDSCs, myeloid suppressor cell, gene network analyses

OPEN ACCESS

Edited by:
Sarah E. Church, NanoString Technologies, United States

Reviewed by:
Shane Curran, EMD Millipore, United States
Patrick Wen, Dana–Farber Cancer Institute, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *02 December 2020*
Accepted: *12 April 2021*
Published: *07 May 2021*

Citation:
Raphael I, Kumar R, McCarl LH, Shoger K, Wang L, Sandlesh P, Sneiderman CT, Allen J, Zhai S, Campagna ML, Foster A, Bruno TC, Agnihotri S, Hu B, Castro BA, Lieberman FS, Broniscer A, Diaz AA, Amankulor NM, Rajasundaram D, Pollack IF and Kohanbash G (2021) TIGIT and PD-1 Immune Checkpoint Pathways Are Associated With Patient Outcome and Anti-Tumor Immunity in Glioblastoma. Front. Immunol. 12:637146. doi: 10.3389/fimmu.2021.637146

INTRODUCTION

Malignant gliomas are the most common primary malignant central nervous system (CNS) tumor in adults (1). Glioblastoma (GBM) are highly aggressive brain cancers and the most common type of high-grade glioma (HGG) (2). The current standard of care for GBM patients include a combination of surgery, radiation therapy, and chemotherapy. However, even with standard of care, the median overall survival times remain less than two years (3, 4). Therefore, identification of novel GBM treatment strategies is warranted.

The immune system can mount specific and durable responses against tumors (5, 6). However, cancer cells, tumor-myeloid cells, and tumor infiltrating regulatory T cells (Tregs) can express negative regulators of the immune system including immune checkpoint (IC) molecules, thereby limiting effective anti-tumor immunity (7, 8). In recent years, the development of immunoregulatory drugs that block IC pathways, such as PD1/PD-L1 inhibitors, have emerged as a promising treatment strategy against a variety of malignancies, including melanoma, lung cancers, and head and neck cancers (9, 10). Although anti-PD1/PD-L1 immunotherapy shows durable response in other types of malignancies, its efficacy is limited to approximately 10% of GBM patients (11–13), thus highlighting the need for more effective and novel approaches, including the combination of additional IC inhibitors (ICIs) to target several IC pathways simultaneously.

T cell immunoreceptor with Ig and ITIM domain (TIGIT) is an IC receptor expressed on activated T cells, NK cells, and Tregs (14). Elevated TIGIT expression on TILs correlates with reduced TIL cytokine production and poor overall survival (14). TIGIT binds with high-affinity to CD155 (PVR) and with low-affinity to CD112 (PVRL2; nectin-2) which are expressed in the tumor microenvironment (TME) by antigen presenting cells (APCs) and tumor cells (15). The binding of TIGIT to CD155 suppresses the activation of TILs. CD155 can also bind CD226, which is expressed on T cells and provides a stimulatory signal which promotes T cell activation, thus competing with TIGIT binding to CD155. However, TIGIT has a significantly greater affinity to CD155 than CD226 (15, 16). While blocking the interaction between TIGIT and CD155 has been identified as a potential therapeutic target in treatment of malignancies, its effects in GBM are poorly understood (17).

MDSCs are myeloid-lineage regulatory cell that act as negative immune regulators in the TME (18). MDSCs consist of two major subtypes based on phenotype: PMN-MDSCs matched with granulocytes, and Mo-MDSC resembling inhibitory monocytes (18). In mice, PMN-MDSC are defined a $CD11b^{+}Ly6C^{low}Ly6G^{high}$, and Mo-MDSC as $CD11b^{+}Ly6C^{high}Ly6G^{low}$ whereas in human, PMN-MDSC are defined as $CD14^{-}CD11b^{+}CD33^{+}CD15^{+}$ and Mo-MDSC as $CD14^{+}CD11b^{+}HLA\text{-}DR^{low}$. Some studies have shown that increased presence of MDSCs within the TME is related to poor clinical outcome in patients treated with ICI (19). Consequently, reduced infiltration of MDSCs in TME has shown enhanced anti-tumor efficacy of ICI in pre-clinical tumor models (20, 21).

In order to identify putative IC targets in GBM, we first analyzed of The Cancer Genome Atlas (TCGA) dataset and identified IC molecules whose expression is associated with poor survival in GBM patients. We found that upregulated expression of *PD1* and *TIGIT*, but not other ICs or their ligands, are associated with reduced patient survival. We demonstrate that dual treatment with αPD1/αTIGIT prolonged survival in a murine GBM model, at least in part by targeting MDSCs. Together, our data provide new insights into mechanisms of immunotherapy in GBM.

MATERIALS AND METHODS

TCGA Data Analysis

The Cancer Genome Atlas (TCGA) database was used to assess survival of patients with GBM in accordance with gene expression levels of immune checkpoint molecules. Survival analysis was performed through the cBioPortal platform using a z-score of 1.0 for all checkpoint receptors and their respective ligands. The correlation of checkpoint gene expression with z score >2.0 was considered upregulated expression. Kaplan-Meier (KM) survival curves were generated to determine overall survival (OS) and disease-free survival (DFS).

RNA Sequencing (RNA-seq) and Pathway Analysis

The study uses RNA-seq datasets of GBM tissue from The Cancer Genome Atlas (TCGA), and the raw expression files were downloaded from TCGA Genomic Data Commons (GDC) Data Portal. Reads were quantified and mapped to human genome (Ensembl GRCh38 Homo sapiens) Salmon version 0.8.2 (22). Transcript-per-kilobase-million (TPM) were used for gene-correlation and pathway analyses. Pearson's rank correlation analysis was performed for *TIGIT* and *PDCD1*. Genes with statistically significant correlation (p value < 0.05 and false discovery rate (FDR) $p < 0.05$) were used to determine pathway enrichment using Gene Ontology (GO) (23) for Reactome (version 65 Released 2020-11-17) (24) and PANTHER Overrepresentation Test (Released 20200728) (25) curated pathways. Pathway enrichment cutoff was set for $p<0.05$ using Fisher's Exact test and FDR $p<0.05$ and enrichment scores greater than 1. Immunological network analysis was performed using ClueGo v2.5.7 (26) and Cytoscape 3.8 (27) with the current parameters: GO ImmuneSystemProcess EBI-UniPort, GO term fusion, network specificity was set to medium-detailed, pathways' p value <0.05 with Benjamini-Hochberg correction. Positively and negatively correlated genes were used to determine positively and negatively associated networks, respectively.

Single Cell RNA-seq (scRNA-seq) Analysis

scRNA-seq data were obtained from Wang et al. (28) and processed as described previously (28). Briefly, the neoplastic cells and non-neoplastic cells were separated *via* copy number variation (CNV). The presence/absence of CNVs was assessed with CONICSmat (29), and the primary cell types of non-neoplastic cells (i.e. monocytes/myeloid) were identified by using ELSA (30). $CD11b^{+}$ monocyte/myeloid cell population was sampled for further analysis using Seurat package on

Bioconductor (R) (31). Following Elbow Plot analysis, the number of principal components analysis (PCA) was set up to 3 with 0.2 resolution for UMAP clustering.

Cell Lines

GL261 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), 1x antimycotic-antimycotic solution (Gibco), 1% L-glutamine, ß-mercaptoethanol, 200 µg sodium pyruvate, and 1x NEAA. Cell lines were kept in a 37°C humidified incubator with 5% CO2. Cell number and viability were measured using the trypan exclusion method (0.4% trypan Blue, Gibco).

Mice

C57BL/6J mice (Stock No. 000664) and B6.Cg-Thy1 a/Cy Tg (TcraTcrb)8Rest/J (PMEL; Stock No. 005023 (32)) were purchased from the Jackson Laboratory and housed in animal facility of the UPMC Children's Hospital of Pittsburgh. Animals were kept in the facility for at least one week prior to performing any procedures to minimize stress-related symptoms. 5–6-week-old female were used in the experiments. All experiments were conducted following protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC).

Intracranial Tumor Model and Antibodies Treatment

Mice were anesthetized by mask inhalation of 1.5% vaporized isoflurane throughout the surgical procedure. GL261 cells (100,000 cells in 2 µL DPBS) were stereotactically implanted into the caudate nucleus using the following coordinates relative to bregma: x = +2.5 mm (lateral), y = +1.5 mm (anterior), and z = 2-3.0 mm (inferior) (33). MRI was performed 7 days post tumor cell implantation to confirm tumor presence, and again at day 40 to measure tumor size growth in control-treated animals and αTIGIT & αPD1 dual blockade-treated animals. All mice were randomized prior to their separation into treatment groups. IgG1 (clone MOPC-21), IgG2a (clone RTK2758), αPD1 (clone RMP1-14) and αTIGIT (clone 1G9) antibodies were obtained from Bio-X-Cell Antibodies were dosed at 200 µg per animal and administered *via* intraperitoneal (i.p.) injection, as described previously (34) for both the survival and immunophenotyping studies. Anti-TIGIT and anti-PD1 treatments were given on the same day twice per week starting on day 8, for a total of 7 doses. Mice were euthanized after receiving seven doses of immune-checkpoint inhibitor therapeutic antibodies (αTIGIT/αPD1) to investigate biological endpoint and immune cell phenotype. Mice were monitored for weight loss and morbidity symptoms for survival study. All survival experiments were repeated in triplicate with at 4-6 animals per group.

Mouse Immune Cell Isolation

For the biological endpoint study, mice were euthanized on day 22 (CO2 asphyxiation followed by cervical dislocation) post-tumor inoculation. Brains were dissected and processed for flow cytometry analysis. Brains were homogenized in Collagenase IV Cocktail (3.2 mg/mL collagenase type IV, 1.0 mg/mL deoxyribonuclease I, 2 mg/mL Soybean Trypsin Inhibitor). Samples were centrifuged for 5 minutes at 1500 rpm, followed by red-blood cell (RBC) lysis using ACK lysing buffer (Lonza). Cell viability was measured using the trypan blue exclusion method. Cells were resuspended in FACS Buffer (DPBS with 1% BSA) and centrifuged for 5 minutes at 300 g, after which the pellet was resuspended in FACS buffer. The cells were then stained with appropriate antibodies and acquired on a BD LSR Fortessa flow cytometer.

Isolation of TILs From GBM Patients

Patient-derived GBM tissue was dissociated, using Accutase (1:10), to form a single cell suspension (SCS). SCS was centrifuged at 1500rpm for 5 mins. The pellet was resuspended in 5mL of 70% Percoll solution. A Percoll gradient of 5mL of 37 and 5mL of 30% Percoll sequentially, was then overlaid onto the tumor-containing 70% Percoll solution. The tumor gradient solution was centrifuged at 2400 rpm for 20 minutes. Immune cells at the interphase were collected and washed once with PBS. The cells were then stained with appropriate antibodies and acquired on a BD LSR Fortessa flow cytometer.

Isolation of PBMCs

Peripheral blood samples were collected in preservative-free heparin tubes (10 U/mL) and layered into an equal volume of Ficoll-Hypaque density gradient solution (Amersham Pharmacia Biotech Ltd., Little Chalfont, UK). Samples were then centrifuged at 2250 rpm for 20 minutes. After removal of the top layer (plasma), the mononuclear cells (PBMCs) were collected and washed twice with PBS (Hyclone™, GE Healthcare). Cell viability was determined by trypan blue exclusion and exceeded 95%. The cells were then stained with appropriate antibodies and acquired on a BD LSR Fortessa flow cytometer.

Generation of Immunosuppressive Myeloid Cells From Bone Marrow (BM)

Immunosuppressive myeloid cells were generated as described previously (35). Briefly, tibia and femur-derived BM cell from C57BL/6j mice were cultured in complete DMEM media supplemented with 10 ng/ml each of GM-CSF and IL-4. On day 3, floating cells were removed, and medium was replaced with 1:1 complete DMEM media to GL261 tumor-derived conditioned media (TCM), supplemented with GM-CSF and IL-4 for 3 additional days prior to use.

Suppression of T Cell Proliferation Assay

T cell suppression assay was performed as described previously (21, 36). In brief, hGP100-restricted (B6.Cg-Thy1a/Cy TCR-transgenic) $CD8^+$ T cells were isolated from PMEL-mice (32) using magnetic bead separation (Miltenyi Biotec) and labeled with Cell-Trace proliferation dye (Invitrogen. Cat. No C34557) according to the manufacture guidelines. Feeder cells (antigen presenting cells) were generated from non-$CD8^+$ cell fraction and were treated with 10 µg/ml of mitomycin at 37°C for 1 hour to cease proliferation (37). T cells and feeder cells were co-cultured with BM-derived immunosuppressive myeloid cells in

the presence of 100 U/mL hIL-2 (PeproTech), 100 μg/mL hGP10025-33 peptide (antigen), and 10 μg/mL of either IgG2a (RTK2758) – as control, or αPD1 (RPMI14) and αTIGIT (1G9). Cells were collected and analyzed on day 4 by flow cytometry.

Flow Cytometry

Prior to cell surface staining, samples were stained with cell viability dyes (GhostDye or 7AAD) in PBS for 20 minutes in 4°C and then washed with FACS buffer. For mouse immune cell staining, the cell suspensions were blocked with 1% anti-mouse Fc-receptor (CD16/CD32) in FACS buffer for 20 minutes, then washed and stained with fluorescently labeled anti-mouse antibodies for 45 minutes in FACS buffer at 4°C. TILs (n=5) and PBMCs from 2 matched, 3 unmatched and 3 healthy donor (HD) patient samples (n=8) were washed with PBS and stained with cell-surface antibodies for 30 minutes at 4°C per the manufacture guidelines. After staining, cells were washed with FACS buffer and fixed with fixation buffer (BD Cytofix/Cytoperm buffer). The cells were washed with FACS buffer, resuspended in FACS buffer and analyzed by flow cytometry. The antibody clones were purchased from BioLegend or eBioscience and used for flow cytometry as follows: For mouse cell staining: CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD45 (30-F11), Gr-1 (RB6-8C5), CD3 (17A2, and 145-2C11), Granzyme B (QA16A02). For human cell staining: CD45 (2D1), CD11b (ICRF44), CD3 (C3e/1308), CD8 (OKT-8), PD-1 (EH12.2H7), PD-L1 (MIH2), CD33 (WM53), CD226 (11A8), TIGIT (A15153G), CD155/PVR (SKII.4). GhostDyes (TONOBO) UV450 and Red-780, and 7AAD were used to stain for cell viability (live/dead) according to the manufacture guidelines. Gating was performed on live $CD45^+$ cells to designate all immune cells. All samples were analyzed on a BD LSRFortessa (BD Biosciences). Data were analyzed using BD FACSDiva (BD Biosciences) and FlowJo V10 data analysis software (FlowJo LLC).

Statistical Analysis and Software

Kaplan-Meier survival curves were generated to determine survival and then compared using the log-rank Mantel Cox test. One-way analysis of variance (ANOVA) with Kruskal Wallis multiple comparisons test was used to compare assays containing more than two groups. Statistical significance was considered as $p < 0.05$. Normal distribution was assumed unless specified overwise in the text or figure legend. The analyses were performed using GraphPad Prism 8 or Bioconductor (R programing) on RStudio.

RESULTS

High Expression Level of Immune Checkpoint Molecules Associated With Overall Survival (OS) and Disease-Free Survival (DFS) in GBM

To identify putative immunotherapy targets for GBM, we evaluated the expression of IC genes and their ligands in RNA sequencing (RNA-seq) data of 153 GBM tumor samples in the TCGA database (38). We first assessed the correlation of 15 established IC gene expression levels with overall survival (OS) and disease-free survival (DFS) (39, 40). Upregulated expression was defined as expression z score greater than 2. Our data demonstrate that upregulated expression (red lines) of *TIGIT* and *PDCD1* (gene encoding PD1) were associated with poor patient outcome and increased mortality as compared with patients who had no change in *TIGIT* and *PDCD1* RNA expression (green lines) (**Figures 1A, B**). Upregulated *ICOS* expression was also associated with reduced OS and DFS, although the data did not reach significance (**Figures 1A, B**). However, upregulated expression of other IC receptor genes, including *CTLA4*, *LAG3*, TIM3 (*HVAC1*), *BTLA4*, and *CD224* were not associated with changes in OS and DFS. Interestingly, expression of genes for *CD155* (PVR), *PD-L1*, and *ICOS-L*, the ligands for TIGIT, PD1, and ICOS, respectively, was also significantly associated with decreased OS and DFS, whereas upregulated expression of other IC gene ligands did not affect these parameters in GBM patients (**Figures 2A, B**). Although our survival analysis assessed patients with elevated expressed based on Z score (i.e. compared to mean expression of that gene), we further examined the absolute expression of each gene to determine the extent of therapeutic utility among all patients. Our data show that a large portion of patients showed to have physiologically relevant expression levels (TPM>1) of the genes encoding to the checkpoint receptors PD-L1 (94%) and CD155 (PVR; 100%) (**Supplemental Figure 1**). Additionally, PD1 and TIGIT were reported to be expressed by large frequencies of GBM $CD4^+$ and $CD8^+$ TILs (34, 41). Taken together these data suggest that PD1/TIGIT-targeted therapy may be relevant for many patients with GBM.

TIGIT and PD1 Are Co-Expressed, Share Common Gene Networks, but Are Also Associated With Distinct Pathways in GBM

Our data revealed that TIGIT/CD155 and PD1/PD-L1 checkpoint genes were significantly associated with GBM clinical outcome, thus we next analyzed RNA-seq data from these patients to identify genes and pathways which may be involved with TIGIT and PD1 expression in GBM. Notably, the expression of *TIGIT* and *PDCD1* were significantly correlated with each other (**Figure 3A**), suggesting a rationale for dual blockade of these checkpoint molecules in GBM patients. Despite their significant correlation, *TIGIT* and *PDCD1* may be associated with unique gene networks and pathways (42). Therefore, we next interrogated the gene networks associated with the expression of *TIGIT* and/or *PDCD1* in GBM. We identified a total of 6347 genes which correlated with *TIGIT* and *PDCD1* expression with high statistical significance ($p<0.05$ and FDR<0.05) (**Figure 3B**). While many genes correlated with both *TIGIT* and *PD1* expression, we also identified a large number of genes and pathways uniquely correlated with either *TIGIT* or *PD1* (**Figures 3B, C**).

TIGIT/PD1 (shared)-associated pathways included immune related pathways, such as Toll-like receptor (TLR) signaling,

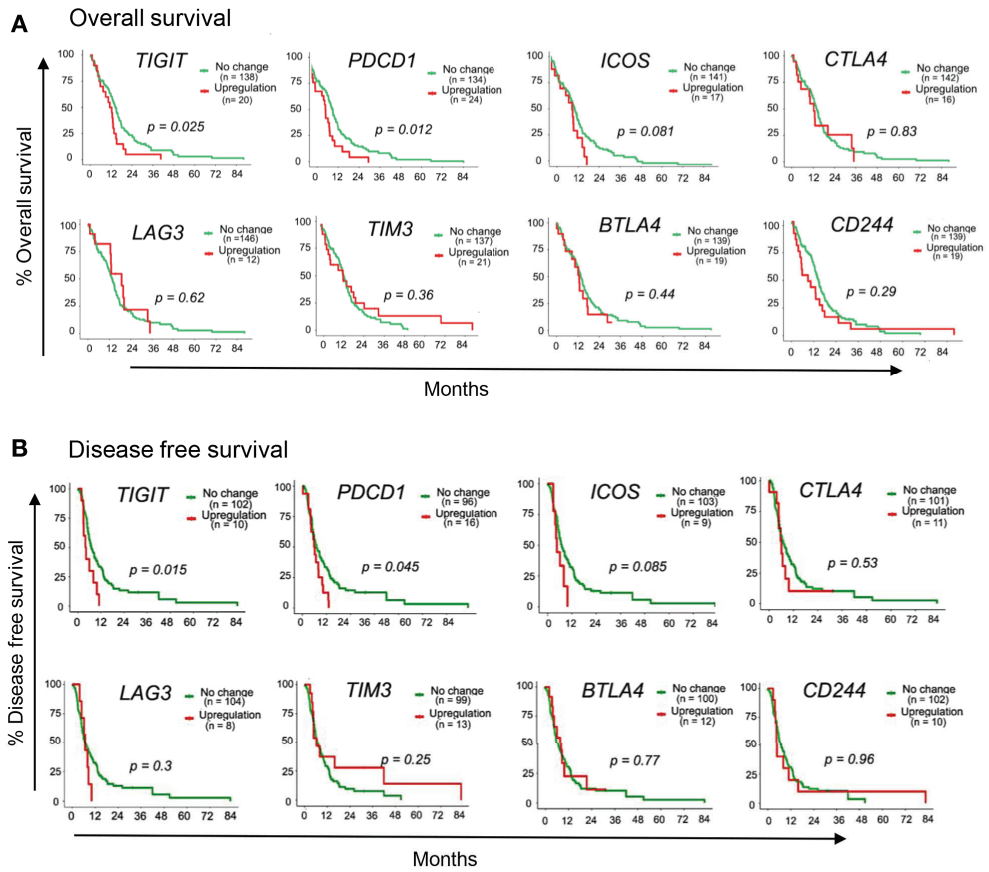


FIGURE 1 | Immune-checkpoint receptor genes associated with GBM patient outcome. TCGA patient survival data obtained from cBioPortal, and patients were grouped based on gene expression z-scores to *upregulated* expression (z ≥2; red line) or *no change* expression (z <2; green line). The **(A)** overall survival rate and **(B)** disease free survival rate, were plotted using Kaplan-Meier survival curves. P values reflect one-way ANOVA with Kruskal Wallis comparison test. n=153.

interleukins signaling (such as IL-10 and IL-2), TCR signaling and T cell activation, and innate immune system pathways (**Figure 3D**). Interestingly, TIGIT-associated pathways included Treg development, MHC class I presentation, caspases and death-receptors signaling, control of cell cycle transition, regulation of TLR and Nf-kB signaling, and p53 regulation (**Figure 3D**). PD1-associated pathways included cell motility, oxidation and phagocytosis, IL-12 mediated Jak-STAT signaling, MHC class II and antigen presentation, and EGF receptor signaling (**Figure 3D**). Immunological network analysis showed that many immune responses were strongly associated with the expression of TIGIT and PD1, mostly T cell activating and regulation of immunity, but also innate immune functions such as leukocytes degranulation, and functions of macrophages and dendritic cells (**Figure 3E**).

Together, these data suggest that upregulated expression of TIGIT and PD1 may confer immunosuppression and tumor aggression in GBM patients through both shared and distinct pathways, and therefore targeting both these pathways may be beneficial for improving clinical outcome of GBM patients.

Combination of αTIGIT and αPD1 Immunotherapy Increases Numbers of TIL Cytolytic CD8$^+$ T Cells and Prolongs Long-Term Survival of GBM-Bearing Mice

Our data suggest a beneficial outcome for IC blockade of TIGIT and/or PD1 in GBM. To investigate this hypothesis, C57BL/6 mice were intracranially injected with syngeneic GL261 cells, followed by 7 doses of immunotherapy with (1) isotype control antibodies, (2) αPD1, (3) αTIGIT, or (4) a combination of αTIGIT/αPD1 therapeutic antibodies, administered twice per-week starting on day 8 post-tumor injection (**Figure 4A**). Analysis of immune cells was performed uniformly across groups on day 22 post-tumor implantation (biological endpoint) followed by MRI analysis for tumor size on day 40 for control and dual αTIGIT/αPD1-treatment groups (**Figure 4A**). Control mice (Isotype; black line) displayed median survival of 33 days (range: 29-51 days) with severe morbidity signs and did not reach long-term survival endpoint (**Figure 4B**). While αTIGIT monotherapy (green line) moderately improved survival, treatment with αPD1 (blue line) or a combination

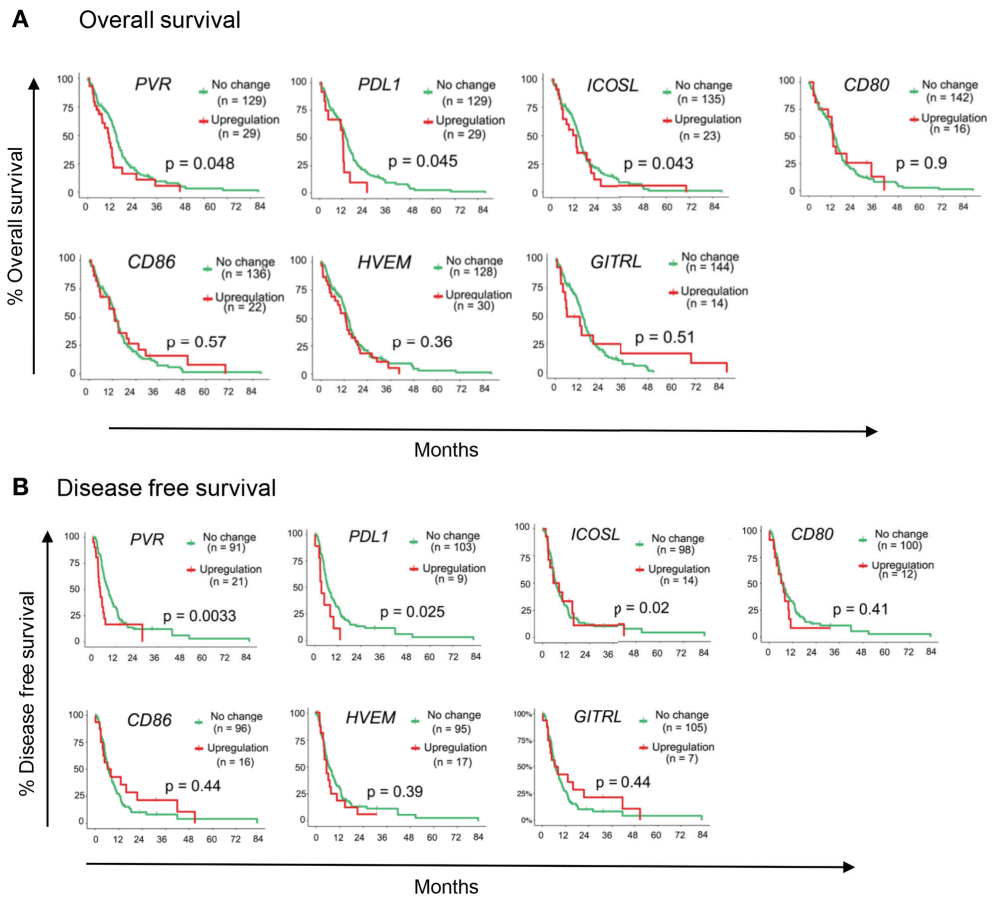


FIGURE 2 | Immune-checkpoint ligand genes associated with GBM patient outcome. TCGA patient survival data obtained from cBioPortal, and patients were grouped based on gene expression z-scores to *upregulated* expression (z ≥2; red line) or *no change* expression (z <2; green line). The **(A)** overall survival rate, and **(B)** disease free survival rates were plotted using Kaplan-Meier survival curves. P values reflect one-way ANOVA with Kruskal Wallis comparison test. n=153.

treatment of αTIGIT/αPD1 (red line) significantly prolonged animal survival as compared with isotype treated animals (**Figure 4B**). The median survival of αTIGIT treatment was 34 days (range: 32-43 days) while αPD1 monotherapy (green line) was 37 days (range 32-74 days). Notably, αTIGIT/αPD1 dual treatment most significantly prolonged mice survival with median survival of 48 days (range 39-74 days) (**Figure 4B**). MRI analysis showed that in αTIGIT/αPD1 treated animals the tumor size was significantly smaller than tumors in isotype-treated animals (**Figure 4C**). These data confirm previous results in which immunotherapy combination of αPD1 with αTIGIT reduced tumor burden and improved survival of mice with glioma (34).

To examine a mechanism by which the combination therapy improved anti-tumor immunity, we explored the effect of treatment on tumor-infiltrating lymphocytes (TILs) and their cytolytic phenotype on day 22 post tumor implantation. Although αPD1 monotherapy did not significantly affected the percentages of $CD4^+$ TILs, treatment with either αTIGIT or αTIGIT/αPD1 resulted in a significant increase of $CD4^+$ TILs as compared with control animals (**Figure 4D**). Additionally, we noted a significant increase in percentages of $CD8^+$ TILs in tumors in animals treated with αPD1 or αTIGIT/αPD1, but not in αTIGIT-monotherapy treated animals (**Figure 4E**). Analysis showed that treatment with either αPD1 or αTIGIT monotherapy resulted in a mild increase in the percentages of $CD8^+$ granzyme-B^+ TILs, while this effect was significantly increased in αTIGIT/αPD1 combination treatment (**Figure 4F**). These data are complementary to previous results showing that αPD1 or αTIGIT immunotherapy enhances the expression of TNFα and IFNγ in TILs from GBM (34) as well as other cancers (43), and suggest that the therapeutic effect of checkpoint blockade with αTIGIT/αPD1 may work through distinct mechanisms to affect $CD4^+$ and $CD8^+$ TILs and promote anti-glioma immunity.

Dual Blockade of TIGIT and PD1 Regulates MDSCs in GBM Murine Model

Previous reports have shown that MDSCs stimulate suppressive mechanism to develop a pre-metastatic niche, promote tumor growth, inhibit anti-tumor function of TILs, and negate immunotherapy which results as resistance to IC blockade (44,

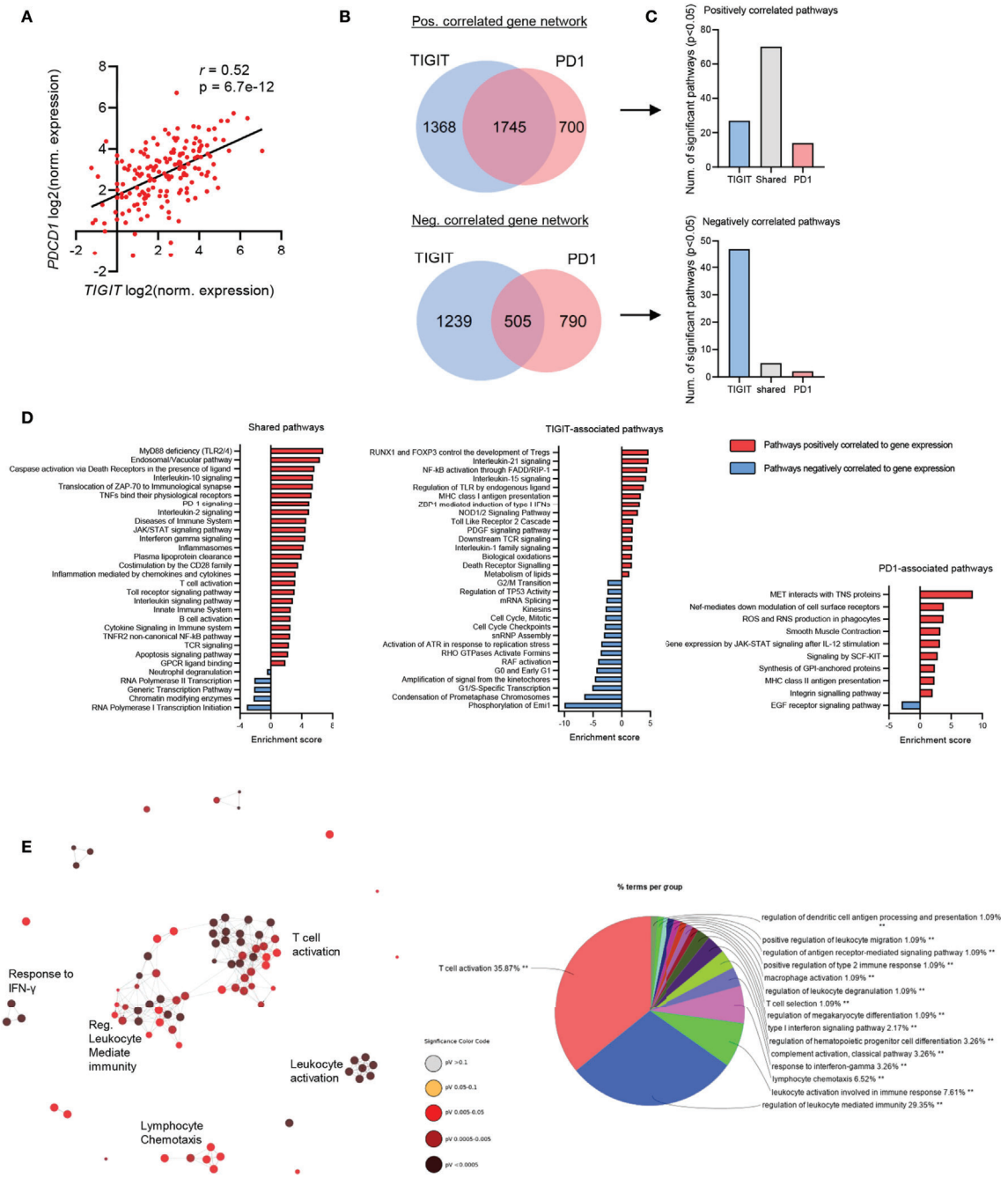


FIGURE 3 | *TIGIT* and *PDCD1* (PD1) exhibit shared immunological networks but have unique regulatory pathways in GBM. GBM patients' RNA-seq data was obtained from TCGA, transcript per million (TPM) normalized reads were calculated per each patient and Pearson's correlation analysis was performed. n=153. Genes with a statistically significant ($p<0.05$ and FDR<0.05) positive correlation and negative correlation to *TIGIT* and *PDCD1* expression were identified. **(A)** Pearson's correlation analysis of *TIGIT* and *PDCD1* expression. **(B)** Venn diagrams showing number of statistically significant correlated genes unique and overlapping within TIGIT and PD1 gene groups. **(C)** Number of statistically significant ($p<0.05$ and FDR<0.05) pathway enriched in each corresponding gene group. **(D)** Representative pathways which are positively and negatively enriched in the shared-gene group, TIGIT-associated group, and PD1-associated group. **(E)** Network analysis for Gene Ontology (GO) Immunological Processes associated with *TIGIT* and *PDCD1* positively correlated gene network. Statistically significant gene correlation and pathway enrichments were corrected for false discovery rate (FDR) using Benjamini-Hochberg test.

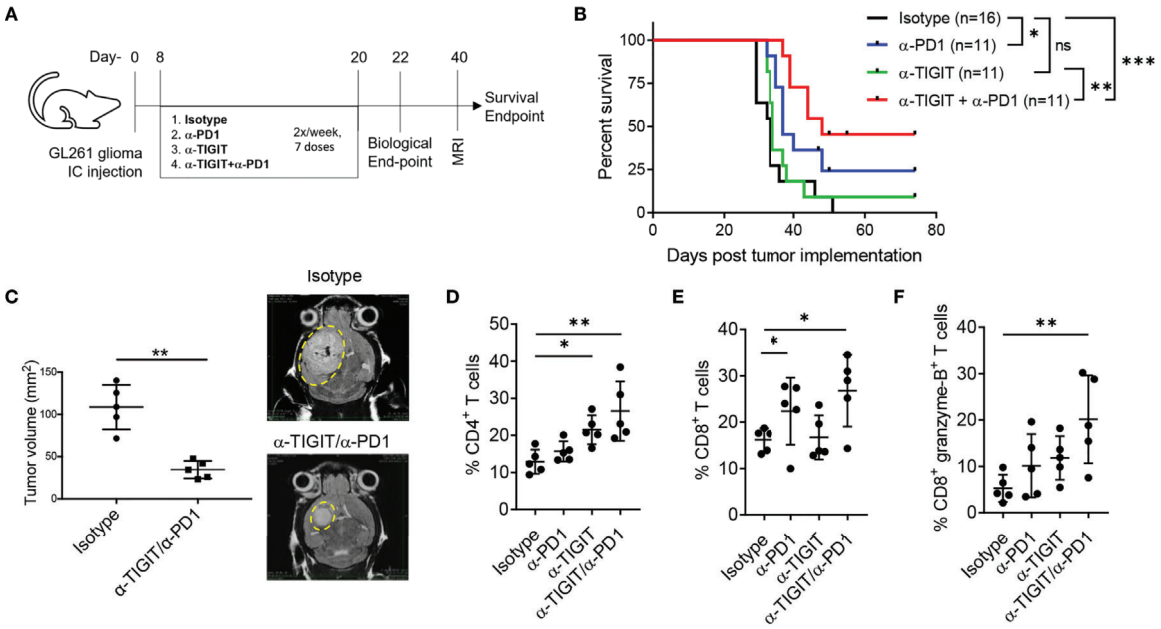


FIGURE 4 | Anti-TIGIT and anti-PD1 combination improves survival of GL261 glioma bearing mice. GL261 glioma cells were injected stereotactically in the caudate putamen of C56BL/6J mice followed by immunotherapy treatment starting on day 8 post tumor injection. Mice were evaluated for T cell responses on day 22 (biological endpoint) and for tumor size by day MRI on day 40. **(A)** Schematic showing induction of GL261 glioma in mice following treatment regimen using anti-PD1 and anti-TIGIT immunotherapies. **(B)** Survival curves with Log-rank (Mantel-Cox) curve comparison test. Pooled data from 3 independent experiments. **(C)** Pooled data and representative MRI images of tumor growth in murine GL261 glioma model in anti-PD1/anti-TIGIT treated group and isotype (control) treated animals. Unpaired *t* test with Welch's correction. n=5 per group. **(D–F)** Percentages (%) of CD45$^+$ glioma-infiltrating CD4$^+$ T cells **(D)**, CD8$^+$ T cells **(E)**, and CD8$^+$ granzyme B$^+$ T cells **(F)**, on day 22 following anti-TIGIT/anti-PD1 immunotherapy. Representative data of 3 experiments. n=5 per group. One-way ANOVA with multiple comparisons test corrected for false discovery rate (FDR) using Benjamini-Hochberg test. *P* values are as followed: *≤0.05, **≤0.01, ***≤0.001. NS, not significant.

45). Furthermore, MDSCs have been shown to contribute to immunosuppressive microenvironment in gliomas, including GBM (45–47). MDSCs were shown to express PD-L1 (48). Additionally, inhibition of TIGIT was reported to abrogate MDSC immunosuppressive capacity *in vitro* (49). Together, these data suggest that targeting PD1 and TIGIT pathways may affect MDSCs in GBM. However, the effects of these checkpoint on MDSC infiltration in gliomas are ill defined (45). We, therefore, investigated if MDSCs were affected by immunotherapy in our model on day 22 (biological endpoint; end of immunotherapy). Shown in **Figure 5A**, glioma infiltrating MDSC subsets were characterized by the expression of Gr1 and CD11b as follows: PMN MDSCs were defined as CD11b$^+$Gr1^{high} cells, whereas Mo MDSCs were defined as CD11b$^+$Gr1$^{intermediate\ (int)}$ cells (18). We evaluated the levels of MDSCs and their subsets following immunotherapy (**Figure 5A**; lower panel). Our data show, that compared with isotype treatment, both αTIGIT monotherapy and dual blockade of TIGIT & PD1 significantly reduced the frequencies of GL261 glioma infiltrating MDSCs (CD45$^+$CD11b$^+$Gr1$^+$ cells), most strikingly for αTIGIT/αPD1 combination therapy (**Figure 5B**). Treatment with αPD1 showed a trend of decreasing MDSC percentages, though the results did not achieve statistical significance (**Figure 5B**). Analysis of MDSC subsets revealed that αTIGIT/αPD1 dual treatment significantly decreased the frequencies of PMN MDSCs (**Figure 5C**), while Mo MDSCs levels remained mostly unaltered (**Figure 5D**). Furthermore, we observed a statistically significant increase in ratios of CD8$^+$ T cells over total MDSCs in tumors when mice were treated with αTIGIT monotherapy or αTIGIT/αPD1 combination therapy (**Figure 5E**). Blockade of PD1 alone did not significantly increase the CD8$^+$ T cells/MDSCs ratios (**Figure 5E**). Together, our data reveal a mechanism of TIGIT/PD1 blockade in glioma and suggest distinct roles of these ICs on MDSC subsets and in regulating tumor immunity.

Myeloid Cells Upregulate PD-L1 and TIGIT-Ligands in GBM Which Inhibit T Cell Functions

We next evaluated the potential of αTIGIT/PD1-immunotheraphy to impact MDSC-like cell in GBM patients. For that, we first analyzed single-cell (sc)RNA-seq data of CD11b$^+$ myeloid cells from GBM patients (28) for the expression of PD1, TIGIT, and their ligands. Myeloid cells were confirmed based on the expression of CD45 (*PTPRC*), *CD14*, and *CSF1R* genes (**Figure 6A**) (28, 50). Of note, we identified 4 unique clusters of tumor-associated myeloid/macrophage cells (TAMs) in GBM, which had distinct expression profiles (**Figure 6A** and **Supplemental Figure 2A**). The expression of CD33, an hematopoietic progenitor cell marker which commonly used to identify pan-MDSCs (51), was distributed throughout the TAM clusters. Nonetheless, the expression of inhibitory and suppressive markers, including genes for IL-4R, IL-10, IL-6, VEGFA, CCL2 and IL-1β (**Figure 6A** and

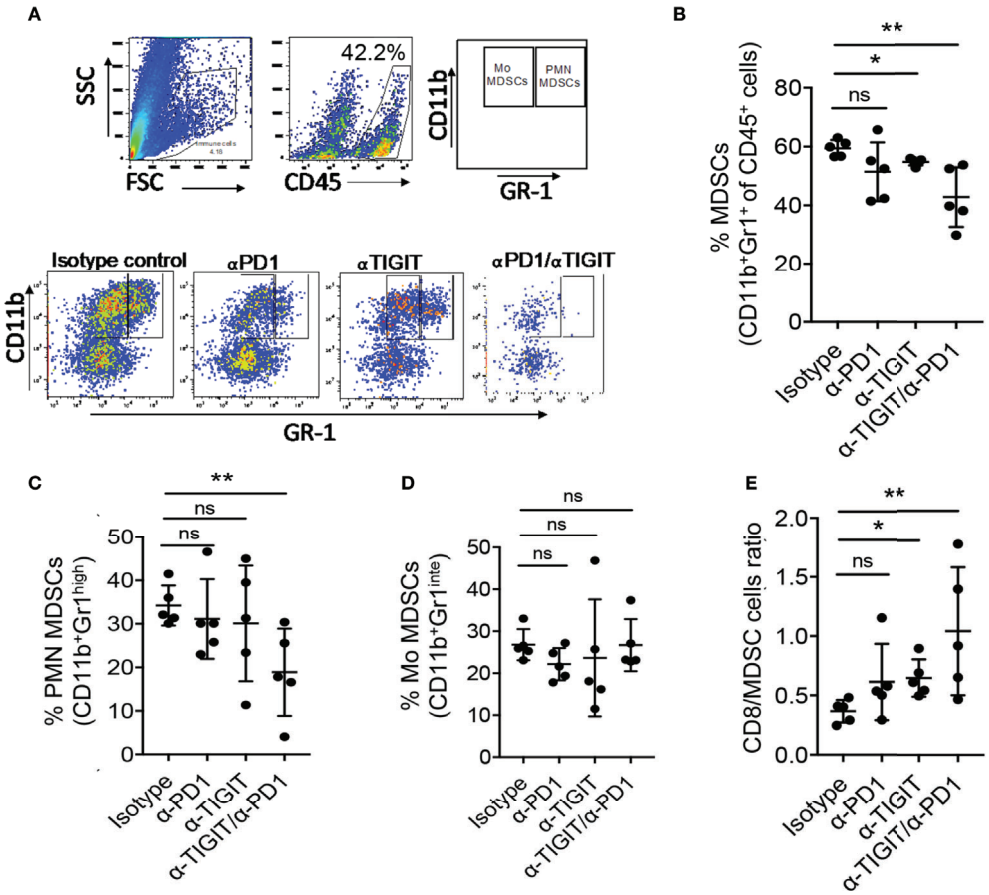
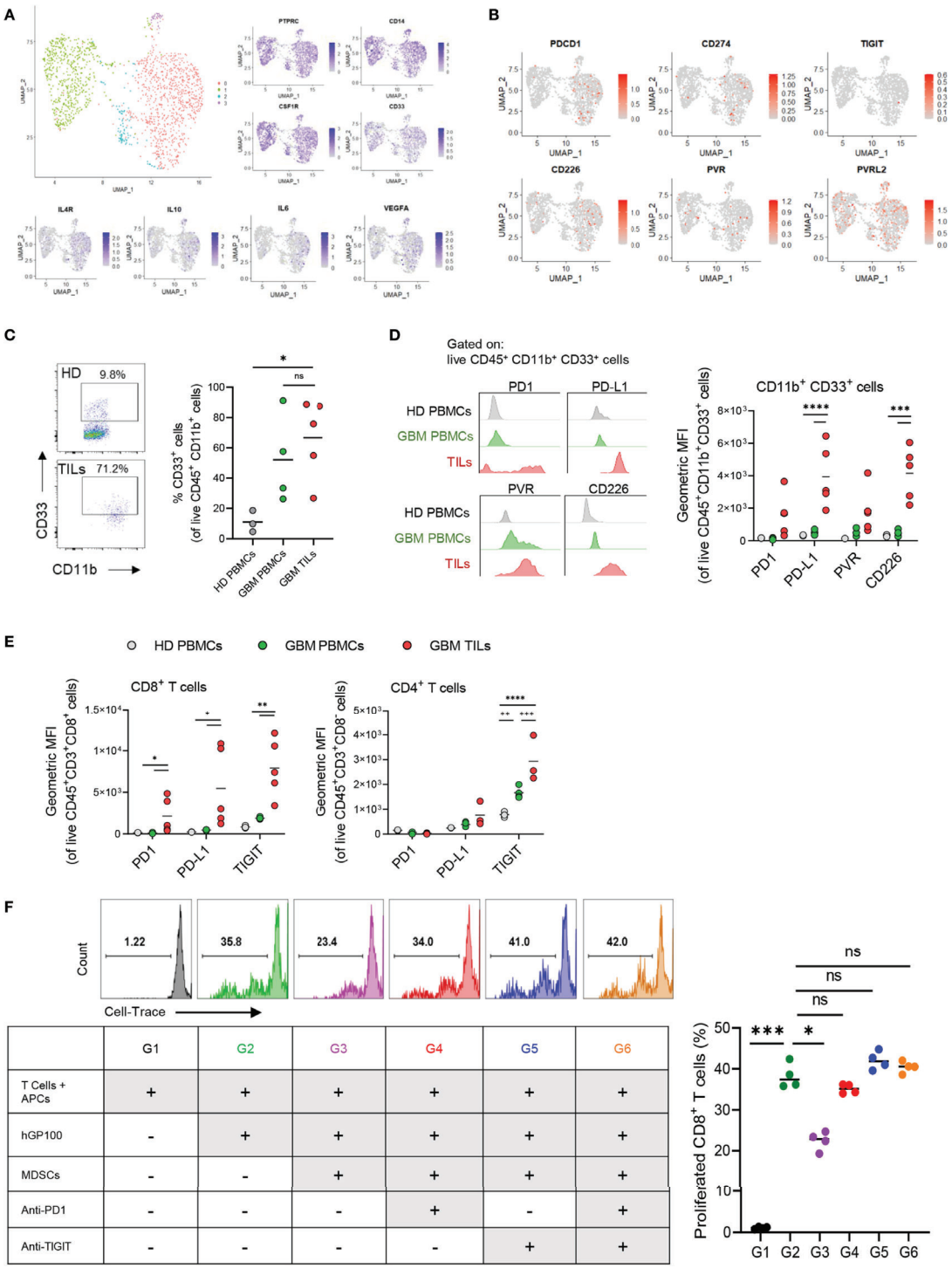


FIGURE 5 | Anti-PD1/TIGIT immunotherapy is associated with altered myeloid-derived suppressor cells (MDSCs) in GL261 murine model. GL261 glioma cells were injected stereotactically in the caudate putamen of C56BL/6J mice followed by immunotherapy treatment starting on day 8 post tumor injection, and the frequencies of MDSCs were determined on day 22. **(A)** Representative flow cytometry plots showing gating strategy of PMN MDCSs and Mo MDCSs based on the expression of Gr1 and CD11b. **(B–D)** Percentages (%) of $CD45^+$ glioma-infiltrating total MDSCs ($CD11b^+$ $Gr1^+$) **(B)**, PMN MDSCs ($CD11b^+$ $Gr1^{high}$) **(C)**, and Mo MDSCs ($CD11b^+$ $Gr1^{low}$) **(D)**, on day 22 following treatment. **(E)** Ratios of tumor infiltrating CD8+ T cell to total MDSCs. Representative data of 3 experiments. n=5 per group. One-way ANOVA with multiple comparisons test corrected for false discovery rate (FDR) using Benjamini-Hochberg test. *P* values are as followed: *≤0.05, **≤0.01, NS, not significant.

Supplemental Figure 2A), were mostly expressed by cluster 0, suggesting that these cells had a tumor-promoting and immune-suppressing functions, which resemble MDSC-like cells (52). Interestingly, *PDCD1* (PD1), *CD274* (PD-L1), and *CD226*, were also predominantly expressed by cluster 0 (**Figure 6B** and **Supplemental Figure 2B**). CD155 (PVR) was also associated and expressed by cluster 0, although less frequent than CD226, PD1, and PD-L1 (**Figure 6B** and **Supplemental Figure 2B**). PVRL2, another inhibitory receptor that bind to TIGIT (53), was also expressed by TAMs, with highest expression levels in cluster 0 (**Figure 6B** and **Supplemental Figure 2B**). We did not detect TIGIT expression in TAMs by scRNA-seq (**Figure 6B**). Additionally, we noted high expression and associated of ICOS-L with cluster 0 (**Supplemental Figure 2B**), which interestingly was also associated with GBM patient OS and DFS (**Figures 2A, B**). These data suggest that immunosuppressive TAMs, such as MDSCs, express genes for PD1, PD-L1 and TIGIT-ligands. Therefore, evaluated the protein expression of these markers on $CD45^+$ immune cells in patient derived GBM tissue and PBMCs, and healthy donor (HD) PBMCs. The frequencies of $CD11b^+$ $CD33^+$ cells in GBM TILs were on average higher compared to GBM PBMCs, and were significantly higher than of HD PBMCs (**Figure 6C** and **Supplemental Figure 2C**), suggesting that MDSCs are present at high levels in GBM and could contribute to the TME immunosuppression (46). Consistent with our scRNA-seq data, $CD11b^+$ $CD33^+$ cells had higher expression levels of PD1, PD-L1, PVR, and CD226 in TILs, as compared with $CD11b^+$ $CD33^+$ cells from PBMCs of GBM patients and HDs, most notably for PD-L1 and CD226 expression (**Figure 6D**). Moreover, as compared to PBMCs samples we noted an increased expression of PD1, PD-L1 and TIGIT on $CD8^+$ TILs, while $CD4^+$ TILs had mostly upregulated expression of TIGIT (**Figure 6E**). Together, these data suggest that $CD11b^+$ $CD33^+$ TAMs may promote immunosuppressive functions at-least in part through expression of PD1/TIGIT-checkpoint ligands. To test this hypothesis, hGP100-restricted naïve T cells isolated from pmel mice (32) were activated *in vitro* with $hGP100_{25–33}$ peptide and feeder cell



	G1	G2	G3	G4	G5	G6
T Cells + APCs	+	+	+	+	+	+
hGP100	-	+	+	+	+	+
MDSCs	-	-	+	+	+	+
Anti-PD1	-	-	-	+	-	+
Anti-TIGIT	-	-	-	-	+	+

FIGURE 6 | PD1, PD-L1 and TIGIT-ligands are expressed on myeloid suppressor cells in GBM and contribute to T cell dysfunction. Single cell (sc) RNA-seq analysis was performed on myeloid cells from GBM patients. **(A)** UMAP clustering and expression (z-scores) of suppressive myeloid cell markers. **(B)** Expression z-scores of PD1/TIGIT-associated checkpoint molecules in the scRNA-seq clusters. **(C–E)** Healthy donor (HD) PBMCs and GBM patient PBMCs and TILs analyzed flow cytometry for myeloid cells, T cells, and IC markers. n = 4 HD; n = 5 GBM patients. **(C)** Representative flow cytometry plots and percentages (%) of $CD11b^+$ $CD33^+$ myeloid cells. **(D)** Representative histograms and mean fluorescence intensity (MFI) of PD1, PD-L1, PVR, and CD226 on $CD11b^+$ $CD33^+$ cells. **(E)** MFI of PD1, PD-L1, PVR, and CD226 on $CD8^+$ T cells and $CD4^+$ T cells. **(F)** T cell proliferation assay of murine hGP100-reactive $CD8^+$ T cells cultured with immunosuppressive myeloid cells with αTIGIT and αPD1. Representative histogram plots and percentages (%) of proliferated $CD8^+$ T cells at different culture conditions as indicated in the table legend. n=4 per group. One-way ANOVA with Tukey multiple comparisons correction. ns, not significant. p = *<0.05, **<0.01, ***<0.001, ****<0.0001.

(antigen presenting cells; APCs) in the presence of bone-marrow derived myeloid cells (putatively MDSC-like cells) cultured in GL261 cell-derived tumor-conditioned media and treated with αTIGIT and αPD1. Our data indicated that glioma-conditioned immunosuppressive myeloid cells significantly inhibited $CD8^+$ T cell proliferation, which was restored by the addition of αTIGIT or αPD1 (**Figure 6F**). In summary, these new data suggest that immunosuppressive myeloid cells, and presumably MDSCs, suppress anti-tumor immunity by inhibiting antigen-specific T cell function in GBM, at least in part *via* TIGIT and PD1 pathways, which may have major implication to patient treatments by immunotherapy.

DISCUSSION

In cancer, the dysregulation of immune checkpoints, such as TIGIT and PD1, is directly associated with tumor progression and enhanced immune evasion (54–57). In the past decade, an increasing number of IC-targeted immunotherapies have proven to have substantially beneficial outcomes for a wide variety of malignancies and provide durable tumor immunity and long-term patient survival (58). Nonetheless, evidence supporting the efficacy of IC immunotherapy in glioma remains insufficient (11).

In this study we interrogated RNA-seq data of 153 GBM patients in the TCGA database to identify IC genes whose upregulated expression is associated with poor outcome. We found that upregulated expression of *TIGIT* and *PDCD1*, as well as their ligands *CD155* (PVR) and *PD-L1* (respectively), was significantly correlated with poor DFS and OS. Other checkpoint pathways with inhibitors currently in development, including LAG3 and TIM3, were not associated with either patient OS or DFS. We posit that interrogation of TIGIT and PD1 -associated regulatory gene networks in responding and non-responding GBM patients would be of great interest to identify biomarker of ICIs.

PD1 is an immune checkpoint expressed on activated immune cells, including $CD4^+$ and $CD8^+$ TILs. The binding of PD1 to its ligand, PD-L1 on tumor and stromal cells, delivers a signal that inhibits effector functions such as cytokine production and cytolytic activity in the tumor microenvironment (TME) (59). PD-L1, like many other IC ligands, is hijacked by tumor cells in order to evade anti-tumor immunity. Accordingly, blockade of PD1/PD-L1 pathway with antibodies have been shown to improve T cell function and reduce tumor burden in several types of tumors (60, 61). Previous studies demonstrated elevated levels of TIGIT expression in human gliomas (34); however, the therapeutic effects of targeting this pathway in glioma patients remain poorly understood. TIGIT has recently emerged as an important checkpoint that is also expressed by activated $CD4^+$ and $CD8^+$ TILs. TIGIT has a higher binding affinity to CD155 than CD226; thus, once TIGIT is upregulated, the inhibitory signal becomes more dominant (62–64). Similarly, interfering with TIGIT/CD155 interaction has been identified as a potential therapeutic target for malignancies (65). Interestingly, blocking PD1/PD-L1 signaling was shown to increase the expression of TIGIT on Tregs in head and neck squamous cell carcinoma (HNSCC) patients (49), suggesting a resistant mechanism for αPD1 immunotherapy mediated by TIGIT.

Accordingly, our data support prior studies that combination immunotherapy treatment targeting the PD1 and TIGIT pathways leads to prolonged survival in GBM murine models (34, 66). Furthermore, we showed that αTIGIT/αPD1 dual treatment increased the numbers of $CD8^+$ TILs and enhanced their lytic function in GBM, supporting previous findings that this treatment can enhances IFN-γ expression in glioma-infiltrating T cells (34). Importantly, our data indicate that combined immunotherapy with αTIGIT/αPD1 affects MDSCs in the glioma TME.

MDSCs are a heterogenous population of immature myeloid cells that contribute to tumor growth, accumulation of additional immunosuppressive cells, and immunotherapy resistance (66, 67). Furthermore, MDSCs express large amounts of immunosuppressive factors, multiple anti-inflammatory cytokines and chemokines that directly stimulate tumor progression (68). Notably, a long-term survival study in melanoma patients showed that elevated numbers of MDSCs were highly associated with ICI resistance and negative therapeutic outcomes (69). Additionally, elevated numbers of tumor infiltrating MDSCs are correlated with $CD8^+$ TIL dysfunction and induced tumor cell expression of IC ligands; thus, MDSCs may promote and sustain an immunosuppressive glioma TME (70–72). Here, we showed that TIGIT blockade stimulated anti-tumor cytotoxic T cell (CTL) responses and reduced the immunosuppressive MDSCs in a murine model of GBM. Moreover, we found that PMN MDSC, but not Mo MDSC accumulation was reduced by dual blockade of TIGIT and PD1, compared with controls. Thus, our data suggest that PMN and Mo MDSCs might have different mechanisms to confer resistance against ICI immunotherapy, but may also be a target of ICI in glioma. We posited that future studies should focus on unveiling the crosstalk and mechanisms by which ICIs affect MDSCs in glioma. Along these lines, we showed that suppressive myeloid cells express PD1, PD-L1, and TIGIT-ligands in human GBM tissue. Moreover, we demonstrated that antigen specific T cell proliferation is inhibited by immunosuppressive myeloid cells can be restored by TIGIT/PD1 blockade. This suggests that CTL exhaustion might be regulated at least in part by the expression of IC ligands on MDSCs in GBM.

Treg cells are major components of the immune suppressive TME which express many ICs (73). The expression of TIGIT and PD1 by Treg cells was shown to enhances their immunosuppressive functions and contribute to tumor progression both in glioma murine models and GBM patients (74). Importantly, Treg cells are major source of IL-10 in GBM (74, 75), and IL-10 can induce MDSC development and enhance their suppressive functions (76, 77), as well as increasing the expression of PD1 myeloid cells (78). Additionally, TIGIT is important for IL-10 expression by Treg cells (55). Therefore, it is possible that αTIGIT might also regulate MDSC cell numbers and functions by suppressing Treg expression of IL-10. Future studies should focus on the mechanisms and crosstalk between Treg cells and MDSCs *via* checkpoint molecules in the GBM TME and their contribution to ICI resistant.

In summary, our data support the concept of treating GBM patients with dual blockade of PD1 and TIGIT and provides new insights into mechanisms of GBM immunotherapy to facilitates the development of novel treatments.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in https://www.ncbi.nlm.nih.gov/gap/ online repository, under accession number phs000178.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

Performed experiments and collected data: IR, RK, LM, KS, PS, CS, JN, NA, MLC, AF and TB. Formal data analysis and figures: IR and RK. Statistical analysis: IR, SZ, and DR. RNA-seq and single cell RNA-seq analysis: IR, LW, and DR. Resources, concepts and/or manuscript revisions: IR, BH, SA, AB, FSL, IP, NA, BC, AD and GK. IR wrote the manuscript. IR revised the manuscript with assistance from GK. IR and GK designed the experiments. GK supervised and financed the study. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by National Institute of Health (NIH)/National Cancer Institute (NCI) grants R01CA244520 and NIH R01CA222804 (to GK), NIH/National Institute of Biomedical Imaging and Bioengineering (NIBIB) R21EB029650 (to GK), The Walter L.Copeland Fund of The Pittsburgh Foundation (to IR, GK) and The Brain Tumor Funders' Collaborative (GK). IR was supported by a fellowship from UPMC Children's Hospital of Pittsburgh. This work utilized the Hillman Cancer Center Flow Cytometry Core, a shared resource at the University of Pittsburgh supported by the CCSG P30 CA047904.

ACKNOWLEDGMENTS

The results shown here are in whole or part based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga. The authors would like to thank Dr. Yijen Wu, director of Rangos Research Center Animal Imaging Core for assistance with MRI imaging and analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.637146/full#supplementary-material

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

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frontiers in Immunology

REVIEW
published: 11 May 2021
doi: 10.3389/fimmu.2021.674192

The Immunology of Hormone Receptor Positive Breast Cancer

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OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 28 February 2021
Accepted: 13 April 2021
Published: 11 May 2021

Citation:
Goldberg J, Pastorello RG, Vallius T, Davis J, Cui YX, Agudo J, Waks AG, Keenan T, McAllister SS, Tolaney SM, Mittendorf EA and Guerriero JL (2021) The Immunology of Hormone Receptor Positive Breast Cancer. Front. Immunol. 12:674192. doi: 10.3389/fimmu.2021.674192

Immune checkpoint blockade (ICB) has revolutionized the treatment of cancer patients. The main focus of ICB has been on reinvigorating the adaptive immune response, namely, activating cytotoxic T cells. ICB has demonstrated only modest benefit against advanced breast cancer, as breast tumors typically establish an immune suppressive tumor microenvironment (TME). Triple-negative breast cancer (TNBC) is associated with infiltration of tumor infiltrating lymphocytes (TILs) and patients with TNBC have shown clinical responses to ICB. In contrast, hormone receptor positive (HR+) breast cancer is characterized by low TIL infiltration and minimal response to ICB. Here we review how HR+ breast tumors establish a TME devoid of TILs, have low HLA class I expression, and recruit immune cells, other than T cells, which impact response to therapy. In addition, we review emerging technologies that have been employed to characterize components of the TME to reveal that tumor associated macrophages (TAMs) are abundant in HR+ cancer, are highly immune-suppressive, associated with tumor progression, chemotherapy and ICB-resistance, metastasis and poor survival. We reveal novel therapeutic targets and possible combinations with ICB to enhance anti-tumor immune responses, which may have great potential in HR+ breast cancer.

Keywords: hormone receptor (HR), breast cancer, immunotherapy, immune exclusion, T-cell exclusion, antigen presentation, clinical trial

INTRODUCTION

Immunotherapy represents a paradigm shift in oncology. In particular, immune checkpoint blockade (ICB) has emerged as an efficacious treatment option for many tumor types, providing new therapeutic options for previously untreatable cancers. ICB therapy involves the use of humanized antibodies to target and neutralize immune checkpoint proteins with the goal of

invigorating T cell activation and anti-tumor responses. Targeting immune inhibitory molecules, including cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-1 (PD-1) and its ligand, PD-L1, aims to reinvigorate exhausted T cells, thus enabling improved tumor antigen recognition and cytotoxic activity (1). The benefits of ICB, however, are not equally realized among different cancer types. In general, cancers that respond to ICB have at least one of these three key features: high tumor mutational burden (TMB), high numbers of tumor-infiltrating-lymphocytes (TILs) and/or high PD-L1 expression (2). Tumors from melanoma and lung cancer patients generally exhibit all of these features and have demonstrated superior responses to ICB (3–5). In contrast, breast tumors generally have low TMB, are often poorly infiltrated by TILs, have low levels of PD-L1 expression, and are thus considered to be nonimmunogenic and less responsive to ICB (6–9).

Breast cancer is a histopathologically and molecularly heterogeneous disease, ranging from the more indolent luminal A tumors, which are generally estrogen receptor positive (ER+) and human epidermal growth factor receptor 2 (HER2)-negative, to the highly aggressive, basal-like triple-negative tumors, which are negative for the ER, progesterone receptor (PR) and HER2. Triple-negative breast cancer (TNBC) generally has a higher mutational load, greater TIL infiltrate and higher PD-L1 expression relative to other breast cancer subtypes (10–12). Consistent with those immune features, the greatest successes reported to date of ICB in breast cancer clinical trials have been in patients with TNBC. However, chemotherapy combinations may prove effective, particularly for breast cancers that are not innately sensitive to ICB (13). Two agents, the anti-PD-L1 agent atezolizumab and the anti-PD-1 agent pembrolizumab, have been approved for use in combination with nab-paclitaxel or chemotherapy, respectively, for the treatment of metastatic TNBC following the results of phase 3 clinical trials showing improvement in progression free survival (PFS) with the use of these agents (14–16). Importantly, atezolizumab and nab-paclitaxel also led to a clinically meaningful improvement in overall survival (OS) in patients with PD-L1 positive disease (15). To date ICB has not been approved for the treatment of other subtypes of breast cancer. Given that TNBC comprises only 15% of all breast cancer cases (17), there is an urgent need to better understand the underlying basis of diminished immune responses to these other subtypes, with the goal of making those subtypes susceptible to ICB or other agents that act by enhancing anti-tumor immune responses.

Hormone receptor positive (HR+) breast cancer comprises approximately 70% of breast cancers and is characterized by dependence on ER signaling (17). HR+ breast cancer is generally a more indolent breast cancer subtype, has a low TMB and low PD-L1 expression (9, 18). Importantly, among the different breast cancer subtypes, HR+ tumors tend to have the lowest numbers of TILs (8, 15, 19–25). There are currently no FDA approved ICB agents for the treatment of HR+ breast cancer, however in the past several years new evidence has emerged showing immunogenic subsets of HR+ tumors (26) and that ICB might be effective in combination with the right chemotherapy (13). In addition, advances in single cell sequencing and imaging technologies have revealed a wide diversity of both immune and non-immune cells that comprise the HR+ TME. Nevertheless, with respect to HR+ breast cancer, there remains a gap in knowledge as to how baseline immune contexture affects a patient's prognosis and how individualized treatments can be developed based on the characteristics of a patient's TME. In this review, we summarize what is known about the immunogenicity of HR+ breast cancer and the opportunities to target HR+ tumors with ICB and other immune-activating therapies.

THE ROLE OF TUMOR-INFILTRATING LYMPHOCYTES (TILS) IN HR+ BREAST CANCER

Lymphocytes, which are white blood cells including T cells, B cells and natural killer cells, were first correlated with breast cancer outcome in the early 1990s (27). Since then TILs have been studied extensively in breast cancer and have been shown to have both prognostic and predictive value (28), yet their role in HR+ breast cancer is more elusive (**Table 1**). TIL analysis in clinical laboratories is performed using a continuous parameter on a single hematoxylin and eosin (H&E)-stained tumor section and criteria described by Denkert et al. is used to score infiltrating TILs (40). Intratumoral TILs (iTILs) are defined as intraepithelial mononuclear cells within tumor cell nests or in direct contact with tumor cells, and stromal TILs (sTILs) as lymphocytes in the tumor stroma without direct contact with tumor cells. While stromal and iTILs are generally correlated, iTILS are far less abundant and more difficult to identify on H&E sections and new guidelines advocate to quantify only sTILs on H&E-stained tumor sections (54). Interestingly, a study conducted by the International Immuno-Oncology Biomarker Working Group demonstrated that a software-guided image evaluation approach could improve inter-observer variability (55). These efforts have focused on standardizing an approach to establish TILs as a predictive and prognostic biomarker to guide the clinical management of breast cancer. However, as described in **Table 1** there are different methods of TIL assessment that has been reported which may account for differences observed between studies.

TILs in Breast Tumors Before and After Neoadjuvant Systemic Therapy

Over a decade ago, it was shown that the presence of TILs is an independent predictor of response to neoadjuvant chemotherapy (NAC) in all subsets of breast cancer, where high levels of TILs were associated with increased pathological complete response (pCR) rates compared to tumors that demonstrated absence of TILs (40). Subsequently, tumors from the BIG 02-98 trial revealed that TILs are associated with clinical benefit from adjuvant chemotherapy in patients with TNBC and HER2-positive (HER2+) breast cancer (19). In addition, this trial demonstrated that TILs were significantly lower in HR+/HER2- tumors

TABLE 1 | Summary of the association of tumor infiltrating lymphocytes (TILs) in HR+ breast cancer with clinical outcome.

Publication	Study Design (Trial Name)	Number of Evaluable HR+ Samples	TIL Assay	Findings
Adjuvant Setting				
Loi et al. (19)	Prospectively defined, retrospective (BIG 02-98 trial)	1078	sTILs, iTILs in H&E	No association found between TILs and DFS or TILs and OS
Loi et al. (29)	Prospectively defined, retrospective (FinHER trial)	694	sTILs, iTILs in H&E	No association found between TILs and distant DFS or TILs and OS
Dieci et al. (30)	Prospectively defined, retrospective (Two French multicentric trials)	501	sTILs, iTILs in H&E	No association found between TILs and OS
Carbognin et al. (31)	Sensitivity analysis of data from Loi et al. (19, 29) & Dieci et al. (30)	2132	sTILs, iTILs in H&E	No association found between TILs and OS
Krishnamurti et al. (32)	Retrospective, archival tissues	187	sTILs in H&E	Negative association found between TILs and Oncotype DX recurrence score
Miyoshi et al. (33)	Retrospective, multicentric	639	sTILs in H&E	No association found between TILs and timing of recurrence
Fujimoto et al. (34)	Retrospective, archival tissues	519	sTILs, iTILs in H&E	Ki67-low group: high-TILs showed significant unfavorable DFS Ki67-high group: high-TILs showed nonsignificant favorable DFS
Ali et al. (35)	Prospectively defined, retrospective (SEARCH, BCCA, NBCS, NEAT trials)	6714	IHC staining for CD8+ and FOXP3+ sTILs and iTILs	Intratumoral CD8+ lymphocytes not associated with outcome
Sobral-Leite et al. (36)	Prospectively defined, retrospective (IKA trial)	563	IHC staining for CD4, CD8, and FOXP3	High CD8+ T cell infiltration associated with increased risk of recurrence
Gu-Trantien et al. (37)	Retrospective (fresh and archival tissues)	510	Gene expression	An 8-gene Tfh signature showed significant prognostic values in luminal tumors
Liu et al. (38)	Retrospective, archival tissues	2351	IHC staining for FOXP3+ sTILs and iTILs	FOXP3+ regulatory T cells were associated with poor prognosis
Koletsa et al. (39)	Prospectively defined, retrospective (HE10/97, HE10/00 trials)	600	IHC staining for CD3+, CD8+ and FOXP3+ sTILs, iTILs and total TILs	Assessment of CD3+, CD8+ and FOXP3+ lymphocytes densities adds no value over a traditional stromal TILs assment
Post-neoadjuvant Setting				
Denkert et al. (40)	Prospectively defined, retrospective (GeparDuo and GeparTrio trials)	659	sTILs, iTILs in H&E, immune mRNA markers	Increased TILs associated with pCR
Issa-Nummer et al. (41)	Prospectively defined, retrospective (PREDICT trial)	209	sTILs, iTILs in H&E	Validation of results in Denkert et al. (40)
Denkert et al. (10)	Prospectively defined, retrospective (GeparDuo, GeparTrio, GeparQuattro, GeparQuinto, GeparSixto, and GeparSepto trials)	832	sTILs in H&E	Increased TILs associated with shorter OS
Skriver et al. (42)	Prospectively defined, retrospective (phase II Danish Breast Cancer Group trial)	106	sTILs in H&E	Increased TILs from baseline associated with poor treatment response
Ono et al. (43)	Retrospective, archival tissues	46	sTILs in H&E	No correlation found between TILs and pCR
Hwang et al. (44)	Retrospective, archival tissues	131	sTILs in H&E	No correlation found between TILs and pCR
Russo et al. (45)	Retrospective, archival tissues	119	sTILs in H&E	No correlation between TILs and survival
Ali et al. (46)	Prospectively defined, retrospective (ARTemis trial)	446	computational pathology of sTILs in H&E	Lymphocyte density associated with pCR in multivariate analysis
Seo et al. (47)	Retrospective, archival tissues	100	IHC staining for CD4+, CD8+ and FOXP3+ sTILs and iTILs	CD8+ TILs were independent predictors for pCR
Brown et al. (48)	Retrospective, archival tissues	58	Quantitative IF for CD3, CD8, and CD20 sTILs	CD20+, but not CD3+ or CD8+ lymphocytes predict pCR
Post-neoadjuvant Setting				
Watanabe et al. (49)	Retrospective, archival tissues	Pre-Tx: 91 Post-Tx: 80	iTILs in H&E	Low TILs associated with improved RFS only in post-Tx group
Pelekanou et al. (50)	Retrospective, archival tissues	46	sTILs in H&E	Increased TILs post-Tx associated with longer 5-year RFS
Hamy et al. (51)	Retrospective, archival tissues	223	sTILs in H&E	No association between TILs and DFS
Ladoire et al. (52)	Retrospective, archival tissues	88	IHC staining for CD8+ and FOXP3+ sTILs	High CD8+ and low FOXP3+ lymphocyte infiltrates associated with improved RFS and OS
Asano et al. (53)	Retrospective, archival tissues	80	sTILs in H&E	RCB-TILs score predicts recurrence, may be a more sensitive indicator than TILs alone

sTIL, stromal tumor infiltrating lymphocytes; iTIL intratumoral tumor infiltrating lymphocytes; H&E, hematoxylin and eosin; DFS, disease free survival; OS, overall survival; pCR, pathological complete response.

compared to other subtypes (19). The extent of clinical response to NAC is a prognostic factor for TNBC, HR+ and HER2+ breast cancer, with the best clinical outcomes seen in patients that experience a pCR (56, 57). A meta-analysis of six randomized trials by the German Breast Group showed that increased TILs were predictive for more favorable response to NAC for all breast cancer subtypes, where higher pCR rates were observed when tumors were categorized as high TILs. In this study, TILs were analyzed as predefined groups of low (0-10% immune cells in stromal tissue within the tumor), intermediate (11-59%), and high TILs (≥60%). A univariable analysis revealed that a 10% increase in TILs was associated with longer DFS in TNBC and HER2+ breast cancer but not in luminal-HER2- tumors. Interestingly, an increase in TILs was associated with longer OS in TNBC, had no association with HER2+ breast cancer and was associated with shorter survival in luminal-HER2- tumors (10). The finding that TILs have a positive short-term prognostic value (as measured by response at surgery) whereas they have a negative long-term prognostic value highlights the complexity of TILs in the TME. Previous work by this same group had shown the positive association with short-term responses in HR+ breast cancer and these findings were confirmed by the same group in the PREDICT study; and should be noted that TNBC was associated with higher TILs compared to HR+ breast cancer (10, 41, 58, 59). In another study evaluating baseline biopsies prior to chemotherapy, where both areas of stroma infiltrated by lymphocytes (proportional score) and intensity of lymphatic infiltration (intensity score) were taken into consideration, high TILs score was associated with pCR in TNBC but not for Her2+ or HR+ tumors (43). Other retrospective cohorts evaluating pre-NAC TILs association with pCR similarly failed to find a significant correlation, most likely due to the limited number of HR+ tumors used in pCR prediction (44, 45, 60). The significance of TILs in patients treated with neoadjuvant hormonal therapy has been recently studied as part of a nationwide phase II trial conducted by the Danish Breast Cancer Group. The group evaluated pretreatment core biopsies and surgical specimens for percentage of TILs and pathological complete response was assessed using Residual Cancer Burden (RCB) index. The group reported that increasing TILs during letrozole treatment was significantly associated with a poor treatment response (42). Interestingly they propose that an increase in TILs during endocrine therapy might imply immunogenicity, and these patients could be targetable by immunotherapy (42).

Unconventional approaches to measure lymphocyte infiltration have also revealed interesting results from analysis of baseline tumors. In a cohort of TNBC patients, stromal TILs and TILs measured by tumor infiltrating lymphocyte volume (TILV) were significantly correlated with pCR (61). In that study TILV were calculated using the formula TILV = % stroma in tumor x % stromal TILs; where stromal TILs were assessed according to the standardization and guidelines of the international TILs working group (54). In an analysis of the ARTemis trial using computational pathology, lymphocyte density was significantly associated with pCR in multivariate analysis but there was no association between pre-treatment lymphocyte density and survival in either HR+ or HR- patients treated with NAC (46). Subset analyses of lymphocyte infiltrates have been described in breast cancer where TILs are largely composed of CD4+ and CD8+ T cells (62). In a retrospective study, CD8+ TILs in pre-chemotherapeutic biopsy specimens were found to be independent predictors for pCR irrespective of breast cancer subtype (47). Conversely, in another study, CD20+ lymphocytes (generally thought to be B cells) scored by quantitative immunofluorescence positively predicted pCR in response to NAC irrespective of HR and HER2 status, whereas CD3+ and CD8+ lymphocytes did not (48).

In addition to the value of TILs as a potential biomarker predicting response to NAC, there is an interest from the International Immuno-Oncology Biomarker Working Group on Breast Cancer (IIBWG) in evaluating the utility of TILs to refine risk stratification in patients with residual disease following neoadjuvant treatment (63). A retrospective multicenter study with TNBC patients concluded that the presence of TILs in residual disease following NAC was a strong favorable prognostic factor for both metastasis-free and OS in this subtype of breast cancer (64); less work has been done in HR+ disease. Watanabe and colleagues evaluated TILs in HR+/HER2- primary breast cancers before and after NAC, and concluded that low TILs following NAC, but not at baseline, were associated with a significantly better recurrence free survival (RFS) (49). In another cohort that included all breast cancer subtypes, increased TIL infiltration after NAC compared to baseline was associated with longer 5-year RFS (50). Furthermore, Ladoire and colleagues found the association of both high CD8+ and low FOXP3+ lymphocyte infiltrates following NAC was linked with improved RFS and OS in a cohort that included all breast cancer subtypes (52). In addition, a combined score associating CD8/FOXP3 ratio and pathological AJCC staging isolated a subgroup of patients with a long-term overall survival of 100% (52). In contrast, in a retrospective French cohort, high post-NAC TILs were associated with worse disease-free survival (DFS) in HER2+ patients, but not in TNBC and HR+ patients (51). Asano and colleagues (53), combined the residual cancer burden (RCB) index (57) and TILs ("RCB-TILs") to predict survival after NAC. In their multivariate analysis, RCB-TILs was an independent factor for recurrence overall and within each of the breast cancer subtypes, suggesting RCB-TILs may be a more sensitive prognostic marker than TILs or RCB alone (53). The IIBWG has recently launched an international effort to include TILs in a new version of the RCB index to better stratify patients post-NAC (63).

TILs in HR+ Breast Tumors Managed With Adjuvant Systemic Therapy

Studies that have evaluated TILs in early-stage treatment-naïve breast tumors managed with adjuvant systemic therapy have so far generally failed to demonstrate prognostic value in HR+ tumors (19, 29, 32, 65, 66). In the BIG 02-98 trial, in which patients were randomized to a doxorubicin-based regimen with or without docetaxel, TILs were not significantly associated with DFS or OS in HR+/HER2- (19). These findings were confirmed in HR+/HER2- cases from the FinHER trial, in which patients were randomized to adjuvant docetaxel or vinorelbine regimens, followed by fluorouracil, epirubicin and cyclophosphamide (29).

Similarly, by combining patients from two French multicentric trials, randomized by addition of adjuvant anthracycline-based therapy, a significant association between TILs and OS was not identified in HR+/HER2- breast tumors (30). The aforementioned studies were included in a sensitivity analysis of randomized trials in the adjuvant and neoadjuvant setting, which confirmed there was no association between baseline TILs and OS for HR+/HER2- tumors (31). In a more recent study, patients who underwent mastectomy without neoadjuvant treatments were evaluated for TILs. In HR+ breast cancer, there was a negative association between Oncotype DX recurrence score and both overall and peripheral TILs, where peripheral TILs were evaluated as the percentage of stromal lymphocytes encountered in the entire circumferential invasive tumor front. The negative association between TILs and Oncotype DX score may indicate the possible prognostic value of TILs in HR+ breast cancer. However, peripheral TILs were significantly associated with OS and DFS in TNBC but not in HR+ breast cancer (32).

It is noteworthy that several studies have identified a link between subpopulations of T cells in HR+ breast tumors and long-term outcomes following adjuvant systemic therapy. In Ki67-high breast cancers, high TILs were associated with favorable DFS, irrespective of subtype, but increasing TIL levels correlated with worse DFS in the Ki67-low group (defined as ≤ 25%) with the HR+/HER2- subtype. These results highlight variation in TIL prognostic significance between Ki67-high and -low breast cancers, particularly for the HR+/HER2- subtype (34). In a large study of 12,439 patients, assessment of T cell infiltration in breast cancer indicated that intratumoral CD8+ lymphocytes were associated with worse outcomes in HR+/HER2- patients, however the association did not remain significant in multivariate analysis (35). Similarly, in a retrospective analysis of a prospective randomized trial in HR+ breast cancer in which postmenopausal patients with early stage HR+/HER2- breast cancer were randomized to tamoxifen treatment or no adjuvant therapy, it was found that tumors with high CD8+ T cell infiltrates were associated with increased recurrence risk (36). Other T cell subsets have also been examined in early-stage HR+ breast tumors treated with adjuvant systemic therapy. To better understand CD4+ follicular helper T cells (Tfh), an 8-gene Tfh signature was reported, which was consistently prognostic in luminal tumors, as well as in other subtypes (37). Conversely, FOXP3+ regulatory T cells assessed in treatment naïve tumors were shown to be an indicator of poor prognosis in HR+ breast cancer, but of favorable prognosis in HR-/HER2+ tumors (38). However, recent work including all subtypes of breast carcinomas concluded that CD3+, CD8+ and FOXP3+ lymphocyte densities did not add prognostic information over stromal TILs assessed on H&E in early intermediate/high-risk breast cancer treated with adjuvant chemotherapy (39). These findings confirm the complexity of the TME in HR+ breast cancer and taken together, indicate that further investigation is necessary to determine the predictive and prognostic values of TILs in HR+ breast cancer.

Location of TILs in Breast Tumors

The location and organization of TILs, in particular, T cells in tumors may be important in their ability to become activated and exert anti-tumor effects as well as B cells in tertiary lymphoid structures (reviewed in the next section). Important work has been done to investigate the spatial location of T cells in TNBC which led to a tumor immune microenvironment (TIME) classification to group tumors into patterns according to $CD8^+$ TIL spatial distribution (67). Immunoreactive TMEs were identified that consisted of tumoral infiltration of granzyme B+CD8+ T cells (GzmB+CD8+ T cells), a type 1 IFN signature, and elevated expression of immune inhibitory molecules such as indoleamine 2,3-dioxygenase (IDO) and PD-L1, which correlated with favorable clinical outcomes. This same group showed that "immune-cold" TMEs, which had absence of tumoral CD8+ T cells, were defined by elevated expression of the immunosuppressive marker B7-H4, signatures of fibrotic stroma and poor outcomes (67). Interestingly, a significant accumulation of proinflammatory CD68+CD206- macrophages were found in tumors with high infiltration of CD8+ T cells compared to TNBC with less CD8+ T cells (67). Indeed, localization and composition of T-cells in TNBC has demonstrated that the immunomodulatory subtypes are associated with the highest expression of adaptive immune-related gene signatures and a fully inflamed spatial pattern (68). Other work in TNBC has focused on exclusion of T cells from tumor cell clusters and spatial-profile analysis and mathematical modeling suggests a possible inhibitory signal inside tumor cell clusters, which prevents CD8+ T cells from infiltrating into tumor cell clusters (69). The location of T cells may help understand responses to ICB and identify tumors with high likelihood of response in TNBC and may extend to HR+ breast cancer. However, characterization of the spatial organization of T cells and other immune cells in HR+ breast cancer remains an unmet need.

Tumor-Associated Tertiary Lymphoid Structures (TLS)

TLS are ectopic lymphoid organs, composed of lymphoid cells that arise in chronic inflammatory states, including tumors (70). These structures have considerable morphological overlap with secondary lymphoid organs (SLO), particularly lymph nodes, although "TLS" can refer to structures of varying complexities, from simple lymphocytic clusters to elaborate formations highly reminiscent of a SLO (71). TLS exhibit characteristics of structures in the lymph nodes associated with the generation of an adaptive immune response, including a T cell zone with mature dendritic cells, a germinal center with follicular dendritic cells and proliferating B cells, and high endothelial venules (72). There is increasing interest in studying tumor-associated TLS as recent work has revealed these structures to be valuable biomarkers in multiple tumor types, including breast cancer (73–78). The presence of TLS structures has demonstrated both prognostic and predictive value in breast carcinomas, although data is discrepant on whether these structures are associated with favorable or detrimental outcomes. Martinet and colleagues found that high densities of tumor-associated high endothelial venules, a common constituent of TLSs, were independently associated with longer DFS and OS in breast cancer patients, irrespective of HR status (79). In a study conducted by Liu and

colleagues, TLSs were significantly associated with favorable DFS in patients with HER2+ breast cancer, independent of TIL status (76). In contrast, a recent analysis of all breast cancer subtypes reported that the presence and density of peritumoral TLSs were not independently associated with DFS and OS (80). Interestingly, TLSs have been demonstrated to be significant predictors of pCR in TNBC patients treated with NAC (78). It is important to note that tumor-associated TLS assessment has not been standardized, although it should preferably be performed in full-face sections, as biopsies or tissue microarrays likely cannot accurately reflect TLS status (81). In addition, H&E evaluation underestimates the presence of these structures compared to immunohistochemistry (IHC). Using IHC, one group has identified TLS by staining for CD45 to identify leukocytes and CD20/CD3 to identify B cell follicles surrounded/adjacent to T cell zones, respectively. The study revealed that and intra- and inter-observer agreement is superior using IHC compared to H&E (81). Modern multiplex imaging technologies are emerging as an improved modality to study these structures as evident in several recent publications (73, 77, 82).

Antigen Presentation in HR+ Breast Cancer

As we discussed above, the number of infiltrating TILs within a breast tumor has both prognostic and predictive implications. In order for anti-tumor T cell responses to be generated, tumor antigens must be presented complexed with human leukocyte antigen (HLA) molecules at the cell surface for recognition by T cells (83). Studies have shown that HLA downregulation is an important mechanism of immune evasion that has been observed in multiple tumor types (84–86). The true frequency of HLA downregulation in cancer is controversial, in part due to differing antibodies used to detect HLA-class I (HLA-I) molecules. In the past decade, the EMR8-5 antibody has emerged as the method of choice to detect surface HLA-I expression on tumor cells (87–89). Torigoe and colleagues used the EMR8-5 antibody by IHC to assess the frequency of HLA class I downregulation in various cancer tissues (n=246). Using criteria established by the HLA and Cancer Component of the 12th International Histocompatibility Workshop (90), HLA expression was scored based on cell expression and intensity. The group found that HLA-I was decreased in 20-42% of lung, liver, colon, renal and urothelial cancer cases (91) whereas 85% of breast cancer cases had loss of or decreased HLA-I expression (91). Another report from Kaneko and colleagues reported HLA-I downregulation in 32.5% of breast tumors and was significantly associated with worse clinical features (nodal involvement and stage) as well as worse disease-free interval (84). Similarly, using multiple antibodies against HLA, Garrido and colleagues revealed various types of HLA-I alterations in 79 of 98 (81%) of breast tumors, including complete HLA-I loss in 53 (54%) of the samples (92). HLA-I downregulation may be particularly important in HR+ breast cancer as Sinn and colleagues measured HLA-I expression in 863 breast cancer cases from the GeparTrio trial, including all subtypes of breast cancer. The group found that HR+/HER2- cancers had the lowest level of HLA class I expression compared to other subtypes (93). Furthermore, a negative correlation between mRNA expression of the estrogen receptor 1 (ESR1) gene and HLA was also found in the Cancer Cell Line Encyclopedia (CCLE) (94). Importantly, in a study of The Cancer Genome Atlas (TCGA), ESR1 expression was found to be inversely correlated with HLA-A and CD8B gene expression (94). These studies suggest that HLA expression may be inversely correlated with ER expression and positively correlated with T cell infiltration. However, the mechanism underlying this relationship has not yet been elucidated (95, 96). Taken together these data suggest that HLA downregulation may be an important mechanism of immune evasion in breast cancer and in particular in HR+ breast cancer.

Prior to presentation of antigen complexed with an HLA molecule, that antigen must undergo processing. Components of the antigen-processing machinery (APM) have also been evaluated in breast cancer. Liu and colleagues found differential expression of antigen-processing molecules between primary breast tumors with and without associated brain metastases (n=65, 49 HR+) (96). In particular, primary breast lesions in patients who later developed brain metastases showed lower beta 2 microglobulin (B2M; the co-receptor for HLA) expression as well as other APM components, such as transporter associated with antigen processing 1 and 2 (TAP1/2), and calnexin, which are essential components for antigen processing and loading on HLA. In addition, CD8 T cell infiltration was significantly higher in primary breast lesions without an associated brain metastasis and was correlated with TAP1 expression. Preclinical data further support these findings. Murine tumor cells stably transfected with silencing hairpin (sh)RNA for TAP1 demonstrated a decreased susceptibility to cytotoxic T lymphocytes *in vitro* and an increased frequency of spontaneous brain metastasis *in vivo* (96). These data suggest that a deficiency in antigen-processing machinery may increase the likelihood of metastasis through deficient immune surveillance.

The value of HLA downregulation as a biomarker in breast cancer has been assessed in several studies of early-stage and metastatic disease. Although the data are conflicting, the majority of studies indicate that HLA-I downregulation is associated with poor prognosis. In a large retrospective study, the correlation of HLA-I expression with clinical outcome was assessed in 465 surgically resected breast cancer specimens including 310 primary HR+ tumors (97). Complete loss of HLA-I was observed in about 18% of both the HR+ and HR- subsets and survival analysis revealed that HLA-I expression loss was significantly correlated with worse disease-specific survival (DSS). In addition, HLA-I was found to be an independent prognostic factor for adverse DSS in patients with stage II-IV breast cancer. Interestingly, in contrast to the previously mentioned studies, in a study of 439 invasive primary breast cancers including all subtypes, Madjd and colleagues found strong HLA-I staining correlated with the development of metastasis and HLA-I downregulation to be associated with improved clinical outcomes (98).

Natural Killer Cells in HR+ Breast Cancer

While low expression of MHC-I may limit CD8 T cell recognition and response to HR+ breast tumors, the lack of MHC-I molecules should in turn promote NK cell activation, representing an alternate immunotherapeutic target (99–101). In general, NK cells account for a small portion of infiltrating lymphocytes in breast tumors (102, 103). Interestingly, analyses of TCGA and METABRIC samples revealed HR+ tumors have lower NK cell gene expression compared to TNBC tumors and immune-rich HR+ tumors have a lower proportions of NK cells compared to immune-rich TNBC tumors (104, 105). The combination of MHC-I downregulation and NK cell exclusion has not been analyzed in the literature and is an active line of investigation in our lab. Although NK cell infiltration is limited in HR+ tumors, HR+ breast cancer cell lines are more susceptible to IL-2 stimulated NK cell lysis than are TNBC or HER2+ cell lines (106–108), indicating that potential strategies to target HR+ tumors may include adoptive transfer of exogenously stimulated or genetically altered NK cells. Multiple pre-clinical investigations showed efficacy of NK-CAR cells targeting HER2 in HER2+ breast cancer (109, 110), tissue-factor in TNBC (111), epithelial cell adhesion molecule (EpCAM) in both HER2+ and TNBC (112) and epidermal growth factor (EGFR) in all breast cancer subtypes (113). Importantly, EpCAM is highly expressed in all breast cancer subtypes and thus can serve as a potential NK-CAR target in HR+ tumors (114). Overall, the majority of NK cell-based immunotherapy investigations have centered around HER2+ breast cancer as HER2-targeting monoclonal antibodies work, in part, through antibody-dependent cellular cytotoxicity, of which NK cells play a crucial role (115). NK cell immunotherapy has gained traction in TNBC, including a phase 1 investigation of PD-1 inhibition in combination with a novel inhibitor of the NK cell checkpoint poliovirus receptor related immunoglobulin domain containing (PVRIG) (NCT03667716). Given that HR+ tumors have low MHC-I and HR+ cell lines are highly susceptible to NK cell cytotoxicity, there may be great opportunity for NK cell-based therapy in HR+ breast cancer and further pre-clinical and clinical investigations are warranted.

Beyond TILs: Tumor Associated Macrophages (TAMs) in the TME

Historically, HR+ breast tumors have been considered immunologically cold as there are relatively few T cells associated with these tumors (26). However, other immune cells are associated with the TME in breast cancer. Beyond T cell subsets (cytotoxic T cells, T regulatory T cells), and other lymphocytes (natural killer cells and B cells), myeloid cells (macrophages and dendritic cells), plasmacytoid dendritic cells, and neutrophils have been identified in breast tumors, all of which are known to play critical roles in immunomodulation of cancer progression (116). In an analysis of 11,000 HR+ breast tumors, the immune cell type that correlated most significantly with poor clinical outcome was the presence of TAMs (117, 118). TAMs are a heterogeneous population of cells, generally characterized into an M2/M1 phenotypic and functional dichotomy, although TAMS are phenotypically much more dynamic and diverse. "M2-like" macrophages promote tissue remodeling and repair, secrete anti-inflammatory cytokines, and attract T regulatory and Th2 T cell subsets devoid of cytotoxic functions. TAMs are generally more "M2-like" and show pro-tumor functions by promoting tumor survival, proliferation, angiogenesis, and dissemination (119–125). Alternatively, "M1-like" macrophages are potent effector cells that kill microorganisms and tumor cells and can recruit cytotoxic T lymphocytes (CTLs) to activate adaptive immune responses. They can mediate phagocytosis and cross presentation of antigen to T cells. Clinically, the presence of TAMs is associated with metastasis (119) and poor survival (120, 121, 124), and has been shown to induce endocrine resistance in HR+ breast cancer cells *in vitro* and *in vivo* through NF-κB and IL-6-dependent signaling pathways (126). Importantly, a higher fraction of "M1"-like TAMs in HR+ breast cancer correlated with a higher pCR rate as well as prolonged DFS and OS (118). We recently reported that in HR+/HER2- breast tumors analyzed before and after NAC, sTIL and CD8+ cells were significantly decreased after treatment, whereas expression analyses revealed that there was increased expression of immunosuppressive (M2-like) macrophage-specific genes after chemotherapy. Macrophage biology and mechanisms of immune suppression in breast cancer has been recently reviewed by Mehta and colleagues (127). Macrophage reprogramming has shown tolerability and promise in solid tumors including breast cancer (128), and has been recently reviewed by Mehta and colleagues (127). Further work to identify strategies to harness the anti-tumor potential of macrophages may offer potential opportunities for the treatment of HR+ breast cancer.

IMMUNE CHECKPOINTS AND IMMUNOTHERAPY TRIALS IN HR+ BREAST CANCER

The first clinical target of ICB therapy was the T cell inhibitory molecule, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; CD152) (129–131). Subsequently, ICB agents targeting the T cell inhibitory molecule, programmed cell death protein 1 (PD-1; CD279) (132), and its ligand, PD-L1 (CD247) (133), were developed for the clinic. PD-1 is a receptor expressed mainly by T cells. Its ligand, PD-L1, is a transmembrane protein that plays a crucial role in shutting down active T cell responses and can be expressed on both tumor and immune cells (12, 134). PD-L1 binding to PD-1 functions as an adaptive mechanism for T cell inhibition, and in the context of cancer, induces tumor immune-suppression (2, 135). Sobral-Leite and colleagues characterized PD-L1 expression in 410 primary, treatment-naïve, breast tumors (162 HR+/HER2-, 101 HER2+ and 147 TNBC). PD-L1 positivity was defined as > 1% of immune or tumor cells as assessed by the E1L3N antibody clone. HR+/HER2- tumors had the lowest TIL density and PD-L1 expression. PD-L1-positivity was observed in 53.1% of HR+/HER2-, 73.3% of HER2+, and 84.4% of TNBC tumors and PD-L1 expression showed a strong correlation with TIL density (25).

Similar to TILs, PD-L1 expression has been found to be a prognostic marker in breast cancer, with studies demonstrating an association between PD-L1 expression and improved prognosis in TNBC but not HR+ breast cancer (25, 136–138). There are some data however, indicating that PD-L1 gene expression is associated with improved distant metastasis-free interval, progression-free interval and overall survival in HR+/HER2- breast cancer. In a recent study of 562 breast tumors, PD-L1 protein and gene expression was shown to be associated with a favorable prognosis in early stage invasive HR+/HER2- breast cancer (139). In addition, PD-L1 gene expression added prognostic value to currently validated 21- and 70-gene expression signatures in the same cohort as well as in an additional cohort of 1,081 patients (139).

Despite the limited number of TILs, low PD-L1 expression and low mutational burden in HR+ breast cancer (20), there has been an effort to determine if ICB has a role in HR+ disease (**Table 2**). While, to date, clinical trials testing ICB in HR+ breast cancer have not yet translated to FDA approval, there is opportunity to learn from both past and ongoing trials to identify the ideal therapeutic sequencing, combination strategies and patient population to extract value in this "immunologically cold" subtype of breast cancer, as reviewed below.

ICB Monotherapy in HR+ Breast Cancer

The first trials evaluating ICB as monotherapy in metastatic HR+ disease resulted in only modest response rates. For example, in the KEYNOTE-028 phase 1b trial, 25 heavily pretreated patients with metastatic, PD-L1+, HR+/HER2- breast cancer were administered pembrolizumab monotherapy (140). The objective response rate (ORR) in this cohort was 12% (partial response (PR; n=3), complete response (CR; n=0) with a clinical benefit rate [defined as CR, PR, or stable disease (SD) ≥ 24 weeks] of 20%. The median duration of response reached 12 months, which was higher than expected in this cohort of patients who were chemotherapy and/or endocrine therapy resistant. Of note, two of the three responders had a histology of invasive lobular carcinoma. The ORR in the HR+ cohort (ORR = 12%) was lower than that found for PD-L1+ TNBC patients (ORR = 18.5%) in the KEYNOTE-012 study (141), suggesting this treatment strategy may be more effective in a subset of patients with TNBC. Interestingly, the variation in PD-L1 expression between TNBC and HR+/HER2- breast cancer was also evident in screening participants for the KEYNOTE-012 TNBC study, in which 59% of the total screened had PD-L1+ tumors (141) compared to the KEYNOTE-028 HR+/HER2- study, where only 19% of the total screened were PD-L1+ (140). It is worth noting that not all PD-L1+ TNBC patients derive benefit from ICB and additional work is warranted for novel biomarkers that can predict immunotherapeutic responses and/or strategies that improve response to ICB (142). Importantly, PD-L1 IHC was performed similarly on FFPE archival (KEYNOTE-012) or excisional biopsy specimens (KEYNOTE-028) with a central laboratory that used the 22C3 anti-human PD-L1 antibody (Merck & Co.) PD-L1 expression was determined by combined positive score (CPS) defined as the number of PD-L1+ cells (tumor cells, lymphocytes, and macrophages) divided by the total number of tumor cells, multiplied by 100. According to that assay, a tumor is considered to have positive PD-L1 expression when CPS is greater than or equal to 1.

In the phase 1b JAVELIN trial, 168 heavily pretreated patients with metastatic breast cancer, regardless of subtype or PD-L1 status, were treated with the PD-L1 inhibitor, avelumab (143). Of the 168 patients, 72 had HR+/HER2- disease and the ORR for this group was 2.8% (2/72) compared to 5.2% (3/58) in the TNBC group. The median duration of response was not reached. In addition, subgroup analysis by PD-L1 status did not reveal any trend in efficacy. Given the low ORR, avelumab was determined to have limited therapeutic benefit as monotherapy in patients with metastatic HR+/HER2- breast cancer. Altogether, the KEYNOTE-028 and JAVELIN trials revealed the limited single-agent efficacy of ICB in HR+ breast cancer, particularly in heavily pretreated disease. The limited response to ICB monotherapy led to the inclusion of chemotherapy and other systemic therapeutics that may have synergism with ICB, a strategy used in TNBC.

ICB in Combination With Chemotherapy for HR+ Breast Cancer

Although chemotherapy has historically been considered immunosuppressive (144), robust preclinical and clinical data show that cytotoxic drugs enhance tumor immunity and have synergism with ICB. It is thought that after exposure to chemotherapy, release of tumor cell neoantigens from dying cancer cells can activate an anti-tumor immune response by inducing CD8+ T cell infiltration and activation. Those findings are important because, as discussed earlier, TILs are an independent predictor of response to chemotherapy (40). Preclinical models have shown that the tubulin-targeting drug, paclitaxel, increases tumor cell permeability to granzyme-B (released from CTLs) (145) and upregulates major histocompatibility complex (MHC) class I expression on cancer cell lines (146) to induce tumor cell immunogenicity. Importantly, in the phase III IMpassion130 trial, which tested adding atezolizumab (anti-PD-L1) to nab-paclitaxel (albumin-bound paclitaxel) demonstrated a significant improvement in PFS and a clinically meaningful improvement in OS in first-line treatment of PD-L1+ metastatic TNBC (16). Those results led to the FDA approval of atezolizumab in combination with nab-paclitaxel in PD-L1+ (SP142 IC≥1) metastatic TNBC, establishing the first ICB approval in breast cancer. More recently, pembrolizumab, in combination with different chemotherapy agents, was also approved for the treatment of locally advanced or metastatic TNBC, based on results from the KEYNOTE-355 trial (14). ICB in combination with nab-paclitaxel or chemotherapy is only approved for PD-L1-positive locally recurrent/advanced or metastatic TNBC, and while there are responses, the majority of patients eventually experience disease progression (147, 148).

Given the promising results using chemotherapy with ICB in TNBC, there has been an effort to replicate similar strategies in HR+ breast cancer. Like the early monotherapy trials, the initial chemotherapy plus ICB combination trials focused on heavily pretreated patients in the metastatic setting. The first of these trials used eribulin as a combination agent. Eribulin is a

TABLE 2 | Clinical trials in HR+ breast cancer assessing the safety and efficacy of ICB as monotherapy or in combination with chemotherapy and other treatment modalities.

Trial Identifier (name)	Treatments	Number of Evaluable HR + Samples	Patient Population	Phase	Results
Monotherapy					
NCT02054806 (KEYNOTE-028)	Pembrolizumab	25	Metastatic PDL1+ Heavily pretreated	1b	ORR of 12% (3/25), CBR was 20% with a median duration of response of 12 months
NCT01772004 (JAVELIN)	Avelumab	72	Metastatic Heavily pretreated	1b	ORR of 2.8% (2/72). Lack of response was irrespective of PDL1 status
Chemotherapy Combination					
NCT03051659	Pembrolizumab Eribulin	44	Metastatic Moderately pretreated	II	Addition of Pembrolizumab did not effect mPFS (4.1 *vs* 4.2). No trend with PDL1 status, TILs, TMB. Grade 3-4 AEs seen in 54.6% of patients
NCT03044730	Pembrolizumab Capecitabine	14 (16 TNBC)	Metastatic Endocrine resistant	II	Of the 29 evaluable patients, ORR was 14%, CBR was 28% with a median PFS of 4 months. The response rates did not differ between subtypes
NCT01042379 (ISPY-2)	Pembrolizumab Paclitaxel Doxorubicin Cyclophosphamide	40	Neoadjuvant	II	Addition of Pembrolizumab nearly tripled the PFS (34% *vs* 13%). Likelihood of success in a phase III trial prediction was 99.6%
Other Systemic Therapies					
NCT01042379 (ISPY-2)	Durvalumab Olaparib Paclitaxel	52	Neoadjuvant BRCA+/-	II	Estimated pCR of 28% with a 74.5% likelihood of success in a stage III trial
NCT02734004 (MEDIOLA)	Durvalumab Olaparib	13 (21 TNBC)	Metastatic	II	Of the 30 evaluable patients, at 12 weeks, DCR was 50% with a median PFS of 8.2 months. Survival by subtype was comparable
NCT02779751 (JPCE)	Pembrolizumab Abemaciclib	28	Metastatic Endocrine resistant	Ib	At 12 months, ORR was 28%, DCR was 82% with a median PFS of 8.9 months.
Radiotherapy					
NCT03051672	Pembrolizumab Radiotherapy	18	Metastatic	II	No objective response observed

microtubule inhibitor that, in addition to antimitotic activity, has been shown to reverse epithelial-mesenchymal-transition (EMT) (149) and decreased numbers of FOXP3 and PD-L1 expression as measured through IHC (150). In the phase II trial, eribulin (E) with or without pembrolizumab (P) was evaluated in 88 (44 E+P, 44 E) patients with HR+/HER2- metastatic breast cancer (151). In this cohort, the patients had received at least two prior lines of endocrine therapy and up to two lines of chemotherapy. The addition of pembrolizumab to eribulin did not add any benefit to median PFS (4.1 *vs* 4.2 months, p=0.38). In addition, PD-L1 status, TILs and TMB were not associated with median PFS. Importantly, 54.6% of patients who received E+P experienced grade 3-4 adverse events, including 2 treatment related deaths.

Another trial tested the combination of capecitabine with pembrolizumab (152). Capecitabine is a prodrug of 5-Fluorouricil (5-FU), which inhibits DNA replication. The ability of 5-FU to enhance immune activity is debated. In preclinical studies, 5-FU has been shown to increase expression of carcinoembryonic antigen (CEA) in breast cancer cell lines (153) and reduce the number of myeloid derived suppressor cells (MDSCs) in murine models (154). However, in patients with pancreatic cancer, 5-FU failed to elicit a decrease in MDSCs (155) or a decrease in MDSC promoting cytokines (156). In this phase II trial, 30 patients with metastatic breast cancer and previous endocrine resistance (14 with HR+ disease and 16 with TNBC) were treated with a combination of pembrolizumab and capecitabine (152). Among the 29 evaluable patients, the median PFS was 4 months, the ORR was 14% and the clinical benefit rate (CBR) was 28%. The response rates did not differ between subtypes. Given this relatively modest response rate, this regimen was deemed not worthy of further study in breast cancer.

The lack of clinical benefit in both the eribulin and capecitabine combination trials may indicate that these chemotherapeutic agents do not sufficiently increase tumor immunogenicity to a level that enhances ICB efficacy. Targeted chemotherapy in the form of antibody drug conjugates (ADC) may better augment tumor immunogenicity, as suggested by the efficacy of the anti-Trop-2-SN-38 ADC sacituzumab govitecan in heavily pretreated HR+ metastatic breast cancer refractory to endocrine therapy (157). To test whether ADC therapy synergizes with ICB, the ongoing SACI-IO HR+ trial is investigating whether pembrolizumab added to sacituzumab govitecan improves progression-free survival compared to sacituzumab govitecan alone in PD-L1+ metastatic HR+ disease (NCT04448886). However, an alternate explanation for the lack of efficacy may be the fact that these trials evaluated ICB in heavily pretreated patients with metastatic breast cancer. Compared with metastatic tumors, primary breast cancers have more TILs and higher PD-L1 expression (60, 158), both of which are predictive of response to immunotherapy (2, 25, 136), leading to the hypothesis that ICB could have a more impactful role in the neoadjuvant setting.

In the ISPY-2 trial, 40 HR+/HER2- and 29 TNBC patients were treated in the neoadjuvant setting with pembrolizumab in combination with standard chemotherapy (paclitaxel followed by doxorubicin and cyclophosphamide) (13). pCR was used as the primary endpoint and the study aimed to determine if the combination of pembrolizumab with neoadjuvant chemotherapy was likely to succeed in the phase III clinical trial. In the HR+ subgroup, the addition of pembrolizumab yielded a higher rate of pCR compared to that of the chemotherapy arm (34% *vs* 13%, respectively). Benefit was also seen in the TNBC cohort (60% *vs* 20%). Importantly, the ISPY-2 trial concluded that the predictive probability of this treatment strategy succeeding in a phase III, HR+/HER2- trial was 99.6%. Pembrolizumab was the first agent of ten studied to graduate in the HR+/HER2- subtype in the ISPY-2 trial and may suggest that further stratification or targeting of HR+ patients would reveal which populations would benefit from ICB. With these promising results, the idea of successful implementation of ICB in the "immunologically cold" HR+ subtype was revitalized. Specifically, this arm of the ISPY-2 trial showed that by focusing on patients in the early setting, ICB may have a beneficial role in HR+ disease. Moreover, the results suggest that there may be informed ways to identify the right chemotherapy combinations, particularly for breast cancers that are not innately sensitive to ICB. However, further analyses of long-term outcomes are needed to critically evaluate if the combination of ICB and chemotherapy will provide long-term benefit compared to the potentially life-threatening adverse effects that may be associated with such combinations. Importantly there are two phase III clinical trials evaluating ICB in HR+/HER2- breast cancer in the preoperative setting. In the first, the activity of pembrolizumab in combination with standard chemotherapy and hormone therapy in the preoperative and adjuvant setting versus chemotherapy and hormone therapy alone is being evaluated in stage I-III HR+ breast cancer patients (NCT03725059). Another phase III trial is evaluating the safety and efficacy of adding nivolumab (anti-PD-1) in the preoperative and adjuvant setting in combination with standard therapy in stage II/III HR+/HER2- breast cancer patients (NCT04109066). Early use of ICB in HR+ breast cancer may provide insight into how ICB fits into the clinical care of HR+ breast cancer patients.

ICB in Combination With Other Treatment Modalities for HR+ Breast Cancer

ICB in combination with chemotherapy for the treatment of HR+ breast cancer has shown some success, particularly in the neoadjuvant setting; however, it remains unclear if chemotherapy is sufficient to reverse these immunologically cold tumors. Importantly, there is a wide variety of treatment options for patients with HR+ breast cancer including targeted molecules and radiation. Thus, there has been an interest in the synergistic potential of these other treatment modalities.

PARP inhibitors olaparib (159) and talazoparib (160) are approved for the treatment of advanced breast cancers with BRCA1/2 germline mutations. These drugs block the base excision repair pathway, leading to DNA damage, and induce synthetic lethality in BRCA mutant breast cancers (161). More recently, PARP inhibitors were shown to generate an antitumor response through activation of the STING (stimulator of interferon genes) pathway (162). In murine models, STING-dependent infiltration of CD8+ T cells was demonstrated to be required for response to olaparib (163). Furthermore, PARP inhibitors were found to increase PD-L1 expression in breast cancer cell lines and murine models (164). Thus, PARP inhibitors represent a promising combination therapy with ICB. In the phase II MEDIOLA trial, the efficacy of durvalumab (anti-PD-L1) in combination with olaparib was assessed in 34 patients with metastatic breast cancer (13 had HR+ disease, 21 had triple negative disease) with germline *BRCA*1/2 mutations (165). Of the 30 evaluable patients, this combination strategy achieved (at 12 weeks) was a disease control rate (DCR) of 85% with a median PFS of 8.2 months. Median OS was comparable between the subtypes (HR+ = 22.4; TNBC = 20.5) and was comparable to either agent used as monotherapy. Interestingly, the efficacy was dependent on the extent of prior treatment. Patients with 0-1 prior lines of chemotherapy experienced a longer median duration of response (12.9 months *vs*. 5.5 months) and a longer median PFS (11.7 months *vs* 6.5 months) compared to patients with 2 prior lines of chemotherapy. With the exciting results from the MEDIOLA trial, PARP inhibitors gained much interest as a combination strategy with immunotherapy. In a second arm of the ISPY-2 trial, neoadjuvant durvalumab and olaparib in combination with paclitaxel (DOP) were compared to paclitaxel alone in patients with high risk, HER2- breast cancer (52 HR+ and 21 TNBC), regardless of BRCA status (166). Both subtypes yielded a significant clinical benefit with an estimated pCR of 28% in HR+ patients and 47% in TNBC patients. The estimated probability of success in a phase III clinical trial for DOP in HR+ patients was 74.5%. Importantly, this trial showed PARP inhibitors have synergism with checkpoint blockade, regardless of BCRA status. However new data indicate that PARP inhibitors may negatively modulate the TME by inducing suppressive TAMs and therefore should be further evaluated (167).

CDK4/6 inhibitors (abemaciclib, palbociclib, ribociclib) inhibit cell cycle progression and are approved for patients with HR+/HER2- metastatic breast cancer (168, 169). In addition to cell cycle inhibition, abemaciclib has been shown to enhance immunogenicity within the TME through increased antigen presentation, increased CD8+ T cell infiltration, and decreased T-reg infiltration and proliferation (170) and PD-L1 expression (171). Goel et al. first reported that abemaciclib plus anti-PD-L1 induced durable responses in preclinical models of HR+ breast cancer and mice deemed tumor free were protected from subsequent tumors when re-challenged with tumors, suggesting sustained immune memory (170). Similarly, Schaer and colleagues showed synergism between abemaciclib and PD-L1 inhibitors in murine models (172). These results were confirmed in the NeoPalAna trial, in which patients with primary HR+ breast cancer underwent tumor biopsies prior to palbociclib and then at 2 and 12 weeks of treatment. Gene expression profiling revealed that the addition of palbociclib to endocrine therapy enhanced anti-tumor immunity, as seen in the mouse models (170). Thus, CDK4/6 inhibition is a potential candidate to combine with ICB in patients with HR+ breast cancer.

In cohort C of the phase 1b JPCE trial, the efficacy of abemaciclib in combination with pembrolizumab was assessed in 28 patients with endocrine resistant, metastatic HR+/HER2- disease (173). The inclusion criteria were 1-2 prior treatments with chemotherapy, no previous CDK4/6 or ICB treatments and ECOG PS ≤1. At 24 weeks, 8 patients achieved a confirmed partial response (ORR 28%). The DCR was 82%, median PFS was 8.9 months and OS was 26.3 months. We compared to abemaciclib monotherapy in a similar patient population (MONARCH1) (174), the clinical benefit was not only numerically but also statistically significantly improved. Combination therapy resulted in numerically higher rates of elevated transaminases; however, the overall safety profile was considered generally tolerable. Importantly, in another cohort (cohort D) of the JPCE trial, the safety of abemaciclib in combination with pembrolizumab and the aromatase inhibitor (AI) anastrozole was assessed in 26 patients with locally advanced or metastatic HR+/HER2- breast cancer (175). Preliminary safety results revealed a high level of grade 3/4 AEs including 8 patients with neutropenia, 6 patients with elevated alanine aminotransferase and 2 therapy-related fatalities (pneumonitis). Given the high level of adverse events in cohort D, further development of this triple approach has been discontinued (176).

Radiotherapy is a well-established local therapy that has shown a survival benefit in high risk and early-stage breast cancer (177). Historically, the benefits of radiotherapy were attributed to cell-autonomous death from overwhelming DNA damage. However, further analysis revealed radiation-induced DNA damage can stimulate a systemic immune-mediated anti-tumor response, known as the abscopal effect (178). Importantly, a recent trial in TNBC found the combination of pembrolizumab and radiotherapy resulted in partial and durable responses in 33% of patients (3 of 9) (179). Thus, synergistic effects of radiation with immunotherapy were tested in HR+ breast cancer patients. In a phase II trial, the efficacy of pembrolizumab in combination with palliative radiotherapy was assessed in 8 patients with HR+ metastatic breast cancer (180). There were no objective responses observed among 8 patients, resulting in early closure of the study. In contrast, a similar trial in patients with TNBC demonstrated a partial response in 33% and stable disease in 11% of the 17 patients (181). While the combination of radiotherapy plus pembrolizumab produced no objective responses in the HR+ patient population, it is important to note that the patients in this study were very heavily pretreated, and the number of patients was small, making it difficult to draw definitive conclusions from this trial. Determining the potential benefits of combining systemic ICB treatment with local radiotherapy likely warrants future studies. As with other combination strategies, finding the optimal patient population and sequence of treatment may yield clinical benefit.

Other Checkpoint Inhibitors in HR+ Breast Cancer

Other T cell checkpoints other than CTLA4 and PD-1 have been identified and have been targeted for anti-cancer therapy and has been previously reviewed (182). For example, T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) is upregulated by immune cells, including activated T cells, natural killer cells, and regulatory T cells and T-cell immunoglobulin and mucin domain 3 (Tim-3) is a checkpoint receptor expressed by a wide variety of immune cells as well as leukemic stem cells. Both TIGIT and Tim-3 are promising new target for cancer immunotherapy (183, 184). There are currently Phase I trials evaluating TIGIT and Tim-3 including for patients with breast cancer. Future work will determine if these other checkpoints will be relevant for HR+ breast cancer. However, given the low recruitment of both T cells and NK cells as well as low tumor and immune cell expression of PD-L1 in HR+ breast cancer, it will be important to identify other ways to modulate the TME to successfully activate an anti-tumor immune response in HR+ breast cancer. In a study of approximately 450 HR+ tumors treated with AI, AI-resistant luminal B tumors revealed an upregulation of immune checkpoint components, particularly indoleamine 2, 3-dioxygenase 1 (IDO1), lymphocyte-activation gene 3 (LAG3), and PD-1, which are associated with negative regulation of T cell activation and function (185, 186). Additionally, downregulation of the human mutL homolog 1 (MLH1), which is vital in mismatch DNA repair, was also identified in AI-resistant tumors. IDO1 expression in intraepithelial myeloid cells was strongly associated with PD-L1 expression on carcinoma cells and PD-1 and LAG3 expression on TILs. This study also provided evidence that IDO1+ macrophages correlated with CD8+ T cells and might suggest a mechanism of T cell suppression (187). The IDO1 inhibitor, epacadostat, has been recently tested in clinical trials and has shown both safety and activity, especially in combination with Ipilimumab (anti-CTLA4) in metastatic melanoma (188–190). Taken together, these findings suggest that subsets of HR+ breast cancer may benefit from IDO-targeted treatment and may warrant further study.

There is evidence that estrogens can modulate PD-1/PD-L1 expression in endometrial tissue (191) and on immune cells (192, 193), and PD-L1 expression on HR+ breast cancer cells *in vitro* (194), which may limit the function of T cells in HR+ breast cancer. Anti-estrogen therapy has been shown to amplify immunotherapeutic target expression of α-lactalbumin on breast cancer cells. α-lactalbumin is a lactation protein negatively regulated by estradiol-17β and has been a target of vaccination in TNBC (195). Therefore, anti-estrogen therapy may downregulate PD-L1 expression and increase other targets, acting as a priming event for concurrent therapy to induce an anti-tumor immune response (196, 197). In addition, in preclinical studies, steroid-like selective ER degrader (SERD) fostered immune stimulatory activity by inhibiting suppressive myeloid cells and, in combination with anti-PD-L1 therapy, induced tumor regression and activation of anti-tumor macrophages and T cells (197).

Anti-estrogen therapy has also been shown to regulate CD47 expression. CD47 is a widely expressed cell-surface receptor that inhibits phagocytosis signaling through its engagement with SIRP1α on macrophages. High expression of CD47 correlates

with worse survival in both HR+ and HER2+ breast cancer but not TNBC (198). CD47 is highly expressed in endocrine therapy-resistant tumors, suggesting a new role for CD47 in mediating anti-estrogen resistance (199). Targeting the unfolded protein response, GRP78, re-sensitized tumors to anti-estrogen treatment and correlated with increased levels of calreticulin and high molecular group box 1 (HMGB1) protein, indicating activation of immunogenic cell death pathways (200). Co-expression of GRP78 and CD47 is associated with a significant decrease in survival in HR+/HER2- breast cancer (200). In addition, CD47 has been shown to have increased expression on HR+ breast cancer cells following hypoxia (201). Furthermore, H3K27ac ChIP-Seq profiling revealed downstream super enhancers associated with CD47 in an HR+ breast tumor and HR+ cell lines but not TNBC tumors or cell lines (202). Anti-CD47 therapy has been extensively studied for the treatment of other cancers to eliminate tumor cells through macrophage phagocytosis (203, 204). Such strategies may offer therapeutic utility in the treatment of HR+ breast cancers, especially those resistant to endocrine therapy (119–124). Given that HR+/HER2- tumors generally do not present with the T cell inflamed phenotype, developing alternative strategies for activating anti-tumor immune responses remains an unmet need. In that regard, use of current as well as novel technologies should be employed for deep characterization of HR+ breast tumors with the goal of elucidating immune mechanisms in the TME that can incite the next generation of clinical trials to enhance immune signaling in HR+ disease.

METHODS FOR INTERROGATING THE TME TO REVEAL NOVEL ICB TARGETS

Recent advances in molecular and genomic profiling, as well as multi-plex tissue analysis have allowed a deep understanding of the TME and have revealed novel mechanisms and opportunities to overcome immune suppression in HR+ breast cancer, as reviewed here. Further strategies aimed at more deeply characterizing the TME of HR+ breast cancer and contrasting it to immune rich, ICB-responsive tumors may greatly facilitate development of novel strategies for the use of ICB in HR+ breast cancer. In this section we aim to review current technologies used to explore the TME and include both advantages and disadvantages to each strategy.

Immunohistochemistry

Several studies have demonstrated that high TILs and PD-L1 expression have been linked to predictive benefit of anti-PD-1/L1 therapy in TNBC (16). Importantly, these are assays that require formalin-fixed paraffin-embedded (FFPE) tissue. Significant heterogeneity of PD-L1 protein expression identified by IHC has been reported in several studies (25, 139). This observed heterogeneity could be caused by the wide array of IHC platforms and antibodies, as well as pathological scoring methods and cutoffs. In addition, the use of tissue microarrays (TMAs) may limit conclusions. A recent study revealed that out of 118 tumors used to compare TMA with whole slide observations, 49% of the TMA tumor results were false negatives, whereas whole tissue sections that the TMAs were derived from revealed positive staining (25). In addition, TILs and/or PD-L1 may not hold the best predictive or prognostic value in HR+ breast cancer.

Macrophages comprise a significant portion of the breast TME (205) and have recently been the focus of several studies using IHC to interrogate HR+ breast tumor samples (206–211). To detect macrophages, IHC studies have most commonly used antibodies against CD68 (207–211) and CD163 (206). Notably, Luminal A (LumA) tumors have been shown to have fewer macrophages compared to Luminal B (LumB) tumors (206, 207). The increased numbers of macrophages in LumB tumors have been associated with an increase of Ki67+ proliferative tumor cells (206, 207), high tumor grade (206, 207, 210, 211) and loss of ER (206–210). In addition, tamoxifen-resistant patients have been shown to have increased numbers of CD163+ macrophages in the TME compared to tamoxifen sensitive patients (212). Increased density of macrophages in breast tumors has been suggested to predict poor prognosis (207), although some studies have been unable to confirm this association (209). Tumor cells can evade macrophage phagocytosis by overexpressing the 'don't eat me' signal CD47, inducing immune escape (213). Yuan and colleagues focused on capturing the interaction between CD68+ macrophages and CD47+ tumor cells in 217 primary breast tumor samples (n=96 HR+) (210). CD68+ macrophages were frequently seen within close proximity of CD47+ tumor cells in all breast cancer subtypes. Nearly 40% of HR+ tumors were characterized with high expression density of both CD47 and CD68, which implies potential crosstalk between tumor cells and macrophages, and the formation of an immunosuppressive TME, at least in a subset of HR+ breast tumors. The combined high expression of CD47 and CD68 was associated with poor prognosis in patients with HR- breast tumors, but no association was observed in patients with HR+ tumors (210).

Genomic and Transcriptomic Profiling

Genomic and transcriptomic profiling of bulk tumor tissue has vastly expanded our knowledge of immune cell phenotypes in HR+ breast tumors. Recently, an extensive immunogenomic profiling of cancers analyzed by The Cancer Genome Atlas (TCGA) were characterized for assessment of total lymphocyte infiltrate, immune cell fractions, gene expression, neoantigen prediction as well as T cell receptor and B cell receptor (214). The analysis included 508 LumA and 191 LumB tumors and revealed six clusters of immune subtypes. The study revealed that a majority (86%) of LumA tumors belonged to either the wound-healing, interferon gamma IFNγ dominant or inflammatory immune subtype (214). In contrast, 95% of LumB tumors belonged to either the wound-healing, IFNγ dominant or lymphocyte-depleted subtype. The wound-healing subtype was characterized by an increased expression of angiogenic genes,

a high proliferation rate and a trend toward T helper 2 (Th2) dominant lymphoid infiltrate. The IFNγ dominant subtypes had increased signatures of $CD8^+$ T cells and a substantial number of lymphocytes compared to macrophages. In contrast, the inflammatory subtype was characterized with increased levels of Th17 gene signatures and a balanced macrophage/lymphocyte ratio. Lymphocyte-depleted subtypes had elevated levels of macrophage signatures, notably M2, with Th1 suppressed response (214). This study challenges the previous paradigm of immunologically cold HR+ breast tumors and highlights the importance of various immunosuppressive mechanisms that are active within HR+ breast tumors.

CIBERSORT (215) is a computational method that quantifies the proportion of 22 functional immune subsets within bulk tissue gene expression profiles. Ali and colleagues used CIBERSORT to analyze bulk gene expression profiles of 10,988 breast tumors (n=5,807/53% HR+/HER2-) from 56 publicly available datasets (117). Specifically, this study aimed to determine the relationship between TME composition and molecular subtype, survival and response to chemotherapy. In HR+ tumors, the presence of M0 macrophages and regulatory T cells were associated with poor prognosis (117), which was later confirmed by another group studying the prognostic significance of tumor-infiltrating immune cells in breast cancer (216). Notably, the HR+ tumors lacking immune infiltration were associated with intermediate or similar survival outcomes compared to HR+ tumors with high or low immune infiltrates. Thus, in this large cohort, the presence of immune cells was not prognostic of outcome in HR+ breast tumors (117).

Recent work from Cassetta and colleagues has identified a TAM signature that is highly enriched in aggressive breast cancer subtypes and associated with shorter disease-specific survival, interestingly the signature was found in all subtypes, providing evidence of heterogeneity in each subtype (217). Bense et al. characterized the immune cell composition and functionality of 7,270 breast tumors (n=4,094 HR+/HER2-) (118). This study used raw microarray expression data from primary breast tumors that were publicly available in the Gene Expression Omnibus (GEO) database (218). CIBERSORT was used to estimate immune cell type fractions, and the relationship between the immune cell type fractions and five different immune signatures was determined (37, 219–222). In the HR+/HER2- cohort, a higher fraction of M1 macrophages was predictive of pCR to neoadjuvant chemotherapy and prognostic of DFS and OS. A high CD4+ follicular helper T cell signature score was associated with prolonged DFS and OS (37). Additionally, a high CD8+ T cell exhaustion signature score was associated with shorter DFS in patients with HR+ tumors regardless of HER2 status, suggesting the hypothesis that CD8+ T cell exhaustion could be related to immune evasion in HR+ breast cancer. However, this observation was not confirmed in the subgroup analyses focusing only on HR+/HER2- or HR+/HER2+ tumors.

In another effort to study the complex relationship between ER positivity and inflammatory response, gene expression of 195 breast tumors was compared to matched adjacent normal tissue (223). Surprisingly, HR+ tumors had a decrease in macrophage related gene signatures compared to adjacent normal tissue samples. In addition, there was an inverse correlation between the tumor estrogen pathway expression and the tumor macrophage score, suggesting that high levels of estrogen signaling have suppressive effects on macrophages in the breast tumor microenvironment.

Single-cell Analysis

Single-cell RNA sequencing allows precise cell state mapping and reveals individual immune cell phenotypes within tumors. One of the early efforts to characterize the immune landscape of breast tumors with single-cell RNA sequencing was made by Chung et al., who analyzed a total of 175 immune cells from 11 breast cancer patients (18). The detected TAM populations were enriched for genes related to immunosuppression and promotion of tumorigenesis. Azizi et al. performed more extensive profiling of the breast TME (n=8 primary breast tumors; 5/8 HR+) (224). They observed an increased diversity of immune cell states in breast tumors compared to normal breast tissue. Notably, when focusing on the macrophage populations in these breast tumors, both immunosuppressive and immunostimulatory related gene signatures were frequently expressed in the same cells. The positive correlation of both pro- and anti-tumor associated genes challenges the previously suggested and mutually exclusive M1 and M2 activation states and highlights the continuous spectrum of activation states of TAMs in breast cancer.

Molecular profiling with mass cytometry (CyTOF) of 138 breast cancer patients (39% LumA, 51% LumB) using 34 immune cell targets and 38 tumor-centric antibodies with mass cytometry (225) revealed epithelial, endothelial, fibroblasts and immune cells. The most abundant immune cell types were T cells and myeloid cells. Twenty unique CD4+ and CD8+ T cell clusters were identified. A minor proportion of both LumA and LumB tumors harbored PD-1+ T cells. However, the PD-1+ T cells were more abundant in LumB tumors compared to LumA tumors. When focusing on the co-expression of PD-1, CTLA-4 and activation marker CD38 across the various T cell phenotypes, the authors found PD-1^{int}CTLA-4^-CD38^- T cells were more frequent in LumA tumors compared to LumB tumors. Notably, a minor subset of all HR+ tumors had increased frequencies of PD-1^{high}CTLA-4^+CD38^+ T cells and T regs, suggesting that a specific subset of HR+ breast cancer patients could be candidates and benefit from immune checkpoint blockade therapies. In addition to various T cell phenotypes, 19 unique myeloid cell clusters were identified, which were further divided into five categories: 1) CD14-expressing monocytes ($CD14^{+/int}CD16^{-/+}$), 2) early immigrant macrophages (HLA-$DR^{int}CD192^+$), 3) tissue-resident macrophages ($CD206^+$HLA-DR^{int}), 4) TAMs ($CD64^{high}$HLA-DR^{high}) and 5) myeloid-derived suppressor cells (HLA-$DR^{-/low}$). The composition of these heterogenous myeloid cell categories varied according to the tumor grade and histopathological subtype. When focusing on PD-L1 expression in the myeloid compartment, PD-$L1^+$ TAMs were more abundant in LumB compared with LumA tumors. The frequency of PD-$L1^+$ TAMs was also higher in grade 3 than in grade 2 tumors (225).

Multiplex Tissue Analysis

Although the previously presented studies using CyTOF, bulk RNA and single-cell RNA sequencing provide comprehensive insight on the heterogeneity of cell phenotypes and states across breast cancer subtypes, these methods lack the spatial information of the tissue architecture and do not provide an opportunity to evaluate the relationships of single cells in the spatial context. Several single-cell imaging techniques have been used to address this challenge, including multiplex IHC (226), cyclic immunofluorescence (CyCIF) (227), CODEX (228), multiplexed ion beam imaging (MIBI) (229) and imaging mass cytometry (IMC) (230–233). However, to date, only two publications (234, 235) have focused on HR+ breast tumors with the previously mentioned single-cell pathology techniques.

Jackson et al. studied the complex single-cell phenotypes and their spatial location in breast tumors with IMC (235). The aim was to quantify spatial inter- and intratumor heterogeneity of the breast TME on a single-cell level. In this study, tissue microarrays (TMAs) composed of 352 breast tumors (n=175 HR+/HER2-) were analyzed. Diverse cell phenotypes of endothelial, immune, stromal, and tumor cells were identified using 35 antibodies. Populations of fibroblasts, endothelial, and immune cells were present at similar densities in each breast tumor subtype. When looking at the cell-cell interactions, a subset of microenvironment communities was enriched for only T cells, while communities consisting of large networks of T and B cells across the samples were also identified, possibly implying the existence of TLS. The microenvironment communities that were enriched in fibroblasts had decreased numbers of immune cells, which supports the hypothesis of fibroblasts as mediators of immune exclusion (236). Interestingly, HR+ tumors harbored a range of fibroblast-enriched stromal environments, and only a subset of HR+ tumors contained rare and localized immune-enriched stromal environments (236).

As a follow-up study, the effect of somatic alterations on the cellular composition of breast tumors and the architecture of the tumor microenvironment was studied by coupling single-cell IMC data to the multiplatform genomic profiling with transcriptomic, Copy Number Aberration (CNA) and microRNA data (234). A total of 483 primary breast tumor samples (30.8% LumA, 21.1% LumB) from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort collected between 1985 and 2005 were included in this comprehensive phenogenomic analysis. IMC analysis revealed various epithelial, stromal and immune cell phenotypes. Breast cancer subtypes were determined with PAM50 gene expression profiles. Within this cohort, the only immune cell phenotype enriched in the HR+ tumors were $Vim^{+}Slug^{-}$ macrophages, which were enriched in the LumB subtype. LumA tumors were characterized by enrichment of several distinct fibroblast and myofibroblast phenotypes, that were not found as extensively within the other genomic breast cancer subtypes. The expression of hormone receptors and various cytokeratins within epithelial cells also differed between LumA and B tumors. Beyond cell phenotyping, the authors showed how certain epithelial, stromal, and immune cell phenotypes were linked with underlying driver gene alterations and CNAs. The number of proliferative cells, macrophages and T cells increased with genomic instability. The authors concluded that the cell phenotypes are diverse across the breast cancer genomic subtypes and that the luminal tumors were composed of a mixture of cell phenotypes rather than of a single dominant cell population. The authors noted that the phenotypic compositions of luminal tumors seemed to be largely affected by both somatic alterations and the transcriptional programs induced by ER signaling, which is consistent with previous studies suggesting that endocrine therapy expands the phenotypic clones that are under-presented at the time of diagnosis (237).

The field of single-cell analysis is constantly growing, and these previously mentioned modern techniques (226–230) will greatly contribute to our understanding of the complexity of HR+ breast TME. The evaluation of large tumor areas with high-throughput, whole tissue section imaging methods, such as CyCIF (227) (**Figure 1**), and in the future, 3D modeling of tumor architecture will provide a deeper knowledge of potential novel biomarkers and therapeutic targets in HR+ breast cancer.

DISCUSSION

In this review, we explored the immune microenvironment of HR+ tumors, along with pre-clinical approaches and clinical investigations in HR+ breast immuno-oncology. We shed light that in fact, HR+ tumors are not devoid of immune infiltration. Next generation sequencing and various histologic approaches show that there is an endogenous, albeit limited, immune response to HR+. However, an immunosuppressive TME characterized by TAMs and low levels of tumor HLA-I expression, limits anti-tumor immune activity and may be the culprit for T cell and NK cell exclusion. Additionally, low PD-L1 expression on HR+ tumors and infiltrating immune cells may further limit the efficacy of PD-1/PD-L1 targeted therapy. We further posit that deep mechanistic and functional characterization of the immunologic aspects of the TME in HR+ breast cancer is urgently needed. Comprehensive profiling of HR+ tumors at baseline and on treatment, combined with pre-clinical study, should lead to improved understanding of the TME and reveal mechanisms by which HR+ breast cancers obstruct T cell and NK cell infiltration, evoke low levels of HLA class I expression and are broadly resistant to ICB.

Patients with metastatic HR+ breast cancer have shown limited response to checkpoint inhibition, and clinical investigations into this patient population has thus been limited. Importantly, the ISPY-2 trial (neoadjuvant chemotherapy plus pembrolizumab) revealed, for the first time, a clinically effective immunotherapeutic strategy for patients with HR+ breast cancer. Furthermore, data from PARP and CDK4/6 inhibitor combinations with checkpoint inhibitors are promising. There are currently multiple on-going clinical trials assessing the combination of checkpoint blockade with PARP inhibitors (NCT03594396, NCT02849496) and CDK4/6 inhibitors (NCT02778685, NCT02779751, NCT0314728, NCT03147287, NCT03573648, NCT03294694) in HR+ breast cancer. Identifying the appropriate combination strategy, sequencing of treatment and

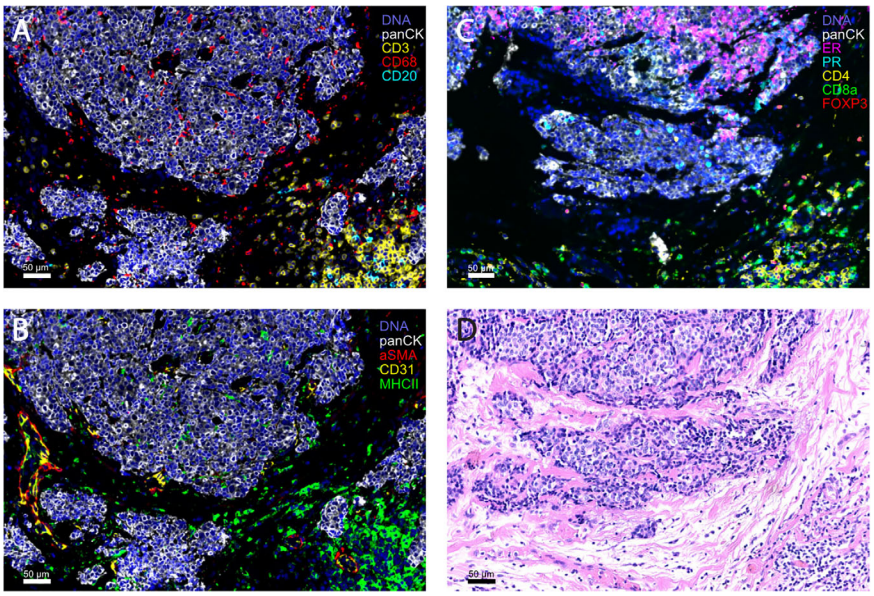


FIGURE 1 | Representative images of HR+ breast tumors obtained with highly multiplex cyclic immunofluorescence (CyCIF) imaging **(A-C)** and the corresponding H&E section **(D)**. CyCIF is a robust tool for the investigation of the complexity of the tumor microenvironment, by linking the cell type with spatial information. **(A, B)** are from the same formalin-fixed, paraffin embedded (FFPE) slide and **(C, D)** are both from serial sections of a primary breast tumor (invasive ductal carcinoma, HR+HER2-).

patient population is critical to the optimal use of ICB in HR+ breast cancer. It is possible that targeting alternative checkpoints such as TIGIT and Tim-3, largely expressed on T cells, as well as therapies against NK cell checkpoints, such as killer cell immunoglobulin-like receptors (KIR; also known as CD158) and NKG2A, will be effective in HR+ breast cancer. However, effective targeting of such checkpoints will likely require appropriate recruitment strategies aimed at getting T cells and NK cells into the tumor.

We present novel immunotherapy strategies that warrant new lines of investigation, such as adding other agents (i.e. targeted therapies such as small molecule drugs or monoclonal antibodies) that impact the TME, thereby increasing TIL (both T cell and NK cell) infiltration and enhancing response to ICB. As an example, it has been shown that CDK4/6 inhibitors result in tumor-expression of cytokines that promote T cell recruitment. We also highlight the role of immune cells other than T cells, such as TAMs, which are abundant in HR+ breast tumors and play an immunosuppressive role in the TME. Further work is needed to better characterize TAMs in HR+ breast cancer, which will inform how to move forward in targeting these cells for anti-cancer therapy. Taken together, these findings will be critical for next generation clinical trials to harness the power of immunotherapy in HR+ breast cancer.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

FUNDING

EM acknowledges the Rob and Karen Hale Distinguished Chair in Surgical Oncology for support. JG is supported by the Dana-Farber/Harvard Cancer Center (DF/HCC) Specialized Program of Research Excellence (SPORE) in Breast Cancer P50 CA1685404 Career Enhancement Award, The Susan G. Komen Foundation Career Catalyst Award CCR18547597, The Terri Brodeur Breast Cancer Foundation, The Saverin Family Foundation at Dana-Farber Cancer Institute and The Ludwig Center at Harvard. SM is supported by a Department of Defense BCRP Era of Hope Expansion Award W81XWH2010472 and a METAvivor Translational Research Award, Chicago Metsquerade Presented in Memory of Lauren Smoke. TV acknowledges grant support from the Finnish Medical Foundation, Relander Foundation, Turku University Foundation, Maud Kuistila Memorial Foundation, the Finnish Society of Oncology, and the Cancer Society of Southwest Finland.

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Conflict of Interest: EM has been compensated for participation on Scientific Advisory Boards for Astra-Zeneca/Medimmune, Celgene, Genentech, Exact Sciences (formerly Genomic Health), Merck, Peregrine Pharmaceuticals, SELLAS Lifescience, and Tapimmune and has had clinical trial support to her former institution (M.D. Anderson Cancer Center) from Astra-Zeneca/Medimmune, EMD-Serono, Galena Biopharma and Genentech, and current institution from Genentech *via* a SU2C grant. EM has also had sponsored Research Support to the laboratory from GSK and Eli Lilly.

JG is a consultant for Glaxo-Smith Kline (GSK), Codagenix, Verseau, Kymera and Array BioPharma and receives sponsored research support from GSK, Array BioPharma and Eli Lilly.

ST receives institutional research funding from AstraZeneca, Lilly, Merck, Nektar, Novartis, Pfizer, Genentech/Roche, Immunomedics, Gilead Exelixis, Bristol-Myers Squibb, Eisai, Nanostring, Cyclacel, Odonate, and Seagen; has served as an advisor/consultant to AstraZeneca, Lilly, Merck, Nektar, Novartis, Pfizer, Genentech/Roche, Immunomedics, Bristol-Myers Squibb, Eisai, Nanostring, Seagen, Puma, Sanofi, Celldex, Paxman, Puma, Silverback Therapeutics, G1 Therapeutics, AbbVie, Anthenex, OncoPep, Outcomes4Me, Kyowa Kirin Pharmaceuticals, Daiichi-Sankyo, Gilead and Samsung Bioepsis Inc. AW receives institutional research funding from Genentech/Roche.

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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frontiers in Immunology

MINI REVIEW
published: 10 June 2021
doi: 10.3389/fimmu.2021.702726

Regulatory T Cells: Barriers of Immune Infiltration Into the Tumor Microenvironment

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Regulatory T cells (T_{regs}) are key immunosuppressive cells that promote tumor growth by hindering the effector immune response. T_{regs} utilize multiple suppressive mechanisms to inhibit pro-inflammatory responses within the tumor microenvironment (TME) by inhibition of effector function and immune cell migration, secretion of inhibitory cytokines, metabolic disruption and promotion of metastasis. In turn, T_{regs} are being targeted in the clinic either alone or in combination with other immunotherapies, in efforts to overcome the immunosuppressive TME and increase anti-tumor effects. However, it is now appreciated that T_{regs} not only suppress cells intratumorally *via* direct engagement, but also serve as key interactors in the peritumor, stroma, vasculature and lymphatics to limit anti-tumor immune responses prior to tumor infiltration. We will review the suppressive mechanisms that T_{regs} utilize to alter immune and non-immune cells outside and within the TME and discuss how these mechanisms collectively allow T_{regs} to create and promote a physical and biological barrier, resulting in an immune-excluded or limited tumor microenvironment.

Keywords: regulatory T cells (Treg), immune infiltration, tumor microenvironment, cancer, vasculature, stroma

OPEN ACCESS

Edited by:
Rieneke Van De Ven,
VU University Medical Center,
Netherlands

Reviewed by:
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Regensburg, Germany
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *29 April 2021*
Accepted: *27 May 2021*
Published: *10 June 2021*

Citation:
Scott EN, Gocher AM, Workman CJ and Vignali DAA (2021) Regulatory T Cells: Barriers of Immune Infiltration Into the Tumor Microenvironment. Front. Immunol. 12:702726. doi: 10.3389/fimmu.2021.702726

INTRODUCTION

Regulatory T cells (T_{regs}) are suppressive $CD4^+$ T cells that are characterized, and largely regulated, by expression of the master transcription factor, forkhead box protein 3 (FoxP3) (1). T_{regs} are critical in the maintenance of peripheral tolerance to prevent autoimmune disease. During pathogenic insults, T_{regs} prevent overt immune activation in efforts to limit tissue damage. T_{regs} are also found in tumors with the ratio of T_{regs} to T cells positively correlating with poor prognosis and response to immunotherapy (2, 3). Strikingly, T_{reg} depletion in murine tumor models results in complete tumor clearance, however these mice ultimately succumb to lethal systemic autoimmune disease (4–7). The drastic effect of T_{regs} on tumor growth has sparked interest in elucidating T_{reg} function within the tumor microenvironment (TME) in efforts to selectively target tumor-infiltrating T_{regs} while sparing peripheral T_{regs} (8).

Immunotherapies designed to target intratumoral Tregs have focused on key surface markers that are highly expressed and contribute to their suppressive functions, such as CTLA-4, CD25,

TIGIT, 4-1BB, OX-40, CCR4, and CCR8. Targeting these markers therapeutically has had some clinical success. The first FDA-approved immunotherapy utilized a blocking monoclonal antibody specific for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 or CD152) (ipilimumab), which preserves T cell activation *via* preventing CTLA-4 binding to CD28 thus allowing for CD28 engagement of CD80/86 (9). Currently, the complete mechanism for ipilimumab is not fully elucidated but may also involve depletion of T_{regs} *via* antibody-dependent cell-mediated cytotoxicity (ADCC) (10). Despite ipilimumab prolonging patient survival and increasing the five-year survival rate, 10-15% of patients experience Grade 3-4 immune-related adverse events, thus investigation of additional T_{reg}-targeting strategies are warranted (11). Monoclonal antibodies against CD25, OX-40 and GITR have produced favorable anti-tumor effects, which were dependent on ADCC mediated T_{reg}-depletion (12). Studies to uncover both novel molecules enriched on tumor infiltrating T_{regs} or mechanisms of suppression unique to the TME are warranted to improve targeted immunotherapy while limiting toxicity.

T_{regs} are found throughout the TME and can even exert suppressive function at a distance, forming physical, metabolic, and trafficking 'barriers' to exclude pro-inflammatory cells from the TME. These barriers can be both 'physical', by limiting the ability of effector T cells to enter into the tumor, and 'functional', by limiting the activity of effector cells already within the TME. Together, these barriers create an immune-excluded TME with studies showing that decreased $CD8^+$ T cells, specifically, within the vicinity of tumor cells correlates with poor outcomes (13). The primary 'barriers' constructed by T_{regs} that prevent the infiltration of pro-inflammatory cells include poor activation of T cells in the periphery, disorganized vasculature, prevention of the formation of lymphatic structures in the TME and a stroma that hinders the migration of cells into and around the tumor bed (14, 15). These barriers of immune exclusion that T_{regs} erect will be discussed herein, starting with the tumor core and working outward through the peri-tumor to the stroma, ending with lymphatic structures and the periphery (**Figure 1**). Investigation of the pro-tumorigenic effects of T_{regs} in the whole tumor (non-micro) environment is necessary to elucidate novel therapeutic strategies to dismantle pro-tumor T_{regs} while maintaining peripheral tolerance.

T_{REGS} AS ANTI-INFLAMMATORY INTRATUMORAL BARRIERS

The frequency and organization of T_{regs} within the TME is diverse in cancer patients; however, high T_{reg} infiltration often correlates with poor prognosis in many cancer types (16–18). The origin of these T_{regs} – either thymically (tT_{regs}) or peripherally-derived (pT_{regs}) – is still being debated (19). T cell receptor (TCR) sequencing studies in carcinogen-induced murine models and in human melanoma, gastrointestinal and ovarian cancers have shown distinct TCR sequences between intratumoral T_{regs} and $FOXP3^-$ conventional $CD4^+$ T cells (20–22). A study using a genetically-driven prostate cancer murine model showed that intratumoral T_{regs} were thymically-derived, had less diverse TCRs, and had TCRs specific for the prostate tissue (23). Conversely, a recent study in breast cancer patients showed 65% TCR overlap of intratumoral T_{regs} with activated conventional $CD4^+$ T cells (24). Overall, T_{reg} conversion in the periphery and upon entry into the TME may be a rare event and may only be observed with the use of TCR transgenic mice or human tumors of specific tissue origins. However, having a TME that contains pT_{regs} and/or tT_{regs} may provide diverse functions (stability, effector and cytokine profile) that may provide a therapeutic opportunity to dedifferentiate T_{regs} to an unstable, non-immunosuppressive state (ex-T_{regs}) (25).

Tumors create an immunosuppressive environment that attracts T_{regs} and also support their anti-tumor function. Tumors secrete the CC chemokine ligand 22 (CCL22) and CCL17, which recruit T_{regs} to the tumor *via* T_{reg} expression of the CC chemokine receptor 4 (CCR4) (26). Use of mogamulizumab (anti-CCR4) in patients with cutaneous T cell lymphoma or solid tumors, reduced the levels of circulating or intratumoral $CCR4^+$ $T_{regs,}$ respectively, but did not induce potent antitumor effects (27, 28). Combination of mogamulizumab with nivolumab (anti-PD1) in phase I clinical studies was tolerable and increased intratumoral $CD8^+$ T cells and decreased T_{regs} in patients with solid tumors, making this therapeutic combination an effective option (29). Under hypoxic conditions, tumors secret CCL28 which recruits T_{regs} *via* CCR10 (30). Additionally, tumors secrete CCL5 which recruits T_{regs} *via* CCR5 and pre-clinical studies with CCR5 inhibitors have decreased T_{reg} tumor infiltration and tumor growth (31, 32).

Conventionally, T_{regs} have higher affinity to self-antigen compared to other T cells which allows for suppression of autoreactive T cells and prevention of autoimmune disease. Tumors express self-antigens that are over expressed, inappropriately expressed, or mutated and preferentially promotes the activation and sequestration of T_{regs} as seen by the expansion of a few T_{reg} clones specific for tumor antigens in cancer patients (33–35). A study using non-TCR transgenic mice showed that the TCRs of intratumoral T_{regs} are also found on T_{regs} from tumor draining lymph nodes (dLN), suggesting that T_{regs} are activated in the dLN, clonally expand, and migrate to the tumor where they accumulate (36). Although these data strongly suggest that T_{regs} recognize specific tumor antigens, albeit lower diversity compared to activated intratumoral conventional $CD4^+$ cells, not all T_{regs} in the TME have tumor antigen-specific TCRs.

The high proliferation index of cancer cells creates a high energy demand, which forces the tumor to switch from oxidative phosphorylation to glycolysis (referred to as the Warburg effect), which generates a lactic acid-rich, glucose-poor, and hypoxic TME (37). Uptake of glucose by intratumoral T_{regs} promotes instability and loss of suppressive function. Instead, intratumoral T_{regs} upregulate pathways involved in lactic acid metabolism, and lactate uptake is required for maintenance of suppressive function of intratumoral, but not peripheral, T_{regs} (38). Mechanistically, Foxp3 promotes glycolysis *via* binding to the

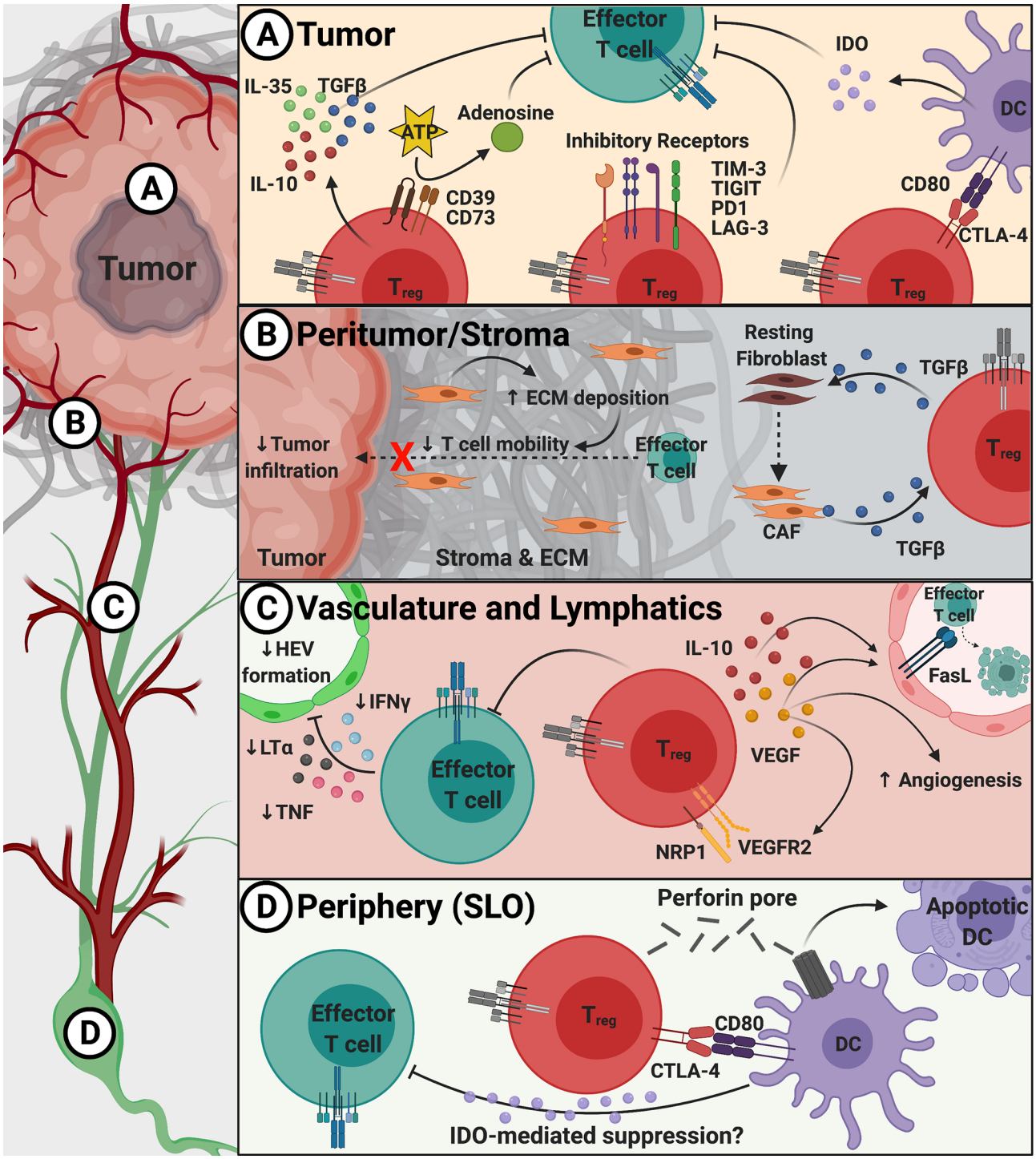


FIGURE 1 | Overview of suppressive mechanisms used by T_{regs} to create barriers to immune infiltration into tumors. Panel **(A)** Within the TME, T_{regs} utilize inhibitory receptors (TIM-3, TIGIT, PD1, and LAG-3), inhibitory cytokines (TGFβ, IL-10, and IL-35), DC modulation (via CTLA-4 and LAG-3), and metabolic disruption (via CD39/CD73) to suppress the anti-tumor T cell response. **(B)** T_{reg}-derived TGFβ induces cancer-associated fibroblast (CAF) development that increases extracellular matrix (ECM) production and deposition within the peritumoral space (stroma) to inhibit effector T cell migration. **(C)** T_{regs} block entry of effector T cells through preventing proper cytokine signals that promote high endothelial venule (HEV) formation as well as production of inhibitory IL-10 and VEGF to promote dysregulated angiogenesis. **(D)** In the periphery and secondary lymphoid organs (SLO), T_{regs} can modulate DC maturity and induce apoptosis to prevent proper effector T cell activation.

promoter of Myc and inducing expression (39). Deletion of hypoxia-inducible factor 2α (HIF-2α) from murine T_{regs} destabilized T_{regs} and prevented growth of MC38 colon adenocarcinoma (40). Collectively, consumption of glucose and oxygen by the proliferating tumor constructs a favorable metabolic landscape for T_{regs} to stably thrive in the TME.

Once in the tumor, T_{regs} suppress the anti-tumor response through contact-dependent and contact-independent mechanisms. Contact-dependent mechanisms utilizing CTLA-4, lymphocyte-activation gene 3 (LAG-3), and T cell immunoglobulin and ITIM domain (TIGIT) prevent activation and maturation of dendritic cells (DCs) thus preventing an effective anti-tumor T cell response (**Figure 1A**). CTLA-4 on T_{regs} binds CD80 molecules on DCs to induce transendocytosis and downregulation of CD80 expression and production of the inhibitory molecule indoleamine 2,3-dioxygenase (IDO) (41–44). While the intracellular domain of CTLA-4 is not thought to have a signaling function, it is important for the regulation of endocytosis and trafficking (45, 46). Specifically, a mouse model of T_{reg}-specific CTLA-4 deletion resulted in fatal lymphoproliferative and autoimmune diseases while drastically limiting tumor progression, illustrating the importance of CTLA-4 in mediating T_{reg} function through transendocytosis of CD80 and CD86 (44, 47, 48). LAG-3 binding to major histocompatibility complex class II on DCs reduces the expression levels of the costimulatory molecule CD86 and IL-12 cytokine production (49). TIGIT ligation of CD155 on DCs increased production of IL-10 and lowered IL-2, supporting an immunosuppressive environment (50, 51). While programmed cell death 1 (PD1) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) are highly expressed on T_{regs} and important for suppressive function, the mechanisms are unknown (52, 53).

Contact-independent mechanisms of T_{regs} include the secretion of the inhibitory cytokines IL-10, IL-35, and transforming growth factor-β (TGFβ), which suppress the activity of effector cells (**Figure 1A**). IL-10 suppresses *via* inhibition of CD28 tyrosine phosphorylation and induction of $CD8^+$ T cell exhaustion *via* upregulation of B lymphocyte-induced maturation protein-1 (BLIMP1) (54, 55). IL-35 limits the proliferation and memory formation, and promotes exhaustion in $CD8^+$ T cells similarly to IL-10 by expression of BLIMP1 and downstream inhibitory receptors (54, 56). TGFβ decreases effector function *via* inhibiting the transcription of proinflammatory cytokines (interferon gamma [IFNγ]) and granzyme B, and T helper cell transcription factors (T-box transcription factor and GATA binding protein 3), although the precise mechanism of action remains unknown (57–60). While these activities represent the general role of TGFβ, it is important to understand that different isoforms may have differing functions based on the expression pattern in various cancers (61–63). Thus, secretion of these cytokines by T_{regs} acts as a functional 'barrier' that prevents the function and expansion of surrounding effector T cells.

T_{regs} in the TME also suppress anti-tumor immunity through metabolic disruption *via* CD25/IL-2, CD39/CD73, and IDO (**Figure 1A**). IL-2 is required for effector T cell differentiation and fate upon immune activation and is critical for the development, regulation, proliferation and maintenance of T_{regs} (64). T_{regs} express high levels of the IL-2 receptor, CD25, which also deprives surrounding effector T cells of IL-2 (65). T_{reg} expression of the ectonucleotidases CD39 and CD73 convert ATP and ADP into adenosine, which suppresses effector T cells *via* the adenosine receptor 2A (66, 67). Interestingly, T_{reg} ligation of CD80/CD86 on dendritic cells (DCs) *via* CTLA-4, increases the production of IDO (47) (**Figure 1A**). IDO metabolizes the essential amino acid tryptophan, limiting its availability, into different suppressive metabolites including kynurenine which inhibits T cell proliferation (43, 68). Despite promising findings in murine models and human *in vitro* studies, a Phase III clinical study with the IDO1 inhibitor epacadostat in combination with pembrolizumab (anti-PD1) in melanoma was disappointing (69). The lack of epacadostat efficacy in the clinic may be due to low initial levels of tryptophan and kynurenine in the TME, the presence of other enzymes able to catabolize tryptophan such as IDO2 and tryptophan 2,3-dioxygenase (TDO2), inefficient inhibition of IDO1, or adaptive resistance.

Through the expression of inhibitory receptors, inhibitory cytokines and metabolic disruptors, T_{regs} impose a terminal functional barrier within the TME to inhibit the infiltrated effector cells. However, T_{regs} also reside on the perimeter where the tumor meets the stroma (peritumor) and act as a functional and physical barrier to tumor immune infiltration.

T_{REGS} AS PERITUMORAL ANTI-INFLAMMATORY BARRIERS

The non-tumor cells within the TME make up the stromal compartment and include different lineages of fibroblasts that secrete various types and amounts of extracellular matrix (ECM) proteins that influence T cells migration. Among these proteins are fibronectin (FN) and collagen (COL), with COL being more abundant in the tumor stroma and having increased stiffness which impedes T cell motility (70). T_{regs} are found in the stroma of various tumors types and correlates with poor outcome (71–74) (**Figure 1B**). Using 3D *in vitro* culture of T_{regs} in a COL gel matrices, T_{reg} markers were shown to be upregulated in high-density, compared to a low density, COL matrix, and also associated with decreased cytolytic activity (75). However, the interplay between T_{regs} and COL needs to be further defined. In a model of radiation-induced pulmonary fibrosis, T_{regs} promoted epithelium-to-mesenchymal transition (EMT) *via* β-catenin (76). In support of this, ectopic expression of Foxp3 by murine non-small cell lung cancer cells promoted EMT and tumor metastasis (77). Further studies to determine the direct role of T_{regs} in COL deposition and EMT are warranted.

IDO induces T_{reg} differentiation through the generation of tryptophan metabolites and subsequent aryl hydrocarbon receptor signaling (78, 79). IDO inhibits effector T cell activity and it has been shown in gastric cancer cell lines to be associated with ECM, COL metabolic and catabolic processes. Specifically *IDO1* and *COL12A1*

synergistically promoted cell migration *in vitro* (80). In a B16 melanoma model, the IDO1 inhibitor LW106 decreased tumor-associated stromal cells and COL deposition, and increased infiltration of effector cells. Additionally, LW106 decreased T_{regs} and delayed tumor growth, suggesting a potential role for T_{regs} in LW106 efficacy, however the direct impact of LW106 on T_{reg} differentiation was undefined (81).

Fibroblasts isolated from tissue of invasive breast cancer patients had increased growth and invasion rate when treated with TGFβ, which was hypothesized to foster tumor invasion. Head and neck cancer patient-derived xenografts showed upregulation of TGFβ signaling in patients that progressed with cetuximab, an epidermal growth factor receptor inhibitor, compared to sensitive patients (82). This latter study showed elevated TGFβ1 signaling in cancer-associated fibroblasts (CAFs) in cetuximab progressors (83). In a model of pancreatic cancer, CAFs were found to express lower levels of *Col* and *Fn1* mRNA when T_{regs} were deleted, which was accompanied by an increase in effector $CD4^+$ and $CD8^+$ T cell infiltration, and was proposed to result from the loss of *Tgfb1* produced by T_{regs} (84). It is hypothesized that T_{reg} production of TGFβ1 promotes fibroblast differentiation into CAFs (**Figure 1B**).

Collectively, these findings suggest a role for stromal T_{regs} in the promotion of COL and CAF formation, EMT and metastasis which creates a 'rigid' barrier to tumor immune infiltration. Ultimately, T_{regs} support an immunosuppressive stroma, and favor metastasis and disease progression. However, the mechanisms T_{regs} utilize to execute these pro-tumor effects and the therapeutic strategies to selectively inhibit these stromal T_{regs}, remain obscure.

T_{REGS} AS BARRIERS TO TUMOR INFILTRATION BY AUGMENTING TUMOR ANGIOGENESIS

Blood supply into the TME is critical for the survival and growth of tumors, and angiogenesis positively correlates with disease progression (85). Metabolically active tumors utilize conserved angiogenic mechanisms found in wound healing to mediate growth of new blood vessels. Hallmarks of tumor vasculature includes disorganized and immature vessels that lack vessel hierarchy and have increased permeability (86). Additionally, lymphatic vessels in the TME are dilated and leaky, which results in the accumulation of fluid and waste products. However, functional lymphatics exist at the tumor margin and are sufficient to mediate metastasis (87). The consequences of these features include metastasis and poor delivery of cancer therapies, but of interest is the inability for tumor infiltration of anti-tumor immune cells.

Tumor angiogenesis is driven by high levels of pro-angiogenic molecules, such as members of the vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-B) and TGFβ families, as well as hypoxia (86) (**Figure 1C**). VEGF-A is produced upon binding of the hypoxia-inducible factor 1 (HIF-1) α and β heterodimer to the *VEGF* promoter (88). VEGF-A produced by intratumoral $CCR10^+$ T_{regs} in a CCL28-expressing murine ovarian tumor model, increased angiogenesis and tumor growth (30). Similarly, $Helios^+$ T_{regs} in a lymphoblastic leukemia model induced angiogenesis *via* the VEGF-VEGFR2 pathway (89). VEGF-C also utilizes VEGFR2 and VEGFR3 to induce lymphangiogenesis (90). Although T_{regs} do not produce VEGF-C, the lymphatic system represents another avenue in which T_{regs} prevent proper effector T cell tumor infiltration.

Another feature of tumor-associated vessels is the ability to communicate with the immune milieu. Endothelial cells induce Fas ligand (FasL) expression upon exposure to prostaglandin E_2 (PGE_2), hypoxia and T_{reg}-produced VEGF and IL-10 to mediate T cell apoptosis (91, 92). Endothelial FasL preferentially kills $CD8^+$ T cells, while sparing T_{regs} due to T_{reg} expression of the anti-apoptotic gene, FADD-like IL-1β-converting enzyme (92) (**Figure 1C**). A feed-forward loop may exist in which VEGF-A and IL-10-producing T_{regs} in the TME promotes $CD8^+$ T cell exclusion yet favors T_{reg} infiltration, which further adds to the VEGF-A and IL-10 pools.

Targeting T_{regs} through inhibition of the VEGF pathway may be advantageous as T_{regs} not only produce, but also respond to, VEGF through expression of VEGFR2 and its co-receptor Neuropilin-1 (NRP1), the latter of which is highly expressed on murine and intratumoral human T_{regs} (93–95) (**Figure 1C**). Strikingly, a NRP1 antagonist increased $CD8^+$ T cell infiltration and decreased tumor growth in a murine model (96, 97). The addition of a VEGF blocking antibody to a model of adoptive cell therapy led to increased tumor infiltration of transferred cells and a reduction in tumor growth (98). Use of the immunomodulatory drug thalidomide in chronic lymphocytic leukemia decreased NRP1 expression on T_{regs}, which may contribute to the reported antiangiogenic properties (99). However, efficacy of these therapies may vary depending on the organization and location of the blood vessels within and around the tumor bed. For example, location of the vasculature within the tumor, either throughout the tumor mass (tumor vessels) or within the stroma (stromal vessels), dictated the efficacy of VEGFR2-blocking antibodies, with only the former producing a significant anti-tumor response (100). In this study, stromal vessels mediated extravasation of immune cells directly to the stroma where they were trapped in the dense architecture surrounding the tumor mass, whereas tumor vessels mediated extravasation of immune cells directly to the tumor. The difference in therapeutic response may be attributed to the spatial distribution of vessels and T_{regs} and/or that this is simply reflective of a more immune-impacted tumor, which is known to be a positive prognostic indicator (101–103). Collectively, this may explain the seemingly paradoxical findings that T_{regs} may in certain circumstances appear to be a positive prognostic factor of survival

In summary, T_{regs} support pro-tumor angiogenesis in the TME *via* secretion of VEGF-A and IL-10, and expression of NRP1 (**Figure 1C**). Studies to further assess the impact of T_{regs} on the efficacy of VEGF/VEGFR inhibition/blockage and anti-NRP1, and the reorganization of the immune landscape of the TME post-therapy, will be critical to improving therapeutic response.

T_{REGS} AS BARRIERS TO IMMUNE CELL EGRESS IN THE STROMA AND PERIPHERY

T_{regs} are also found within tumor-associated tertiary lymphoid structures (TLS), in which case the positive prognostic value of mature TLS now predicts worse outcomes and relapse in many cancer types (104–106). T_{regs} in TLS of a lung adenocarcinoma model prevented an anti-tumor response, and T_{reg} depletion resulted in increased proliferation and tumor infiltration of effector T cells (107). Similarly, $CD8^+$ T cells and natural killer (NK) cells secrete IFNγ, tumor necrosis factor (TNF-α) and lymphotoxin α3, which induce neogenesis of high endothelial venules (HEV) that resemble lymph node (LN)-like vasculature and mediate T cell infiltration (108) (**Figure 1C**). A study showed that HEV formed when T_{regs} were depleted, and attributed HEV formation to increased TNF-α from T cells (109, 110) (**Figure 1C**). However, a study of colorectal cancer patients showed a positive correlation of TNF-α expression with positive LN stage and tumor recurrence (111). These studies illustrate the divergent role of lymphatics in the TME, thus more research is needed to understand the intricacies of TLS and HEV formation to therapeutically exploit their anti-tumoral role.

Tregs utilize unique mechanisms in the draining secondary lymphoid tissues to prevent recruitment to the TME. T_{regs} found in the peritumoral LN of a pancreatic ductal carcinoma model expressed CTLA-4, and CTLA-4/CD80 ligation with DCs inhibited conventional $CD4^+$ T cell tumor infiltration (112). Although the mechanism is unclear, T_{reg} : DC interaction decreases CD80/CD86 expression on DCs and induces production of IDO to suppress effector function (43, 47, 68) (**Figure 1D**). Similarly, T_{regs} utilize perforin to directly kill DCs in tumor-draining LN (113) (**Figure 1D**). Altogether, T_{reg} suppression of DCs prevents effector T cell activation and lymphatic egress to the tumor site, thus promoting impaired anti-tumor immunity.

Collectively, T_{regs} in the stroma and periphery prevent tumor infiltration of immune cells by suppressing HEV formation, interfering with T cell activation by APCs and suppressing the production of proinflammatory cytokines by effector T cells. The anti-tumor effects seen with immunotherapies that block T_{reg}-mediated suppression of the T cell/APC synapse and ultimately increase proinflammatory cytokines, may concurrently promote HEV formation and restructuring of the stroma, therefore the need for complimentary spatial and functional T_{reg} studies is pertinent.

CONCLUSIONS

T_{regs} have diverse mechanisms to maintain tumor immune exclusion by affecting immune and non-immune cells, inside and outside of the tumor mass. Foundational studies interrogating intratumoral T_{regs} along with mechanisms of action for cancer immunotherapies have highlighted the impact intratumoral T_{regs} have on suppressing the anti-tumor response. However, mechanistic details of how to overcome these barriers are incomplete, leading to the following key questions:

(1) *What is the extent of T_{reg} and stromal cell interactions, and how do these interactions impact the composition of the stroma?* Initial findings suggest that T_{regs} and stromal cells work together to prevent tumor immune infiltration via induction of CAFs by T_{reg}-derived TGFβ. CAFs increase deposition of COL and FN and maintain T_{reg} suppressive functions. However, it is unclear if CAFs and T_{regs} need to directly interact for this feedback loop to occur and if other signaling events are needed to establish this suppressive peritumoral barrier. If this is a contact-dependent mechanism, it may be advantageous to develop therapeutics (i.e. blocking antibodies or inhibitors) that prevent the interaction of these two cell types within the stroma.

(2) *What are the mechanisms that retain T_{regs} in the stoma?* CAFs support physical barriers that hinder effector T cells propagation in the stroma, where T_{regs} are abundant. Human T_H2-like T_{regs} ($GATA3^+CCR4^+$) have the highest chemotaxis, viability and suppressive function, and are enriched in melanoma and colorectal cancer (114). GATA3 has been shown to bind to the promoter/enhancer of the IL-7 receptor and lL-7 signaling in T_{regs} is critical for development, expansion and peripheral homeostasis (115, 116). Additionally TGFβ promotes IL-7 receptor expression (117). One may then hypothesize that since CAFs produce IL-7, CAFs may support the proliferation of T_H2-like T_{regs} in the stroma, thus maintaining an immunosuppressive stroma. Additionally, CAFs from hepatocellular carcinoma induce IDO in regulatory DCs, which promotes T_{reg} proliferation (118). Collectively, these factors may provide a stromal environment favorable to T_{regs}, a notion strengthened by the observation that T_{reg}-rich adenocarcinomas expressed higher TGFβ and VEGF which may reinforce T_{reg} suppressive function and stability, respectively (119). These observations support the need for further investigation into the effects of anti-TGFβ and VEGF therapies on the stromal compartment and distribution of T_{regs} throughout. However, anti-VEGF therapy in this context may be detrimental if the stroma is heavily vascularized.

(3) *Do Tregs utilize a common pathway to promote angiogenesis and lymphangiogenesis, and can this pathway be therapeutically inhibited to normalize tumor vascularization and increase immune infiltration?* Peritumoral and intratumoral vasculature and lymphatics greatly dictates tumor infiltration of effector cells, however, specific mechanisms T_{regs} use to alter these structures is incomplete. Anti-angiogenic molecules in the clinic, such as sunitinib (receptor tyrosine kinase inhibitor) and bevacizumab (anti-VEGF), prevent the accumulation and function of T_{regs} by reducing their proliferative capacity and production of IL-10 and TGFβ, respectively (120, 121). As a VEGF co-receptor, NRP1 is a promising therapeutic as T_{reg}-restricted deletion of NRP1 not only results in loss of suppressive function but also a gain of effector function via the expression of T-bet and

production of IFNγ (95). The high expression of NRP1 by human tumoral T_{regs} in contrast to peripheral T_{regs} makes the VEGF/NRP1 axis a promising therapeutic target in order to normalize the vasculature and enhance effector T cell responses (122).

(4) *Do T_{regs} utilize one suppressive mechanism preferentially to create multiple barriers to effective T cell infiltration, and if so, can this be targeted therapeutically to curtail multiple barriers of immune exclusion simultaneously?* T_{regs} suppress the anti-tumor immune response through numerous mechanisms however, there are some recurring elements that when targeted could ameliorate multiple barriers (123). Of particular interest are IDO and NRP1. IDO inhibition may recruit peripheral effector T cells and reinvigorate intratumoral effector T cells, allowing for effective immune infiltration and anti-tumor activity, respectively. IDO inhibition in the peritumoral stroma may lower COL deposition which would increase the tumor infiltration of effector T cells. NRP1 blockade may lower the suppressive function of intratumoral T_{regs} and suppress angiogenesis. Targeting IDO and/or NRP1 may promote tumor infiltration and generate a less suppressive TME.

In summary, future studies must utilize mechanistic and spatial approaches to dissect the suppressive mechanisms employed by T_{regs} at various locations in the TME. These spatially-mapped functional studies will aid in the development of novel immunotherapies that aim to dismantle the T_{reg}-induced physical, metabolic and trafficking barriers within the TME.

AUTHOR CONTRIBUTIONS

ES, AG, CW, and DV wrote the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Institutes of Health [F32 CA247004 to AG, T32 CA082084 to AG and ES; P01 AI108545, R01 CA203689 and P30 CA047904 to DV].

ACKNOWLEDGMENTS

We thank everyone in the DV Lab (Vignali-lab.com; @Vignali_Lab) for all their constructive comments and advice.

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Conflict of Interest: DV: cofounder and stockholder – Novasenta, Tizona and Trishula; stockholder – Oncorus, Werewolf and Apeximmune; patents licensed and royalties - Astellas, BMS; scientific advisory board member - Tizona, Werewolf, F-Star, Bicara, Apeximmune; consultant - Astellas, BMS, Incyte, Almirall, G1 Therapeutics; research funding – Novasenta, BMS and Astellas.

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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