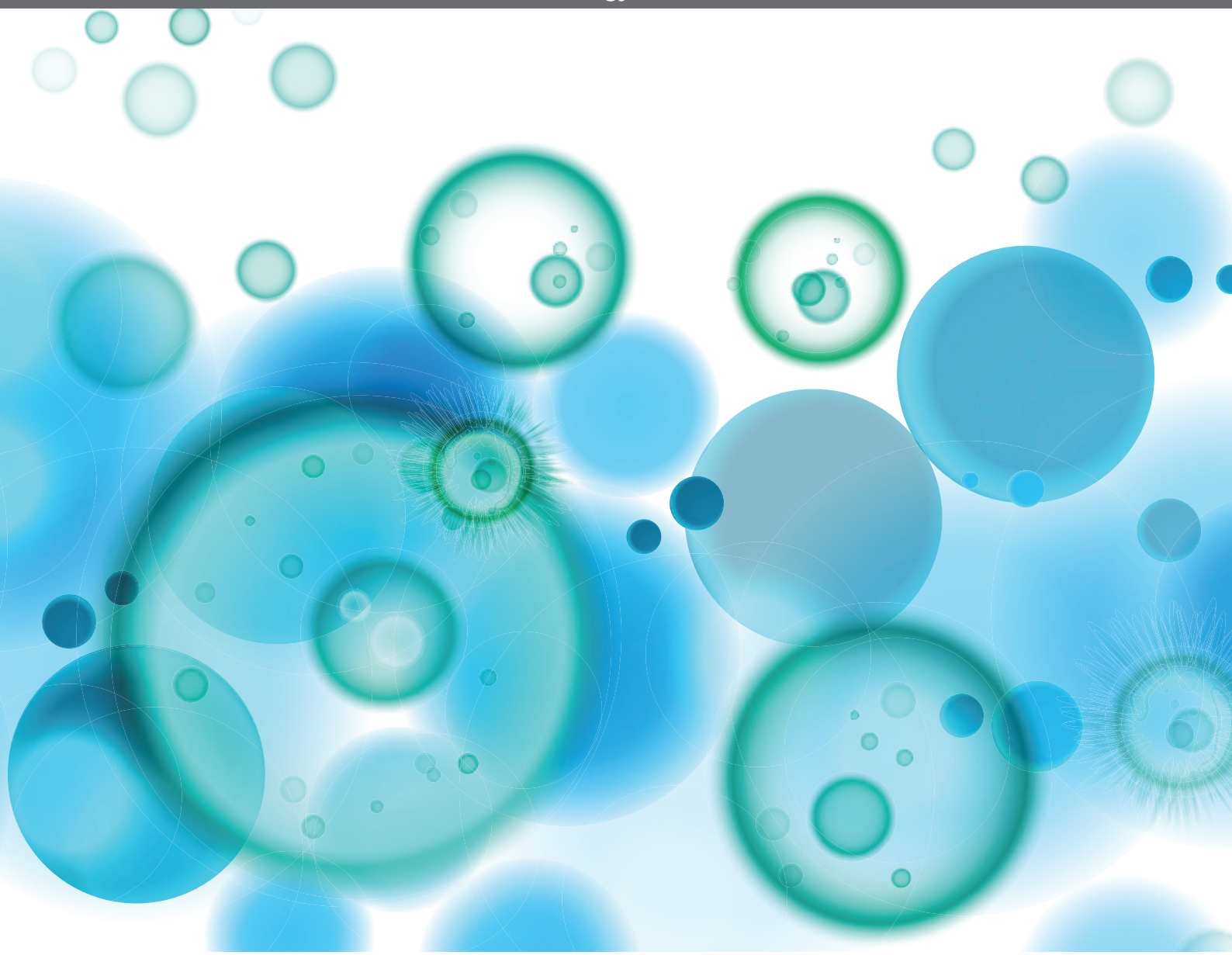
IMMUNE CELL LINEAGE REPROGRAMMING IN CANCER

EDITED BY: Jianmei Wu Leavenworth, Lewis Z. Shi, Xi Wang and Haiming Wei
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88974-473-2
DOI 10.3389/978-2-88974-473-2

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IMMUNE CELL LINEAGE REPROGRAMMING IN CANCER

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Topic Editor Dr. Lewis Shi received financial support from Varian Medical System, Inc. The other Topic Editors declare no competing interests with regard to the Research Topic subject.

Citation: Leavenworth, J. W., Shi, L. Z., Wang, X., Wei, H., eds. (2022). Immune Cell Lineage Reprogramming in Cancer. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-473-2

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frontiers in Immunology

EDITORIAL
published: 19 January 2022
doi: 10.3389/fimmu.2021.838464

Editorial: Immune Cell Lineage Reprogramming in Cancer

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Keywords: tumor immunity, regulatory T-cells, helper T-cells, tumor-associated macrophages, natural killer cells, epigenetic regulation, lineage reprogramming, cancer immunotherapy

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Edited and reviewed by:
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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 2021*
Accepted: *31 December 2021*
Published: *19 January 2022*

Citation:
Leavenworth JW, Shi LZ, Wang X and Wei H (2022) Editorial: Immune Cell Lineage Reprogramming in Cancer. Front. Immunol. 12:838464. doi: 10.3389/fimmu.2021.838464

Editorial on the Research Topic

Immune Cell Lineage Reprogramming in Cancer

Cancer immune evasion, as a result of prominent immunosuppression, is a major barrier to effective anti-tumor immunity and immunotherapy. Both adaptive and innate immune cells in cancer have shown phenotypic and functional instability by reprogramming into different cell subsets or states that impact tumor growth, progression or metastasis. Our Research Topic has attracted 18 contributions from 145 authors, which collectively cast a largely complete picture of our current understanding of the immune cell reprogramming and associated mechanisms in cancer, with or without therapeutic interventions.

REPROGRAMMING ADAPTIVE IMMUNE CELLS IN CANCER

As one of the major anti-tumor cytotoxic T lymphocytes (CTLs), $CD8^+$ T-cells generally reside in the tumor with exhausted and dysfunctional states (1). $CD8^+$ T-cell exhaustion is a contentious topic in the field of cancer research, as two models are proposed to explain this formation: one, the attrition of effector cells upon chronic antigen stimulation, and two, early bifurcation of an exhausted lineage in tumorigenesis (1, 2). Using two distinct T-cell receptor (TCR) transgenic and transplantable tumor models, Sullivan et al. demonstrate that although both tumor-specific and tumor-nonspecific bystander $CD8^+$ T-cells traffic to solid tumors *via* the chemokine receptor CXCR3, the former cells are exhausted, while the latter cells within the same tumor microenvironment (TME) retain memory and functional activity, which supports the notion that chronic TCR stimulation is the central driver of T-cell exhaustion. In contrast, Busselaar et al. provide a new perspective that the early priming without $CD4^+$ T-cell help differentiates $CD8^+$ T-cells into a predysfunctional state to express the transcription factor TCF-1 and coinhibitory

receptors, such as PD-1 (3). Subsequent antigen stimulation drives their differentiation into TCF-1$^-$ terminally exhausted cells dependent on the transcription factor TOX (4, 5). Importantly, PD-1 blockade along with CD27 costimulation and other alternative approaches that recapitulate CD4$^+$ T-cell help could fully rescue the predysfunctional state, suggesting new strategies for cancer immunotherapy. Interestingly, memory bystander CD8$^+$ T-cells reported by Sullivan et al. do not express high levels of PD-1. It is not clear if these cells respond to PD-1 blockade as efficiently as predysfunctional CD8$^+$ T-cells. Nevertheless, these studies highlight the plasticity of intratumoral CD8$^+$ T-cells that could be exploited for cancer immunotherapy.

CD4$^+$ T-cells not only provide help to CD8$^+$ T-cells to optimize CTL response, but also directly regulate the magnitude and quality of anti-tumor immunity (6). In addition, emerging studies have demonstrated that CD4$^+$ T-cells provide help to B-cells to induce anti-tumor humoral antibody response and the formation of tumoral tertiary lymphoid structures, which serve as predictive and prognostic factors in patients with cancer and those receiving immunotherapies (7, 8). Conversely, accumulation of CD4$^+$ regulatory T-cells (Tregs) in many tumors is a hallmark of immunosuppressive TME (9). The versatility of CD4$^+$ T-cell functional activity lies at heterogenous subsets and states of these cells, as reviewed by DiToro and Basu, who also provide a comprehensive review of the complex transcriptional networks and dynamic responses of CD4$^+$ T-cell subsets in intestinal inflammation and colorectal cancer. Additionally, they address therapeutic targeting *via* CD4$^+$ T-cell functional plasticity, including manipulation of the colonic microbiota. In a study conducted by Fraga et al., some patients with oral squamous cell carcinoma (OSCC) have increased tumor-infiltrating T helper (T_H) 2-like and CCR8$^+$ effector T-cells (Teff) and Tregs, which are subsets associated with poor prognosis. Co-culture assays and proteomic analysis of the secretome from OSCC have further identified an important link with increased production of prostaglandin E2 and activated vitamin D signaling to the T_H2-like Treg and Teff phenotype and induction of CCR8 but inhibition of cytokine secretion in Teff. Moreover, malignant OSCC samples express elevated CCL18, the CCR8 ligand, to promote CCR8 upregulation in Teff, forming an immunosuppressive feedback loop. A more focused review of Tregs is provided by Dixon et al., who have discussed the stability and suppressive function of tumoral Tregs, including a subset of effector Tregs, follicular regulatory T (T_{FR}) cells that are implicated in the regulation of anti-tumor humoral response (10), and the therapeutic potential by targeting Treg reprogramming for cancer treatments.

REPROGRAMMING INNATE IMMUNE CELLS IN CANCER

In addition to the adaptive immune system, components of innate immune system contribute to tumor growth, progression and response to immunotherapy. There are diverse types of innate immune cells. Some display tumor-killing capacity, while others exhibit pro-tumoral property. Natural killer (NK) cells by virtue of their natural cytotoxicity are crucial in the control of various types of cancer. Hu et al. provide an overview of how the TME alters NK cell phenotype, function, metabolism and migration, while Xia et al. focus on the epigenetic regulation of NK cell heterogeneity in cancer, and discuss epi-drugs used to target NK-mediated anti-tumor immunity. Like suppressive lymphocytes, innate myeloid cells, including myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAMs), also accumulate in many types of tumors. Several transcription factors, such as C/EBPβ and c-Rel, are reported to regulate MDSC differentiation and function (11, 12), but the lineage-specific regulator remains unclear. Fultang et al. propose a c-Rel-C/EBPβ enhanceosome containing these known transcription factors in myeloid precursors as a unified mechanism for the regulation of MDSC signature genes during their differentiation in response to aberrant inflammatory cytokine signals, suggesting potential therapeutic strategies *via* specifically targeting MDSC. A detailed review of TAMs is presented by both Ricketts et al. and Pan et al., who have discussed the TAM plasticity and approaches targeting TAMs to improve the anti-tumor response. The former has also presented interesting proactive questions by pointing out that the *in vitro* M1/M2 experimental model cannot accurately represent the intra-tumoral TAM heterogeneity, while new technologies, such as single-cell RNA-sequencing and spatial localization, would help refine our understanding of TAMs. Although this collection cannot provide an exhausted list of innate immune cells, the above studies highlight the importance of innate regulation of tumor immunity, and the potential to harness the plasticity of these innate immune cells for cancer therapy.

REPROGRAMMING THE TUMOR MICROENVIRONMENT

Cancer is increasingly viewed as a "tumor ecosystem" in which tumor cells interact with other tumor cells, stromal cells and all kinds of immune cells to constitute an immunosuppressive TME that is a major obstacle to effective anti-cancer immunity. Instead of focusing on a specific type of immune cells, Yang and Wang have discussed the epigenetic regulation of tumor cells, intratumoral immune cells, tumor-immune crosstalk and the heterogeneity of TME from a systemic view, proposing that combined epi-drugs and immunotherapy is an effective strategy for cancer therapy. This review has also briefly presented how microbiota-derived signals or metabolites could epigenetically regulate the TME, an open area for future exploration. The TME creates a condition that is disadvantageous to the nutrient uptake and metabolism of immune effector cells. Li Y et al. have discussed how TME-derived metabolites reprogram immune cells *via* epigenetic regulation, supporting a strategy to enhance the efficacy of immunotherapy using metabolic modifiers. An overview of the ovarian cancer TME by Luo et al. has also described tumor-infiltrating immune cells that are modulated by genetic and epigenetic factors, particularly noncoding RNAs,

intrinsically or extrinsically from tumor cells. The cytokine signaling and components like JAK-STATs that mediate tumor-immune interactions in the TME are also a focus of this review. The complexity and plasticity of TME is impacted by the genomic heterogeneity of tumor cells, which can be assessed *via* targeted next-generation sequencing. Using this technology, Lin et al. are able to define the spatial heterogeneity of multiple tumors of resected multifocal hepatocellular carcinoma. Moreover, circulating-free DNA from matched preoperative peripheral blood effectively captures these genomic alterations, serving as a promising tool to inform cancer progression and to potentially guide the selection of best treatments, including immunotherapies, for cancer patients.

REPROGRAMMING IMMUNE CELLS AND TME IN RESPONSE TO CANCER THERAPY

Cancer therapies that are aimed to converting the TME from immunosuppressive (cold) to immune-supportive (hot) are expected to induce the immune cell lineage reprogramming, which is potentially targetable for new therapeutic interventions due to its reversibility. Various cancer immunotherapeutic approaches are currently being employed in the clinic of which immune checkpoint inhibitors (ICIs) targeting PD-1, PD-L1 and CTLA4 have shown the most promising results, despite that the overall response rates remain at low levels in many types of cancer, especially for those cancers with high levels of immunosuppressive cells in the TME or insufficient infiltration of effector cells into tumor. Based on this potential mechanistic link, combined treatments with ICIs and angiogenesis inhibitors that can reduce immunosuppression but enhance effector cell infiltration into tumor to reprogram the TME could improve the outcome of ICI-based therapy (Ren et al.). This review has also summarized the preclinical and clinical studies of using the combined approach for the treatment of advanced non-small cell lung cancer, in addition to a detailed discussion of the mechanisms of vascular endothelial growth factor signaling in tumor immune evasion and progression. In contrast to the beneficial effects, immune-related adverse effects are one of the major concerns for ICI-based therapy. Kim et al. report that IFNγ^{+}IL-17^{-} CD8^{+} T and CXCR3^{+}CCR6^{+} T_H17/T_H1 cells were enriched and clonally expanded in the bronchoalveolar lavage fluid from 11 patients with acute myeloid leukemia and myelodysplastic syndrome after ICI-based therapy, suggesting that these cells may contribute to ICI-related pulmonary complications and serve as predictive and diagnostic biomarkers for these adverse effects.

It is interesting that the involvement of immune regulation is also identified in the standard-of-care treatments like surgical resection and chemotherapy. Shibuya et al. identified a tissue-repair-promoting Ym1^{+}Ly6C^{hi} monocyte subset that results from the inflammation post-resection of primary tumor and promotes lung metastasis of circulating tumor cells at least partly *via* expressing metalloproteinase-9 and CXCR4. These findings suggest this specific immunomodulatory monocyte subset as a predictive biomarker for metastatic recurrence after primary tumor resection. It is known that cisplatin chemotherapy is widely used in multiple tumors, but it produces severe side effects including neurotoxicity and immunosuppression. A safe and effective complementary treatment is required to prevent toxicity and preserve bone marrow hematopoiesis and peripheral immune responses. Li S et al. revealed that electroacupuncture can induce PAC1-mediated neuromodulation of hematopoiesis and alleviate immunosuppression in naïve and tumor-bearing mice during cisplatin treatments. This study may open an interesting research avenue in which the neuro-immune axis can be manipulated for the treatment of cancer and therapy-related side effects.

CONCLUSIONS

This Research Topic "*Immune Cell Lineage Reprogramming in Cancer*" provides updates on the influences of immune cell lineage reprogramming on tumor initiation, progression, and outcomes of therapy. Although cancer immunotherapy has emerged as a promising modality for cancer patients, much remains to be learned given the importance of TME regulation that is complicated by the plasticity and heterogeneity of immune cells and tumor cells. We (the editors) strongly believe that each article published under this Research Topic will help in the discovery of new cellular and molecular candidates or pathways for the development of strategies against cancer.

AUTHOR CONTRIBUTIONS

JWL initiated and organized the Research Topic. All authors made substantial, direct and intellectual contributions to the work, and approved it for publication.

FUNDING

JWL is supported by the University of Alabama at Birmingham (UAB) faculty start-up funds, DoD W81XWH-18-1-0315 and NIH R01AI148711. XW is supported by grants from the Ministry of Science and Technology of People's Republic of China (2014CB910100) and the National Natural Science Foundation of China (81972652). LZS has received research fund from the V Foundation for Cancer Research (V2018-023), American Cancer Society Institutional Research Grant (91-022-19), Varian (a Siemens Healthineers company), NIH R21CA230475, R21CA259721-A1 and Start-up fund from the Department of Radiation Oncology at UAB.

ACKNOWLEDGMENTS

We would like to thank all authors for their contributions to this Research Topic.

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Conflict of Interest: LZS received financial support from Varian, a Siemens Healthineers company.

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frontiers in Immunology

HYPOTHESIS AND THEORY
published: 06 October 2020
doi: 10.3389/fimmu.2020.592569

Helpless Priming Sends CD8$^+$ T Cells on the Road to Exhaustion

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Persistent antigen exposure in chronic infection and cancer has been proposed to lead to cytotoxic T lymphocyte (CTL) "exhaustion", i.e., loss of effector function and disease control. Recent work identifies a population of poorly differentiated TCF-1$^+$PD-1$^+$ CD8$^+$ T cells as precursors of the terminally exhausted CTL pool. These "predysfunctional" CTLs are suggested to respond to PD-1 targeted therapy by giving rise to a pool of functional CTLs. Supported by gene expression analyses, we present a model in which lack of CD4$^+$ T cell help during CD8$^+$ T cell priming results in the formation of predysfunctional CTLs. Our model implies that predysfunctional CTLs are formed during priming and that the remedy for CTL dysfunction is to provide "help" signals for generation of optimal CTL effectors. We substantiate that this may be achieved by engaging CD4$^+$ T cells in new CD8$^+$ T cell priming, or by combined PD-1 blocking and CD27 agonism with available immunotherapeutic antibodies.

Keywords: CD8$^+$ T cell, CD4$^+$ T cell, exhaustion, dysfunction, cancer, infection

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: *07 August 2020*
Accepted: *21 September 2020*
Published: *06 October 2020*

Citation:
Busselaar J, Tian S, van Eenennaam H and Borst J (2020) Helpless Priming Sends CD8$^+$ T Cells on the Road to Exhaustion. Front. Immunol. 11:592569. doi: 10.3389/fimmu.2020.592569

INTRODUCTION

In chronic infection and cancer, CD8$^+$ T cells upregulate coinhibitory receptors and display impaired proliferative and cytotoxic capacities, a phenomenon described as "T cell exhaustion". T cell exhaustion is considered a crucial factor in limiting clinical responses to immunotherapy, but this T cell state is not well understood. Some experts do not envision functions for exhausted T cells, while others surmise a role in host protection (1). Recent data illuminate how exhausted CD8$^+$ T cells are formed. The original model proposed that exhausted CD8$^+$ T cells develop from effector T cells as a result of chronic stimulation *via* their T cell antigen receptor (TCR) (2). However, new transcriptomic analyses, that include TCR-based lineage tracing, argue that exhausted CD8$^+$ T cells are not derived from functional effector cells. Rather, CD8$^+$ T cells can attain a "predysfunctional" state early after infection or tumorigenesis that may progress into a terminally exhausted state. It is considered that predysfunctional cells may also be "reinvigorated" to become CTL effectors. Blockade of the PD-1/PD-L1 coinhibitory axis may lead to such reinvigoration. Knowledge about the exact molecular and cellular mechanisms underlying CD8$^+$ T cell predysfunction, exhaustion and reinvigoration are clinically relevant in chronic infection and cancer, and likely also in autoimmune and inflammatory diseases.

Here, we first discuss the recent literature on $CD8^+$ T cell predysfunction and exhaustion in a key mouse model of chronic virus infection. This work has recently led to the concept that predysfunction and exhaustion represent aspects of a $CD8^+$ T cell differentiation pathway, distinct from effector and memory differentiation. By connecting studies on infection and cancer, we integrate supporting arguments for this concept. We synthesize these recent insights into a model of progressive fate commitment of primed $CD8^+$ T cells. Supported by gene expression analyses, we introduce the novel perspective that the predysfunctional differentiation state results from $CD8^+$ T cell priming in the absence of $CD4^+$ T cell help. This viewpoint implies that reinvigoration of predysfunctional $CD8^+$ T cells may be achieved by addition of "help" signals. We rationalize that PD-1 targeted checkpoint blockade may lead to delivery of help signals and may be supported by engagement of specific T cell costimulatory receptors.

METHODS

No Help $CD8^+$ T Cell Gene Expression Signature

RNAseq fastq files of samples of helped $CD8^+$ T cells (n = 3) and samples of non-helped $CD8^+$ T cells (n = 3) were retrieved from GEO database (GSE89665) (3). FASTQ files were aligned to the mouse genome mm10 (GRCm38.77) using HISAT2 v2.1.0 (4), and number of reads was assigned to genes by using featureCounts v1.6.1 (5). Reads mapped to genes were normalized and differentially expressed gene analysis between non-helped $CD8^+$ T cells and helped $CD8^+$ T cell was performed using edgeR package in R Bioconductor (6). The false discovery rate (FDR) < 0.01 was used as the criteria to select statistically differentially expressed gene lists. In total, a list of 1,331 genes were found differentially expressed between non-helped condition and helped conditions (FDR < 0.01), which represents the No Help signature.

Calculation of No Help Score in Published CD8 T Cell Expression Signatures

RNAseq fastq files were retrieved from GEO database (GSE99531, GSE122713) (7, 8). FASTQ files were aligned to the mouse genome mm10 using HISAT2 v2.1.0 (4), and number of reads was assigned to genes by using featureCounts v1.6.1 (5). Genes with all zero counts were removed. The raw counts were normalized by count per million (CPM) methods (6). For each sample, a "No Help score" was determined by the nearest centroid method on the 1331 genes from the No Help signature. In short, the No Help score was calculated as the difference of Pearson correlations in normalized read counts between a given population and No Help or Help vaccination settings. A higher No Help score indicates greater transcriptional similarity to helpless $CD8^+$ T cells.

Gene Set Enrichment Analysis

RNAseq files of helped or non-helped CD8 T cells, aligned to the mouse genome mm10, were imported into Qlucore Omics Explorer. Genes with less than 5 reads in at least one of the samples were discarded. Mapping quality threshold was set to 10. TNM normalization method was applied. Gene Set Enrichment Analysis was performed using published gene sets of the top 200 up- and downregulated genes from *Tcf7*-GFP^+ versus *Tcf7*-GFP^- P14 cells in chronic LCMV infection (9) or B16-gp33 tumor model (10).

Statistical Analysis

Data was analyzed with GraphPad Prism software using unpaired two-tailed Student's t-test, or repeated measures one-way ANOVA with Tukey's multiple comparison test. A P value < 0.05 was considered statistically significant; *P < 0.05, **P < 0.01, and ***P < 0.001.

Illustrations

Illustrations in **Figures 1–4** were created with BioRender.

HELP DELIVERY DURING $CD8^+$ T CELL PRIMING

Priming of $CD4^+$ and $CD8^+$ T cells relies on three key signals: TCR engagement by peptide/MHC complexes, costimulation by CD28 and members of the TNF receptor family, as well as specific cytokine signaling. Dendritic cells (DCs) can supply these signals, provided that the DC is of the appropriate subset and adequately activated, by pathogen- or danger-derived signals or by $CD4^+$ T cells. In secondary lymphoid organs, $CD4^+$ and $CD8^+$ T cells engage in successive antigen-specific interactions with different DC subtypes. Migratory DCs deliver the antigen from the site of infection, while lymph node-resident DCs pick up the antigen locally. $CD4^+$ and $CD8^+$ T cells are initially activated independent from each other, in different regions of the lymph node by migratory conventional (c)DC1 and cDC2 subsets (12–14). After this first step of priming, a second step of priming takes place on lymph node-resident cDC1s. In this interaction, $CD4^+$ T cell help is delivered that is essential for optimal differentiation of $CD8^+$ T cells into CTL effector and memory cells (11) (**Figure 1**). $CD4^+$ and $CD8^+$ T cells that have undergone the first step of priming produce specific chemokines that attract lymph node-resident cDC1 (12, 13, 15). In case the cDC1 co-presents recognizable MHC class II- and MHC class I-restricted antigens, it can relay help signals from the $CD4^+$ T cell to the $CD8^+$ T cell. Plasmacytoid (p)DCs likely promote this scenario by the production of type I interferon (IFN), which optimizes maturation and antigen crosspresentation by cDC1s (16).

Upon cognate contact with the $CD4^+$ T cell, the lymph node-resident cDC1 gains expression of various cytokines and co-stimulatory ligands that in concert optimize the $CD8^+$ T cell response (11). Interaction between CD40 ligand on the $CD4^+$ T cell and CD40 on the cDC1 amplifies production of IL-12 and IL-15 by the DC, which improves clonal expansion and effector differentiation of $CD8^+$ T cells (17, 18). Furthermore, CD40 signaling in DCs upregulates CD80/CD86 and CD70, which

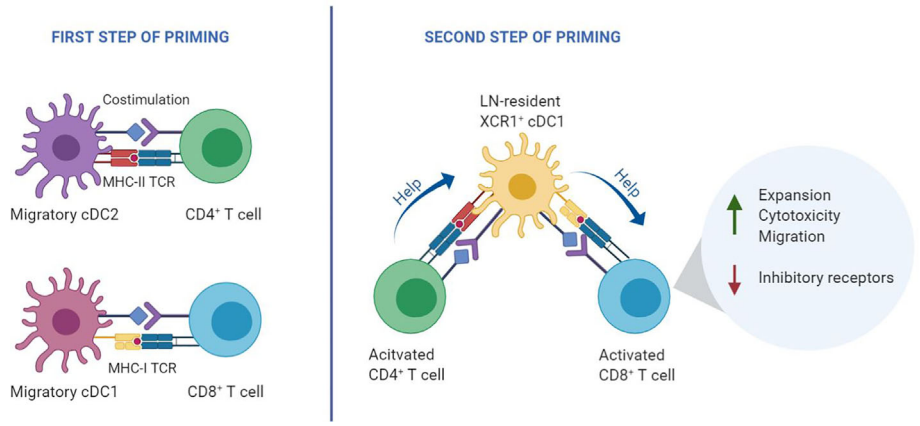

FIGURE 1 | Two-step priming model. During the first step of T cell priming (left), $CD8^+$ T cells and $CD4^+$ T cells are initially activated independently by different DC subtypes that present antigen on MHC class I and class II, respectively. In the second step of priming (right), recently activated $CD4^+$ and $CD8^+$ T cells interact with the same lymph node-resident cDC1 co-expressing MHC class I and MHC class II epitopes. Helped $CD8^+$ T cells undergo optimal priming by signaling *via* various costimulatory and cytokine signals that emerge from the helped cDC1, resulting in an optimal CTL effector program (11).

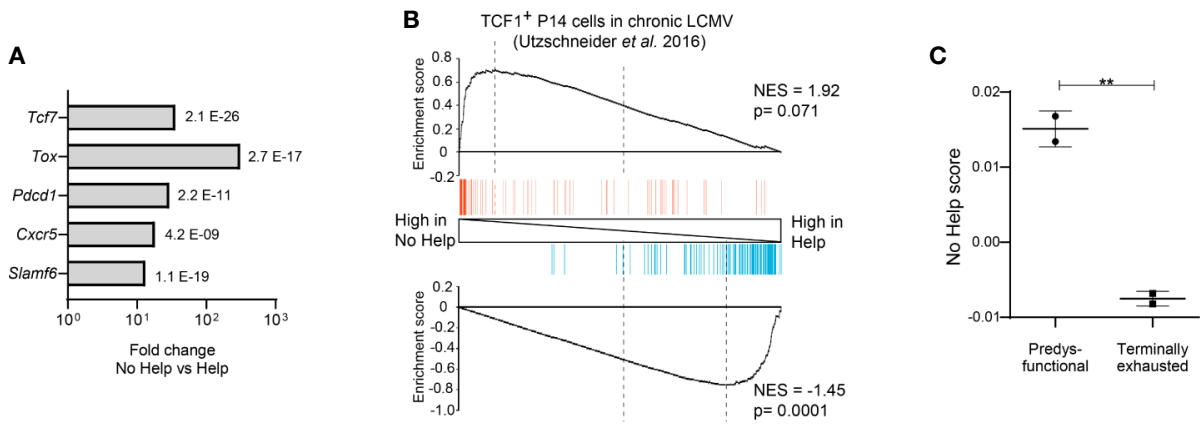

FIGURE 2 | Predysfunctional TCF-1^+ $CD8^+$ T cells in a chronic LCMV infection model display a gene expression signature characteristic of helpless antigen-specific $CD8^+$ T cells in a vaccination model. The transcriptional "No Help" signature was determined by differential gene expression (False discovery rate (FDR) < 0.01) of antigen-specific CTLs raised in No Help versus Help vaccination settings (GEO database GSE89665) (3). **(A)** Differential expression of selected genes characteristic of predysfunctional TCF-1^+CD8^+ T cells (**Table 1**) in No Help versus Help settings. FDR is depicted per gene. **(B)** GSEA of the top 200 upregulated (red)- or downregulated (blue) genes from $TCF1^+$ versus $TCF1^-$ virus-specific $CD8^+$ T cells in chronic LCMV infection (9) within the gene expression profiles of $CD8^+$ T cells from the No Help versus Help vaccination settings. NES, normalized enrichment score. **(C)** No Help score in predysfunctional TCF-1^+TIM3^- and terminally exhausted TCF-1^-TIM3^+ $CD8^+$ T cells from a setting of chronic LCMV infection (GEO database GSE122713) (7). The No Help score was calculated as the difference of correlations in gene expression between a given population and No Help or Help vaccination settings. A higher No-Help score indicates greater transcriptional similarity to helpless $CD8^+$ T cells. **P < 0.01 by Student's t-test.

relay costimulatory signals *via* CD28 and CD27, respectively (19–21). In both $CD4^+$ and $CD8^+$ T cells, CD28 costimulation amplifies the TCR signal and drives cell division (22), while CD27 costimulation promotes cell survival and effector differentiation (3, 23–25). CD27 costimulation of $CD8^+$ T cells is a key effector pathway of $CD4^+$ T cell help. It promotes CTL differentiation and survival, likely directly, but also by increasing expression of the IL-2 receptor alpha chain, IL-2 and the IL-12 receptor, leading to autocrine IL-2 signaling and responsiveness to DC-derived IL-12 (3, 26–28). IL-21 production by $CD4^+$ T cells also promotes CTL effector differentiation (29).

By transcriptomic analyses in mice, we have discovered how help signals impact effector and memory gene expression programs of $CD8^+$ T cells (3, 30). At the effector stage, "helped" versus "helpless" CTLs differentially expressed about 1,000 transcripts, encoding proteins enabling critical CTL functions, such as cytotoxicity and migratory abilities. From

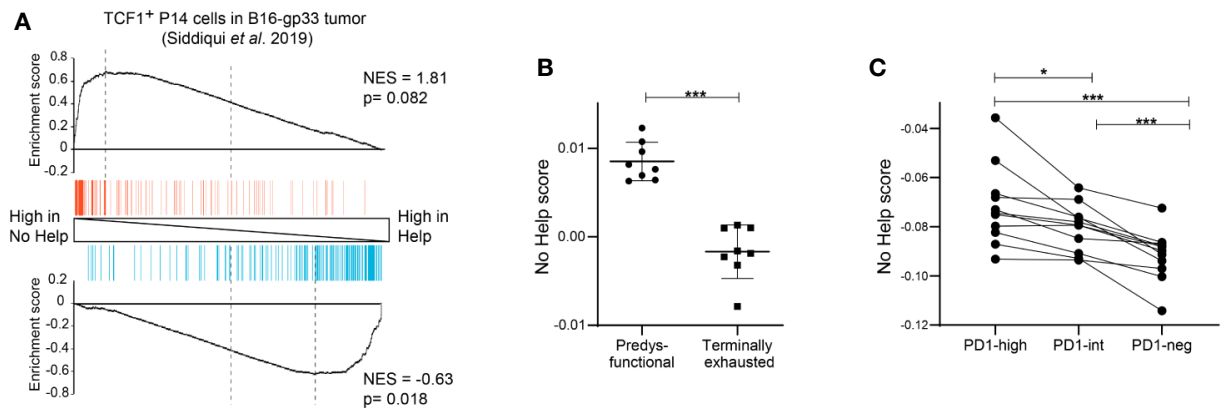

FIGURE 3 | Predysfunctional TCF-1$^+$ CD8$^+$ T cells in human cancer display a gene expression signature characteristic of helpless antigen-specific CD8$^+$ T cells in a mouse vaccination model. **(A)** GSEA showing enrichment of the top 200 upregulated (red) or downregulated (blue) genes in gp33-specific TCF-1$^+$ CD8$^+$ T cells in a murine B16-gp33 tumor model (10) within the gene expression profiles of vaccine antigen-specific CD8$^+$ T cells in No Help versus Help settings (3). **(B)** No Help scores, defined in our vaccination model, determined in the transcriptomes of predysfunctional TCF-1$^+$TIM3$^-$ and terminally exhausted TCF-1$^-$TIM3$^+$ tumor antigen-specific CD8$^+$ T cells from a murine B16-OVA tumor model (GEO database GSE122713) (7). **(C)** No Help score defined as in **(B)**, determined in the transcriptome of patient-matched PD-1-high, PD-1-intermediate, and PD-1-negative CD8$^+$ TILs in human melanoma (GEO database GSE99531) (8). *P < 0.05, ***P < 0.001 by Student's t-test **(B)** or one-way ANOVA **(C)**.

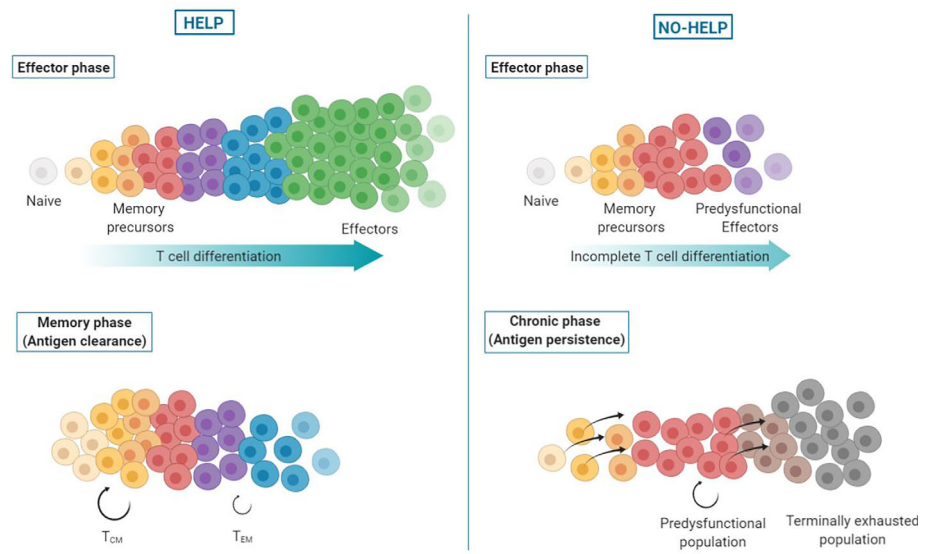

FIGURE 4 | Helpless dysfunction model. Upon priming of CD8$^+$ T cells, a differentiation spectrum is formed, ranging from uncommitted memory precursors to terminally differentiated effector cells. In presence of CD4$^+$ T cell help signals (left), the antigen-specific CD8$^+$ T cell population attains higher differentiation states, with the majority of cells becoming terminally differentiated, short-lived effector CTLs. These helped CTLs clear the antigen source and die. When antigen wanes, memory precursor cells persist and form helped central (T_{CM}) and effector memory (T_{EM}) CD8$^+$ T cells. In absence of help signals (right), antigen-specific CD8$^+$ T cells undergo incomplete effector differentiation and terminally differentiated effector CTLs are lacking. Instead, predysfunctional effector CTLs are formed that are less committed ("memory-like"), i.e., have not fully unfolded their effector program and express coinhibitory receptors. In addition, formation of effector memory CD8$^+$ T cells is impaired. As a result, antigen persists and continuous TCR stimulation of memory precursor cells drives their differentiation into predysfunctional CTLs that self-maintain or differentiate into terminally exhausted cells.

functional studies in a tumor model, we concluded that CD4$^+$ T cell help confers upon CTLs the exact properties desired for effective anti-tumor immunity, as defined by Chen and Mellman in "The cancer immunity cycle" (31). Conversely, helpless CTLs proved to have a dysfunctional phenotype characterized by low cytotoxic capacity and high expression of PD-1 and other co-inhibitory receptors (3), classifying them as "exhausted", according to the original definition. Other authors defined by

micro-array similar gene expression features in helpless CTLs, which proved to resemble exhausted CTLs, as defined in a mouse model of chronic LCMV infection (32). In conclusion, there appears to be a connection between helpless priming of $CD8^+$ T cells and acquisition of the exhausted state. This connection will be clarified in this Hypothesis and Theory article.

ANTIGEN-SPECIFIC $CD8^+$ T CELL FATES IN CHRONIC INFECTION

Exhaustion

Exhaustion of antigen-specific $CD8^+$ T cells was first described in mouse models of chronic infection with LCMV (33). Exhausted virus-specific $CD8^+$ T cells were defined by a diminished ability to display effector functions such as IFNγ production, and high expression of coinhibitory receptors such as PD-1. It was proposed that virus-specific effector $CD8^+$ T cells gradually turn into exhausted cells upon chronic engagement of the TCR by persistent viral antigen. Observations that TCR-regulated transcription factors contribute to exhaustion led to this idea (34–36). In agreement with TCR signaling driving exhaustion, the exhausted virus-specific $CD8^+$ T cell fraction was found to increase in time upon viral persistence (37). However, virus-specific $CD8^+$ T cells can already show impaired effector functions from the beginning of a chronic infection, suggesting causes other than chronic antigen exposure (37). Adoptive transfer experiments demonstrated that exhausted $CD8^+$ T cells in chronic LCMV infections derive from the same progenitors as memory cells and not from terminally differentiated ($KLRG1^{hi}$) effector T cells (38). This finding suggested that exhausted $CD8^+$ T cells in chronic infection do not follow a normal effector differentiation path (39).

Predysfunction

Despite the persistence of viral antigen, not all virus-specific $CD8^+$ T cells in chronic infection acquire a terminally exhausted phenotype. A subset of virus-specific $CD8^+$ T cells in chronic LCMV infection was found to proliferate and give rise to terminally exhausted cells (40). Other authors defined in the same model a small "memory-like" subpopulation within the virus-specific $CD8^+$ T cell pool that retained proliferative capacities and could re-expand upon secondary infection in an antigen-free host (41). Later, this proliferative population was found to express the transcription factor TCF-1 (9, 42) and the chemokine receptor CXCR5 (43, 44). These studies report that $TCF\text{-}1^+$ $CXCR5^+$ $CD8^+$ T population is self-sustaining and constantly replenishes the exhausted $CD8^+$ T cell pool. This population is described by different nomenclature (**Table 1**), but throughout this article, we will use the term "predysfunctional". The predysfunctional population is established early in chronic infection with LCMV strain clone 13, before the peak of the T cell response, but is not seen in acute infection with LCMV strain Armstrong (51). TCF-1 is also expressed in memory T cells in acute infection, but predysfunctional $TCF1^+$ T cells in chronic infection can be identified by co-expression of CXCR5, Slamf6 and PD-1 (29, 44). TCF-1 signaling represses effector differentiation and is thereby essential for generation and maintenance of predysfunctional T cells (42, 57, 59).

TABLE 1 | Definitions of predysfunctional $CD8^+$ T cell populations in chronic infection and cancer.

Population name	Markers	Source	References
Memory-like	$TCF\text{-}1^+$	LCMV-c13	(9, 39, 41, 45)
		Human HCV	(9)
		Human melanoma	(46)
Stem-like	$CXCR5^+TIM3^-$	LCMV-c13	(44, 47)
		Human NSCLC	(48)
	$PD\text{-}1^+CD101^-TIM3^-$	LCMV-c13	(49)
	$TIM3^-CD28^+$	Human kidney cancer	(50)
	$TCF\text{-}1^+$	B16-gp33	(10)
Progenitor-like	$TCF\text{-}1^{high}TIM3^{low}$	LCMV-c13	(42)
		Human melanoma	(42)
	$Tcf7^+Tox^+$	LCMV-c13	(51)
Progenitor	$TCF\text{-}1^+$	LCMV-c13	(52–55)
	$Ly108^+$ ($Slamf6^+$)	LCMV-c13	(29)
Progenitor exhausted	$Slamf6^+TIM3^-$	LCMV-c13; B16-OVA	(7)
	$TCF\text{-}1^+PD\text{-}1^+$	Human melanoma	(7)
Precursor	$T\text{-}bet^{high}Eomes^{low}$	LCMV-c13	(40)
Memory precursor-like	$PD\text{-}1^-TCF\text{-}1^+$	MC38-OVA	(56)
Precursor exhausted	$KLRG1^-PD\text{-}1^+$ $Ly108^+$	LCMV-c13	(57)
	$TCF\text{-}1^+$	LCMV-c13	(58)
Stem cell-like exhausted	$CXCR5^+TIM3^-$	LCMV-c13	(59)
Pre-exhausted	*GZMK*$^+$, *ZNF683*$^+$	Human NSCLC	(60)
Predysfunctional	multiple	Human cancers	(61)
Early dysfunctional	$CD38^{low}CD101^{low}$	ASTxCre-ER^{T2}	(62, 63)
Transitional	*GZMK*$^+$	Human melanoma	(64)
		Human HCC	(65)
Follicular cytotoxic	$CXCR5^+$	LCMV-13	(43, 66)
		LCMV-DOCILE; HIV	(67)
		Human CHB	(68)

The listed populations have in common that they sustain the CTL response in presence of persistent antigen, and form the progenitors of the terminally exhausted population, as originally shown by Utzschneider et al. (9), Wu et al. (42), He et al. (43), and Im et al. (44) and corroborated by Miller et al. (7) and Zander et al. (29). Other cited papers consider the defined population to be predysfunctional based on the markers and the proliferative/ "stem-like" phenotype described in the original papers. In the papers describing human single cell RNAseq data, the predysfunctional population is defined by intermediate expression of inhibitory receptor genes, low expression of effector-associated genes, and TCR sharing with the terminally exhausted population.

From Predysfunction to Exhaustion

Antigenic stimulation of predysfunctional $TCF\text{-}1^+CXCR5^+$ $CD8^+$ T cells can drive their differentiation into $TCF\text{-}1^-$ $CXCR5^-$ "terminally exhausted" cells (40, 49, 69). During this differentiation process, predysfunctional cells transiently acquire a more effector-like gene signature (49, 57, 70). Terminally exhausted $CD8^+$ T cells are short-lived and display higher expression of coinhibitory receptors than $TCF\text{-}1^+$ predysfunctional cells (9, 42–44). Conversion from a predysfunctional to a terminally exhausted state is associated with epigenetic and transcriptional changes involving genes encoding coinhibitory receptors, effector molecules and effector-associated transcription factors (7, 47, 70).

The transcription factor TOX plays a critical role in epigenetic imprinting of dysfunction in the TCF-1$^+$ subset and induces fate commitment to a terminally exhausted phenotype (51, 52, 71–73). Both the establishment of the predysfunctional population and the TOX-driven commitment to exhaustion are part of a differentiation path that is separate from effector differentiation, occurring in early stages of chronic LCMV infection (51, 57, 71). Together, these findings provide strong support for the notion that terminally exhausted T cells found in chronic infections are derived from a population of predysfunctional cells, instead of from functional effectors. Similar processes likely take place in human, where virus-specific predysfunctional and terminally exhausted CD8$^+$ T cell populations have been identified in patients with chronic hepatitis C virus (HCV) infection (9). Also, CXCR5$^+$ CD8$^+$ T cells were found in patients infected with human immunodeficiency virus (HIV) or chronic hepatitis B virus (HBV) (67, 68).

Reinvigoration

Importantly, PD-1 blockade unleashes the expansion potential of predysfunctional, but not terminally exhausted virus-specific CD8$^+$ T cells (9, 43, 44). Predysfunctional TCF-1$^+$ CD8$^+$ T cells express PD-1 that supports the maintenance of this population early during chronic LCMV infection (57). Chronic virus infections (LCMV clone 13, HIV) induce chromatin accessibility and permanent demethylation of the *Pdcd1* locus (encoding PD-1), causing exhausted CD8$^+$ T cells to stably express PD-1 at high levels (74, 75). Terminally exhausted CD8$^+$ T cells express higher levels of PD-1 and other coinhibitory receptors than predysfunctional cells (9, 42, 43). In the terminally exhausted population, efficacy and durability of virus-specific CD8$^+$ T cell reinvigoration by PD-1 blockade proved to be limited by the epigenetic landscape, including chromatin accessibility and *de novo* DNA methylation (76, 77). Taken together, these results argue that the predysfunctional virus-specific CD8$^+$ T cell population in chronic infection is reinvigorated by PD-1 blockade. Predysfunctional cells respond to PD-(L)1 blockade by undergoing proliferation, as well as differentiation toward a terminally exhausted phenotype (7). During this differentiation, cells pass through an intermediate or "transitory" state, characterized by a transcriptional signature that resembles that of effector CTLs (49, 70). While these effector-like CD8$^+$ T cells that are reinvigorated by PD-1 blockade are able to produce cytokines and contribute to virus control, they retain expression of inhibitory receptors and eventually convert to a terminally exhausted state upon persistent antigen exposure (49).

PROPOSITION: HELPLESS PRIMING GENERATES PREDYSFUNCTIONAL CD8$^+$ T CELLS

Establishing a chronic infection in mouse models is often aided by depleting CD4$^+$ T cells (33, 37, 44, 77, 78), suggesting a link between the absence of CD4$^+$ T cell help and infections persisting chronically. Decreased antigen presentation and decreased costimulatory signaling by DCs during priming promote the formation of TCF-1$^+$ cells, suggesting that this population may be generated as a result of suboptimal priming (45). Importantly, CD4$^+$ T cell depletion in chronic LCMV infection impaired the generation of terminally differentiated effector CD8$^+$ T cells, but not of predysfunctional TCF-1$^+$ CD8$^+$ T cells (53). This finding indicates that the predysfunctional TCF-1$^+$ CD8$^+$ T cell population is formed independently of CD4$^+$ T cell help. We propose that this population is formed as a result of helpless priming and provide supporting evidence in this article.

As a model to study CD4$^+$ T cell help for the CTL response, our group made use of a therapeutic DNA vaccination scheme in mice. We used a comparative setting with two vaccines that encode an immunodominant MHC-I restricted peptide from the human papillomavirus (HPV) E7 protein to prime CD8$^+$ T cells, either with or without HPV-unrelated immunodominant MHC-II restricted peptides to induce CD4$^+$ T cell help (79). Genome-wide mRNA deep sequencing of HPV-E7-specific CD8$^+$ T cells at the effector stage of the CTL response yielded "Help" and "No Help" signatures (3). Helpless CTLs expressed many genes characteristic of the predysfunctional CD8$^+$ T cell subset at a higher level than helped CTLs, including *Tcf7* (encoding TCF-1), *Tox*, *Pdcd1*, *Cxcr5*, and *Slamf6* **(Figure 2A)** (3). We therefore hypothesized that predysfunctional CD8$^+$ T cells found in chronic LCMV infection are cells that have not experienced CD4$^+$ T cell help during priming. To test this, we determined how predysfunctional CD8$^+$ T cells defined in literature and helpless CD8$^+$ T cells defined in our study are related at the gene expression level, by Gene Set Enrichment Analysis (GSEA). A published gene expression signature characteristic for the predysfunctional TCF-1$^+$ CD8$^+$ T cell population in chronic LCMV infection (9) in mice thus proved to be enriched in the No Help gene expression signature of antigen-specific CD8$^+$ T cells from our vaccination study **(Figure 2B)**. Additionally, using another published dataset from chronic LCMV infection (7), we determined a "No Help score" as a measure of correlation with our No Help gene expression signature. This analysis demonstrated that predysfunctional TCF-1$^+$ CD8$^+$ T cells display a higher No Help score than TCF-1$^-$ terminally exhausted cells, indicating that predysfunctional CD8$^+$ T cells are transcriptionally more similar to helpless CD8$^+$ T cells **(Figure 2C)**.

CD8$^+$ T CELL DYSFUNCTION IN CANCER

The Parallel

In cancer, tumor antigen-specific CD8$^+$ T cells may be chronically stimulated within the tumor micro-environment (TME), which theoretically can lead to exhaustion, as it does in mouse models of chronic virus infection. However, in the LCMV models, infection is systemic and analysis is generally focused on CD8$^+$ T cells from the spleen. This milieu is distinct from the TME in partially undefined aspects. In both environments, specific conditions are created by interplay between infected cells or growing tumor cells, immune cells and non-immune cells. Intratumoral CD8$^+$ T cells are known to be exposed to

various suppressive immune cell types, inhibitory molecules, hypoxia, metabolites and nutrient deprivation (2).

Mouse Models

Using a mouse model of tamoxifen-inducible liver cancer, it was shown that tumor antigen-specific $CD8^+$ T cells taken from the TME early during tumorigenesis could be reinvigorated by PD-1 blockade or recall in an antigen-free host. Late in tumor development, however, these cells could no longer be rendered functional. It was found that tumor-specific $CD8^+$ T cells in the TME over time acquire a fixed dysfunctional phenotype (62). Follow-up research in this model showed that tumor-specific $CD8^+$ T cells in the TME first attain a reversible dysfunctional state and next enter a epigenetically fixed dysfunctional state (63). These data are in agreement with a transition from predysfunction to exhaustion.

In a murine melanoma model, single-cell transcriptomics revealed that among $CD8^+$ tumor infiltrating lymphocytes (TILs), TCF-1^+ predysfunctional and TCF-1^- terminally exhausted cell subsets can be discerned that are analogous to those defined in chronic LCMV infection. Adoptive transfer experiments demonstrated that TCF-1^+ $CD8^+$ T cells can persist long-term inside a tumor and give rise to terminally exhausted cells (7). Like in chronic infection, transcriptional and epigenetic changes underlying this conversion depended on the transcription factor TOX (72, 73).

Human Cancer

Also in human cancer, there is increasing evidence for the existence of predysfunctional and terminally exhausted $CD8^+$ T cell populations. In non-small cell lung cancer (NSCLC), CXCR5 expression was selectively found on $CD8^+$ TILs and not on $CD8^+$ T cells from healthy tissue or blood (48). In kidney cancer, TCF-1^+TIM3^-CD28^+ predysfunctional TILs were found to reside in niches that are rich in antigen-presenting cells, while PD-1^+TIM3^+ terminally exhausted cells were distributed throughout the tumor tissue. Transcriptional and epigenetic profiles of these human TIL subsets proved to be similar to those described in the mouse. Importantly, TCR repertoire overlap between the two populations indicated that TCF-1^+ predysfunctional TILs are indeed the progenitors of terminally exhausted TILs (50). TCR repertoire overlap between a terminally exhausted TIL population, characterized by high expression of coinhibitory receptor genes, and a predysfunctional TIL population, characterized by expression of *GZMK*, was also found in human melanoma (64), NSCLC (60), colorectal cancer (CRC) (80) and hepatocellular carcinoma (HCC) (65). These findings are consistent with a model where also in human cancer, exhausted TILs derive from a predysfunctional population. However, a strict division of the human TIL pool into predysfunctional or terminally exhausted may be an oversimplification. Rather, CTL dysfunction in human TILs covers a spectrum of differentiation states, ranging from predysfunctional to terminally exhausted (61).

The question remains whether the active CTLs that display effector functions in human tumors are generated from a separate $CD8^+$ T cell pool, or are connected to the (pre) dysfunctional pool. In CRC, HCC and NSCLC studies, TCR sharing was found between *GZMK*$^+$ predysfunctional TILs and *CX3CR1*$^+$ effector populations from blood and normal tissue (60, 65, 80). These results support a model in which the predysfunctional population forms a branchpoint from which differentiation trajectories of effector versus exhausted $CD8^+$ T cells emanate, possibly reflecting $CD8^+$ T cells after the first step of priming that subsequently receive $CD4^+$ T cell help, versus $CD8^+$ T cells that do not. However, it was not determined in those studies whether the T cells that shared TCRs were tumor-specific. In melanoma, intratumoral *GZMH*$^+$ effector CTLs did not share TCRs with the predysfunctional or exhausted $CD8^+$ TIL population, indicating that they formed a separate lineage (64). Interestingly, in this study, tumor reactivity was enriched in the dysfunctional but not in the cytotoxic TIL population, suggesting that the cytotoxic population consists of bystander cells that do not recognize the tumor, as was demonstrated before (81, 82). These data argue that in melanoma, persistent tumor antigen recognition drives the conversion of helpless tumor-specific TILs from the predysfunctional to the terminally exhausted state, while the tumor may also harbor helped bystander cells with an effector phenotype (61). Whether tumor-specific dysfunctional TILs can differentiate within the TME into competent effector CTLs remains to be investigated.

Reinvigoration

In mouse models of melanoma, the TCF-1^+ predysfunctional $CD8^+$ TILs proved to be the responders to PD-1 blockade and necessary for tumor control (7, 10, 56). In melanoma patients, an increased fraction of TCF-1^+ predysfunctional $CD8^+$ TILs is a positive predictor for response to PD-(L)1 targeted therapy (7, 46). In a murine liver cancer model, CD101 and CD38 marked predysfunctional versus terminally exhausted TILs. These markers were heterogeneously expressed by PD-1^{high} TILs from melanoma and NSCLC patients, suggesting that the human PD-1^{high} TIL population consists of a mixture of predysfunctional and terminally exhausted cells (63).

HELPLESSNESS AND PREDYSFUNCTION IN CANCER

$CD4^+$ T cell help is less likely to be delivered in cancer than in infection for the following reasons: Tumor cells generally do not express PAMPs and may only exude DAMPs under specific circumstances. Therefore, they are less likely to activate migratory DCs than infected cells. Furthermore, in the suppressive TME, migratory cDC2s, which are essential for the priming of $CD4^+$ T cells (83), are reportedly suppressed by Tregs, resulting in suboptimal priming of $CD4^+$ helper T cells in the tumor-draining lymph node (84). Also, DC-activating signals such as type I IFN that promote crosspresentation functions of the lymph node-resident cDC1 (16), are often lacking. In the blood of melanoma patients, tumor reactivity of CTLs was found to be enriched in the PD-1^+ population (85). These data led us to hypothesize that helpless priming may contribute to the dysfunctional phenotype of $CD8^+$ T cells in cancer.

To test this hypothesis, we performed bioinformatic analyses using our previously defined No Help versus Help signatures of mouse $CD8^+$ T cells and datasets from mouse and human cancer. GSEA showed that gene sets characteristic of predysfunctional TCF-1^+ $CD8^+$ TILs from a gp33 antigen bearing B16 melanoma mouse model (10) were enriched in the No Help gene expression signature **(Figure 3A)**. In an ovalbumin (OVA) antigen-bearing B16 melanoma model from a different research group (7), TCF-1^+ $CD8^+$ TILs displayed a higher No Help score than TCF-1^- $CD8^+$ TILs **(Figure 3B)**. These results indicate that also in mouse cancer models, dysfunctional TCF-1^+ $CD8^+$ TILs display a gene expression profile that resembles that of helpless cells. In NSCLC patients, the presence of PD-1^{high} TILs was a positive predictor of response to PD-1 blockade therapy. Importantly, PD-1^{high} TIL displayed higher intrinsic tumor reactivity compared to TIL populations with intermediate or no PD-1 expression from the same tumor (8). We used the published gene expression profiles from these matched TIL subsets to calculate their No Help score. Among these patients' TIL populations, the transcriptome of PD-1^{high} TILs was most similar to that of helpless vaccine antigen-specific $CD8^+$ T cells **(Figure 3C)**. These data from human cancer support our hypothesis that dysfunctional tumor-reactive $CD8^+$ T cells are cells that have lacked help during priming.

HELPLESS DYSFUNCTION MODEL

We present a novel model posing that virus-specific or tumor-specific, predysfunctional TCF-1^+ $CD8^+$ T cells in chronic infection or cancer result from priming in the absence of $CD4^+$ T cell help. $CD4^+$ T cell help delivered during priming optimizes effector differentiation of antigen-specific $CD8^+$ T cells (3, 53). Additionally, $CD4^+$ T cell help promotes effector memory $CD8^+$ T cell (T_{EM}) generation, and renders these T_{EM} cells more effector-like on a per-cell basis (30). These results are in line with a previously proposed progressive differentiation model for primed $CD8^+$ T cells (86), adding that $CD4^+$ T cell help shifts differentiation of primed $CD8^+$ T cells toward a more effector-like state **(Figure 4)**.

By optimizing CTL function, $CD4^+$ T cell help contributes to antigen clearance, which is necessary for proper memory formation (87, 88). $CD4^+$ T cell help also promotes the long-term maintenance of T_{CM} cells and is necessary for open configuration of gene loci encoding CTL effector molecules in memory $CD8^+$ T cells (30, 89, 90). The epigenetic imprinting induced by help signals during priming allows memory cells to rapidly exert effector functions upon reactivation in a $CD4^+$ T helper cell-independent manner (30, 91).

In the absence of $CD4^+$ T cell help, effector differentiation of $CD8^+$ T cells is incomplete, resulting in predysfunctional CTLs that have limited cytotoxic and migratory potential and express coinhibitory receptors (3, 32), which prohibits antigen clearance. The chronic stimulation of memory precursor cells impairs the formation of a memory pool and instead drives their differentiation into predysfunctional CTLs, as seen in chronic infection and cancer (39, 54). These predysfunctional TCF-1^+ cells have self-maintaining properties and form the progenitors of the terminally exhausted TCF-1^- $CD8^+$ T cell pool (58). Exhausted $CD8^+$ T cells differ in their epigenetic and transcriptional states from predysfunctional $CD8^+$ T cells. They have a further developed effector differentiation program, but are fixed in their dysfunctional state (55).

OVERCOMING CTL DYSFUNCTION BY HELP SIGNALS

Based on our model, we propose that in chronic infection and cancer, CTL dysfunction can be overcome by help signals. In that scenario, help signals would enable the CTLs to progress further toward a terminal effector differentiation state. Adoptive transfer of $CD4^+$ T cells has been shown to increase proliferation of pre-existing TCF-1^+ $CD8^+$ T cells in chronic LCMV infection (53). Also, adoptive transfer of IL-21-producing $CD4^+$ T cells into tumor-bearing mice induced generation of a $CX3CR1^+$ effector $CD8^+$ T cell pool, leading to improved tumor control (29). Using help signals to alleviate CTL dysfunction is not yet incorporated into clinical protocols. In the clinic, PD-1 blockade is used as method to "reinvigorate" dysfunctional CTLs.

We here propose that PD-1 blockade recapitulates aspects of $CD4^+$ T cell help and acts on the predysfunctional/helpless $CD8^+$ T cell population. As reviewed in the preceding sections, in chronic LCMV infection and cancer, PD-1 blockade induced proliferation of predysfunctional TCF-1^+ $CD8^+$ T cells. The question is whether PD-1 blockade is sufficient to overcome lack of help and—by association—to convert predysfunctional CTLs into fully functional effectors. In chronic LCMV infection, established through transient $CD4^+$ T cell depletion, PD-L1 blockade promoted differentiation of predysfunctional $CD8^+$ T cells into transitional cells that displayed a more effector-like phenotype and contributed to virus control. However, eventually these cells became terminally exhausted (49). Blockade of the PD-L1/PD-1 axis in a helpless setting increases the magnitude of the antigen-specific $CD8^+$ T cell response, but in contrast to $CD4^+$ T cell help, it did not rescue the formation of the effector population that conferred protection against chronic infection and cancer (29). These results suggest that predysfunctional/helpless cells cannot be rescued by PD-1 blockade alone.

The prevailing view is that PD-1 blockade relieves pre-existing dysfunctional CTLs from suppression in the TME. However, accumulating data argue that PD-1 blockade can also facilitate *de novo* CTL priming. Firstly, PD-(L)1 targeted immunotherapy can be effective while PD-L1 is not expressed in the tumor (92). Secondly, PD-1 signaling impedes TCR as well as CD28 signaling, indicating that it can also impact on costimulation at the T cell/DC interface (93). In agreement with this, tumor regression upon PD-1 blockade in mouse colon carcinoma depended on CD28 co-stimulation (94). Thirdly, the response to PD-1 blockade in mouse colon carcinoma was found to depend on influx of newly activated $CD8^+$ T cells from tumor draining lymph nodes (95).

Recent data from human cancer also argue that PD-1 blockade promotes $CD8^+$ T cell priming: In basal cell carcinoma, new $CD8^+$ T cell clones entered the upon tumor PD-1 blockade (96). TCR repertoire analysis argued that these clones pre-existed in blood and entered the tumor after treatment (97). PD-1 is expressed rapidly after stimulation of naive $CD8^+$ T cells, and inhibits effector differentiation during priming (98). We found that in the $CD4^+$ T cell help-dependent second step of priming, $CD8^+$ T cells downregulate PD-1, whereas helpless cells maintain PD-1 expression (3). This supports a model in which PD-1 serves as a checkpoint in the two-step T cell priming process.

We have shown in the mouse vaccination model, that the effects of $CD4^+$ T cell help on the CTL response could be mimicked by combined PD-1-blockade and CD27 agonism (99). We and others have shown that delivery of $CD4^+$ T cell help is highly dependent on CD70-CD27 signaling and CD27 agonism installs a large part of the Help gene signature into $CD8^+$ T cells during priming (3, 20, 24, 25). The combined effect of PD-1 blockade and CD27 agonism likely recapitulates combined CD28 and CD27 costimulation that are known to complement each other in generation of the CTL effector pool (23). The collective data make a strong case for combining CD27 agonism with PD-(L)1 blockade in cancer immunotherapy.

CONCLUDING REMARKS

We here present our hypothesis that $CD8^+$ T cell priming in the absence of $CD4^+$ T cell help leads to $CD8^+$ T cell dysfunction. We pose that exhausted antigen-specific $CD8^+$ T cells observed in infection and cancer derive not from previously active CTLs, but from helpless $CD8^+$ T cells that emerge from the priming process in a dysfunctional state. We pose that provision of $CD4^+$ T cell help, or the key signals that recapitulate help for $CD8^+$ T cells will be crucial for the development of effective immunotherapeutic strategies in chronic infection and cancer. In immunotherapy, reverting exhausted cells back to a functional phenotype is considered an important challenge (1). Alternatively, we argue that in patients with immunogenic cancer types, *de novo* priming of helped $CD8^+$ T cells will be beneficial for tumor control. For this purpose, potential approaches are antigen-agnostic PD-1/CD27 targeting or antigen-informed therapeutic vaccination. Such vaccines should contain MHC-I and MHC-II epitopes to activate both $CD8^+$ and $CD4^+$ T cells. Other strategies include specific targeting of antigens and activation signals to $XCR1^+$ cDC1s. In these approaches, evaluation of the transcriptional help signature in tumor-specific $CD8^+$ T cells is a potential diagnostic tool.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/.

AUTHOR CONTRIBUTIONS

JuB performed data analysis and wrote the paper. ST performed data analysis. JaB contributed to writing the paper. HE critically reviewed the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by grant 11079 from the Dutch Cancer Society.

ACKNOWLEDGMENTS

We thank Paul Vink and Lars Guelen (Aduro Biotech) for their support of this project.

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Conflict of Interest: HE and ST were employees of Aduro Biotech. HE has stocks and/or stock options in Aduro Biotech, Inc.

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: 03 December 2020
doi: 10.3389/fimmu.2020.583084

Tumor-Associated Macrophages in Tumor Immunity

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Tumor-associated macrophages (TAMs) represent one of the main tumor-infiltrating immune cell types and are generally categorized into either of two functionally contrasting subtypes, namely classical activated M1 macrophages and alternatively activated M2 macrophages. The former typically exerts anti-tumor functions, including directly mediate cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) to kill tumor cells; the latter can promote the occurrence and metastasis of tumor cells, inhibit T cell-mediated anti-tumor immune response, promote tumor angiogenesis, and lead to tumor progression. Both M1 and M2 macrophages have high degree of plasticity and thus can be converted into each other upon tumor microenvironment changes or therapeutic interventions. As the relationship between TAMs and malignant tumors becoming clearer, TAMs have become a promising target for developing new cancer treatment. In this review, we summarize the origin and types of TAMs, TAMs interaction with tumors and tumor microenvironment, and up-to-date treatment strategies targeting TAMs.

Keywords: tumor-associated macrophages, regulation, immunosuppression, tumor microenvironment, tumor therapy

OPEN ACCESS

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

Received: *14 July 2020*
Accepted: *04 November 2020*
Published: *03 December 2020*

Citation:
Pan Y, Yu Y, Wang X and Zhang T (2020) Tumor-Associated Macrophages in Tumor Immunity. Front. Immunol. 11:583084. doi: 10.3389/fimmu.2020.583084

INTRODUCTION

Macrophages play critical roles in both innate and adaptive immunity and are known for their remarkable phenotypic heterogeneity and functional diversity. Embryonic hematopoietic stem cells in a variety of tissues during fetal development and differentiate into tissue-specific resident macrophages, including Kupffer cells in the liver, alveolar macrophages in the lung, and osteoclasts in bone tissue. After birth, bone marrow-derived precursors in particular circulating monocytes can also differentiate into macrophages in steady state or during tissue inflammation (1). Macrophages are involved in tissue and systemic inflammation and immunity, as well as tissue reconstruction. They have a wide range of functions, including phagocytosis, antigen presentation, defense against microbial cytotoxicity, and secretion of cytokines, complement components, etc. (2). It is worth noting that the broad biological activities of macrophages often have diametrically opposite characteristics, such as inflammatory response and anti-inflammatory activity; immunogenic and inducing immune tolerance; causing tissue destruction and repairing (3).

Tumor-associated macrophages (TAMs) are macrophages that participate in the formation of the tumor microenvironment. TAMs are widely present in various tumors (4). TAMs can promote tumor growth, invasion, metastasis, and drug resistance (5). It has been proposed that functional

difference of macrophages is closely related to the plasticity of macrophages, and its functional phenotype is regulated by molecules in tumor microenvironments.

In this review, we discuss the origins and types of TAMs, the interaction between tumors and the tumor microenvironment, and review the emerging strategies for cancer treatment *via* targeting TAMs.

ORIGINS AND TYPES OF TAMs

Origins

For a long period of time, it is believed that macrophages in tumors are exclusively recruited from the periphery by chemotaxis and generated by monocytic precursors in the local environment. However, more recent evidence shows that at least certain tumors, tissue-specific embryonic-derived resident macrophages infiltrate tumor tissues and thus represent a nonnegligible input source of TAMs (6). Although there have been studies showing that monocytic-derived but not embryonic-derived resident macrophages are capable in supporting the growing body of TAMs in the inflammatory environment of tumor, the potentially different roles of monocytic- versus embryonic-derived TAMs on tumor development and/or progress remains an intriguing question that is largely unanswered (2).

M-MDSCs (monocyte-related myeloid-derived suppressor cells) are currently known as another main circulating precursor of TAMs. MDSCs are a type of myeloid leukocytes that is related to immunosuppression (7). Based on surface markers Ly6C+/Ly6C- and Ly6C-/Ly6G+, MDSCs can be divided into monocyte (M)-related and granulocyte (G)-related MDSC. Among them, M-MDSCs are induced into TAMs by various chemokines (8).

It is all know that macrophages derive from bone marrow-derived monocytes. In tumors, TAMs mainly originate from bone marrow monocytes, but recent evidence suggests that, recruitment of circulating monocytes is essential for TAMs accumulation. Circulating inflammatory monocytes could be recruited by multiple chemokines (CCL2 and CCL5) and cytokines (CSF-1 and members of the VEGF family) to tumor (9). Tumor growth can also induce the differentiation of CCR2+ monocytes into TAMs (10).

Furthermore, complement components, particularly C5a, are an important mediator of the recruitment and functional polarization of TAMs (11). Indeed, such chemokines do more than attractants do because they activate transcription programs that help macrophages tilt toward the functional of a particular phenotype (12). At the same time, CSF-1 is a monocyte attractant, as well as macrophage survival and polarization signals, which drive TAM to immunosuppressive differentiation M2 macrophages (13). Unlike CSF-1, GM-CSF activates macrophage function associated with antitumor activity (14).

Types

Macrophages undergo specific differentiation in different tissue environments, and can be divided into two different polarization states: M1 type macrophages (M1) and M2 type macrophages (M2).

M1 can respond to dangerous signals transmitted by bacterial products or IFN-γ, which attracting and activating cells of the adaptive immune system; an important feature of M1 is that it can express nitric oxide synthase (iNOS) and reactive oxygen species (ROS) (15–17) and cytokine IL-12 (18). M1 also has the function of engulfing and killing target cells.

M2 expresses a large number of scavenger receptors, which is related to the high-intensity expression of IL-10, IL-1β, VEGF and matrix metalloprotein (MMP) (19, 20). M2 has the function of removing debris, promoting angiogenesis, tissue reconstruction and injury repairments, as well as promoting tumorigenesis and development (4).

It is worth noting that the polarization of macrophages into M2 appears to be oversimplified. Some people have classified M2 macrophages into M2a (induced by IL-4 or IL-13), M2b (induced by immune complexes combined with IL-1β or LPS) and M2c (induced by IL-10, TGFβ, or glucocorticoid), and M2d (conventional M2 macrophages that exert immunosuppression) (21, 22).

THE ROLE OF TAMs IN TUMOR PROGRESS

Current studies have shown that TAM population is in a state of constant transition between the two forms of M1 and M2 type. The proportion of each form is determined by the type and concentration of different signals in the tumor environment (**Figure 1**).

M1 Macrophages and Tumor Suppression

M1-type macrophages have anti-tumor effects, which can distinguish tumor cells from normal cells. By identifying tumor cells and ultimately killing tumor cells, studies have found that M1 type macrophages have two different effects on killing tumor cells mechanism. M1 type macrophages directly mediate cytotoxicity to kill tumor cells: macrophage-mediated cytotoxicity is a slow process (generally requires 1 to 3 days) and involves multiple mechanisms. For example, macrophages release tumor killing molecules such as ROS and NO, which have cytotoxic effects on tumor cells (23). The other is antibody-dependent cell-mediated cytotoxicity (ADCC) killing tumor cells: ADCC requires less time to kill tumor cells (generally within a few hours) and requires the participation of anti-tumor antibodies (24).

M2 Macrophages Promote Tumor Cell Proliferation and Invasion

TAM infiltration is closely related to tumor cell proliferation. Many studies have shown that TAMs can express a variety of cytokines that stimulate tumor cell proliferation and survival, including epithelial growth factor (EGF), platelet-derived growth factor (PDGF), TGF-β1, hepatocyte growth factor (HGF), and epithelial growth ligands of the factor receptor (EGFR) family

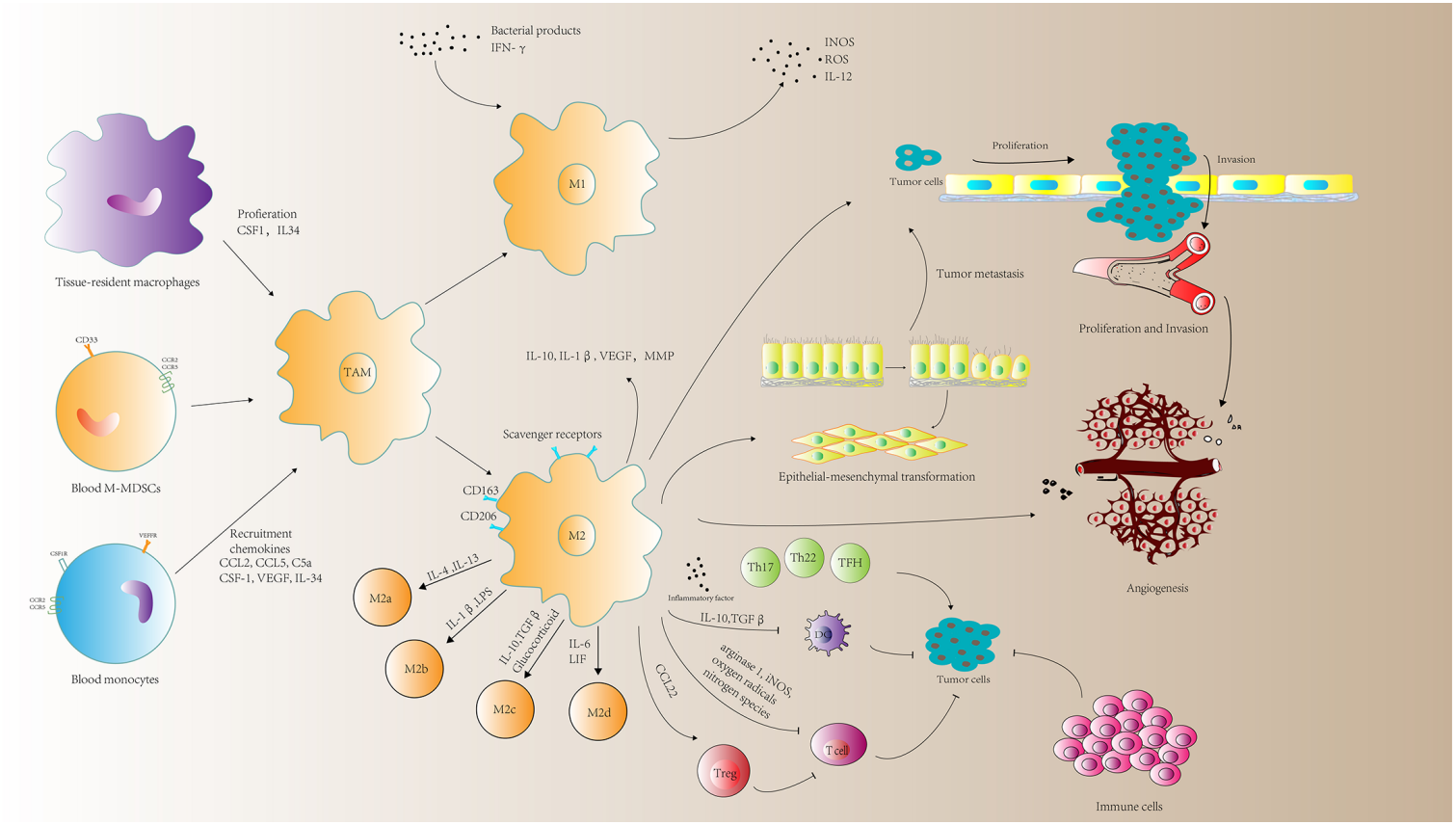

FIGURE 1 | A schematic representation of the roles of tumor-associated macrophages (TAMs) in tumor progression. TAMs can mediate immune response, tumor cell proliferation and invasion, angiogenesis and metastasis. MMP, matrix metalloprotein; M-MDSCs, monocyte-related myeloid-derived suppressor cells; CSF1, macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; INOS, nitric oxide synthases; LIF, leukocytosis induced factor.

and basic fibroblast growth factor (BFGF) (25). The ligands of the EGFR family play an important role in tumorigenesis, especially breast and lung cancers. Members of this family can form homo- or heterodimers on the cell surface, mediating the transduction of cell proliferation signals. In all, TAMs are an important cell source for EGF secretion in tumor tissues (25).

As for invasion, in glioma cells, extracellular adenosine deaminase protein cat eye syndrome critical region protein 1 (CECR1) has been shown to regulate the maturation of macrophages. CECR1 is induced by M2-like TAM secretory effects activate MAPK signaling and stimulate the proliferation and migration of glioma cells (26). Another investigation shows that a positive feedback loop of CCL5 and CCL18 between TAMs and myofibroblast is constituted to drive the malignant invasion of phyllodes tumor (PT). CCL5 binds to CCR5, and activates the AKT signal to recruit and repolarize TAMs. TAMs release CCL18 to further induce the invasion of malignant PTs by differentiating the mesenchymal fibroblasts to myofibroblast, causing the malignancy of PTs (27).

TAMs Promote Tumor Metastasis

Tumor metastasis is an important feature of poor prognosis after tumor therapy. The main reason for tumor cell migration and metastasis is the degradation and damage of tumor tissue endothelial cell basement membrane. It has been reported that activated TAMs exert a direct effect on promoting metastasis *via* directly producing soluble factors (28). M2 macrophages can destroy matrix membrane of endothelial cells by secreting matrix metalloproteinases (MMPs), serine proteases, cathepsins, and decompose various collagen and other components of extracellular matrix, thereby helping the migration of tumor cells and tumor stromal cells (19, 20). Epithelial-mesenchymal transition (EMT) is the basis of tumor metastasis (29). This process enables tumor cells to acquire the ability to migrate and endows them with the properties of stem cells (30). Besides, cytokines produced by tumor cells also promote the differentiation process of TAMs, thus forming a positive feedback loop between TAMs and EMT (31).

M2 Macrophages Promoting Angiogenesis

TAMs are enriched in hypoxic areas with poor blood supply (1). Proangiogenic effects by TAMs involves the coordinated regulation of a wide range of cytokines, including BFGF, VEGF, IL-1, IL-8, TNF-α, MMP-9, MMP-2, and nitric oxide (NO). The coordinated expression of these molecules promotes the proliferation of endothelial cells, matrix remodeling and vascularization in time and space. Macrophages can release the angiogenic molecules and express a series of enzymes involved in the regulation of angiogenesis, including MMP-2, MMP-7, MMP-9, MMP-12, and cyclooxygenase-2 (20, 32).

However, metabolism still exists in angiogenesis, and it is still unknown whether changes in metabolism affect these functions. Hypoxic TAM strongly up-regulates the expression of mTOR's negative regulator REDD1. REDD1-mediated mTOR inhibition can hinder glycolysis in TAM and reduce its excessive angiogenic response, thereby forming abnormal blood vessels (33).

Immune Regulation by TAMs

TAM can regulate the killing effect of T cells and NK cells on tumor cells. M1 macrophages increased the number of total and activated natural killer (NK) cells in fibrotic liver, released TNF-related apoptosis-inducing ligand (TRAIL), and induced HSC apoptosis (34). HCC-derived exosomes induced macrophages to upregulate the expression of IFN-γ and TNF-α in T cells, while the expression of inhibitory receptors PD-1 and CTLA-4 was upregulated (35). In mesothelioma, the macrophages isolated from pleural effusion showed the M2 phenotype were negatively correlated with T cells *in vivo*, which emphasized the use of macrophages as treatments in mesothelioma Target potential (36).

In addition to these functions, TAMs can also directly inhibit $CD8^+$ T-cell proliferation through metabolism of L-arginine *via* arginase 1, iNOS, oxygen radicals or nitrogen species (37–39). Besides, TAMs recruit Tregs through CCL22 (40), which further suppress the antitumor immune response of T-cells. Conditional TAM ablation blocks Treg cell recruitment and inhibits tumor growth by lowering the CCL20 level of xenograft mice (41).

Substantial evidence indicates that the inflammatory reaction at a tumor site can promote tumor growth and progression. Inflammation and immune evasion are considered as hallmarks of cancer. It has been reported that TAMs can also contribute to cancer-related inflammation that leads to tumorigenesis by generation of inflammatory Th subset such as TFH (42). Toll-like receptor 4 (TLR4)-induced monocyte inflammation is important for induction of IL21+ TFH-like cells, which operate in IL21-IFNγ-dependent pathways to induce plasma cell differentiation and thereby create ideal conditions for M2b macrophage and cancer progression (42) (**Figure 1**). These suggest that strategies to influence functional activities of inflammatory cells may benefit anticancer therapy.

FACTORS REGULATING TAMs FUNCTIONS

TAMs are a collection of multiple cell types with a wide range of functional effects under steady state and pathological conditions. This diversity is regulated by many different factors, such as the tumor cell-derived soluble molecules, tumor metabolic alterations, other immune cells and other factors (**Figure 2**).

Tumor Cell-Derived Soluble Molecules

TAMs can be activated and polarized by tumor cell-derived soluble molecules, thereby promoting tumor progression and metastasis. Tumor cells secrete the sonic hedgehog (SHH), and tumor-derived SHH drives TAM M2 polarization. Hh-dependent polarization of TAM suppresses the recruitment of $CD8^+$ T cells to TME *via* inhibiting CXCL9 and CXCL10, mediating TAM immunosuppression mechanism (43). In addition, kynurenine produced by glioblastoma cells can activate the aromatic hydrocarbon receptor (AHR) in TAMs, and AHR can drive KLF4 expression and inhibit NF-κB

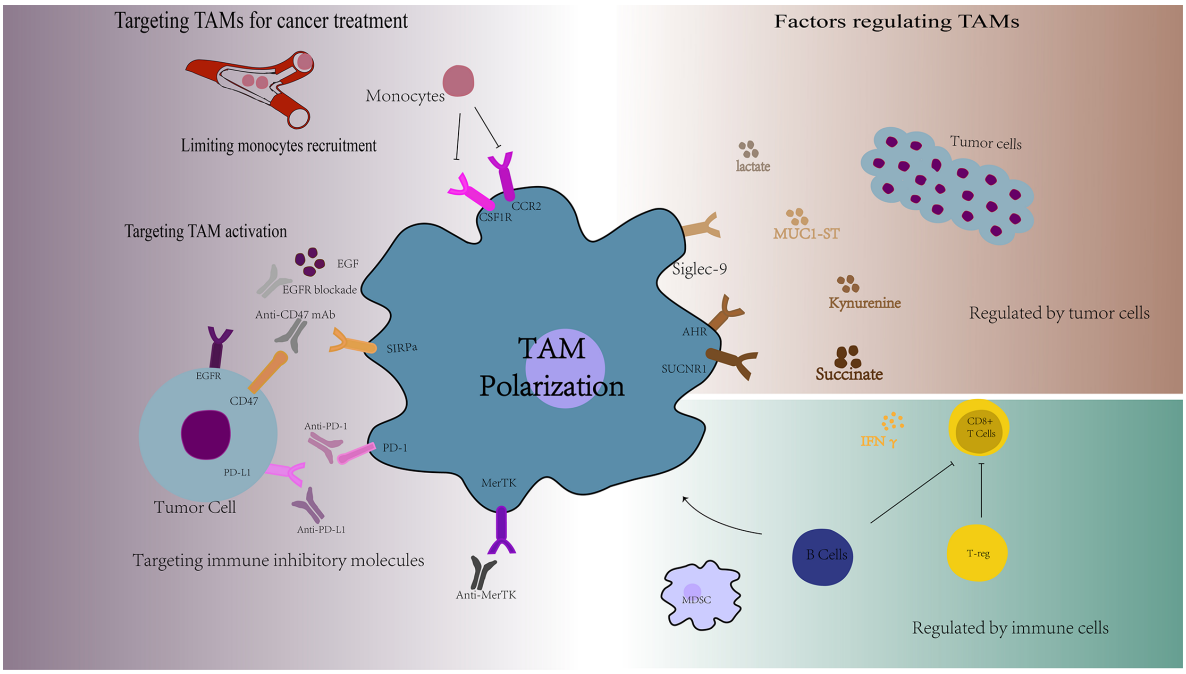

FIGURE 2 | Overview of the factors regulating TAMs functions and the targets of TAMs for cancer treatment. TAMs are a collection of multiple cell types with a wide range of functional effects, which are regulated by many different factors, such as the tumor cell-derived soluble molecules, tumor metabolic alterations, and other immune cells. Targeting TAMs is a new cancer treatment strategy, including limiting monocytes recruitment, targeting TAMs activation, and targeting TAMs specific markers. AHR, aromatic hydrocarbon receptor; SUCNR1, succinate Receptor 1; EGF, epidermal cell growth factor; SIRPα, signal regulatory protein alpha.

activation in TAMs, which regulate TAM function and T cell immunity (44). Cancer cells can also release succinate into their microenvironment and activate the succinate receptor (SUCNR1) signal, thereby polarizing macrophages to TAMs (45). Meanwhile, there is a positive correlation between the expression of osteopontin (OPN) in tumor cells and TAMs infiltration. OPN promotes chemotaxis migration and activation of TAMs (46). Also, when mucin MUC1 is expressed on cancer cells and is decorated with multiple short, sialylated O-linked glycans (MUC1-ST), which will induce TAM to express M2-like phenotype (47).

Tumor Metabolic Alterations

It is worth noting that macrophage polarization is correlated with distinct metabolic characteristics pertaining to glucose metabolism (48, 49), lipid metabolism (50), and glutamine metabolism (51). Such metabolic alterations can also determine the phenotype and function of TAMs in promoting the cancer progression (52).

Cancer cells can utilize metabolic byproducts to take the control of tumor-infiltrating immune cells to their own benefit. For example, lactate secreted by glycolysis in cancer cells, which transfers the polarization of TAMs from a pro-inflammatory (M1-like) to an anti-inflammatory (M2-like) phenotype (53, 54). Another research shows that membrane cholesterol efflux drives TAM reprogramming and tumor progression. Ovarian cancer cells promote membrane cholesterol efflux, and increased cholesterol efflux promotes IL-4 mediated signaling in TAMs, which will promote tumor invasion and metastasis (55). In addition, glutamate-ammonia ligase (GLUL) favors M2-like TAMs polarization by catalyzing the conversion of glutamate into glutamine, and GLUL inhibition can transfer M2-like TAMs into M1-like phenotype by increasing glycolytic flux and succinate availability (51).

Regulated by Immune Cells

TAMs can be regulated by other immune cells, such as Treg cells, MDSCs and B cells. IFN-γ is the main cytokines responsible for inhibiting M2-like TAM. Treg cells can inhibit IFN-γ secreted by $CD8^+$ T cells, which will prevent the activation of fatty acid synthesis that mediated by sterol regulatory element binding protein 1 (SREBP1) in immunosuppressive M2-like TAM. Therefore, Treg cells indirectly but selectively maintain M2-like TAM metabolic adaptability, mitochondrial integrity and survival rate (56). In addition, MDSCs also regulate TAM differentiation and promote tumor proliferation by downregulation of STAT3 (57). Besides, B cells are the key factors determining the tumor promoting function of TAMs. B cells can induce M2b macrophage polarization in human HCC (58), as well as suppress other immune cells, such as CD8+ T cells and M1 macrophages in the tumor microenvironment and promote the proliferation of cancer cell (59). Depletion of B cells prevented generation of M2b, increased the activity of anti-tumor T cell response, and reduced tumor growth.

Regulation by Other Factors

There are also some other factors of tumor microenvironment that can regulate TAMs function. Autophagy in the tumor microenvironment can provide essential nutrients, nucleotides, and amino acids to the tumor cells, facilitating tumor growth (60). Autophagy proteins in myeloid cells in the tumor microenvironment help to activate TAM by influencing LAP and mediate immunosuppression of T lymphocytes (61). In non-alcoholic fatty liver disease (NAFLD), NLRC4 contributes to the polarization of TAM to M2 type and the production of IL-1β and VEGF, thereby promoting the growth of tumor (62). Moreover, C-Maf transcription factor is the main regulator of cancer-promoting TAM polarization. C-Maf can promote the immunosuppressive activity of TAMs and control its metabolic process (63).

TARGETING TAMs FOR CANCER TREATMENT

TAMs are one of the most important components of the tumor immunosuppression microenvironment with high degree of plasticity. TAMs have both M1 and M2 type and have the potential ability of repolarization to M1 type macrophages. Therefore, targeting TAMs is a new cancer treatment strategy, including limiting monocytes recruitment, targeting TAMs activation, reprogramming TAMs into anti-tumor activity, and targeting TAMs specific markers (**Figure 2**).

Limiting Monocyte Recruitment

One of the strategies for targeting TAMs is to block monocyte recruit to tumor tissue. Tumor cells recruit CCR2-expressing monocytes from the peripheral blood to the tumor site by releasing CCL2 and these recruit CCR2-expressing monocytes will finally mature into TAMs, which accelerate the tumor progress. Thus, targeting CCL2-CCR2 axis is a very effective method of cancer therapy. Blocking the CCL2-CCR2 axis could greatly reduce the incidence of tumors by preventing TAMs recruitment and enhance the anti-tumor efficacy of CD8+ T cells in the tumor microenvironment (64).

CSF1 signaling pathway plays a key role in the production of bone marrow monocytes and the polarization of TAMs in tumor tissues. CSF1 produced by tumor cells caused down-regulation of granulocyte-specific chemokine expression in HDAC2-mediated cancer-associated fibroblasts (CAF), thereby limiting the migration of monocytes to tumors. The combination of CSF1R inhibitor and CXCR2 antagonist can prevent granulocytes from infiltrating the tumor, showing a strong anti-tumor effect (65). Also, combination of anti-PD-1 and anti-CSF1R antibodies induces melanoma regression in mice (66).

Targeting TAM Activation

Targeted activation of TAMs is an effective tumor treatment method. One of them is inhibiting TAMs from promoting tumor cell activation. Epidermal cell growth factor (EGF) secreted by TAM activates EGFR on tumor cells, which in turn upregulates VEGF (vascular endothelial growth factor)/VEGFR signaling in surrounding tumor cells, thereby promoting the proliferation and migration of tumor cells. EGFR blockade or ICAM-1 (intercellular adhesion molecule) antibody neutralization in TAM reduced the occurrence of ovarian cancer in mice (25).

Another effective tumor treatment method is blocking inhibitory receptor signals on TAMs that promote phagocytosis and antigen presentation function. Tumor cells highly express CD47, which restricts the ability of macrophages to engulf tumor cells through the signal regulatory protein alpha (SIRPα) -CD47 signal. The destruction of the SIRPα-CD47 signal axis is effective against various brain tumors including glioblastoma multiforme (GBM) by inducing tumor phagocytosis (67). Leukocyte immunoglobulin-like receptor subfamily B (LILRB) family is a class of inhibitory receptors expressed by myeloid cells, and its ligands are MHCI-like molecules (68). LILRB1 is up-regulated on the surface of TAM, and the MHCI-like component β2-microglobulin expressed by cancer cells can directly protect it from being engulfed. Therefore, blocking MHC I molecules or LILRB1 can enhance TAM phagocytosis (69).

Targeting pre-tumor myeloid cells at the metabolic level is another therapeutic strategy. Immunosuppressive phenotype of TAMs is controlled by long-chain fatty acid metabolism (especially unsaturated fatty acids), which makes BMDMs polarized into M2 phenotypes with strong inhibitory ability. Therefore, chemical inhibitors can effectively block TAM polarization *in vitro* and tumor growth *in vivo* (70).

Reprogramming TAMs Into Anti-Tumor Activity

One of the key characteristics of macrophages is their plasticity, which allows them to change the phenotype according to the tumor microenvironment. Therefore, reprogramming TAMs into an anti-tumor phenotype is a very promising tumor treatment strategy. Anti-tumor macrophages (M1 type) have abilities to clear and destroy tumor cells. RP-182 can selectively induce conformational switching of the mannose receptor CD206 expressed on TAM expressing the M2 phenotype, reprogramming M2-like TAM into anti-tumor M1-like TAM phenotype (71). Another finding shows that serine/threonine protein kinase 1 (RIP1) interacting with receptors in TAMs in pancreatic ductal adenocarcinoma (PDA) is up-regulated. Targeting RIP1, which act as a checkpoint kinase, reprogram TAM toward $MHCII^{hi}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$ phenotype (72).

Targeting Immune Inhibitory Molecules on TAMs

Targeting immune inhibitory molecules on TAMs is also an effective method. Blocking of MerTK leads to the accumulation of apoptotic cells in tumor cells and triggers a type I interferon response. MerTK blockade increases tumor immunogenicity and enhances anti-tumor immunity. Treatment of tumor-bearing mice with anti-MerTK antibodies can stimulate T cell activation and synergize with anti-PD-1 or anti-PD-L1 therapy (73). PD-1-PD-L1 therapy can also work by direct action on macrophages. Both mouse and human TAM express PD-1. The

expression of TAM PD-1 is negatively correlated with the phagocytic ability against tumor cells, and blocking PD-1-PD-L1 *in vivo* will increase the phagocytosis of macrophages, reduce tumor growth, and rely on macrophage-dependent ways to prolong the survival of mice in cancer models (74).

CONCLUDING REMARKS

Under the effect of the tumor microenvironment, TAMs are tamed by tumor cells and has become a promoter of tumor growth. Studies have shown that TAMs have a significant role in promoting the development and progress of tumors. Therefore, how to inhibit the tumor-promoting roles of TAMs will provide new clues for future tumor therapy. However, a number of key questions remain to be answered, including mechanisms of TAM development, key factors that drive phenotypic changes of TAMs in the tumor microenvironment. Recent pre-clinical and clinical studies aiming at targeting TAMs for cancer treatment have shown inspiring results. TAM-targeting therapy represents a promising treatment of cancer patients in the future.

AUTHOR CONTRIBUTIONS

YP and YY analyzed the data and wrote the paper. XW and TZ edited the manuscripts. 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

MINI REVIEW
published: 14 January 2021
doi: 10.3389/fimmu.2020.619253

Myeloid-Derived Suppressor Cell Differentiation in Cancer: Transcriptional Regulators and Enhanceosome-Mediated Mechanisms

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OPEN ACCESS

Edited by:
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Reviewed by:
Katherine Chiappinelli, George Washington University, United States
Justin Lathia, Case Western Reserve 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: *19 October 2020*
Accepted: *30 November 2020*
Published: *14 January 2021*

Citation:
Fultang N, Li X, Li T and Chen YH (2021) Myeloid-Derived Suppressor Cell Differentiation in Cancer: Transcriptional Regulators and Enhanceosome-Mediated Mechanisms. Front. Immunol. 11:619253. doi: 10.3389/fimmu.2020.619253

Myeloid-derived Suppressor Cells (MDSCs) are a sub-population of leukocytes that are important for carcinogenesis and cancer immunotherapy. During carcinogenesis or severe infections, inflammatory mediators induce MDSCs *via* aberrant differentiation of myeloid precursors. Although several transcription factors, including C/EBPβ, STAT3, c-Rel, STAT5, and IRF8, have been reported to regulate MDSC differentiation, none of them are specifically expressed in MDSCs. How these lineage-non-specific transcription factors specify MDSC differentiation in a lineage-specific manner is unclear. The recent discovery of the c-Rel–C/EBPβ enhanceosome in MDSCs may help explain these context-dependent roles. In this review, we examine several transcriptional regulators of MDSC differentiation, and discuss the concept of non-modular regulation of MDSC signature gene expression by transcription factors such as c-Rel and C/EBPß.

Keywords: myeloid-derived suppressor cell, immunosuppression, enhanceosome, aberrant myelopoiesis, tumor immunobiology

Abbreviations: AKT, Protein kinase B; ATF, Activating transcription factor; C/EBPβ, CCAAT-enhancer-binding protein ß; COX-2, Cyclooxygenase 2; CREB, cAMP response element-binding protein; ERK, Extracellular-signal-regulated kinase; G-CSF, Granulocyte colony-stimulating factor; GCN2, General control nonderepressible 2; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HDAC2, Histone Deacetylase 2; HMG I/Y, High mobility group protein; IL-1ß/6/10/23, Interleukin -1ß/6/10/23; IRF8, Interferon Regulatory Factor 8; LAP/LAP*, Liver-enriched activating protein – C/EBPß isoforms; LIF, Leukemia inhibitory factor; LIP, Liver-enriched inhibitory protein; MAPK, Mitogen-activated protein kinase; MDSC, Myeloid-derived suppressor cell; NFAT, Nuclear factor of activated T-cells; NFI-A, Nuclear factor 1 A-type; NOX2, NADPH oxidase 2; PGE2, Prostaglandin E2; PI3K, Phosphoinositide 3-kinase; RAGE, Receptor for advanced glycation endproducts; RB, Retinoblastoma protein; ReCHIP, Re-Chromatin immunoprecipitation; S100A8/9, S100 Calcium Binding Protein A8; STAT3/5, Signal transducer and activator of transcription 3/5; TIPE2, TNF alpha induced protein 8 like 2; TNF, Tumor necrosis factor; VEGF, Vascular endothelial growth factor; c-Rel, Cellular Avian Reticuloendotheliosis Viral Oncogene Homolog; iNOS/NOS2, inducible NO synthase/Nitric Oxide Synthase 2.

INTRODUCTION

Tumor immune evasion is an essential feature of tumorigenesis (1, 2). To successfully establish themselves within a host, tumor cells leverage biochemical signals and rogue immune cells to hide from and repress host immune responses (1–3). Immunotherapy, which can restore immune response and anti-cancer immunity, has revolutionized cancer therapy. However, rogue immunosuppressive cells, including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), regulatory T-cells (Tregs), regulatory dendritic cells (RegDCs), cancer-associated fibroblasts, and myeloid-derived suppressor cells (MDSCs), still represent significant impediments to immunotherapy, contributing to therapy failure and poor clinical outcomes (4–8). Of these pro-tumoral cell types, MDSCs are perhaps the least well characterized.

MDSCs are a heterogenous population of immunosuppressive pro-tumoral leukocytes which arise as a result of defects in myelopoiesis (9). Under physiological conditions, progenitor myeloid cells differentiate into macrophages, dendritic cells or granulocytes. Under pathological conditions like cancer or chronic infections, aberrant myelopoiesis allows the accumulation and expansion of immature myeloid cells with strong immunosuppressive capabilities (10–16). While these cells possess many phenotypic and morphological hallmarks of anti-tumor myeloid-lineage cells like monocytes and neutrophils, they differ significantly in their activation programs and function to inhibit anti-tumor immunity by producing immunosuppressive factors like arginase, nitrogen species and reactive oxygen species, among others (10, 17–19). MDSCs are a significant obstacle to immunotherapies including checkpoint inhibitors; accumulation of MDSCs populations within circulating and tumor-infiltrating leukocytes have been observed in patients who fail to respond to checkpoint inhibitor therapy (18, 19).

There are two major subsets of MDSCs– granulocytic or polymorphonuclear MDSCs (G-MDSCs or PMN-MDSCs), which are phenotypically similar to granulocytes, and monocytic or mononuclear MDSCs (M-MDSCs), which are phenotypically similar to monocytes. PMN-MDSCs have a $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ phenotype in mice and a $CD11b^{+}CD14^{-}CD15^{+}/CD66b^{+}$ phenotype in humans while M-MDSCs are identified as $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ in mice, and $CD11b^{+}CD14^{+}HLA^{-}DR^{-/lo}CD15^{-}$ in humans (20, 21). MDSC markers were recently reviewed here (21). A third mixed population of MDSCs, early-stage MDSC (e-MDSC), with phenotype Lin^{-} (including CD3, CD14, CD15, CD19, and CD56) $HLA^{-}DR^{-}CD33^{+}$ was recently proposed in humans (22). e-MDSCs also contain immature progenitor myeloid cells and their equivalent in mice is yet to be identified (22).

While a lot is known about the phenotypic and morphological delineations of MDSCs, the biochemical markers and effectors underlying their development and function are still poorly understood. As such, the identification of these drivers of pathological MDSC expansion and immunosuppressive activity has been the subject of intensive research in recent years. Recently identified MDSC effectors, mostly transcription factors (TFs) and apoptotic regulators, include IRF8 (23), STAT3 (23–26), C/EBPß (27, 28), S100A8/9 (29), TIPE2 (30, 31), GCN2 (32), among others (**Table 1**). Of all these regulators, C/EBPß has emerged as an essential "master" regulator of MDSC expansion and immunosuppressive activity. Most of the known MDSC regulators drive expansion and immunosuppressive activity in C/EBPß-dependent mechanisms. Additionally, C/EBPß deletion alone in myeloid cells was sufficient to halt MDSC generation and immunosuppressive activity (27). Recent evidence, however, suggests that c-Rel, a member of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) family of transcription factors, regulates C/EBPß activity and expression in MDSCs (33). In this review we describe c-Rel and C/EBPß as master effectors of MDSC biology and highlight how a non-modular c-Rel-C/EBPß "enhanceosome" drives MDSC development and function in cancer.

KNOWN MDSC EFFECTORS

MDSCs arise when sustained pathologic inflammation induces an aberrant differentiation program in myeloid precursors giving rise to immunosuppressive cells (10–16). This is mediated by activation of complex transcriptional machinery within these cells by inflammatory cytokines including GM-CSF, IL-6, G-CSF, IL-1ß, PGE2, TNFα, and VEGF (10–16). Currently known transcriptional regulators of MDSC biology include STAT3, CEBP/β, STAT5, IRF8, S100A8/9, RB, TIPE2 and GCN2 (**Table 1**).

STAT3 is a key repressor of antitumor immunity (39, 40). It impairs antigen presentation and inhibits the production of immunostimulatory cytokines while promoting the expression of immunosuppressive molecules. It is highly active in most cancers where it promotes the production of inflammatory cytokines and growth factors like IL-6, IL-10, IL-23, LIF, VEGF, and HGF (39, 41). These molecules induce STAT3 activation in myeloid precursors which drives cell survival, transcription of immunosuppressive enzymes (ARG1 and iNOS), and aberrant differentiation into MDSCs. It also interacts with C/EBPß at promoter sites to regulate transcription (33, 34). Intriguingly, a decrease in MDSC STAT3 activity in the tumor environment is associated with differentiation into TAMs (42). Within myeloid precursors, STAT3 and STAT5 also inhibit IRF8, a crucial transcription factor for normal myeloid differentiation into monocytes and dendritic cells (23). IRF8 functions as a negative regulator of MDSCs and its downregulation is necessary for pathologic MDSC expansion (23).

S100A8/9 produced by tumors binds to RAGE receptors on myeloid precursors inducing activation of an NF-κB-C/EBPß-STAT3 axis (29). This promotes production of S100A8/9 in MDSCs and drives both expansion and chemotactic migration to tumor sites for immunosuppression. The MDSC-secreted S100A8/9 creates an autocrine feedback loop that exacerbates MDSC accumulation.

High reactive oxygen species (ROS) associated within tumor microenvironments and IL-6 induce TIPE2 in myeloid precursors (30, 31). Active TIPE2 promotes the expression of C/EBPß and STAT3 *via* the PI3K/AKT and MAPK/ERK pathways. This leads to MDSC accumulation and polarization into an immunosuppressive phenotype. In the absence of TIPE2 MDSCs became anti-tumoral indicating TIPE2 functions as a molecular polarity switch in MDSCs (30). GCN2 similarly functions as a polarity switch in MDSCs. It alters myeloid

TABLE 1 | Known effectors or regulators of MDSC biology.

Effectors	Mechanisms	References
STAT3	Stimulates inflammatory cytokines, activates transcription of immunosuppressive enzymes with C/EBPß. Downregulates IRF8	(23, 33, 34)
STAT5	Downregulates IRF8, promoting aberrant myeloid differentiation	(23)
C/EBPß	Master regulator. Promotes transcription of immunosuppressive enzymes and inflammatory cytokines in tumor microenvironment	(27, 35–37)
IRF8	Crucial for normal myeloid differentiation. Negative regulator of MDSCs. Downregulated by STAT3/5	(23)
S100A8/9	Produced by tumors. Binds to RAGE receptors in myeloid precursors and activates immunosuppressive NF-κB-C/EBPß-STAT3 signaling axis.	(29)
RB	Epigenetically silenced by HDAC6 in MDSCs. Negatively regulates myeloid differentiation into PMN-MDSCs.	(38)
TIPE2	Induced by IL-6 and high ROS in tumor microenvironment. Activates C/EBPß and STAT3 which promote immunosuppressive activity.	(30, 31)
GCN2	Polarity switch. Expression correlates with immunosuppressive activity. Induces C/EBPß and CREB2/ATF4 promoting immunosuppression.	(31)

function by inducing C/EBPß and CREB-2/ATF4 which promote MDSC expansion and immunosuppressive activity (32). Epigenetic silencing of Rb by HDAC-2 in myeloid precursors also promotes accumulation of PMN-MDSCs (38).

C/EBPß appears to be an essential player among these effectors in MDSCs.

C/EBP PROTEIN FAMILY

C/EBPß is the second member of the CCAAT/Enhancer Binding Protein (C/EBP) family of transcription factors (28). C/EBP proteins are basic-region-leucine zipper transcription factors which regulate both emergency and steady state myelopoiesis (35, 43–45). C/EBPα, the first member of the family, regulates steady state myelopoiesis. C/EBPα is highly expressed early identified n the myeloid differentiation process and is an essential molecular switch for the transition from common myeloid precursors to granulocyte macrophage progenitors (46). The role of other C/EBP family proteins, including C/EBPδ and CHOP, are less clear but they are all thought to similarly regulate myelopoiesis as well as modulate the activity of other C/EBP proteins (28). C/EBPδ regulates the expression of inflammatory cytokines including COX-2, iNOS, G-CSF, IL-1β, IL-6, and TNF-α, and has been implicated in MDSC expansion (47, 48). CHOP on the other hand, lacks DNA-binding activity but can form heterodimers with C/EBPß isoforms and other family members, regulating their activity (49). It has similarly been implicated in MDSC expansion *via* these regulatory events (50).

Within the context of MDSC development and function, C/EBPß (also known as IL6-DBP, CRP2, NF-IL6, NF-M or TCF5) is the most important C/EBP (**Figure 1**). It has three isoforms with diverse, context-dependent roles (28, 51). The first two, LAP and LAP*, contain both a DNA-binding domain and an activation domain. The third isoform, LIP, lacks an activation domain and attenuates transcriptional activity *via* heterodimerization with LAP/LAP* (35, 45, 52). C/EBPß controls emergency myelopoiesis, which is a characteristic feature of many solid tumors due to chronic tumor-induced inflammation (53–55). Deregulations of C/EBPß activity are thus a significant contributing factor to aberrant myelopoiesis and MDSC expansion under pathological conditions (27, 28).

Stimulation with inflammatory cytokines like G-CSF, GM-CSF and IL-6 drives an increase in C/EBPß expression and DNA-binding activity (27, 35, 56). Upregulated LAP and LAP* isoforms of C/EBPß function as mediators of cytokine-induced inflammatory response *via* transcriptional activation of inflammatory genes IL-6, TNF and G-CSF, exacerbating the response (45). Under pathological conditions, this sustained inflammatory activation promotes aberrant myeloid development and differentiation into immunosuppressive phenotypes (27, 35, 36). Following IL-6 stimulation, C/EBPß, in concert with STAT3, also promotes miR-21 and miR-181b, which induce NFI-A to promote MDSC accumulation in the bone marrow and spleen (34).

Within tumors, aerobic glycolysis, a hallmark of cancer, leads to an increase in LAP which promotes G-CSF+GM-CSF expression and secretion (37). Li et al. showed that in breast cancer cells, preferential activation of aerobic glycolysis over oxidative phosphorylation, inhibits AMPK-ULK1 and autophagy signaling, allowing stabilization and activity of LAP (37). Cytokines, induced by LAP, travel to the myeloid compartment where they promote expansion of MDSC precursors and direct their differentiation into suppressor cells. Within MDSCs, activated C/EBPß directly binds to and promotes the transcription of immunosuppressive enzymes including Arg1, Nos2, Nox2, and Cox2 (27, 36, 57). These enzymes are crucial members of the MDSC immunosuppressive machinery. Arg1 and Nos2 deplete environmental L-arginine, a crucial amino acid for T-cell survival and anti-tumor activity (58–61). Nox2 increases ROS which block T-cell activation and activity (62, 63). The COX-2-PGE2 cascade suppresses both dendritic and natural killer cell activity, while promoting the expression of immunity repressor PD-L1 (64, 65). It is also plausible that activated C/EBPß in myeloid precursors similarly induces the production of GM-CSF and IL-6 which drive MDSC accumulation and function in autocrine signaling mechanisms.

In macrophages, PI3Kγ activates C/EBPß, which serves as a critical polarization switch from an immunostimulatory to an immunosuppressive phenotype during tumor progression (66). This suggests C/EBPß could also regulate MDSC differentiation into TAMs in the tumor microenvironment.

Seminal work by Marigo et al. showed that C/EBPß deletion in all hematopoietic lineage cells was enough to halt MDSC genesis and completely abrogate their immunosuppressive activity on antigen activated T-cells (27). They also observed significant reduction in both Arg1 and Nos2 expression and activity. C/EBPß deletion potentiated adoptive T-cell therapy resulting in a complete cure for 60% of mice bearing subcutaneous fibrosarcoma. Their work and subsequent studies suggest C/EBPß is an essential mediator of MDSC development and activity (36, 67, 68).

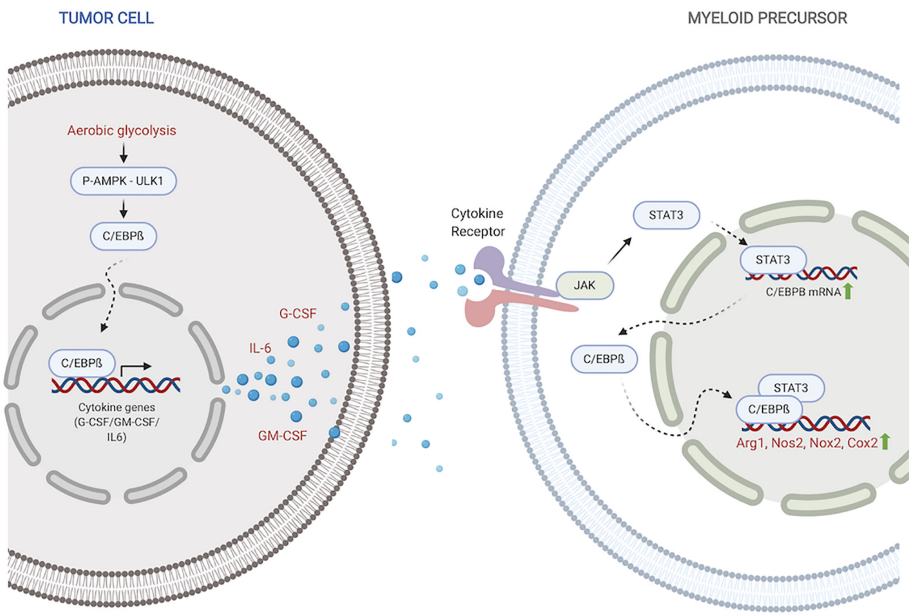

FIGURE 1 | C/EBPß regulates MDSC expansion and function. Within the tumor, C/EBPß promotes transcription of inflammatory cytokines. Inflammatory cytokines then reciprocally induce C/EBPß in myeloid compartment which promotes transcription of immunosuppressive molecules. Created with BioRender.com.

Perhaps unsurprisingly, many studies into molecular effectors of MDSCs have focused on upstream regulators of C/EBPß. Of these recently found effectors, c-Rel, appears to be an essential regulatory partner for C/EBPß in MDSC.

C-REL, A NEW REGULATOR OF MDSC DIFFERENTIATION AND FUNCTION

c-Rel, is a member of the NF-κB family of TFs which regulate a variety of molecular processes from embryogenesis to hematopoiesis and inflammation (69, 70). Being a class 2 member of the family, it contains both an N-terminal Rel-homology domain (RHD) and a transactivation domain (TAD) (70, 71). c-Rel's RHD mediates interactions with other proteins and transcriptional regulators at promoter sites where its TAD recognizes and binds to consensus GGGCTTTCC sequences (69, 72). These interactions, especially with other NF-κB members to form heterodimers, are essential for c-Rel transcriptional activity. c-Rel's TAD also contains several serine residues which are readily phosphorylated, regulating c-Rel nuclear localization, transactivation and DNA binding activity (73–76).

c-Rel is an important regulator of immune cell function. It is crucial for normal B- and T- cell activation and proliferation (77–81). Upon lymphocyte activation, c-Rel induces IRF-4 in B-cells which promotes cell cycle progression and proliferation. IRF-4 has κB elements in its promoter region to which a c-Rel:p50 heterodimer binds. B-cell proliferation defects have been observed in c-Rel deficient mice (82). Similar defects in T-cell activation and proliferation following stimulation have been observed in c-Rel knockout mice (77).

c-Rel is a key regulator of autoimmunity *via* its role in promoting the generation of Th1, Th17 and Foxp3$^+$ regulatory T cells (T_{regs}) (83–87). c-Rel is responsible for assembling a transcriptional enhanceosome including RelA, NFAT, SMAD and CREB that binds and transcribes *Foxp3*, a master regulator of T_{reg} immunosuppression (84). c-Rel also directly regulates the expression of many proinflammatory cytokines *via* its context-dependent binding events at promoter sequences (79, 80, 88). Intriguingly, despite its significant roles in both inflammation and autoimmunity, the effects of c-Rel deficiency on immune homeostasis appear to be mostly minor (77).

Although previously thought to primarily function in the lymphoid compartment, mounting evidence suggests a significant role for c-Rel in myeloid cells. We recently showed that c-Rel regulates MDSC expansion and function in cancer (57). Both global and myeloid-specific c-Rel deletion blocked tumor growth and markedly decreased MDSC accumulation in melanoma and lymphoma mice models. The few MDSCs that were generated in the c-Rel knockout mice were defective in suppression when compared to MDSCs from Wild-type mice. c-Rel deletion also altered MDSC metabolism, reducing mitochondrial respiration and glycolysis, inducing a Warburg-like metabolic state. We also observed downregulation of signature MDSC genes in c-Rel knockout mice including Arg1, Nos2, and C/EBPß, key members of the MDSC immunosuppression machinery. There was also heightened inflammatory gene expression in c-Rel deficient MDSCs compared to wild type, a phenotype that was rescued by C/EBPß overexpression. This suggests that c-Rel's effect in MDSCs is C/EBPß dependent.

Mechanistically, c-Rel directly regulates the transcription of these MDSC signature genes (57). Upon stimulation with

GM-CSF and IL-6, c-Rel binds to the promoters of *Arg1* and *Cebpb* where it forms a transcriptional complex with pSTAT3, C/EBPß and p65. ReChIP analyses showed that these factors all bind to the same promoter element, suggesting the formation of a single enhanceosome complex which drives MDSC biology. c-Rel-C/EBPß enhanceosomes have previously been identified as transcriptional regulators in hepatocytes (89, 90).

ENHANCEOSOMES

Enhanceosomes are high-order protein complexes, usually transcription factors, that bind cooperatively at a gene's promoter or enhancer regions to activate transcription (91, 92). Many cis-regulatory elements, including promoters and enhancers, contain overlapping DNA binding sites for various transcription factors. This allows the formation of elaborate protein complexes which alter chromatin architecture and recruit the RNA polymerase transcription machinery, regulating gene expression as a functional, nucleoprotein unit (91, 92). These enhanceosome complexes effectively function as "on" and "off" transcriptional switches, specifying key developmental and cell lineage-determining gene regulation events (91, 92). Enhanceosomes could comprise any number of multifunctional transcriptional regulators in an almost limitless number of combinations, specifying the varied cell differentiation programs found in multicellular organisms. An increasing number of enhanceosomes are being described, shifting previously established transcription paradigms.

Fiedler et al. recently described a "Wnt enhanceosome" consisting of ChiLS, Runt/RUNX2, ARID1 and Groucho/TLE which is integrated by Pygo at TCF enhancers to drive Wnt signaling in *Drosophila* (93). Additionally, the Wnt enhanceosome could incorporate a number of factors in a lineage-dependent manner and be switched "off" by Notch. This allows context-dependent regulation of TCF/LEF target genes to simultaneously promote embryogenesis and development while preventing hyperproliferation and cancer. Pawlus et al. similarly described a multifactorial HIF enhanceosome comprising of HIF1, HIF2, RNA poll II and varied transcription factors at enhancer sites for HIF target genes (94). These context-dependent enhanceosomes help explain the dual oncogenic and tumor-suppressive role of HIF-mediate hypoxia. Scotto et al. also showed that multidrug resistance in cancer is governed by an MDR1 enhanceosome at the *MDR1* promoter which can be activated by a variety of stimuli including differentiation agents like retinoic acid, UV radiation and chemotherapy (95). The MDR1 enhanceosome included NF-Y, Sp family transcription factors and histone acetyltransferase PCAF and could be targeted to reverse multidrug resistance.

The assembly and disassembly of enhanceosomes is essential for tight gene regulation in a cell. Because the assembly of a functional enhanceosome complex depends on several factors including local DNA conformation, protein availability and modifications, gene regulation *via* enhanceosomes can be very cell-specific. The absence of any one factor disrupts enhanceosome activity, preventing transactivation. In the case of MDSCs, enhanceosomes at regulatory sites for MDSC signature genes are compelling as key effectors of aberrant MDSC development under pathological conditions.

THE C-REL-C/EBPß ENHANCEOSOME

It is plausible that higher levels of active c-Rel and C/EBPß within the nucleus of pathologically activated myeloid cells drive the formation of altered enhanceosomes at regulatory regions for *Arg1, Nos2, Nox2, Cebpb*, and other MDSC genes. Previous work has identified enhanceosomes for several immunosuppressive mediators including Nos2, Arg1, and Nox2 that do not contain either C/EBPß or c-Rel (96–98). We recently showed abundant c-Rel and C/EBPß accumulation at the gene promoters of both *Arg1* and *C/EBPß* following stimulation with GM-CSF and IL-6 (57). In this c-Rel-C/EBPß MDSC enhanceosome model, c-Rel is recruited first to the promoter site and in its absence, the enhanceosome fails to assemble. Following c-Rel binding, pSTAT3, p65 and C/EBPß are recruited to the promoter site to drive transcription and differentiation into immunosuppressive MDSCs (**Figure 2**).

A similar c-Rel-C/EBPß enhanceosome was previously described (89, 90). Cha-Molstad et al. showed that in hepatocytes, cytokine stimulation promotes c-Rel-mediated recruitment of C/EBPß and STAT3 to the *CRP* gene promoter to activate transcription (89). Intriguingly, c-Rel itself was not directly bound to the DNA sequence. c-Rel DNA binding activity is regulated by phosphorylation of the many serine residues within its TAD (73–76). Because we found c-Rel binding to DNA forming the MDSC enhanceosome, it is plausible highly active kinases within pathologically activated myeloid cells contribute to the formation of the MDSC-specific c-Rel enhanceosome. Other post-translational modifications, specific to myeloid cells under pathological activation, that modulate protein-protein interactions and protein-DNA interactions, might drive the formation of MDSC enhanceosomes. Other NF-κB proteins, including p50, have been reported to be involved in MDSC expansion following stimulation by tumor-derived PGE2 (99). We previously showed that c-Rel could bind p50 in MDSCs (57). P50 could similarly be incorporated into the MDSC enhanceosome during tumorigenesis to drive MDSC expansion and activity. The c-Rel-C/EBPß enhanceosome might also contain other nuclear proteins including co-regulators, deacetylases, architectural proteins like HMG I/Y and nucleosome remodeling proteins.

The c-Rel-C/EBPß enhanceosome is also a promising candidate as a biochemical marker for MDSCs. A significant constraint in MDSC research is the lack of reliable markers to characterize this highly heterogeneous cell population (22). Because yields are often low when isolating MDSCs, especially from *in vivo* systems, most studies lack functional validation of immunoregulatory activity. Improved biochemical markers, specific to MDSCs, would provide a simple validatable phenotype for MDSCs. The individual factors within the enhanceosome are not specific to MDSCs: C/EBPß is enriched in monocytes/macrophages (100, 101). c-Rel and p65 are pervasive regulators of B- and T- cell proliferation (77–81). pSTAT3 is a ubiquitous transcription factor within eukaryotic cells (25, 41). However, concurrent activation of all four, as well

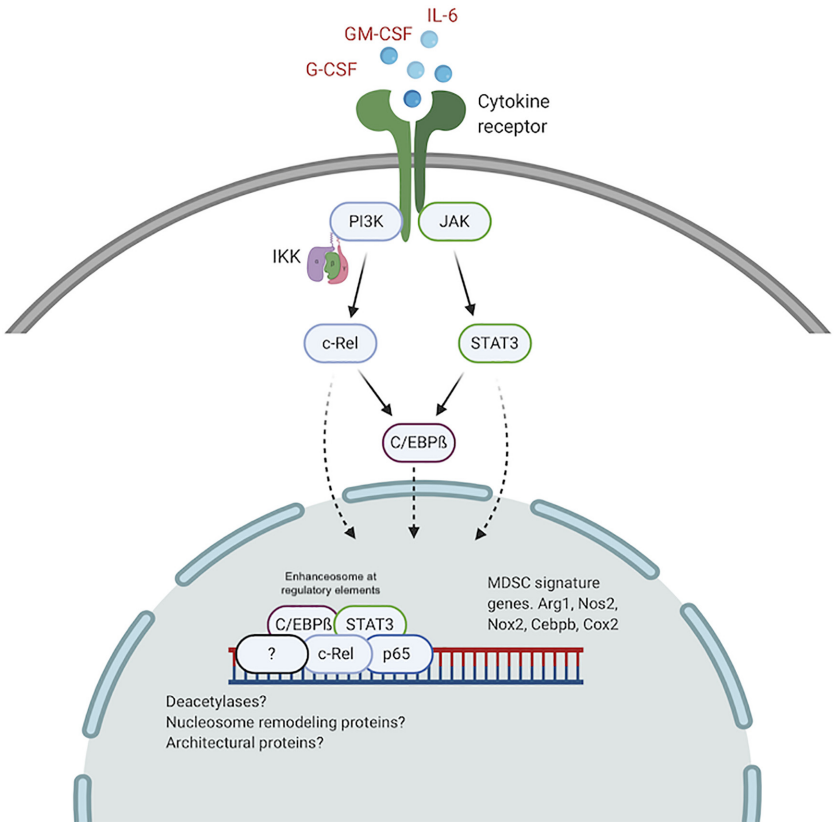

FIGURE 2 | The c-Rel/C/EBPß enhanceosome in MDSCs. c-Rel and C/EBPß induced by tumor secreted cytokines, translocate to the nucleus and assemble an enhanceosome containing STAT3, p65 and other regulators at enhancer sites for immunosuppressive molecules. Created with BioRender.com.

as other putative members of the enhanceosome, could be indicative of an MDSC phenotype. Monitoring assembly and activation of the c-Rel-C/EBPß enhanceosome could thus be a testable marker for MDSC activation and expansion.

This also provides an exciting therapeutic avenue. We showed that a small molecule inhibitor of c-Rel abrogated MDSC development and immunosuppression *via* disruption of the c-Rel complex (57). Similar approaches targeting individual members, aiming to disrupt their interactions in the MDSC enhanceosome, could have thrilling outcomes. Lee et al. showed that cerulenin, a small molecule inhibitor of the NF-κB enhanceosome in macrophages, might disrupt the assembly of the enhanceosome, suppressing pro-inflammatory activation and sepsis (102). Cerulenin specifically disrupted the p65-TonEBP-p300 complex without affecting their expression or DNA-binding. It had no detectable toxicity and animals could tolerate high doses for several weeks (103). Additionally, our c-Rel inhibitor enhanced the anti-tumor effect of anti-PD-1 antibodies suggesting combinatorial restoration of T cell function (via MDSC inhibition) and activation (via PD-1 inhibition) as a viable clinical strategy (57). The development of a novel class of enhanceosome inhibitors targeting MDSCs could represent an exciting approach to potentiate immunotherapy.

CONCLUSION

MDSCs are a product of sustained pathologic inflammation, which develop as a result of aberrant cytokine-mediated activation of complex transcriptional machinery in myeloid precursors (9, 10). They are involved in the pathogenesis of a host of human diseases from cancers to acute infections. In cancer, tumor-produced cytokines mediated by C/EBPß induce c-Rel and C/EBPß in the myeloid compartment, which drives the formation of a c-Rel-C/EBPß-pSTAT3-p65 MDSC enhanceosome. This enhanceosome promotes the transcription of immunosuppressive enzymes and other MDSC signature genes, guiding their differentiation into immunosuppressive cell populations. Because this putative enhanceosome is MDSC-specific, it can be targeted to repress MDSC expansion and immunosuppression. It is thus imperative to further characterize this enhanceosome and develop modalities to inhibit it. Additionally, further studies into other complex transcription programs underlying spatiotemporal gene regulation during aberrant myeloid cell differentiation are warranted. These would identify novel mechanisms and therapeutic targets, which could be blocked clinically to enhance the efficacy of immunotherapies like checkpoint blockade.

AUTHOR CONTRIBUTIONS

NF drafted the manuscript and designed the figures. XL and TI reviewed the manuscript structure and science. YC reviewed the manuscript structure, ideas and science. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by grants from the National Institutes of Health (nos. R01-AI152195, R01-AI099216, R01-AI121166, R01-AI143676, and R01-AI136945 to YC); XL was partially supported by grant no. NIH-T32-DK007780.

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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: 21 January 2021
doi: 10.3389/fimmu.2020.590494

Distinct Immunophenotypes of T Cells in Bronchoalveolar Lavage Fluid From Leukemia Patients With Immune Checkpoint Inhibitors-Related Pulmonary Complications

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Patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) treated with immune checkpoint inhibitors (ICIs) are at risk of pneumonitis as well as pneumonia (combined henceforth as ICI-related pulmonary complications). Little is known about the cellular and molecular mechanisms underlying ICI-related pulmonary complications. We characterized lymphocytes from bronchoalveolar lavage (BAL) fluid and peripheral blood from seven AML/MDS patients with pulmonary symptoms after ICI-based therapy (ICI group) and four ICI-naïve AML/MDS patients with extracellular bacterial or fungal pneumonias (controls). BAL T cells in the ICI group were clonally expanded, and BAL IFNγ$^+$ IL-17$^-$ CD8$^+$ T and CXCR3$^+$ CCR6$^+$ Th17/Th1 cells were enriched in the ICI group. Our data suggest that these cells may play a critical role in the pathophysiology of ICI-related pulmonary complications. Understanding of these cell populations may also provide predictive and diagnostic biomarkers of ICI-related pulmonary complications, eventually enabling differentiation of pneumonitis from pneumonia in AML/MDS patients receiving ICI-based therapies.

Keywords: pneumonitis, Th17/Th1 cells, checkpoint inhibitor, acute myeloid leukemia, immune-related adverse event

OPEN ACCESS

Edited by:
Xi Wang,
Capital Medical University, China

Reviewed by:
Penghui Zhou,
Dana–Farber Cancer Institute, United States
Thomas Cluzeau,
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *01 August 2020*
Accepted: *07 December 2020*
Published: *21 January 2021*

Citation:
Kim ST, Sheshadri A, Shannon V, Kontoyiannis DP, Kantarjian H, Garcia-Manero G, Ravandi F, Im JS, Boddu P, Bashoura L, Balachandran DD, Evans SE, Faiz S, Ruiz Vazquez W, Divenko M, Mathur R, Tippen SP, Gumbs C, Neelapu SS, Naing A, Wang L, Diab A, Futreal A, Nurieva R and Daver N (2021) Distinct Immunophenotypes of T Cells in Bronchoalveolar Lavage Fluid From Leukemia Patients With Immune Checkpoint Inhibitors-Related Pulmonary Complications. Front. Immunol. 11:590494. doi: 10.3389/fimmu.2020.590494

HIGHLIGHTS

- Th17/Th1 and IFNγ^+ IL-17$^-$ CD8$^+$ T cells were enriched in bronchoalveolar lavage fluid from leukemia patients with ICI-related pulmonary complications.
- Bronchoalveolar lavage T cells were clonally expanded in patients with ICI-related complications compared with controls in terms of T cell receptor repertoire.

INTRODUCTION

Patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) are susceptible to serious infections, including pneumonia. Although immune checkpoint inhibitor (ICI)-based therapies, specifically epigenetic agent azacytidine in combination with a PD-1 inhibitor, have demonstrated encouraging responses and improved overall survival in patients with frontline or relapsed MDS or relapsed AML, ICIs are associated with immune-related adverse events, including pneumonitis (1–5). Studies have demonstrated that 10–12% of patients with a hematologic malignancy treated with ICI(s) developed pneumonitis (1, 6). Thus, AML/MDS patients receiving ICI-based therapies are at risk to develop pneumonia (due to disease and treatment-related neutropenia and immunosuppression) as well as pneumonitis (combined henceforth as ICI-related pulmonary complications). Because ICI-related pulmonary complications are life-threatening (7), understanding the pathophysiology is critical for prompt diagnosis and early intervention. Detailed characterization of the immune cells in the inflamed lung and peripheral blood (PB) from patients with AML/MDS treated with ICI-based therapies, the first step in elucidating these pathophysiologic mechanisms, would be particularly valuable. In the current study, we characterized lymphoid immune cell populations in bronchoalveolar lavage (BAL) fluid and in PB from AML/MDS patients who received ICI(s), developed pulmonary symptoms, and underwent a diagnostic bronchoscopy. As a control, we analyzed BAL fluid and PB from ICI-naïve AML/MDS patients with pulmonary symptoms who had a confirmed extracellular bacterial or fungal pneumonia.

MATERIALS AND METHODS

Patient Selection

From March 2017 to January 2018, we reviewed for inclusion in our study 40 AML/MDS patients who underwent diagnostic bronchoscopy due to radiographic abnormalities and/or pulmonary symptoms, including fever, cough, and shortness of breath. We excluded six patients who had undergone stem cell transplantation and five patients who had received non-ICI immunotherapy. Another four patients declined to participate. Among the remaining 25 patients, 10 had received ICI therapy and 15 had not. Three of the 10 patients who had received ICI therapy were excluded; one had had pneumonia 6 weeks prior to the bronchoscopy, one had completed ICI therapy more than 12 weeks prior to the bronchoscopy, and one had lung lesions that turned out to be lymphoma. Thus, the ICI group comprised seven patients. An expert multidisciplinary committee consisting of two pulmonologists (AS and VS), one rheumatologist (SK), one infectious disease specialist (DK), and one hematologist (ND) adjudicated the presence of pneumonitis or pneumonia in these seven patients. Pneumonitis was considered the leading diagnosis if 1) radiologic patterns favored pneumonitis over pneumonia (e.g., diffuse ground-glass opacities), 2) the natural history and type of symptoms were more consistent with pneumonitis, 3) there was a clear response to corticosteroids but not antibiotics, or 4) there was histopathologic confirmation of pneumonitis or organizing pneumonia in the absence of microbiological cultures. Pneumonia was considered the leading diagnosis if 1) radiologic patterns favored pneumonia over pneumonitis (e.g., lobar consolidation), 2) the natural history and type of symptoms were more consistent with pneumonia, 3) there was a clear response to antibiotics but not corticosteroids, or 4) there was a positive microbiological culture from a lower respiratory specimen. Four patients in the ICI group met the criteria for pneumonia (hereafter, ICI-pneumonia) and three patients were determined to have pneumonitis (hereafter, ICI-pneumonitis). Two patients in the ICI-pneumonitis group had positive BAL culture results (one for *Stenotrophomonas* and one for *Enterococcus faecalis*), but the expert multidisciplinary committee determined that these were colonizations rather than active infections.

Of the 15 patients who had not received ICI therapy, eight patients were excluded because the BAL culture results were negative. Because the immune response in viral infections is distinct from that of extracellular bacterial/fungal infections, we excluded another two patients whose BAL culture results were positive for a virus. Another patient was excluded because the positive BAL culture result was clinically determined to be colonization by the expert multidisciplinary committee. The remaining four patients, whose extracellular bacterial/fungal infection was confirmed microbiologically and clinically, comprised the control group.

The patient selection process is summarized in **Figure 1**. Samples were collected and distributed under protocol PA15-0551 approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center and all patients provided written informed consent.

Sample Collection

Residual BAL fluid (10–35 ml) from all participants was obtained and transported on ice to the laboratory. PB samples (15–30 ml) were collected from available patients 3 ± 3 days (mean ± SD) after the bronchoscopy. One participant in the control group (Cont_2) declined to provide a PB sample.

Cell Isolation

After centrifugation at 1,600 rpm, the BAL fluid was stored at −80°C. BAL cells were washed with 1× phosphate buffered saline

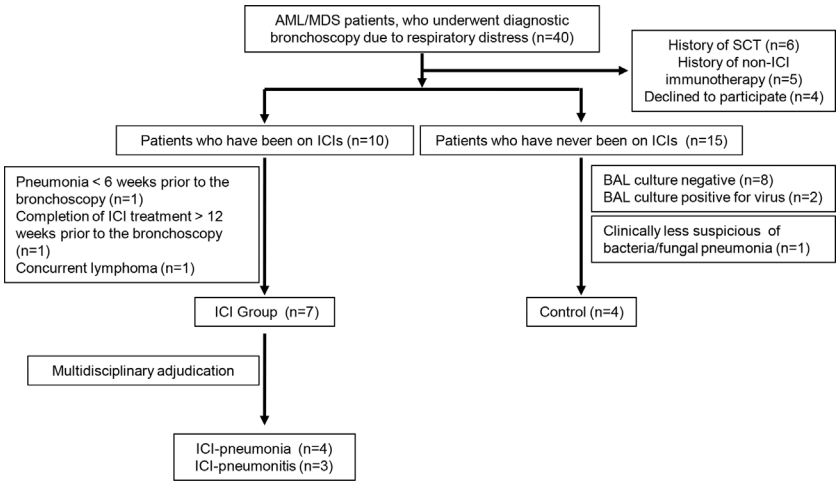

FIGURE 1 | Selection process for patients in the immune checkpoint inhibitor (ICI) group and the control group. AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; SCT, stem cell transplantation; BAL, bronchoalveolar lavage.

(Gibco) and cryopreserved in the presence of 90% fetal bovine serum and 10% dimethyl sulfoxide (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll gradient technique (Sigma-Aldrich) and cryopreserved like BAL cells.

T Cell Receptor Sequencing

DNA was extracted from cryopreserved BAL cells and PBMCs using the QIAamp DNA Mini Kit (Qiagen), and the complementarity determining region 3 of the T cell receptor beta (TCRβ) chain was amplified using the ImmunoSeq hsTCRB Kit (Adaptive Biotechnologies) and sequenced using the MiSeq platform (Illumina). Sequencing data were analyzed using the ImmunoSEQ Analyzer (Adaptive Biotechnologies), by which a series of diversity metrics were generated, including observed richness, Pielou evenness, and Simpson D (8). The clonality metric was defined as 1-Pielou evenness, where the values of clonality approach 0 when all sequences are equally abundant and perfectly even, and the values approach 1 when a single sequence makes up the entire sample.

Flow Cytometry

Cryopreserved BAL cells and PBMCs were thawed, washed, and stained with flow cytometry antibodies to CD3, CD4, CD8, CD19, CD25, CD27, CD45A, CD56, CD127, CCR4, CCR6, CCR7, CXCR3, CXCR5, PD-1, and γδTCR. For intracellular staining, BAL cells and PBMCs were stimulated with cell activation cocktail (BioLegend) containing phorbol 12-myristate 13-acetate (PMA), ionomycin, and bredfeldin for 4 h. Cells were stained for surface molecules, fixed with BD CytoFix/CytoPerm, permeabilized with BD PERM/wash solution, and stained with antibodies to IFNγ and IL-17A. For transcription factor analysis, cells were first stained for surface molecules, then fixed and permeabilized with eBioscience™ FoxP3/transcription staining buffer set. After permeabilization, cells were stained with T-bet, GATA3, RORγt, and FoxP3. Stained samples were acquired using an LSR II FORTESSA X-20 (BD Biosciences) and analyzed using FlowJo software (TreeStar). Detailed information about the flow cytometry antibodies is available in **Supplementary Table 1**.

Identification of Immune Cell Subsets

Live immune cells were detected by gating live-dead. The lymphocytes were further examined by forward scatter and side scatter (SSC). Natural killer (NK) and NK T cells were identified by CD56 and CD3 expression. Within the $CD3^+$ $CD56^-$ cell population, we further gated to identify $CD4^+$ T cells and $CD8^+$ T cells. For $CD4^+$ T cells, after gating regulatory T (Treg) cells ($CD25^{hi}$ $CD127^{lo}$) (9), we further divided non-Treg cells into $CD45RA^+$ naïve T cells, CXCR5-expressing follicular helper T cells (Tfh) (10), and $CD45RA^-$ $CXCR5^-$ cells (non-Tfh effector cells). Non-Tfh effector cells were further divided into effector subsets on the basis of CXCR3, CCR4, and CCR6 expression: Th1 ($CXCR3^+$ $CCR6^-$), Th2 ($CXCR3^-$ $CCR6^-$ $CCR4^+$), Th17 ($CXCR3^-$ $CCR6^+$), and Th17/Th1 ($CXCR3^+$ $CCR6^+$) cells (11–13). $CD8^+$ T cells were examined by CD45RA and CCR7 staining to detect naive, central memory (Tcm), effector memory (Tem), and terminally differentiated effector memory (Tem) cells (14). Within the $CD3^-$ $CD56^-$ population, CD19-expressing B cells were gated. Gating strategies are shown in **Supplementary Figures 1** and **2A**. In parallel, we analyzed IFNγ- and/or IL-17A-producing T cells in BAL fluid and PB samples.

Cytokine Measurement

IFNγ, IL-6, and IL-17A in BAL fluid were measured by multiplex ELISA, using commercially available kits (U-Plex Th17 Combo 2, Meso Scale Discovery).

Statistical Analysis

Significant differences in means between groups were determined by the two-tailed Mann-Whitney U test, two-tailed Wilcoxon paired rank test, or one-way ANOVA. $P < 0.05$ was considered statistically significant. All statistical analyses were done using Prism software.

RESULTS

Patient Demographic Features

The clinical characteristics of the patients are summarized in **Table 1**. Ten of the 11 patients had AML, and most (10/11) had intermediate or advanced cytogenetic characteristics. Most patients (5/7 in the ICI group; 4/4 in the control group) also had leukopenia, with a median white blood cell count of 0.4×10^3/ml. Most patients in the ICI group (6/7) were receiving azacytidine in addition to the ICIs. Four patients were receiving a PD-1 inhibitor and three patients were receiving a combination of CTLA-4 and PD-1 inhibitors; two patients were receiving avelumab (3 mg/kg every 2 weeks), two patients were receiving nivolumab (3 mg/kg every 2 weeks), two patients were receiving ipilimumab (1 mg/kg every 12 weeks) plus nivolumab (3 mg/kg every 2 weeks), and one patient was receiving ipilimumab (3 mg/kg every 4 weeks) plus nivolumab (3 mg/kg every 2 weeks). All patients were on prophylactic regimen including quinolone, azol-antifungal agent, and antiviral nucleoside analogue. Patients in the ICI group developed respiratory symptoms at a median of 2.5 weeks after the initiation of ICIs; however, the range was broad (0.5 to 27.5 weeks). Three patients in the ICI group were receiving steroids at the time of bronchoscopy, at a median dose of 125 mg prednisone (or equivalent), and four patients were receiving steroids at the time of PB collection, at a median dose of 62.5 mg. Four patients in the ICI group had a positive BAL culture result, indicating extracellular bacteria with or without a virus.

Manual Differentials of Bronchoalveolar Lavage and Peripheral Blood

For all patients, manual leukocyte differentials of BAL and PB cells were counted as standard of care (**Table 2**). The differentiation tests of PB from four patients, two in the ICI group and two in the control group, could not be performed owing to severe leukopenia. The frequency of BAL lymphocytes was significantly higher in the ICI group than in the control group (mean ± SD; ICI *vs.* control; 26.4 ± 15.0 *vs.* 3.8 ± 3.6; P = 0.01), whereas the mean frequency of BAL macrophages was significantly lower in the ICI group than in the control group (mean ± SD; ICI *vs.* control; 64.7 ± 15.0 *vs.* 86.5 ± 7.1; P = 0.03). This trend was more prominent in the ICI-pneumonia group than in the ICI-pneumonitis group (**Figure 2**). Consistently, the mean frequency of PB lymphocytes in the ICI group was higher than in the control group; however, the differences did not reach statistical significance (mean ± SD; ICI *vs.* control; 44.8 ± 15.0 *vs.* 19.0± 11.3; P = 0.09).

Distinct Immune Landscape of Bronchoalveolar Lavage T Cells in the Immune Checkpoint Inhibitor Group

Given the enrichment of lymphocytes in BAL fluid and PB, we focused on characterizing lymphocytes and enumerating major lymphocytic subsets in both BAL fluid and PB (**Figure 3**; **Supplementary Table 2**). The absolute number of lymphocytes per 1 ml BAL fluid was higher in the ICI group than in the control group (mean ± SD; ICI *vs.* control: 23,791 ± 41,142 cells *vs.* 1,285 ± 1,051 cells; P = 0.01; **Supplementary Table 2**). The proportions of NK cells ($CD3^-$ $CD56^+$), NK T cells ($CD3^+$ $CD56^+$), B cells ($CD3^-$ $CD56^-$ $CD19^+$), and $CD4^+$ T cells ($CD3^+$ $CD4^+$ $CD8^-$) were similar between the ICI and control groups (**Figure 3A**; **Supplementary Figure 1**). BAL $CD8^+$ T cells ($CD3^+$ $CD4^-$ $CD8^+$) were significantly expanded in the ICI group compared with the control group in terms of frequencies and numbers (frequency: mean ± SD; ICI *vs.* control; 28.4 ± 13.0% *vs.* 5.8 ± 1.2%; P = 0.006) (absolute cell numbers: mean ± SD; ICI *vs.* control; 28.4 ± 13.0% *vs.* 5.8 ± 1.2%). Most of these $CD8^+$ T cells were $CD45RA^-$ $CCR7^-$ effector memory cells (64.2 ± 30.7%; **Figure 3B**), suggesting that these cells play a role in ICI-related pulmonary complications. The frequencies and absolute number of cells for lymphocytic immune subsets in the PB samples were similar between the ICI and control groups (**Figure 3C**).

Next, we delineated $CD4^+$ T cell subsets on the basis of chemokine/cytokine receptor expression, including regulatory T cells, naïve T cells, follicular helper T cells, Th1, Th2, Th17, and Th17/Th1 cells (9–13) (**Figure 3D**; **Supplementary Figure 2**). Although the proportions of PB $CD4^+$ T cell subsets were similar between the ICI and control groups (**Supplementary Figure 3**), BAL Th17/Th1 cells were significantly expanded in the ICI group compared with the control group (mean ± SD; ICI *vs.* control; 43.8 ± 20.5% *vs.* 13.3 ± 8.8%; P = 0.04; **Figure 3D**). For selected patients (n=3 in control; n=3 in ICI-pneumonia; n=2 in ICI-pneumonitis), along with chemokine/cytokine receptors, we also investigated expression of key transcription factors including T-bet (Th1), GATA3 (Th2), RoRγT (Th17), and FoxP3 (Treg) (**Figure 3E**; **Supplementary Figure 3**) (10). Consistent with data in **Figure 3D**, we observed enrichment of T-bet^+ $ROR\gamma t^+$ (Th1) and $CXCR3^+$ T-bet^+ $CCR6^+$ $ROR\gamma t^+$ (Th1/Th17) cells in BAL $CD4^+$ T cells in the ICI group. Most (48.0 ± 22.5%) BAL Th17/Th1 cells expressed PD-1 (**Figure 3F**), suggesting that these cells had persistent antigen exposure (15).

To evaluate the functionality of the T cells, we performed intracellular staining to assess IFNγ- and/or IL-17-producing T cells (**Figure 3G**; **Supplementary Figure 4**). In BAL fluid, the absolute number of IFNγ- and/or IL-17-producing $CD4^+$ T cells was higher in the ICI group than in the control group. In addition to the number of cells, the frequency of $IFN\gamma^+$ $IL\text{-}17^+$ $CD4^+$ T cells in BAL fluid was significantly higher in the ICI group than in the control group (mean ± SD; ICI *vs.* control; 4.1 ± 2.4% *vs.* 0.7 ± 1.3%; P = 0.03; **Figure 3G**). Consistently, although proportions of $IFN\gamma^+$ $IL\text{-}17^-$ $CD8^+$ cells in BAL fluid were similar between the two groups, the absolute number of these cells was higher in the ICI group than in the control group (mean ± SD; ICI *vs.* control; 2135.0 ± 2203.0 cells *vs.* 30.0 ± 44.0

TABLE 1 | Basic characteristics of study patients.

Characteristic		ICI group (n=7)	ICI-pneumonia (n=4)	ICI-pneumonitis (n=3)	Controls (n=4)
Age, years, median (range)		69 (25–81)	63 (25–81)	77 (52–79)	62.5 (55–79)
Sex (male/female)		2/5	0/4	2/1	3/1
Primary tumor					
AML		6	3	3	4
MDS		1	1	0	0
ECOG performance status, median (range)		1.5 (1–2)	1.5 (1–2)	1.5 (1–2)	1.5 (1–2)
Patients with antecedent hematologic disorder		1	1	0	2
Cytogenetic group	Adverse	5	4	1	1
	Intermediate	2	0	2	2
	Favorable	0	0	0	1
Molecular mutations (minimum ≥ 2 cases)	TP53	3	2	1	0
	FLT3	2	1	1	0
	DNMT3A	0	0	0	2
Peripheral blood WBC count at bronchoscopy, $\times 10^3$/ml, median (range)		0.4 (0.2–17.5)	0.5 (0.2–6.2)	0.4 (0.4–17.5)	0.4 (0.1–3.2)
Peripheral blood blasts at bronchoscopy, %, median (range)		25 (0–68) (n=5)	16 (7–25) (n=2)	40 (0–68) (n=3)	2.5 (0–5) (n=2)
BM blasts on most recent BM biopsy prior to bronchoscopy, %, median (range)		44 (10–90)	23.5 (10–90)	58 (44–84)	34 (1–87)
ICI treatment status (frontline/salvage)		3/4	3/1	0/3	n/a
Treatment regimen					
Azacytidine + ICI-based		6	3	3	0
Non-azacytidine + ICI-based		1	1	0	0
Fludarabine + cytarabine + idarubicin + sorafenib		0	0	0	1
Cytarabine + idarubicin		0	0	0	1
Non-immune investigational small molecule(s)		0	0	0	2
Best response to treatment regimen (CR or CRp)		1	0	1	1
Patients actively on ICI treatment at bronchoscopy		7	4	3	n/a
Discontinuation of ICI protocol prior to bronchoscopy		0	0	0	n/a
ICI regimen					
PD-1 inhibitor		4	2	2	n/a
CTLA-4 inhibitor		0	0	0	n/a
Combined PD-1 and CTLA-4 inhibitors		3	2	1	n/a
Admission status at bronchoscopy, routine floor/ICU		7	4/0	3/0	4/0
Patients receiving steroid at time of bronchoscopy					
Dose of prednisone (or equivalent) at time of bronchoscopy, mg, median (range)		125 (50–300) (n=3)	50 (n=1)	212.5 (125–300) (n=2)	n/a (n=0)
Patients receiving steroid at time of blood draw					
Dose of prednisone (or equivalent) at time of blood draw, mg, median (range)		62.5 (30,150) (n=4)	35 (20–50) (n=2)	112.5 (75–150) (n=2)	n/a (n=0)
Duration, weeks, median (range)					
From first ICI infusion to respiratory symptoms		2.5 (0.5–27.5)	1.5 (0.5–3.5)	3.5 (0.5–27.5)	n/a
From first ICI infusion to bronchoscopy		4 (0.5–28)	3.5 (0.5–6.5)	4 (2–28)	n/a
From last ICI infusion to respiratory symptoms		0.5 (0.5–2)	0.5 (0.5–1.5)	2 (0.5–2)	n/a
From last ICI infusion to bronchoscopy		2 (0.5–5.5)	1.5 (0.5–5.5)	2 (1.5–2)	n/a
Patients receiving prophylactic antibiotic at time of bronchoscopy		7	4	3	4
Antibacterial agent					
Levofloxacin		7	4	3	2
Ciprofloxacin		0	0	0	2
Antifungal agent					
Fluconazole		2	1	1	2
Voriconazole		3	2	1	0
Posaconazole		1	1	0	1
Isavuconazole		1	0	1	0
Esavuconazole		0	0	0	1
Antiviral agent					
Valaciclovir		6	3	3	4
Acyclovir		1	1	0	0
Patients admitted ≤6 weeks prior to bronchoscopy		3	2	1	2
BAL fluid culture results					
Negative		3	2	1	0
Virus		0	0	0	0
Extracellular bacteria		3	1	2	2
Fungi		0	0	0	2
Extracellular bacteria and virus		1	1	0	0

(Continued)

TABLE 1 | Continued

Characteristic	ICI group (n=7)	ICI-pneumonia (n=4)	ICI-pneumonitis (n=3)	Controls (n=4)
Findings on chest CT				
Infectious pneumonia	4	4	0	4
Hypersensitivity pneumonitis	2	0	2	0
Organizing pneumonia	1	0	1	0

ICI, immune checkpoint inhibitor; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ECOG, Eastern Cooperative Oncology Group; WBC, white blood cells; BM, bone marrow; n/a, not applicable; CR, complete remission; CRp, complete remission without platelet recovery; ICU, intensive care unit; BAL, bronchoalveolar lavage; CT, computed tomography.

TABLE 2 | Manual differentiations of bronchoalveolar lavage (BAL) and peripheral blood (PB) samples.

BAL	ICI group (n=7)	ICI-pneumonia (n=4)	ICI-pneumonitis (n=3)	Control (n=4)
Cell subsets, %, median (range)				
Lymphocyte	32 (8–46)	36 (32–46)	13 (8–14)	2.5 (1–9)
Macrophage	65 (41–84)	56.5 (41–65)	81 (69–84)	84 (81–97)
Neutrophil	0 (0–1)	0.5 (0–1)	0 (0–0)	0 (0–0)
Eosinophil	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Basophil	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Others	9 (1–17)	7 (1–12)	11 (2–17)	11.5 (1–13)
PB*	**ICI group (n=5)**	**ICI-pneumonia (n=2)**	**ICI-pneumonitis (n=3)**	**Control (n=2)**
Cell subsets, %, median (range)				
Lymphocyte	39 (32–67)	46 (39–53)	33 (32–67)	19 (11–27)
Macrophage	5 (0–20)	7 (1–13)	5 (0–20)	47.5 (12–83)
Neutrophil	16 (0–33)	25.5 (18–33)	13 (0–16)	28 (0–56)
Eosinophil	1 (0–1)	1 (1–1)	0 (0–1)	2.5 (0–5)
Basophil	1 (0–4)	1 (1–1)	0 (0–4)	0 (0–0)
Others†	28 (0–68)	19.5 (11–28)	44 (0–68)	3 (0–6)

ICI, immune checkpoint inhibitor; BAL, bronchoalveolar lavage; PB, peripheral blood.
**PB differentiation tests could not be performed in two patients in the ICI-pneumonia group and two patients in the control group owing to severe leukopenia.*
†Blasts are included.

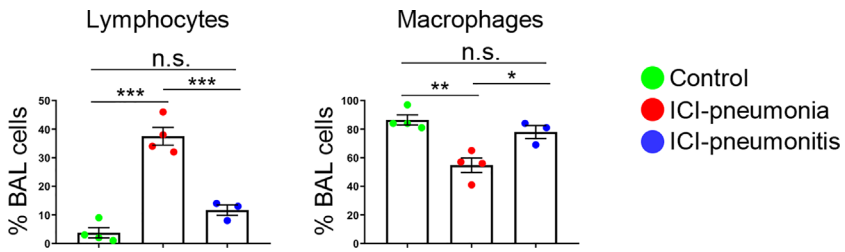

FIGURE 2 | Proportion of major bronchoalveolar lavage (BAL) cell subsets with manual differential tests. Bars indicate the mean and the SEM. One-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant. ICI, immune checkpoint inhibitor.

cells; P = 0.01; **Figure 3G**). Although not statistically significant, the levels of soluble IFNγ, as well as IL-6 and IL-17A, key cytokines for Th17 cell differentiation, plasticity, and function (10, 16), in the BAL fluid were higher in the ICI group than in the control group (**Figure 3H**). IFNγ- and/or IL-17-producing $CD4^+$ and $CD8^+$ T cells in PB were comparable between the ICI and control groups (**Supplementary Figure 4**).

Clonally Expanded Bronchoalveolar Lavage T Cells in the Immune Checkpoint Inhibitor Group

We analyzed the TCR repertoire in 11 matched BAL fluid and PB samples (**Figure 4**). T cells in the ICI group, especially the BAL T cells, were significantly clonally expanded compared with the control group (mean ± SD; ICI *vs.* control; 0.077 ± 0.011 *vs.* 0.014 ± 0.002; P = 0.006). Clonality and diversity of PB T cells was higher in the ICI-pneumonitis group than in the ICI-pneumonia and control groups (clonality: mean ± SD; ICI-pneumonitis *vs.* ICI-pneumonia *vs.* control; 0.16 ± 0.02 *vs.* 0.03 ± 0.03 *vs.* 0.03 ± 0.03, P = 0.001) (diversity: mean ± SD; ICI-pneumonitis *vs.* ICI-pneumonia *vs.* control; 0.02 ± 0.01 *vs.* 0.0004 ± 0.0003 *vs.* 0.0004 ± 0.0006; P = 0.001) (**Figure 4A**). We investigated the overlapped T cell clones in BAL and PB (**Figure 4B**; **Supplementary Figure 6**). Although not reached statistical significance, a greater degree of overlap was observed in the ICI-pneumonitis, compared with ICI-pneumonia and controls

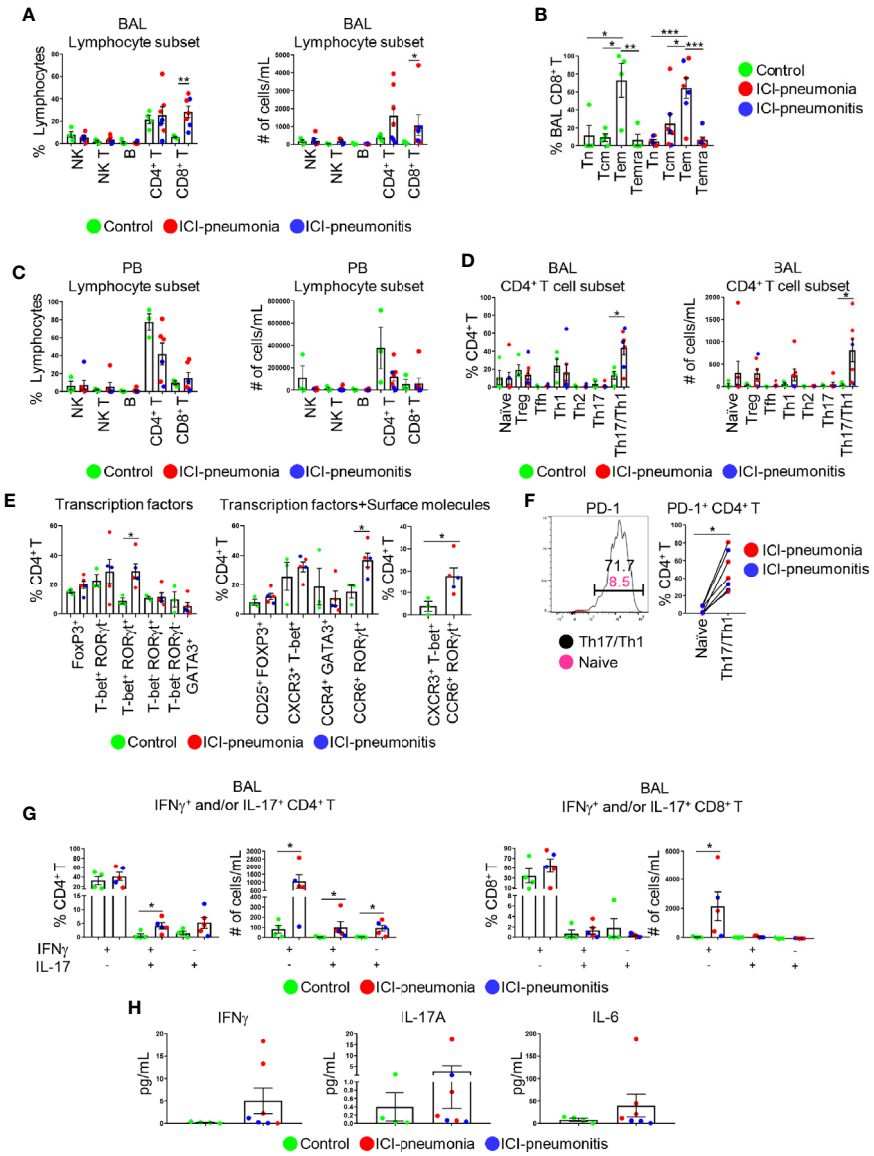

FIGURE 3 | Characterization of lymphoid immune cell subsets in bronchoalveolar lavage (BAL) fluid and peripheral blood (PB). **(A)** Proportions of major BAL immune cell subsets within live lymphocytes and absolute cell numbers in 1 ml BAL fluid. NK, natural killer cells; NK T, natural killer T cells; B, B cells. Bars indicate the mean and the SEM. Mann-Whitney U test. *P<0.05, **P<0.01. **(B)** Proportions of $CD8^+$ T cell subsets within BAL $CD8^+$ T cells. Tn, naïve T cells; Tcm, central memory T cells; Tem, effector memory T cells; Temra, terminally differentiated T cells. Bars indicate the mean and the SEM. One-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. **(C)** Proportions of major PB immune cell subsets within live lymphocytes and absolute cell numbers in 1 ml PB. Bars indicate the mean and the SEM. **(D)** Proportions of $CD4^+$ T cell subsets within $CD4^+$ T cells and absolute cell numbers in 1 ml BAL fluid. Treg, regulatory T cells; Tfh, follicular helper T cells. Bars indicate the mean and the SEM. Mann-Whitney U test. *P<0.05. **(E)** Proportion of BAL $CD4^+$ T cells expressing indicated transcription factors (left), transcription factors and surface molecules (middle and right). Bars indicate the mean and the SEM. Mann-Whitney U test. *P<0.05. **(F)** PD-1 on BAL naïve $CD4^+$ T cells and BAL $CXCR3^+$ $CCR6^+$ Th17/Th1 cells. Left panel shows one of the most representative plots and right panel shows quantification. Wilcoxon paired rank test. *P<0.05. **(G)** Proportions and absolute numbers of IFNγ- and/or IL-17-producing $CD4^+$ and $CD8^+$ T cells in BAL fluid. Bars indicate the mean and the SEM. Mann-Whitney U test. *P<0.05. **(H)** Levels of IFNγ, IL-6, and IL-17A in BAL fluid measured by multiplex ELISA. Bars indicate the mean and the SEM.

(**Figure 4B**), suggesting that ICI-pneumonitis might be a systemic inflammation.

Subgroup analysis of the ICI group based on ICI regimen [PD-1 inhibitor (n=4) compared with combined CTLA-4 and PD-1 inhibitors (n=3)] and concurrent steroid treatment at the time of biospecimen collection [steroid (n=3) compared with no steroid (n=4)] revealed no differences in immunophenotypes or TCR repertoire (data not shown).

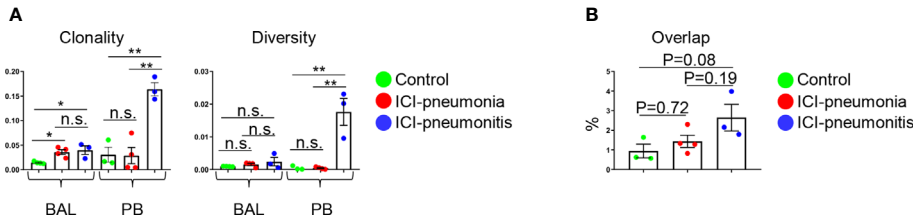

FIGURE 4 | **(A)** Clonality and diversity of T cells in the bronchoalveolar lavage (BAL) fluid and peripheral blood (PB). Bars indicate the mean and the SEM. One-way ANOVA. *P<0.05, **P<0.01, n.s., not significant. **(B)** Quantification of overlapped T-cell receptor sequences between BAL and PB. Bars indicate the mean and the SEM. One-way ANOVA.

DISCUSSION

AML/MDS patients receiving ICIs can develop pneumonia due to their diseases and leukopenia and pneumonitis as an immune-related adverse event. As the first step to investigate mechanisms underlying these ICI-related pulmonary complications, we immunoprofiled BAL fluid and PB samples from AML/MDS patients with pulmonary complications after ICI therapy. Compared with control patients (ICI-naïve AML/MDS patients with bacterial/fungal pneumonia), patients with ICI-related pulmonary complications had enriched lymphocytes, especially Th17/Th1 cells and IFNγ^+ CD8$^+$ T cells, in BAL fluid, as well as clonally expanded BAL T cells. Subgroup analysis of the ICI group revealed that patients with ICI-pneumonia had predominant BAL lymphocytes and patients with ICI-pneumonitis had enhanced T cell clonality and diversity in PB. Combined, our data suggest that distinct T cell responses occur in patients with ICI-related pulmonary complications.

Th17 cells are highly plastic and can be differentiated into CXCR3$^+$ CCR6$^+$ IFNγ^+ IL-17$^+$ Th17/Th1 cells. Studies have shown that Th17/Th1 cells play an important role in the pathogenesis of autoimmune diseases (17). Indeed, Th17/Th1 cells were shown to be enriched in inflammatory sites of autoimmune diseases including the colon in Crohn's disease, cerebrospinal fluid in multiple sclerosis, and synovial fluid in rheumatoid arthritis and juvenile idiopathic arthritis (13, 18–21). Recent studies revealed that these cells are also enriched in the BAL fluid from patients with sarcoidosis (22–24). Based on the studies, we speculate that enrichment of BAL Th17/Th1 cells in our study is not a non-specific finding secondary from inflammation; rather we hypothesize that these BAL Th17/Th1 cells play a key role in the pathogenesis of ICI-related pulmonary complications. Our hypothesis is partially supported by the *in vivo* and *in vitro* observations that genetic or pharmacologic depletion of PD-1 enhanced Th17 responses in a mouse model of allergic asthma (25). Further studies are warranted to investigate the generation and function of Th17/Th1 cells in ICI-related pulmonary complications.

About 3–5% of patients with solid tumors develop pneumonitis after ICI therapy (6). The pneumonitis with solid tumors is one of early immune-related adverse events with onset at a median of 2.8 months, with a wide range (9 days to 19.2 months) (6). Suresh *et al.* recently characterized BAL fluid from patients with solid tumors who developed ICI-induced pneumonitis, and that analysis revealed prominent lymphocytes, especially IFNγ^+ CD8$^+$ T cells (26). Patients in our cohort also developed respiratory symptoms early after the initiation of ICIs (median: 2.5 weeks), and BAL analyses revealed prominent lymphocytes in the ICI group (**Table 2**). Importantly, we observed enrichment of IFNγ^+ CD8$^+$ T cells, but we also observed enrichment of Th17/Th1 cells, suggesting that there are shared and distinct mechanisms underlying ICI-induced pneumonitis depending on the tumor type. Dissection of immune profiles of ICI-induced pneumonitis between patients with solid tumors and those with leukemia would be of future interest.

The difference in immunophenotypes between ICI-pneumonia and ICI-pneumonitis is unclear. Although the difference was not statistically significant, we found that the median onset of respiratory symptoms was shorter in the ICI-pneumonia group than in the ICI-pneumonitis group (ICI-pneumonia *vs.* ICI-pneumonitis; 1.5 *vs.* 3.5 weeks), and proportions of BAL lymphocytes were higher in the ICI-pneumonia group, suggesting that BAL lymphocytes, most likely T cells, of the ICI-pneumonia actively proliferate and/or survive longer compared with BAL T cells of the ICI-pneumonitis. Considering these findings, we speculate that patients with ICI-pneumonia might have more enhanced T cell memory responses than patients with ICI-pneumonitis. Not mutually exclusive, it is also possible that antigens in ICI-pneumonia have heightened antigenicity compared with those in ICI-pneumonitis. In contrast, we found that clonality and diversity of circulating T cells were higher in the ICI-pneumonitis group than in the ICI-pneumonia group. Collectively, we hypothesis that exogenous antigens (bacteria and/or fungus) in the ICI-pneumonia might provide strong TCR and toll-like receptor signal, which induce global and indirect T cell activation/reactivation with prolonged T cell survival. In contrast, endogenous antigens (self-antigens or tumor antigens) might specifically activate T cells recognizing these endogenous antigens, resulting in enhanced TCR clonality. Our hypothesis is supported by the study, showing enhanced TCR clonality in inflamed joints (synovial fluid) and blood of patients with psoriatic arthritis, one of the most common autoimmune diseases (27). In addition, previous studies showed an increase of clonality and diversity of T cells in patients with immune-

related adverse events (28–31). However, given our small sample size and the unstable PB cells in AML/MDS, we could not make any conclusions at present. Future studies investigating cell proliferation (Ki67), apoptosis (annexin V, DAPI), exhaustion (LAG3, TIM3, PD-1, TIGIT), and anti-apoptosis gene expression (Bcl2, Bcl-xL) in BAL/PB cells between ICI-pneumonia and ICI-pneumonitis will enable us to dissect mechanisms of ICI-pneumonia and ICI-pneumonitis. Nevertheless, BAL differentiation counts and/or TCR repertoires in PB might be a potential biomarker to differentiate ICI-pneumonia from ICI-pneumonitis.

Our study has a few limitations. First, because of the small number of patients analyzed, these data are inconclusive. Second, this study does not have a control group comprising patients with solid tumors who developed ICI-induced pneumonitis. In addition, some patients in the ICI group were on azacitidine in addition to ICI and azacitidine can alter immune profiles (32, 33). Indeed, studies revealed increased numbers of Tregs and decreased numbers of $CD8^+$ T cells and Th1 cells after azacitidine therapy (32, 33). Our study showed enrichment of BAL Th17/Th1 cells and $IFN\gamma^+$ $IL\text{-}17^-$ $CD8^+$ T cells in the ICI group while studies showed that stable and decreased numbers of Th17 and $CD8^+$ T cells with azacitidine. Together, we speculated that azacitidine might not have influenced our main observations; however, given that epigenetic mechanisms are critical in regulating T cell lineage commitment (34), ICI-naïve AML/MDS patients with azacitidine monotherapy should also be served as a control group. Third, three participants in the ICI group were receiving steroids at the time of BAL fluid collection and four at the time of PB sample collection, which might have altered the immune profiles.

In this study, the samples were mainly obtained from the pilot phase IB trials initiated in 2017-2018 at the University of Texas MD Anderson Cancer Center, comparing efficacy and safety of ICI-based therapies in patients with AML/MDS. With the initial encouraging results, we have recently opened a number of additional ICI-based trials for AML and MDS including clinical trials of azacitidine + nivolumab + ipilimumab (NCT02397720), azacitidine+venetoclax+ nivolumab (NCT02397720), azacitidine + venetoclax +avelumab (NCT03390296), azacitidine + venetoclax + TIM3 antibody (NCT04150029), with larger numbers of participants (150–180) expected to be enrolled at the MD Anderson across these phase IB/II larger trials. In this manuscript, we aimed to generate hypothesis rather test the hypothesis. Since 10–12% of the AML/MDS patients develops pneumonitis (1, 6), from these upcoming trials, we expect to collect 15–22 BAL and matching PB samples from AML/MDS patients with ICI-pneumonitis (and similar numbers of the samples from AML/MDS patients with ICI-pneumonia as well). Detailed investigation of cell survivals, proliferation, and exhaustion are warranted in future studies to dissect underlying mechanisms between ICI-pneumonia and ICI-pneumonitis. Based on distinct TCR repertoires between ICI-pneumonia and ICI-pneumonitis, analysis of both TCR α and β chains are also needed in the future studies. ICI-naïve AML/MDS patients who develops pulmonary complications after azacitidine monotherapy will be served as a control group in future studies. Additionally, the standard therapy for frontline older AML has now transitioned to azacitidine+venetoclax, and it is possible this will emerge as a more effective therapy in frontline MDS as well. We have a large number of patients treated with azacitidine and venetoclax for both AML and MDS and plan to assess BAL samples on these patients as well to serve as an additional future control. Finally, although we did not see differences of immune profiles of concurrent steroid treatment, the analysis might be underpowered. Future studies should carefully model the use of steroids and standardize BAL collection before steroids are administered. In some cases of life-threatening pneumonitis, steroid therapy is empirically initiated prior to the diagnostic bronchoscopy. Nevertheless, larger numbers of the samples in the future studies will enable us to perform subgroup analysis (steroid *vs*. no steroid) with adequate power. In conclusion, our study showed distinct immunophenotypes of T cells in BAL fluid in AML/MDS patients with ICI-related pulmonary complications. Detailed molecular and cellular characterization of immune cells in a larger number of patients, with appropriate controls, may provide insights into the mechanisms of pneumonitis in AML/MDS treated with ICIs-based therapy, as well as provide diagnostic biomarkers to differentiate pneumonitis from pneumonia and potentially predict the severity of the pneumonitis.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The IRB at the University of Texas MD Anderson Cancer Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK performed experiments, analyzed the data, and wrote the manuscript. AS, VS, HK, GG-M, FR, LB, DB, SE, SF, and ND provided samples. JI, WR-V, MD, SN, RM, LW, ST, CG, and AF performed experiments and discussed results. AD and PB analyzed the data and discussed results. AN discussed the results. SK, AS, VS, DK, and ND were responsible for adjudication of the patients. RN and ND oversaw the study and discussed results. All authors reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by The University of Texas MD Anderson Cancer Center Division of Internal Medicine Developmental Funds (SK), the NIH RO1 grants (RN: R01HL141966 and R01HL143520) and CPRIT grant (RN: RP190326).

ACKNOWLEDGMENTS

The authors thank Jairo Matthews and Wilmer Flores for their support in obtaining human samples. We also thank Jordan Kramer for analyzing the data and reading the manuscript critically. Finally, the authors also thank Editing Services, Research Medical Library at The University of Texas MD Anderson Cancer Center for editing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.590494/full#supplementary-material

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Conflict of Interest: SN has received research support from Kite/Gilead, Cellectis, Poseida, Merck, Acerta, Karus, BMS, Unum Therapeutics, Allogene, and Precision Biosciences; served as consultant and advisory board member for Kite/Gilead, Celgene, Novartis, Unum Therapeutics, Pfizer, Merck, Precision Biosciences, Cell Medica, Incyte, Allogene, Calibr, and Legend Biotech; and has patents related to cell therapy. ND has received research funding from Daiichi Sankyo, Bristol-Myers Squibb, Pfizer, Karyopharm, Sevier, Genentech, and ImmunoGen and has served in a consulting or advisory role for Daiichi Sankyo, Bristol-Myers Squibb, Pfizer, Novartis, Celgene, AbbVie, and Agios.

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: 02 April 2021
doi: 10.3389/fimmu.2021.640369

Role of Epigenetic Regulation in Plasticity of Tumor Immune Microenvironment

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The tumor immune microenvironment (TIME), an immunosuppressive niche, plays a pivotal role in contributing to the development, progression, and immune escape of various types of cancer. Compelling evidence highlights the feasibility of cancer therapy targeting the plasticity of TIME as a strategy to retrain the immunosuppressive immune cells, including innate immune cells and T cells. Epigenetic alterations, such as DNA methylation, histone post-translational modifications, and noncoding RNA-mediated regulation, regulate the expression of many human genes and have been reported to be accurate in the reprogramming of TIME according to vast majority of published results. Recently, mounting evidence has shown that the gut microbiome can also influence the colorectal cancer and even extraintestinal tumors *via* metabolites or microbiota-derived molecules. A tumor is a kind of heterogeneous disease with specificity in time and space, which is not only dependent on genetic regulation, but also regulated by epigenetics. This review summarizes the reprogramming of immune cells by epigenetic modifications in TIME and surveys the recent progress in epigenetic-based cancer clinical therapeutic approaches. We also discuss the ongoing studies and future areas of research that benefits to cancer eradication.

Keywords: DNA methylation, histone modification, ncRNAs, TIME, ITH, epigenetics

OPEN ACCESS

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

Received: *11 December 2020*
Accepted: *15 March 2021*
Published: *02 April 2021*

Citation:
Yang Y and Wang Y (2021) Role of Epigenetic Regulation in Plasticity of Tumor Immune Microenvironment. Front. Immunol. 12:640369. doi: 10.3389/fimmu.2021.640369

INTRODUCTION

Cancer is the leading cause of death worldwide and in China and thus remains as the single biggest stumbling block for extending life expectancy. According to GLOBCAN 2018, there are approximately 18.1 million new cancer cases and 9.6 million new cancer deaths worldwide, 24 and 30% of which occur in China, respectively (1, 2). This suggests a large gap between China and other developed countries, such as the United States, in terms of cancer mortality (1, 3). Thus, new insights into cancer therapy are necessary for the development of novel strategies and efficacious drug combination therapies.

Tumors are not only a group of abnormally proliferative cells, but also a special environment termed as the tumor microenvironment (TME) that contains different cell types, including tumor and immune cells (4). Owing to the large number of immunosuppressive immune cells, the TME is also called TIME. Thus, developing therapeutic approaches targeting the plasticity of TIME has become one of the most attractive area in cancer therapy. Immune checkpoint inhibition (ICI) is a promising strategy that involves the activation of the function of TIME T cells to combat tumor cells

(5, 6). However, the majority of cancer patients exhibited minimal or no clinical response to ICI therapy (5).

Epigenetic changes in genes encoding tumor suppressors, inhibitory cytokines, and immune checkpoint molecules, e.g., PD-L1 and CD47, can lead to impaired anti-cancer immunity, uncontrollable tumor growth, immune escape, and drug resistance, eventually resulting in tumor development, progression, and metastasis (7, 8). Therefore, targeting the epigenetic alterations in cancer cells with epigenetic-associated drugs (epi-drugs) could convert a tumor from an immune suppressive (cold) to an immune permissive (hot) state (9). This could improve the therapeutic effects of other anti-tumor drugs, especially immune checkpoint inhibitors (ICIs). Within the TIME, epigenetic modifications can also be found in tumor-associated immune cells, including myeloid cells, $CD4^+$ T cells, and $CD8^+$ T cells (9–11). During the differentiation from naïve $CD8^+$ T cells to $CD8^+$ effector T cells, epigenetic changes, such as DNA methylation and histone modifications, are involved in the chromatin accessibility (12, 13). The immune checkpoint protein PD-1 expressed on the surface of exhausted T cells is also regulated by DNA methylation (14). Thus, disrupting the unusual epigenetic regulation in cancer can completely shape the TIME by decreasing the populations of immunosuppressive cells, such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) (15), increasing the numbers of $CD8^+$ effector T cells and NK cells (15, 16), elevating the levels of inflammatory cytokines and chemokines (17–19), and upregulating the expression of tumor antigens, such as cancer/testis antigens (CTAs) (20, 21).

Tumor heterogeneity, especially intratumor heterogeneity (ITH), is one of the major hallmarks of cancer. Within TIME, there is diversity in the phenotypes of tumor cells and the infiltration and differentiation status of immune cells, and the diversity is characterized by distinct microscopy fields of a single biopsy. Tumor or TIME is formed from a single mutated cell that abnormally proliferates and accumulates additional mutations through Darwinian evolution (22). This may cause drug resistance to cancer therapy, such as in patients with breast cancer, due to pre-existing resistant subclones within the tumor verified by single-cell sequencing technique (23). Aberrant epigenetic changes occur more frequently than gene mutations in human cancer. Thus, targeting the epigenetic changes in cancer may reverse drug resistance to cancer therapies, particularly immunotherapies, and increase the efficacy of other therapeutic approaches that initially failed to achieve durable responses, which is always attributed to ITH (24).

In this review, we summarize the recent knowledge on the role of epigenetic modifications in TIME and ITH. In addition, the latest clinical therapeutic approaches are discussed. These epigenetic alterations may serve as potential targets for more efficacious therapeutic intervention in cancer.

EPIGENETIC MODIFICATIONS

Epigenetics refers to a special cell events causing heritable phenotypic changes but do not involve alterations in the DNA sequence. Epigenetic modifications involve three different processes, namely DNA methylation, histone modifications, and non-coding RNAs (ncRNAs). They are critical in the regulation of the aberrant expression of tumor-associated genes and encoding of immune checkpoint proteins, tumor suppressors, or oncoproteins in cancer, that contribute to tumor progression and immune invasion (**Figure 1**). Hence, targeting the dysregulation and dynamic nature of epigenetic alterations provides a new strategy for cancer therapy.

DNA Methylation

DNA methylation is a biological process in which methyl groups ($–CH_3$) from S-adenosylmethionine (SAM) are added to the 5' position of the pyrimidine ring of cytosines in the CpG dinucleotide called CpG island. Adenine methylation has been recently observed in mammalian DNA (25), although it has attracted less attention. Gene transcription is silenced when CpG-rich promoters are hypermethylated as these methylated CpGs can impair the binding of transcriptional factors and recruit repressive complexes (26). DNA methylation always represses the expression of tumor-suppressive genes in many types of cancer (27). The process of DNA methylation is mediated by DNA methyltransferases (DNMTs), which include DNMT1, DNMT3a, and DNMT3b (28, 29). DNMT2, a homolog of DNMTs, contains all 10 motifs common to all DNMTs. However, DNMT2 can methylate cytosine-38 in the anticodon loop of aspartic acid transfer RNA (tRNAAsp), instead of DNA (30). In gene promoters, DNA methylation occurs in correlation with gene silencing, whereas in other regions, it modulates enhancer activity, gene activation, and splicing (31, 32). For example, in the promoter region of the *pdcd1* gene, more methylated sites were observed in $PD\text{-}1^{low}$ A20 cells than in $PD1^{high}$ EL4 cells, indicating that DNA methylation occurring in the promoter region silences the expression of PD-1 in T cells (14).

5-methylcytosine (5mC) can be removed *via* oxidation catalyzed by ten-eleven translocation (TET) methylcytosine dioxygenases (TET1, TET2, and TET3), resulting in generation of 5-hydroxymethylcytosine (5hmC), 5-carboxycytosine (5caC), 5-formylcytosine (5fmC), and unmethylated cytosine (33, 34). DNMTs and TETs regulate the gene activation and repression, together maintaining the stability of gene transcription under certain circumstances. Once this balance is interrupted, many genes are abnormally silenced or activated, leading to various pathological conditions, especially cancer (35). In patients with primary breast cancer (PBC) and colorectal cancer (CRC), immune checkpoint proteins PD-L1, CTLA-4, TIGIT and TIM-3 are significantly upregulated with the hypomethylation of promoters because of upregulated TET2 and TET3 (36). The increased levels of immune checkpoint molecules may be one of the causes of repressed activation and function of immune cells in the TIME.

In a pan-cancer analysis result, researchers found that the global loss of DNA methylation is negatively correlated with host immune pathways, including antigen processing and presentation, cytokine–cytokine receptor interaction, and major histocompatibility complex (MHC) (37). In the same study, DNA demethylation has a positive correlation with

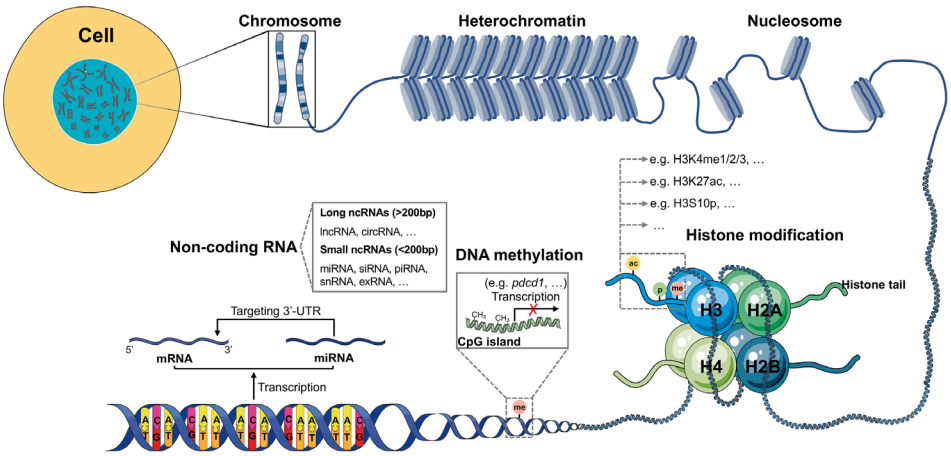

FIGURE 1 | Schematic model of epigenetic regulation. The expression of most human genes is regulated by epigenetic modifications. There are three different epigenetic processes that control gene transcription and expression: DNA methylation, histone modification and ncRNA. DNA methylation always exists in GC-rich areas of the human genome called CpG islands, which can be methylated by DNMTs, resulting in failed transcription of genes, such as *pdcd1*. Histone modification, in which amino acids on four different histone tails (H2A, H2B, H3, H4) can be modified by different enzymes (KMTs, HATs, phosphatases, KDMs, HDACs, among others), results in the regulation of gene expression. In the human genome, many DNA sequences cannot be transcribed into mRNAs but are transcribed as ncRNAs. According to the length, ncRNAs can be divided into small and long ncRNAs. The most investigated ncRNA is miRNA, which targets the 3'-UTR of mRNA, thus contributing to gene silencing.

genomic mutation burden and aneuploidy level, which contributes to tumor cell proliferation (37). Therefore, DNA methylation-modifying agents can be potentially used for cancer therapy or the improvement of the efficacy of cancer immunotherapy. DNA methylation also acts as a modulator of immune cells differentiation. Datasets from the BLUEPRINT Epigenome Project (http://www.blueprint-epigenome,eu) reveal that the global methylation level increases during macrophage differentiation and activation, whereas it acts in an opposite way in T and B cells (38).

Histone Modifications

There are two types of histones: core histones H2A, H2B, H3, H4 and linker histone H1. They can be modified by proteins called "readers," "writers," and "erasers" at the histone tails. The nucleosome core comprises two H2A–H2B dimers and an H3–H4 tetramer. The most frequent histone modifications are methylation, acetylation, and phosphorylation; however, there exist other modifications, including citrullination, ubiquitination, ADP-ribosylation, deamination, formylation, O-GlcNAcylation, propionylation, butyrylation, crotonylation, proline isomerization, and lactylation (39–41). All of these modifications not only activate or repress gene transcription, but also influence several processes, such as DNA repair, DNA replication, and recombination (40). Once histone modifications are aberrantly regulated, the steady state of the cell is disrupted, and diseases, such as cancer initiate, develop, and progress.

Histone Methylation

Unlike DNA methylation, histone methylation involves the addition of methyl groups to mainly lysine (K) (mono-, di-, or trimethylated) and arginine (R) residues (mono- or dimethylated) in the histone tails, which mediate gene transcription, including those cancer progressive and immunosuppressive genes. The six major families of histone lysine methyltransferase complexes (KMT1-6) are responsible for the methylation of lysine residues, mainly on histone H3, followed by H4 (42, 43). The methyl groups added to lysine residues by KMTs can be removed by lysine demethylases (KDMs), which contains six families (KDM1-6) at least (8). The distinct sites or degrees of lysine methylation on histones determine the activation or silencing of many genes. For instance, methylation at lysine 4 on histone H3 (H3K4me1/2/3) and H3K36me2/3 are always involved in the activation of gene transcription, whereas that on H3K9me3 and H3K27me3 exert the opposite function (8, 44). The loss of H3K79me2 in TIME contributes to tumor progression in a mouse model (45). Many immune cell types, such as macrophages, dendritic cells (DCs), and natural killer cells (NKs), can also be regulated by histone methylation in cancer (46–48).

Histone Acetylation

Histone acetylation is involved in the activation of gene transcription by attenuating interactions between histones and DNA *via* the addition of an acetyl group ($-CH_3CO$) from the acetyl coenzyme A (acetyl-CoA) to the α/ε-amino group of lysine side chains, as it neutralizes the positive charge (40, 41, 49, 50). The reversible addition and removal of acetyl groups are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (51). There are two types of HATs (type-A and type-B) found in the human genome, of which, type-B HATs can only acetylate newly synthesized histones, such as H4 at K5 and K12, but not those deposited in the chromatin (40, 52). The well-studied and major families of HATs in humans

include GNAT (HAT1, GCN5, and PCAF), MYST (Tip60, MOF, MOZ, MORF, and HBO1), and p300/CBP (53). On the other hand, the loose chromatin mediated by HATs can be restabilized by HDACs, resulting in transcriptional silencing. HDAC1, a component of the NuRD complex, mediates the histone deacetylation of H3K27 in the promoter region of STAT1, which downregulates STAT1 expression, resulting in type I IFN suppression in TIME (54). HDACs can be classified into four groups (I, II, II, and IV) (53). HDACs, as potential cancer therapeutic targets, have attracted increasing attention due to their role in cancer epigenetics and disease development. Currently, there are four FDA-approved HDAC inhibitors: Vorinostat (SAHA) and Istodax (romidepsin) have been approved for the treatment of cutaneous T-cell lymphoma (CTCL) in 2006 and 2009, respectively; Beleodap has been approved for the treatment of peripheral T-cell lymphomas (PTCL) in 2014; and Panobinostat has been approved for the treatment of patients with multiple myeloma (MM) in 2015 (55). HDAC inhibitors have multiple functions in immunomodulatory activities, including the promotion of the expression of MHC I molecule, tumor antigens, PD-L1, and T cell chemokines, induction of immunogenic cell death hallmarks in tumor cells, and decreasing Treg cells (13, 56, 57). Metabolites, such as butyrate and propionate, produced by the gut microbiome can also inhibit the activity of HDACs (58).

Histone Phosphorylation

Histone phosphorylation, another post-transcriptional modification (PTM) event, occurs mainly at the serine (S), threonine (T), and tyrosine (Y) sites of histone tails and regulates the transcription of genes that are involved in cell cycle and proliferation (27, 59). Histone phosphorylation is correlated with the proliferation and progression of many types of cancer. For instance, decreased H3S10p levels were observed in MDA-MB-231 cells treated with the microRNA-941 inhibitor, which suggests that H3S10p has a potential role in promoting the proliferation of MDA-MB-231 cells (60). The tyrosine 39 of histone H2A.X can be phosphorylated by JMJD6, which leads to triple-negative breast cancer (TNBC) cell growth (61). In castration-resistant prostate cancer (CRPC), researchers have found that histone phosphorylation is positively correlated with cancer cells progression and drug resistance, and its blockade inhibits tumor growth in a CRPC mouse model (62).

Non-Coding RNAs

RNAs that are not translated into proteins are termed as ncRNAs, which represent about 90% of human genome-derived RNAs and contain small ncRNAs, such as microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), small nuclear RNAs (snRNAs), extracellular RNAs (exRNAs), circular RNAs (circRNAs), and long non-coding RNAs (lncRNAs), such as Xist (27, 63). Small ncRNAs are less than 200 bp in length, whereas circRNAs and lncRNAs are more than 200 bp in length (27). The aberrant expression of ncRNAs is always associated with many diseases, including cancer. One of the most widely studied ncRNAs is miRNAs, which are nearly 20 bases long and mediate the cleavage and degradation of mRNAs by targeting the 3'-untranslated region (3'-UTR), thereby leading to translation failure (64).

Thousands of miRNAs have been found to regulate >30% of human genes engaged in the cell cycle, and cell proliferation, differentiation, or apoptosis (65–67). Some miRNAs can act as tumor suppressors by targeting immune checkpoint molecules, such as PD-L1, PD-1, CTLA-4, and TIM-3, in tumor cells, such as ovarian cancer, prostate cancer (PC), and non-small cell lung carcinoma (NSCLC) or immune cells, such as T cells and DCs in the TIME (8). In a glioma mouse model, miR-138 treatment is positively associated with median survival time and negatively correlated with tumor regression (68). While some other miRNAs participate in tumor development. For example, the elevated expression of miR-1269 promotes the formation and progression of gastric cancer and suppresses cell apoptosis by modifying the AKT and Bax/Bcl-2 signaling pathways (69). The overexpression of miR-9 has been confirmed in glioma cells and reported to significantly improve their migration and invasion by targeting COL18A1, THBS2, PTCH1, and PHD3 (70). In cancer immunity, the function of immune cells can also be suppressed by miRNAs (71).

Moreover, emerging evidence has shown that lncRNAs have multiple functions in regulation of cell proliferation, migration, invasion, and apoptosis in cancer progression (72–74). Additionally, lncRNAs may be pivotal regulators of TIME remodeling *via* several mechanisms, including the induction of Treg cells, inhibition of recruitment of macrophages, activation-induced cell death (ACID) of T lymphocytes, and the activation of Ca^{2+}-triggered signaling (75–78).

REPROGRAMING OF IMMUNE CELLS IN TIME

One of the biggest obstacles to cancer therapy is tumor escape from the host immune system. Tumor cells tend to modify the microenvironment around themselves by recruiting and educating immune cells, thereby forming an immunosuppressive area termed as TIME. Immune cells, including innate immune cells and T cells, support tumor expansion *via* various mechanisms, and the critical role of the epigenetic reprograming of these immune cells has been revealed (**Figure 2**). A multi-platform genome-wide dataset of various types of sarcoma demonstrated the correlation between epigenomic alterations and the infiltration of immune cells into the TIME (79).

Innate Immune Cells

Macrophages are a type of white blood cells of the innate immune system that engulf and digest non-self substrates such as cancer cells in a process called phagocytosis. They have also been shown to contribute to tumor growth and progression after epigenetic modification into TAMs, the major infiltrating leukocytes in most malignant tumors. Research groups from the MD Anderson Cancer Center have performed gain-of-function screening of epigenetic regulators in an inducible

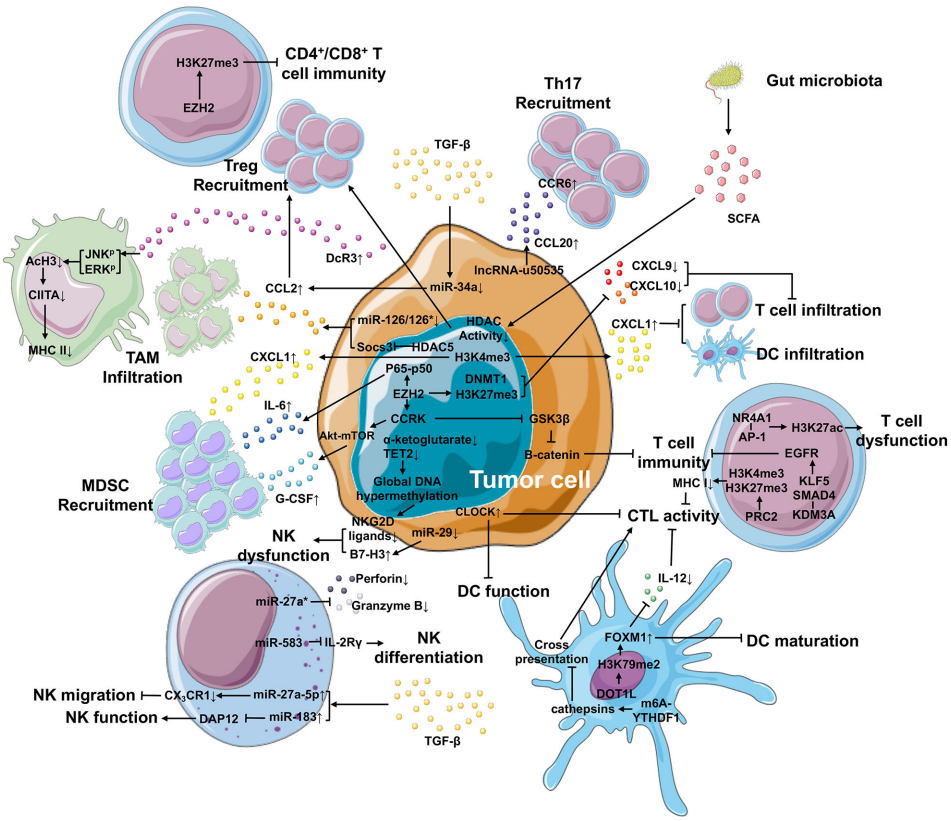

FIGURE 2 | Epigenetic mechanisms in the TIME that contributes to cancer development. Within the TIME, epigenetic regulation plays an important role in generating immunosuppressive environment and facilitating tumor differentiation. In tumor cells, epigenetic regulation is involved in the upregulation of IL-6 and G-CSF and the downregulation of CXCL9 and CXCL10 *via* EZH2, as well as the elevated expression of CXCL1 secreted by tumor cells *via* H3K4me3, leading to improved MDSC recruitment and repressed T cell or DC infiltration, respectively. The expression of CCL2 (responsible for the recruitment of TAM and Treg cells) and CCL20 (responsible for Th17 recruitment) is enhanced by miR-126/126* or miR-34a and lncRNA-u50535, respectively. Furthermore, tumor cells can suppress the function of macrophage-, NK cell-, DC- and T cell-mediated immunity through other epigenetic mechanisms. In the TIME, high TGF-β levels can be produced by not only tumor cells, but also other cell types. TGF-β can regulate the expression of miRNAs in tumor cells and NK cells, suppressing NK migration and function and Treg recruitment. What's more, the gut microbiota releases SCFA that inhibits the activity of HDACs, further improving the recruitment of Treg cells.

$Kras^{G12D}$ p53 null pancreatic ductal adenocarcinoma (PDAC) mouse model and identified that HDAC5 mediates the upregulated expression of chemokine CCL2 by repressing Socs3, resulting in the recruitment of TAMs, which subsequently enables KRAS*-independent tumor growth (80). CCL2 expression is regulated by miR-126/126* in breast cancer cells. Downregulated miR-126/126* by promoter methylation of their host gene *Egfl7* mediates CCL2 upregulation (81). Finally, elevated CCL2 recruit macrophages to promote breast cancer metastasis. MHC II molecules on the surface of macrophages mediate antigen presentation, which is important for the induction of adaptive immune responses. In patients with pancreatic cancer, ERK and JNK induce histone deacetylation at the promoter region of the class II transactivator (CIITA), leading to decoy receptor (DcR3)-mediated downregulation of MHC II expression (82). The loss of MHC II expression impairs the antigen presentation, resulting in TAM-induced immunosuppression (82). The differentiation and polarization of macrophages can also be modulated by the enhancer of zeste homolog 2 (EZH2) (83), a histone methyltransferase and the catalytic subunit of polycomb repressive complex 2 (PRC2), indicating that EZH2 is involved in the reshaping of TIME.

MDSCs ($CD11b^{+}Gr1^{+}$) are a heterogeneous group of immune cells from the myeloid lineage and possess strong immunosuppressive activities in cancer. In breast cancer patients, MDSC levels in the blood are approximately 10-fold higher than healthy individuals (84). Their expansion into the TIME is negatively correlated with poor survival rates due to inhibited $CD8^{+}$ T cell proliferation in hepatocellular carcinoma (HCC) (85). In the same study, upregulated EZH2 interacts with the phosphorylated NF-κB subunit p65, and the EZH2-NF-κB complex binds to the *IL-6* promoter to enhance the expression of IL-6, thereby subsequently inducing MDSC recruitment to the TIME (85). In another study, the Akt-mTOR signaling pathway has been shown to trigger the recruitment of MDSCs to promote tumor initiation (86). And Akt phosphorylation can be mediated by cell cycle-related kinase (CCRK), whose expression can be regulated by EZH2 (87). These findings suggest that MDSCs can

be recruited through distinct mechanisms associated with epigenetic modifications, especially those mediated by EZH2.

DCs are professional antigen-presenting cells (APCs) and act as messengers between the innate and adaptive immune systems. However, their antigen-presenting capacity is abolished in many solid tumors owing to their immature state and low levels of IL-12 production. Mechanistically, forkhead box M1 (FOXM1) expression is enhanced by H3K79me2 that is present in both tumor cells and DCs, which causes abnormal maturation phenotypes of DCs and decreased production of IL-12 in tumor-bearing mice with pancreatic and colon cancers (47). Furthermore, H3K79 is methylated by DOT1-like histone lysine methyltransferase (DOT1L) and the inhibition of DOT1L not only decreased H3K79me2, but also downregulated FOXM1 expression and reversed the immunosuppressive state (47). FOXM1 is reported to be associated with cancer proliferation, angiogenesis, EMT, migration, metastasis, and stemness in many types of cancer (88). A recent study has revealed that the RNA N6-methyladenosine (m^6A) modification is correlated with TIME infiltration in gastric cancer (89). In DCs, the m^6A modification mediated by RNA methyltransferase Mettl3 in the transcripts of CD40, CD80, and TLR4 signaling adaptor Tirap promotes the activation and function of DCs and DC-based T cell response (90). Han et al. have reported that the binding of YTH N6-methyladenosine RNA binding protein 1 (YTHDF1) to the transcripts encoding lysosomal proteases modified by m^6A methylation improved the translational efficiency of lysosomal cathepsins in DCs, whereas the suppression of cathepsins in DCs significantly strengthened its ability to cross-present tumor antigens, which in turn enhanced the tumor infiltrating $CD8^+$ T cell antitumor response (91). Through screening of known epigenetic regulators, the circadian locomotor output cycles kaput (CLOCK), a circadian regulator possessing potential histone acetyltransferase activity, has been shown to have a negative correlation with the function of $CD8^+$ activated T cells and DCs in glioblastoma (GBM) (92). However, further studies are needed to elucidate the epigenetic regulation mechanism of CLOCK in TIME.

NK cells are cytotoxic lymphocytes critical to the innate immune system. Their role is analogous to that of cytotoxic T lymphocytes (CTLs), which recognize target cells such as cancer cells upon the expression of non-self HLA antigens. NKG2D ligands (ULBP1 and ULBP3) on tumor cells are downregulated *via* DNA methylation, resulting in the escape of IDH1 and IDH2 mutant gliomas from NK cells (93). IDH1 and IDH2 mutations cause global DNA hypermethylation because of decreased α-ketoglutarate levels and TET2 function in many cancer types, including acute myelogenous leukemia (AML) (93). The cytotoxicity of NK cells is also regulated by miRNAs. It is well known that B7-H3, a surface glycoprotein, exerts inhibitory effects on NK cells, which abolishes the anti-tumor activity of these cells (94). The downregulation of miR-29 expression in cancer contributes to the B7-H3 upregulation, leading to NK cell dysfunction and tumor immune escape (95, 96). Perforin (Prf1) and granzyme B (GzmB) are key cytotoxic effectors that kill cancer cells for NKs. However, miR-27a* reverses the cytotoxicity of NK cells by silencing Prf1 and GzmB expression (97). Because Prf1 and GzmB are the functional effectors of CTLs, the cytotoxic capacity of CTLs may also be inhibited by miR-27a*. Using a genome-wide mRNA and miRNA database, Yun et al. identified that miR-583 targets the 3'-UTR of the IL2 receptor gamma (IL2Rγ) and acts as a negative regulator of NK cell differentiation (98). The activity of NK cells is strongly repressed by TGF-β, an immunomodulatory cytokine that is released in the TIME. TGF-β induces the overexpression of miR-27a-5p, which targets 3'-UTR of the chemokine receptor CX_3CR1 expressed in several immune cells, resulting in the suppression of the migration ability of NK cells (99). Another TGF-β-induced miRNA is miRNA-183. The miR-183 binds and suppresses the DNAX activating protein 12 kDa (DAP12), an adaptor protein critical for NK cells, to inhibit NK cell function, thus creating an immunosuppressive TIME (100).

T Cells

The key effector cells for tumor eradication are the CD8+ cytotoxic T cells because they directly recognize and kill cells displaying foreign antigens through binding MHC I molecules. The loss of MHC I expression in tumor cells abolishes antigen presentation, thereby contributing to immune evasion. A genome-wide CRISPR/Cas9 screen was performed and identified that PRC2, a complex with histone methyltransferase activity, silences the expression of MHC I *via* bivalent H3K4me3 and H3K27me3 modifications and inhibits the anti-tumor immunity mediated by T cells (101). Simultaneously, the existence of bivalent H3K4me3 and H3K27me3 at the MHC I promoter region in a range of human MHC I-deficient cancers was detected (101). Thus, targeting bivalent H3K4me3 and H3K27me3 may be one of the potent therapeutic approaches in cancer treatment. Another *in vivo* CRISPR screen in a PDA mouse model identified that KDM3A potentially blocks T cell-mediated immune response *via* regulating the expression of epidermal growth factor receptor (EGFR) through the Krueppel-like factor 5 (KLF5) and SMAD family member 4 (SMAD4) (102), which makes KDM3A a potential target for cancer therapy.

ICI therapy for various cancers has revolutionized the standard of care and achieved significant clinical outcomes. Nevertheless, only a limited subset of patients harbors positive feedback after ICI treatment (103). The main reason for this is that the expression of immune checkpoint molecules/ligands is always regulated by epigenetic alterations, including DNA methylation, histone modification, and ncRNAs. Epigenetic regulation of immune checkpoint proteins on T cells can lead to an immunosuppressive TIME through the following effects: less responsive T cells, increased Treg cells, MDSC recruitment, and impaired release of effector cytokines (104). The Cancer Genome Atlas (TCGA) Level 1 methylation data from 30 solid tumor types have revealed that hypermethylated costimulatory genes and hypomethylated immune checkpoint genes are negatively associated with functional T cell recruitment to the TIME (105). To promote the therapeutic efficacy of ICI treatment, methods that can be used to restimulate the expression of immune checkpoint proteins and costimulatory molecules are one of the solutions in cancer therapy.

As mentioned above, EZH2 epigenetically upregulates the expression of CCRK, and CCRK inactivates GSK3β *via* phosphorylation, thus further activating β-catenin in HCC cells (87, 106). In addition, β-catenin signaling in melanoma samples is correlated with the absence of a T cell gene expression signature (107). These results suggest a relationship between EZH2 and $CD8^+$ T cell infiltration within the TIME in melanoma. Regarding to T cell infiltration, CXCL1 overexpression in PDA tumors can diminish the number of infiltrated T cells (108). In this study, a library of congenic cell clones from KPCY tumors was established, and the immune microenvironment was analyzed. In brief, they found that H3K4me3 modification at the *Cxcl1* promoter enhances the expression of CXCL1 in PDA tumor cell clones, leading to low infiltration of T cells and DCs, and the recruitment of MDSCs, which shapes the TIME and influences the outcome of immunotherapy (108). Effector T-cell trafficking to the TIME is mediated by T helper 1 (T_H1)-type chemokines CXCL9 and CXCL10. Whereas, in a human ovarian cancer model, H3K27me3 induced by EZH2 and DNA methylation catalyzed by DNMT1 at their promoter regions repress the expression of CXCL9 and CXCL10 in tumor cells (109). Furthermore, the expression of EZH2 and DNMT1 in tumors is negatively correlated with $CD8^+$ T cell infiltration within the TIME, as well as patient prognosis (109). Therefore, EZH2 can serves as a cancer therapeutic target. Infiltrated T cells may be dysfunctional because of different mechanisms, which may include nuclear receptor subfamily 4 group A member 1 (NR4A1) regulation. NR4A1 is highly expressed in tolerant T cells and can bind to activator protein 1 (AP-1) to promote H3K27ac, which leads to the activation of tolerance-related genes (110).

In the healthy state, Treg cells play a pivotal role in maintaining host immune homeostasis. However, in HCC tumors, TGF-β stimulation leads to the low expression of miR-34a, upregulates CCL2 and finally recruits more Treg cells to the TIME (111). EZH2, an important methyltransferase, is considered as a potent therapeutic target in many cancers. The distinct expression level of EZH2 in Treg cells depends on their locations. Particularly, Treg cells in tumor tissues specifically express high levels of EZH2 and its histone modification H3K27me3 compared with those in non-lymphoid tissues, resulting in tumor tolerance (112). In addition, the EZH2 and H3K27me3 levels are increased only in Treg cells when compared to $CD4^+Foxp3^-$ T cells in tumor tissues (112). Targeting EZH2 in Treg cells remodels the TIME by improving recruitment and function of $CD4^+$ and $CD8^+$ effector T cells that guide antitumor immunity (112). The presence of Th17 cells (a group of $CD4^+$ T cells characterized by RORγ expression and IL-17 production) in the TIME is correlated with poor prognosis in colorectal cancer patients. Th17 cells can be recruited to the TIME *via* the CCR6-CCL20 pathway in cervical cancer due to upregulated CCL20 in tumor tissues and high expression of CCR6 on Th17 cells aggregated within tumor tissues (113). It is a possible that Th17 cells are recruited into the TIME *via* the CCR6-CCL20 axis, thereby contributing to the lncRNA u50535-mediated tumor growth and metastasis of CRC (114, 115). In addition to $CD8^+$ T cells, how to regulate the Treg cells and Th17 cells in TIME is also a viable option to improve the clinical outcome of cancer therapy.

In recent years, the gut microbiota has received increasing interest as they have been revealed ton interact with many human diseases, including cancer not only limited to colorectal cancer but also extraintestinal tumors (116). The gut microbiota can affect the DNA methylation patterns, chromatin structure, and miRNA activity to maintain the host immune system and homeostasis through the microbes themselves or metabolites (117–119). Butyrate, a short chain fatty acid (SCFA) derived by gut microorganisms, inhibits HDAC activities and induces an abundance of Treg cells, leading to tumor suppression in colitis-associated cancer (CAC), a major subset of CRC (120, 121). However, the relationship between HDAC inhibition and Treg cell recruitment in CRC needs to be clarified. Cancer immunotherapy requires microbiota-derived signals because the function of DCs for priming $CD8^+$ T cells is controlled by the gut microbiota through H3K4me3, which activates genes related to immune responses (122, 123). There is limited evidence illustrating the mechanism of epigenetic modification between gut microbiota and TIME, which makes this area being an interesting field for researchers to investigate.

EPIGENETICS IN INTRATUMORAL HETEROGENEITY

ITH is termed as subpopulations of cancer cells with different phenotypes and molecular features within a tumor and also contains heterogeneity of the TIME, resulting in tumor metastasis, drug resistance and tumor relapse (**Figure 3**). Cancer stem cells (CSCs), a small population of stem-like cancer cells within the TIME, are one of the two major frameworks for interpreting the causes of ITH (22). Accumulating evidence suggests that CSCs represent a heterogeneous population of cells that can be regulated by epigenetics, possessing tumorigenicity and metastasis. In breast cancer, the MLL4-mediated H3K4me2 and the CBP/p300-c-Myc complex-mediated H3ac contribute to self-renewal of CSCs by regulating the expression of epithelial–mesenchymal transition (EMT) regulators, such as SNAIL, ZEB1, and ZEB2, in the absence of KDM6A (124). KDM6A (also known as UTX), a component of the MLL complex, recruits LSD1, HDAC1, and DNMTs to form a complex that inhibits H3K4me2 and H3ac, and enhances DNA methylation at the promoter regions of SNAIL, ZEB1, and ZEB2, thereby resulting in abolished CSC self-renewal, tumor proliferation, and migration (124). However, the role of KDM6A in breast cancer remains controversy, and whether KDM6A can serve as a therapeutic target needs to be further investigated. The expansion of CSCs is also promoted by TWIST1, whose expression is elevated by the CBP-mediated H3ac at the promoter, in which CBP degradation is repressed by MTDH, a protein always associated with tumor progression, metastasis, and drug resistance (125). Several epigenetic inhibitors were

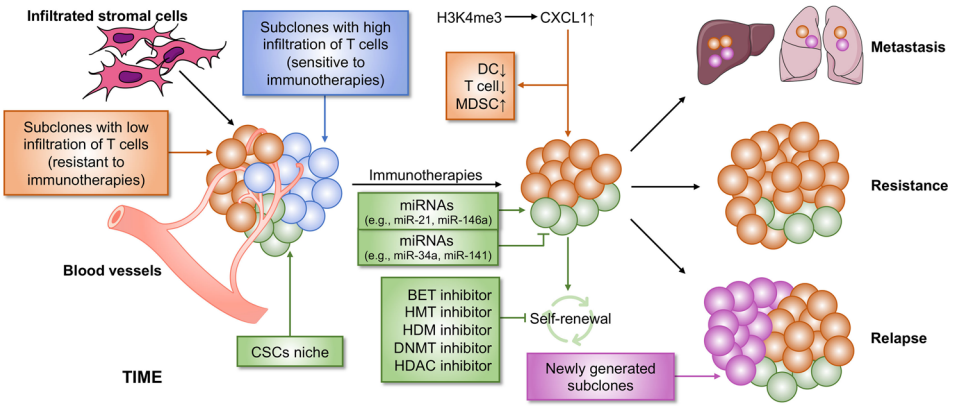

FIGURE 3 | Epigenetic regulation of ITH that contributes to immunotherapy resistance. Cancer is a heterogeneous disease with a complicated TIME, leading to immunotherapy failure. Two main reasons contribute to immunotherapy resistance. First, within the TIME, there are some subpopulations of tumor that are non-responsive to immunotherapy, which causes resistance to immunotherapy. Second, CSCs cause poor clinical outcome in immunotherapy. CSCs may be not completely eliminated by immunotherapy, which subsequently lead to tumor relapse and metastasis. Both the two processes can be regulated by epigenetics, suggesting that a combination of immunotherapy and epi-drugs may be an effective strategy for cancer therapy. BET, bromodomain and extra-terminal domain; HMT, histone methyltransferase; HDM, histone demethylase; DNMT, DNA methyltransferase; HDAC, histone deacetylase.

investigated to block self-renewal of CSCs, including DNMT, HDAC, histone methyltransferase (HMT), histone demethylase (HDM), and bromodomain and extra-terminal domain (BET) inhibitors (126, 127). Additionally, epigenetic regulators, miRNAs, have an ability in modifying CSC development. For example, both miR-34a and miR-141 inhibit prostate cancer stem cells and metastasis by targeting CD44, a CSC marker (128, 129).

CSCs are demonstrated to be involved in immune resistance by multiple lines of evidence in many cancer types and therefore contribute to immunosuppressive TIME. One of the main CSC regulators, c-Myc, that is commonly expressed in many human cancers, can upregulate the expression of immune checkpoint molecules CD47 and PD-L1 (130, 131). Non-autonomously, CSCs from many solid tumors have been proven to be able to release a majority of immunosuppressive factors or cytokines, such as VEGF, TGF-β, IL-4, IL-6, IL10, PD-1, and others, among which many can help recruit suppressive immune cells, including TAMs, Treg cells, and MDSCs, and impair $CD8^+$ T cell function (132, 133). Collectively, CSCs play a pivotal role in the remodeling of TIME to establish an immunosuppressive environment. Multiple therapeutic methods targeting CSCs have sprung up like mushrooms, such as NK cells, CSC-based DC vaccine, CSC-based T cells (including CAR-T), and monoclonal antibodies (133). Overall, targeting CSC-based immunotherapies is a potential effective strategy for cancer treatment.

Another major framework for interpreting the causes of ITH is clonal evolution (22). The concept clonal evolution was proposed by Nowell in 1976 for the first time (134). Throughout the process of tumor development, clonal evolution preferably proceeds in a branching rather than in a linear manner, and this leads to clonal and (epi)genetic diversity in different subpopulations (22). Cancer therapeutic responses in clinical are largely determined by the evolution of resistant subpopulations and the changes in cellular phenotypes (135). Moreover, cancer immunotherapy is mainly dependent on the degree of functional infiltrated T cells, which positively correlates with clinical outcome. However, the number of infiltrated T cells is discriminated among different subclones originating from a single tumor tissue isolated from a PDA mouse model and associated with epigenetic regulation (108).

First, an autochthonous mouse model, including mutated *Kras* and *p53*, of PDA expressing the YFP lineage tag (KPCY) was established. Then, tumor was isolated from KPCY mice and experienced a limiting dilution to generate tumor cell clones. The data showed that TIME is diverse among separated clones, in which low T cell clones correlated with low DC infiltration and high MDSC recruitment. Tumors formed from clones with low T cell infiltration negatively correlated with immunotherapeutic responses, demonstrating that ITH could induce tumor relapse in patients responsive to immunotherapy. Mechanistically, CXCL1 was highly expressed in the tumor clones with low T cell infiltration due to the high levels of H3K4me3 enriched at the promoter region of the *Cxcl1* gene. G-CSF, responsible for MDSC recruitment, was also expressed at high levels in the T cell low tumor clones. However, the exact number of Treg cells was also higher in T cell high clones than in low clones, suggesting a correlation between Treg cells and immunotherapy response, which needs to be further explored. The inhibition of H3K4me3 might be a potential method for eliminating T cell low tumor clones and could be combined with immunotherapy to completely eliminate whole tumor in PDA patients.

CLINICAL TRIALS

The antitumor efficacy of epi-drugs has been proved in preclinical experiments with elevated antitumor immunity.

Many epi-drugs have been applied to clinical trials, and their ability to eradicate cancer has been investigated. Here, we discuss the recent results of clinical trials involved in epi-drugs (**Table 1**).

DNMT Inhibitor

Guadecitabine (SGI-110), a next-generation DNMT inhibitor, is under investigation in clinical trials for its ability of resistance to degradation by cytidine deaminase, leading to a prolonged activity *in vivo*. It has been confirmed that SGI-110 is able to improve the expression of HLA class I molecule on melanoma cells and the number of CD8+ T cells and CD20+ B cells, which demonstrated that SGI-110 has promising immunomodulatory and antitumor capacity (153).

In a phase I clinical trial for PK/PD analysis, 20 patients with recurrent, platinum-resistant ovarian cancer were enrolled and administered with guadecitabine and carboplatin (136). The first six patients treated with 45 mg/m^2 of guadecitabine and carboplatin AUC5 reported neutropenia and thrombocytopenia, while the remaining 14 patients who were treated with 30 mg/m^2 of guadecitabine and carboplatin AUC4 reported no such toxicity. Furthermore, three patients had a partial response (PR) and 15% clinical benefit rate (CBR), and six patients performed stable disease (SD) for more than 3 months with 45% CBR. Additionally, a CA-125 reduction of at least 50% was observed in 5/15 evaluable patients. In summary, this phase I clinical trial demonstrated the efficacy and safety of guadecitabine and carboplatin combination therapy in a platinum-resistant ovarian cancer cohort, supporting a completed Phase II trial (137).

Another phase I trial on guadecitabine was conducted in 22 previously irinotecan-treated patients with metastatic colorectal cancer (mCRC) (138). They were treated across four doses: guadecitabine 30 mg/m^2 with or without growth factor support (GFS) and guadecitabine 45 mg/m^2 with or without GFS. Each patient received 125 mg/m^2 irinotecan at days 8 and 15. At the endpoint of this trial, the median overall survival (OS) was 10.7 months, and 17 patients were evaluable, among which, 12 had SD as the best response and five had PD. Using LINE-1 analysis, global DNA demethylation in tumors was found to be decreased as expected. What's more, guadecitabine 45 mg/m^2 and irinotecan 125 mg/m^2 with GFS showed the least severe side effects in mCRC patients. These findings provide a theoretical basis for a subsequent randomized phase II trial. In elderly non-fit patients with AML, the combination of retinoic acid and decitabine led to a higher remission rate and increased median overall survival, without additional toxicity (147).

HMT Inhibitor

Pinometostat (EPZ-5676) is a first-in-class inhibitor of DOT1L, which plays a central role in Th cell lineage commitment and stability, and has been evaluated as a single agent for the treatment of adult patients with advanced acute leukemia, especially those with mixed-lineage leukemia gene rearrangements (*MLL-r*) leukemia. After treatment, only two patients experienced complete remission at 54 mg/m^2 per day, demonstrating the clinical benefit of EPZ-5676 for *MLL-r* patients (139).

EZH2 is another attractive target for anti-cancer therapy because of its ability in promoting the division and proliferation of cancerous cells and role in regulating immune cells in TIME, including T cells, NK cells, DCs and macrophages (154). Reprograming the TIME by targeting EZH2 is a viable area of cancer research (112, 155). At present, there are three different EZH2 inhibitors, namely tazemetostat, GSK2816126, and CPI-1205, which have been investigated in phase I clinical trials. After treatment with tazemetostat, the most commonly reported adverse event (AE) was asthenia (33%) in 64 patients

TABLE 1 | Recent clinical trials.

Epigenetic inhibitors	Target	NCT number	Conditions	Status	Reference(s)
DNMT inhibitors					
SGI-110	DNMT1	NCT01696032	Ovarian cancer	Phase I	(136, 137)
SGI-110	DNMT1	NCT01896856	Previously treated metastatic colorectal cancer	Phase I/II	(138)
HMT inhibitors					
EPZ-5676	DOT1L	NCT01684150	Advanced hematologic malignancies	Phase I	(139)
GSK2816126	EZH2	NCT02082977	Advanced hematological and solid tumors	Phase I	(140)
CPI-1205	EZH2	NCT02395601	B-cell lymphoma	Phase I	(141)
Tazemetostat	EZH2	NCT01897571	Advanced solid tumors and B-cell lymphomas	Phase I/II	(142, 143)
HDAC inhibitors					
Panobinostat	pan-HDAC	NCT00878436	Recurrent prostate cancer after castration	Phase I/II	(144)
Vorinostat	pan-HDAC	NCT01422499	Relapsed solid tumor, lymphoma or leukemia	Phase I/II	(145)
Vorinostat	pan-HDAC	NCT00731731	Newly diagnosed glioblastoma multiforme	Phase I/II	(146)
Combinations					
Decitabine/Valproic acid/Retinoic acid	DNMT/HDAC	NCT00867672	Acute myeloid leukemia	Phase II	(147)
Romidepsin/5-azacitidine	HDAC/DNMT	NCT01998035	Relapsed/refractory lymphoid malignancies	Phase I/II	(148)
Romidepsin/5-azacitidine	HDAC/DNMT	NCT01537744	Advanced solid tumors	Phase I	(149)
CC-486/pembrolizumab	DNMT/PD-L1	NCT02546986	Advanced or metastatic non-small cell lung cancer	Phase II	(150)
Vorinostat/pembrolizumab	HDAC/PD-L1	NCT02638090	Stage IV non-small cell lung cancer	Phase I/II	(151)
Vorinostat/pembrolizumab	HDAC/PD-L1	NCT02538510	Recurrent squamous cell head and neck cancer or salivary gland cancer	Phase I/II	(152)

(21 with B-cell non-Hodgkin lymphoma and 43 with advanced solid tumors) (142). Among these, no treatment-related deaths occurred, and durable objective response rates were 38 and 5% in patients with B-cell non-Hodgkin lymphoma and solid tumors, respectively (142). GSK2816126, a highly selective inhibitor of EZH2, was applied for the treatment of 41 patients with solid tumors or B cell lymphoma (140). In this trial, 12 (32%) patients had a severe AE, and fatigue (53.7%) and nausea (48.8) were the most common toxicity (140). PK/PD results showed that the half-life of GSK2816126 was approximately 27 h and its maximum tolerated dose (MTD) was 2,400 mg (140). Finally, 14 (34%) patients experienced the best response of SD and 21 (51%) patients had progressive disease (140). CPI-1205, the third selective EZH2 inhibitor, was orally administered twice a day in 32 patients with B-cell lymphomas (141). CPI-1205 had the shortest half-life (~3 h) among the mentioned three EZH2 inhibitors, but induced grade 2 or lower drug-related AEs (141). Among patients, only one achieved a complete response (CR) and five patients had SD (141). Based on these findings, ongoing research needs to be conducted using CPI-1205 in combination in solid tumors (141).

HDAC Inhibitor

HDAC inhibitors have been proved to be able to alter the secretion level of cytokines and chemokines, favoring a Th1 immune response in cancer therapy (156). Panobinostat, a pan-HDAC inhibitor, has been approved by FDA for use in multiple myeloma patients in 2015 and able to improve NK cell-mediated tumor eradication (156, 157). In a phase I/II clinical trial, panobinostat was combined with bicalutamide to treat patients with castration-resistant prostate cancer (CRPC) and restore the resistance to bicalutamide in CRPC patients (n = 64; Phase I: 9; Phase II: 55) (144). In the phase II trial, panobinostat at 40 mg p.o. triweekly was selected as the highest oral dose based on the Phase I trial (144). The median time to PSA progression was 9.4 and 6.3 weeks for the A and B arms, respectively (144). The most common AE for the two arms was fatigue (55 and 65%, respectively), and the toxicity of panobinostat was tolerable with dose reductions (144). Overall, panobinostat, together with bicalutamide, increased rPFS in CRPC patients and reduced androgen receptor-mediated resistance to bicalutamide (144).

HDAC inhibitors can also be combined with DNMT inhibitors for the treatment of lymphomas, AML, and solid tumors. In a phase I study, 5-azacytidine (a DNMT inhibitor) and romidepsin (a HDAC inhibitor) were combined for the treatment of patients with peripheral T-cell lymphoma (PTCL) (148). This combination therapy was well-tolerated in lymphoid malignancy patients and produced a better overall response rate (73%) and complete response rate (55%) in patients with PTCL than in those with non-T-cell lymphoma (148). Combined with the DNMT inhibitor CC-486, romidepsin was investigated in another phase I clinical trial, in which 18 patients with advanced solid tumors were enrolled (149). Although the combination of CC-486 and romidepsin was tolerable, the antitumor effect was not significant (149). Another HDAC inhibitor vorinostat was investigated in two Phase I/II clinical trials as a single agent or in combination therapy (145, 146).

Combination Therapy With ICI

Most patients exhibited no or partial response to ICI therapy, which is attributed to several factors, including tumor mutational burden (TMB), TIME and tumor immune evasion (9). Owing to the function of epigenetic regulation in malignancies, the combination of epi-drugs and ICI therapy may be open a new gate for cancer therapy, especially DNMT inhibitor and HDAC inhibitor (158).

A randomized phase II study was conducted to compare the treatment efficacy and safety of pembrolizumab (PD-L1 mono-antibody) plus CC-486 or placebo in NSCLC patients previously treated with platinum (150). Unfortunately, no improved PFS was shown between pembrolizumab + CC-486 and pembrolizumab + placebo arms. The treatment feasibility might be influenced by AEs, particularly gastrointestinal, thus resulting in non-comparable median OS (11.9 months vs. not estimable) (150).

Two clinical trials, a phase I/Ib and a Phase II, were performed using pembrolizumab and vorinostat combination therapy in patients with NSCLC, and head and neck (HN) and salivary gland cancer (SGC), respectively. The phase I/Ib study demonstrated that pembrolizumab (200 mg) plus vorinostat (400 mg) were the recommended dose which was well tolerated (151). Among the enrolled 33 patients, 30 were evaluable for response: four (13%) had partial response; 16 (53%) had SD; and 10 (33%) had progressive disease (151). In the ICI-pretreated cohort, $CD8^+$ T cell presence in the tumor stromal area was correlated with treatment benefit (151). While MDSCs showed no such association. Another combination therapy involving pembrolizumab and vorinostat was investigated in a phase II trial conducted in 25 HN and 25 SGC patients (152). The toxicities of this combination therapy were more severe than those of pembrolizumab alone reported elsewhere. The median OS and median PFS were 12.6 and 4.5 months and 14 and 6.9 months in the HN and SGC cohorts, respectively. Beneficial responses in SGC were reportedly fewer than those in HN when treated with pembrolizumab and vorinostat, possibly due to the low expression of PD-L1 on SGC.

CONCLUSION

Epigenetic regulation (DNA methylation, histone modification, and ncRNAs) plays a controversial role in cancer initiation and progression, especially in the modification of TIME. Epigenetics-related drugs approved by FDA are proved to be sufficient for cancer therapy, suggesting that targeting epigenetic pathway is a promising strategy for cancer treatment. This strategy can not only induce anti-proliferation of tumor cells, but also shift the TIME from cold to hot. Moreover, the gut microbiota-mediated epigenetic regulation can also influence tumor cells and the host immune system; however, the mechanism by which the microbiota epigenetically shape TIME needs to be further

investigated. Another interesting area of research is the epigenetic regulation of B cell function in tumor development. Because of ITH, therapies targeting each tumor clone and CSCs represent new directions for cancer treatment.

Both pre-clinical and clinical studies have confirmed the antitumor effect of epi-drugs. However, a single epi-drug had not achieved much positive feedback in clinical trials, demonstrating that epi-drugs should be employed in combination with other cancer therapeutic approaches, including chemotherapy, radiotherapy, and immunotherapy, particularly ICI therapy. Due to the toxicity of epi-drugs, ongoing research should focus on how to decrease their side effects. ncRNAs are well-known group of factors that regulate tumor development. Thus, combination of ncRNA-related drugs and immunotherapy may be another potential strategy for cancer treatment in clinical trials.

AUTHOR CONTRIBUTIONS

YY and YW wrote the manuscript. YW critically revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 41931291, No. 81773017 to YW), the Chinese Academy of Medical Science Innovation Fund for Medical Sciences (CIFMS; No. 2019-I2M-1-003), and the Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (2019PT310027).

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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: 13 April 2021
doi: 10.3389/fimmu.2021.641883

Metabolites in the Tumor Microenvironment Reprogram Functions of Immune Effector Cells Through Epigenetic Modifications

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Cellular metabolism of both cancer and immune cells in the acidic, hypoxic, and nutrient-depleted tumor microenvironment (TME) has attracted increasing attention in recent years. Accumulating evidence has shown that cancer cells in TME could outcompete immune cells for nutrients and at the same time, producing inhibitory products that suppress immune effector cell functions. Recent progress revealed that metabolites in the TME could dysregulate gene expression patterns in the differentiation, proliferation, and activation of immune effector cells by interfering with the epigenetic programs and signal transduction networks. Nevertheless, encouraging studies indicated that metabolic plasticity and heterogeneity between cancer and immune effector cells could provide us the opportunity to discover and target the metabolic vulnerabilities of cancer cells while potentiating the anti-tumor functions of immune effector cells. In this review, we will discuss the metabolic impacts on the immune effector cells in TME and explore the therapeutic opportunities for metabolically enhanced immunotherapy.

Keywords: tumor microenvironment, metabolites, immune cell reprogramming, epigenetic modifications, anti-tumor immunity

OPEN ACCESS

Edited by:
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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 December 2020*
Accepted: *15 March 2021*
Published: *13 April 2021*

Citation:
Li Y, Wu Y and Hu Y (2021) Metabolites in the Tumor Microenvironment Reprogram Functions of Immune Effector Cells Through Epigenetic Modifications. Front. Immunol. 12:641883. doi: 10.3389/fimmu.2021.641883

INTRODUCTION

Cancer is one of the leading causes of death globally. Although numerous efforts and progress have been made, curing cancer is still a far-reaching goal thus far. Traditional cancer treatment strategies include surgery, radiation, and chemotherapy. However, other than the common side-effects, studies have shown dire consequences of these strategies, such as higher tumorigenic, metastatic rates, the production of cancer stem cells, the induction of drug resistance, and accelerated aging, etc. (1, 2). Therefore, in recent years, immune cell therapies have attracted increasing attention as one of the best alternative treatment strategies for cancer (3–5). Although promising outcomes have been achieved, such as the application of Chimeric Antigen Receptor (CAR)-T therapy in treating B cell lymphoma (6–8), researchers made limited progress on using immune cell therapy to treat solid tumors. At the same time, our group also developed a new immune cell strategy for cancer immunotherapy, we applied allogeneic Vγ9Vδ2 γδ T cells that originated from healthy donors to treat solid tumors (9, 10) and found that patients respond to this therapy differently. This suggested

that whether adoptively transferred immune cells can function properly in the tumor microenvironment (TME) is the key to successful clinical therapy. Commonly, the negative efficacy can be partly attributed to the complexity and the immunosuppressive nature of the tumor microenvironments (TME). Therefore, to design better immune cell therapies in cancer treatment, scientists need a clear understanding of the multiple aspects that compose and help shape the complexity of TME. It is well known that cancer cells can thrive and meanwhile evade immune cell recognition through "immunoediting" in the TME. Importantly, the acidic, hypoxic, and nutrient-deficient TME provides a competitive advantage to cancer cells to outcompete immune cells (11, 12).

Therefore, an insightful understanding of how TME edits or suppresses infiltrated immune cells is crucial for developing an optimal immune cell strategy to treat solid tumors. Till now, the overview landscape for tumor infiltrated immune cells has been largely established and can be briefly classified into two functional populations, immune suppressive and effector cell. The typical infiltrated suppressive cell includes regulatory T/B cell (T_{reg}/B_{reg}), myeloid-derived suppressor cell (MDSC), M2-like Macrophage, etc., which had been reviewed previously (13–16). As for as infiltrated immune effector cell is concerned, $CD8^+$ cytotoxic T cell, Th1, NK, and γδ T cell are representative populations and have been extensively investigated. In this review, we will mainly focus on current literature of the influence of TME on the immune effector cell, particularly, we are trying to sketch how TME uses metabolites to reprogram infiltrated immune effector cells to accomplish immune escape. Under such context, how cancer cells take advantage of the unique microenvironment to conquer immune cells needs to be briefly introduced at the start of this review.

TME UNIQUELY INHIBITS ANTI-TUMOR IMMUNITY

TME is a Low pH Environment

Malignant cells preferentially use aerobic glycolysis rather than the more energy-efficient mitochondrial phosphorylation as the energy source, known as the "Warburg effect" (17). The end-product of the glycolytic pathway is lactate, the main contributor to the acidic nature of the TME. Studies indicated that lactate could be further used by cancer cells to fuel their metabolism, drive M2 macrophage polarization (18), and severely inhibit the effector functions of cytotoxic, helper T cells (Th1/2, Tc), and natural killer cells in the TME (12, 19–22). Moreover, lactate supports the metabolic need for tumor infiltrated Treg (23, 24), which suppresses effector T cell functions in TME.

Hypoxia is a Hallmark of TME

The uncontrolled cancer cell proliferation inevitably leads to increased oxygen consumption, together with the malformation of the tumor vascular systems, leads to insufficient oxygen supply in the TME, also called hypoxic conditions (25). Hypoxia would further induce Hypoxia-inducible factor-1 alpha (HIF-1α) expression, facilitating the cancer cell adaptation in the oxygen-deficient TME. HIF-1α expression promotes cancer glycolysis and evasion of immunosurveillance, at the same time, tampering with anti-tumor immunity directly by inhibiting NKG2D expression in NK cells (26, 27), reducing $CD4^+$ effector T cell differentiation (28), promoting regulatory T cell differentiation and activity, elevating checkpoint molecule expression (29, 30), as well as inducing T cell apoptosis (31). Moreover, Hypoxia could indirectly drive immunosuppressive metabolites production to support the rapid proliferation of cancer cells (32). Interestingly, the study also demonstrated *in vitro* hypoxic culture conditions would enhance the anti-tumoral functions of $CD8^+$ T cells (33), and research further suggested different T cell subpopulations could respond to hypoxia quite differently. For example, while human $CD8^+$ naïve and central memory T cells were impaired, the functions (proliferation, viability, and cytotoxicity) of effector memory $CD8^+$ T cells could be enhanced in the context of hypoxic conditions (34). These works showed that hypoxia plays various important roles in regulating T cell function (35), and hypoxia-inducible factors (HIF) are involved in mediating the metabolic shift from aerobic respiration to glycolysis as well as enhancing effector function of certain T cell sub-populations in both human and murine (33, 34, 36, 37). Similarly, in mouse $CD4^+$ T cells, augmented HIF activity can promote glycolysis and induce the conversion of Treg into IFN-γ^+ T_H1-like cells (38–40), however, HIF function in human $CD4^+$ T cells remains to be fully addressed. Therefore, a hypoxic condition in TME affects infiltrated immune cells from multiple dimensions. Nevertheless, even though immune effector cells can survive and fulfill functions in hypoxic conditions, functional defects of naive T cell led to failure of its differentiation into the effector T cell, which can eventually compromise the immune balance in the host (**Figure 1**). Additionally, as far as NK is concerned, hypoxia can inhibit the expression of activation-, cytotoxicity-, effector-related molecules of NK cells in both human (41) and murine (42), even though NK cells can still kill target cells *via* antibody-dependent cellular cytotoxicity (ADCC) (41), which suggested HIF-1α behave differently in NK comparing to αβ T cells. Similar to NK, γδ T cells in the TME of mice model also exhibited-hypoxia induced antitumor repression, and HIF-1α also acted adversely (43, 44).

ANTI-TUMOR IMMUNITY OF IMMUNE CELLS IS DISRUPTED IN TME DUE TO LOSS OF THE NUTRITIONAL BATTLE

There is a constant nutrition battle between cancer and immune cells in TME (**Figure 1**). Nutrients such as glucose, amino acids in the TME are often consumed faster by tumor cells than infiltrated immune cells, which thus stripes the energy source that fuels the effector functions of immune cells (45). The imbalance of energy consumption and metabolite productions in the TME further influences the signal transduction and gene expressions among cells in TME,

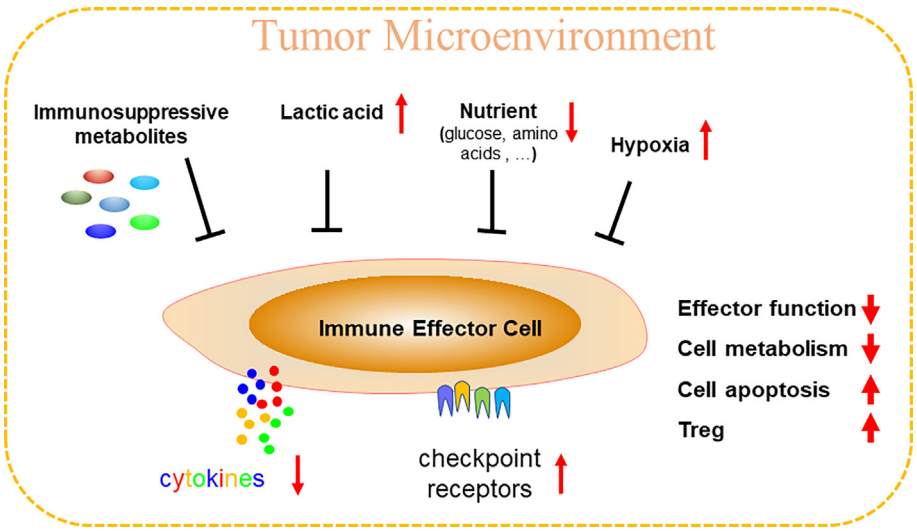

FIGURE 1 | Tumor microenvironment (TME) can specifically inhibit anti-tumor immunity. TME is a hypoxia environment accompanying by high lactic acid and nutritional deficiency, thus produces abundant and various immunosuppressive metabolites. Immune effector cells (cytotoxic T, Th1, NK, γδ T, etc.) in TME are therefore comprehensively inhibited or disrupted, including reducing cytokines release, upregulations of checkpoint receptors, cell cycle arrest, cell metabolism disturbance, increased cell apoptosis, and unfortunately, TME could recruit immunosuppressive immune cells like Treg to reinforce the immunosuppressive microenvironment.

creating an immunosuppressive environment that further supports tumor growth (11). A few elegant studies done by Pearce's group demonstrated that lFN-γ production by effector T cell could be dampened in TME due to the loss of aerobic glycolysis in T cells (46). Their follow-up study further indicated that checkpoint blockade antibodies against CTLA-4, PD-1, and PD-L1 could restore T cell glycolysis and lFN-γ production. Ho et al. showed that glycolytic metabolite phosphoenolpyruvate (PEP) sustains calcium and TCR signaling of effector T cells, increasing PEP production could metabolically reprogram tumor-specific T cell and potentiate their anti-tumor response in TME (47). Such reports suggested that interfering metabolites in TME can rebalance the microenvironment to be suitable for anti-tumor immune effect, and eventually benefit outcomes of tumor immunotherapy. It should be also noted here that inhibited glycolytic metabolism of infiltrated $CD8^+$ cytotoxic T cells in TME does not mean an absolute disaster, because glycolysis inhibition could enhance the generation of neonatal memory $CD8^+$ T cells and antitumor function as well (48, 49). Therefore, the plasticity of infiltrated immune cells should be profoundly understood and be strategically utilized in tumor immunotherapy.

Tuning Amino Acids in TME Regulates Immune Effector Cell Function

Furthermore, amino acid deprivation in TME poses another metabolic challenge to tumor-infiltrated immune cells. For instance, restricting methionine intake from the diet was claimed to effectively slow down tumor growth in the PDX mice model (50), nonetheless, critically impaired T cell effector functions as well as T_H17 differentiation (51, 52). T cell responds to antigenic challenge in the TME by upregulating its amino acid intake to fuel its effector function. This is a process coordinated by the T cell antigen receptor (TCR) and determines T cell differentiation (53). For instance, glutamine is an important amino acid for the proper development of both cancer cells and tumor-infiltrated immune cells. Glutamine regulates mTOR activation (54) and O-GlcNAcylation (55) in effector T cells, which are keys stages for T cell development and function. It is also the main carbon source for the oncometabolite 2-hydroxyglutarate, which regulates the functions and differentiation of effector T cells (56). Nevertheless, conflicting results have been shown on whether limiting glutamine metabolism could strengthen anti-tumor functions of effector T cells (57–59). Recent studies have demonstrated the essential roles of other amino acids such as Arginine (60–62), leucine (63), serine (64) in modulating T cell proliferation and anti-tumor efficacy. However, due to the complexity of tumor infrastructure, the distribution and variation of these nutrients within TME still await further elucidation.

Since there is metabolic plasticity in immune cells, it might be plausible to metabolically target cancer and immune cells (glutamine, methionine, etc.) to enhance the immune effector cell function while inhibiting cancer progression. In this context, it is an urgent need to better understand the roles of different TME metabolites and their related metabolic pathways in TME.

Lipid Metabolism Regulates Immune Effector Cell Function in TME

Lipid metabolism is mainly comprised of fatty acid and cholesterol metabolism (65). Lipid metabolism could regulate tumor-infiltrated immune cells, for example, modulate Treg

functions through influencing mitochondria integrity (66). Effector T cell activation and proliferation require accelerated lipid synthesis and cholesterol uptake since both are crucial components of the cellular membrane. These processes are mediated by transcription factor sterol regulatory element-binding proteins (SREBPs). The lack of functional SREBPs signal in CD8+ T cells leads to attenuated clonal expansion and effector functions (67); as a contrast, increasing cholesterol content in the plasma membrane can enhance CD8+ T cell anti-tumor functions (68). This could be interpreted by a previous report that memory CD8+ T cells rely on cell intrinsic-lipolysis to synthesize fatty acid whereas effector CD8+ T cell (Teff) obtained fatty acids from the external microenvironment (69). Therefore, lipid metabolism was considered to regulate the balance between Treg and Teff in TME (70). Nevertheless, it also showed that high cholesterol in TME could induce CD8+ T cell exhaustion by overexpressing immune checkpoints, such as PD-1, TIM-3, LAG-3, and 2B4, and increasing endoplasmic reticulum (ER) stress (71). Such discrepancy might attribute to the heterogeneity of TME in different cancer types, thus, albeit important for effector T cell metabolism and function, targeting lipid or cholesterol metabolism to potentiate anti-tumor response requires further investigation.

Though metabolic pathways such as glycolysis and oxidative phosphorylation (OXPHOS) are seemingly critical for the thriving of both cancer and infiltrated immune cells, considerable metabolic heterogeneity and plasticity allow us to differentiate the two populations. The advent of single-cell sequencing technologies enables metabolic profiling of TME at a single-cell resolution. For instance, a previous single-cell study revealed a metabolic heterogeneity among cells in TME, with mitochondrial programs being the most distinguishing factor in shaping this heterogeneity in malignant cells and immune cells (72). Metabolites and immunosuppressive characteristics and cellular networks in TME also help shape the metabolic phenotypes and functions of immune cells (**Figure 1**). Therefore, discerning and understanding the diverse metabolic requirements of infiltrated immune cells that work concertedly against cancer cells enable researchers to selectively modulate immune cell functions (73). The knowledge on the minute discrepancy in metabolic dependency between cancer and immune cells provides opportunities for uncovering new therapeutic targets.

TME EPIGENETICALLY REGULATES IMMUNE EFFECTOR CELL FUNCTIONS

"Epi", a prefix from Greek, literally means "upon, over", thus epigenetics is the research focus on sets of instructions directed upon the genome, which is composed of chromosomes. Epigenetics studies focus on understanding the heritable changes in gene expressions that do not involve DNA sequence alteration (74). DNA sequences and histone proteins form nucleosomes, the building blocks of chromosomes. Histones provide structural support to help organize and condense DNA. The epigenetic instructions on the genome are sets of chemical modifications, such as methylation, acetylation, etc. made directly to the DNA bases or histone proteins that wrap around them. Different from genetic coding, epigenetic modifications are reversible and dynamic, allowing changes made as the needs of the cells shift. The existence of epigenome allows the fine-tuning of gene expressions in cells. Normally, epigenetic modifications on the genome are a routine occurrence that maintains the healthy balance of the body by instructing the body to turn "on" or "off" certain genes completely as well as slightly "up" or "down" as required. Therefore, it plays critical roles from determining cell fate to directing cellular functions. Nevertheless, dysregulated epigenetic modifications are common in cancer and other diseases (75–78). Drugs that target cancer cell epigenome also achieve positive outcomes (79–82). Studies in recent years also demonstrated the critical role of epigenetic modifications in immune cell functions (83–86). Progress has been made on developing epigenetic immunotherapy for cancer treatments (85, 87). Therefore, more insightful elucidation of epigenetic regulations of both immune cell function or dysfunction in the TME could inevitably help design more effective immunotherapeutic strategies for cancer.

As for epigenetic modifications, there are at least three epigenetic mechanisms that are under intensive investigation, which include: DNA methylation, histone modifications, and non-coding RNA (ncRNA)-associated gene silencing. ncRNA-associated gene silencing is an emerging field that deserves its own comprehensive review (88, 89). Therefore, in this review, we only focused on illustrating the epigenetic modifications of DNA and histone proteins in TME (**Figure 2**).

TME Stress Induces DNA Methylation of Immune Effector Cells

DNA methylation is the earliest discovered and heavily studied epigenetic modification. It is a chemical process that adds a methyl group ($-CH_3$) to the DNA thereby modifying the expression and functional status of genes. This process is catalyzed by DNA methyltransferase (DNMT) and uses S-adenosyl methionine (SAM) as the methyl group donor (90–92). In a pan-cancer context, Mitra et al. explored and discovered varying levels of CpG methylation of immune cell-type-specific genes that are related to patient survival (93). A comprehensive retrospective paper emphasized the importance of clarifying the DNA methylation sites for the development of cancer biomarkers (94). Point mutation of NADP (+)-dependent isocitrate dehydrogenases IDH1(R132H), which occur frequently in glioblastoma, acute myeloid leukemias, etc., showed a strong correlation between tumorigenesis and specific DNA hypermethylation signatures (95). Moreover, accumulating studies also revealed DNA methylation of cancer cells can modulate both cancer and infiltrated immune cell functions in TME. By analyzing sequencing datasets from BLURORINT Epigenome Project, Schuyler et al. discovered distinctive trends in methylation patterns of innate and adaptive immune cells in TME, suggesting distinct lineage-specific epigenetic mechanisms in regulating tumor infiltrated immune cells functions (96). Specific DNA methylation alterations in the circulating immune cells of cancer patients

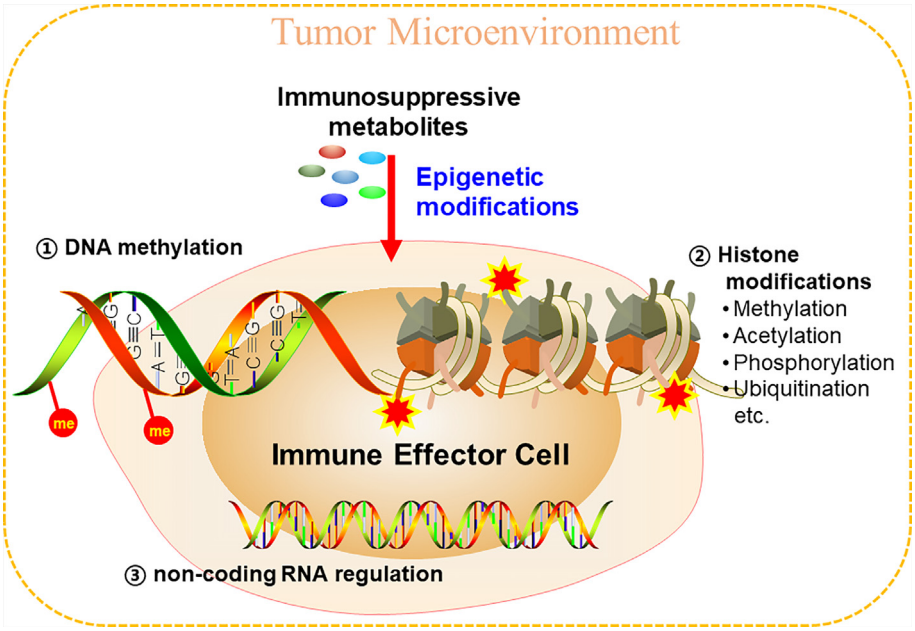

FIGURE 2 | Metabolites in TME could epigenetically reprogram immune cells to inhibit anti-tumor immunity. Epigenetic modifications mainly include three aspects, DNA methylation, histone modifications, and non-coding RNA regulations.

have been observed in head and neck squamous cell carcinoma (HNSCC) (97), ovarian (97, 98), colorectal (99), hepatocellular carcinoma (HCC) (100), and breast cancer (101). Due to their ability to reactivate genes such as tumor suppressors and further elicit immunity towards tumor cells, the development of DNA methylation inhibitors together with immunotherapies, present new cancer treatment opportunities (102).

TME Stress-Induced Histone Modifications of Immune Effector Cells Remain Largely Unclear

Covalent post-translational modification (PTM) modifications of histone, including acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, etc., impacting gene expressions by changing chromatin structures, making it either accessible (euchromatin) or inaccessible (heterochromatin) for gene transcriptions (103, 104). Among these epigenetic modifications on histones, acetylation and methylation gained the most attention. Histone acetylation is the addition of an acetyl group to the lysine residues at histone tails. This reaction is catalyzed by histone acetyltransferases and utilizes acetyl CoA as the acetyl group donor. Upon acetylation, the overall charge on histone tails changes from positive to neutral, weakening the interaction between DNA and histone, therefore facilitating gene transcription. On the other hand, histone deacetylation removes the acetyl group from lysine residues of histone tails, making the chromatin highly condensed and inaccessible for transcription. Thus, the balance between euchromatin and heterochromatin could be tightly regulated by histone acetylation and deacetylation (105, 106). Nonetheless, studies showed that histone acetylation/deacetylation status were dysregulated in cancer development (107, 108), such as cervical cancer (109), breast cancer (110), leukemia (108), and non-small cell lung cancer (111, 112). Like histone acetylation, methylation at the histone tails also regulates gene expression (113, 114). Histone methylation takes place at both arginine and lysine residues at histone tails and comes in three different flavors-monomethylated, dimethylated, and trimethylated. Dysregulation of histone methylation has been shown in causing premature aging and cancers (115), such as colorectal cancer (116, 117), glioblastoma (118), and prostate cancer (119). However, how histone of immune effector cells is modified in TME remains to be further investigated, although Silva-Santos' group investigated the histone methylation patterns and their effect on transcription factors for γδ T cell differentiations in TME of mice model (120). Notably, different inhibitors for histone deacetylase could lead to either suppressed (121) or enhanced (122) human γδ T cell antitumor activity. Thus, histone modification in immune effector cells shall be an interesting research field of antitumor immunity.

TME METABOLITES EPIGENETICALLY REPROGRAM BOTH INNATE AND ADAPTIVE IMMUNE EFFECTOR CELLS

The immunosuppressive nature of TME, mediated by direct comprehensive cell-cell contact and soluble factors such as metabolites, results in alterations in gene expressions in infiltrated immune cells that are partly driven by epigenetic programs. Although extensive efforts have been made on analyzing the histone and DNA epigenetic modifications of cancer cells, little is known about the mechanisms of epigenetic

dysregulation of immune cells in the tumor niche (123, 124). Recent findings indicated that immune cells, especially tumor infiltrated ones, show metabolic reprogramming on their differentiation and effector functions. Ovarian cancers-imposed glucose restriction on tumor infiltrated T cells and dampened their function through epigenetically dysregulating histone methylation patterns (125). It's increasingly considered that both the innate and adaptive arms of the immune network in TME are epigenetically regulated by TME metabolites (e.g., glucose, glutamine, lactate, αKG, 2-HG, etc.).

In the innate arm of the immunity, studies showed that the lineage commitment of myeloid and lymphoid lineage cells is regulated by DNA methylation (126–128). In the myeloid lineage, epigenetic modifiers, including Tet methylcytosine dioxygenase 2 (TET2), isocitrate dehydrogenase 1 (IDH1), IDH2, enhancer of zeste homologue 2 (EZH2) are mutated and lead to defects in DNA and/or histone epigenetic modifications in several myeloid malignancies, such as chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) (129, 130). Zinc Finger E-Box Binding Homeobox 1 (ZEB1), a transcription factor that acts as a tumor suppressor in T-cell acute lymphoblastic leukemia (T-ALL), is repressed due to histone deacetylation and chromatin condensation at its promoter (131).

In the adaptive arm of the immunity, Bian et al. found that by manipulating methionine metabolism in TME, tumor cells lower histone di-methylation at lysine 79 of histone H3 (H3K79me2) in CD8+ T cells, leading to low effector gene expression thus impaired effector T cell immunity. Furthermore, inhibition of the specific and sole methyltransferase for H3K79: DOT1 of CD8+ T cells both *in vitro* and in mice led to the loss of H3K79me2 thus impaired cytotoxicity of CD8+ T cells, which supported their observations in TME (51). Methionine has also been shown to play an essential role in Th17 differentiation and function by regulating histone methylation (52). 2-hydroxyglutarate (2-HG), an oncometabolite caused by IDH mutations that frequently occur in gliomas and acute myeloid leukemia, led to genome-wide histone and DNA methylation alterations (132). S-2-hydroxyglutarate (S-2-HG) in TME could mediate CD8+ T cell differentiation by modulating DNA and histone demethylation status in mice (56). A recent study also indicated that the loss of 2-HG production directly reduced methylation of the Foxp3 gene locus, increasing Fox3 expression, thus reprograms T_H17 differentiation towards Treg cells (133). Moreover, low glucose availability in TME restricts acetyl-CoA level, the acetyl group donor for histone acetylation (134), and Qiu et al. demonstrated that acetate supplementation rescued CD8+ T cell effector function in a glucose restricted environment by promoting histone acetylation and chromatin accessibility thus promoting IFN-γ production of T cells in TME (135). Besides glucose restriction, glutamine deprivation resulted in the differentiation of immunosuppressive regulatory T (Treg) cells from naive CD4+ T cells due to the loss of α-ketoglutarate (αKG), the glutamine-derived metabolite that is needed for DNA demethylation and regulates CD4+ T cell T_H1 differentiation. Nevertheless, the addition of αKG analog could shift the differentiation towards that of a T_H1 phenotype (136). Therefore, although the underlying molecular mechanisms on how TME metabolites serve as activators or inhibitors for epigenetic modifications in immune cells need to be further elucidated, manipulation of metabolic conditions of T cells, particularly effector T cells would provide a potential alternative strategy in the application of T cell-based immunotherapy.

A NEW FRONTIER OF CONDITIONING METABOLISM TO ENHANCE IMMUNE EFFECTOR CELL FUNCTIONS IN IMMUNOTHERAPIES

Recent advances on epigenetic modification strategies in cancer treatment provide us mechanistic insights into the interplay of immune and tumor cells with their environmental cues (80, 87). DNA methylation inhibitors alone or coupled with other inhibitors to target the epigenetic processes, such as histone deacetylases, methylases, and demethylases, are becoming important treatment regimens in certain cancers, especially hematological malignancies. The epigenetic reprogramming of TME in combination with immunotherapies opens a new therapeutic window for more effective cancer therapies (102). Epigenetic therapies that coupled epigenetic immune modulation with immune therapy priming achieve satisfying preclinical and clinical results in various gastrointestinal cancers (117, 137). Combining DNA-demethylating agents with histone deacetylase inhibitors (HDACis) in non-small-cell lung cancer (NSCLC) treatment regimen reversed tumor evasion and led to robust T cell anti-tumor response (138). Zou group demonstrated DNA methylation by enzyme DNMT1 and histone H3 lysine 27 trimethylation (H3K27me3) by enzyme EZH2 in tumor led to epigenetic silencing of T helper 1 (T_H1) type chemokine, and subsequent undermined effector T cell trafficking to TME. Using epigenetic modulators (5-AZA-dC, GSK126, etc.) to target these two enzymes could reprogram T cells for more effective T cell immunotherapy (85).

Studies showed that the functions of chromatin-modifying enzymes such as histone acetyltransferases, deacetylases, and DNMT strongly depend on metabolic signals such as acetyl-CoA, Nicotinamide adenine dinucleotide (NAD), and SAM in TME, epigenetically modulating CD8+T cells activation and exhaustion (139). Moreover, metabolites in TME could also upregulate immune checkpoint molecule expressions (140, 141) and suppress immune cell activation (142–144), leading to dampened efficacy of the immune therapies (145). Therefore, metabolic conditioning of CD8+ or other immune cell functions in TME might help overcome the current weaknesses of immune cell-based immunotherapies. Recent findings in immune cell metabolic reprogramming indicated the possibilities of clinical metabolic interventions for cancer treatment (12, 146). Metabolic intervention by sodium bicarbonate helps neutralize the lactate acidity in AML, leading to improved efficacy of CD8+T cell immunotherapy (147). Pearce group showed that transient glucose restriction (TGR) in CD8+effector T cell before adoptive

transfer metabolically condition effector T cell functions and enhance tumor clearance in mice (148). Additionally, clinical studies on epigenetic therapy for cancer have been previously reviewed (81, 149), showing that targeting epigenetic modifications or regulators in cancer cells would potentiate anti-tumor immune therapy.

SUMMARY

In this review, we focused on immune effector cells in TME and reviewed literature about how epigenetic modifications, in the form of DNA methylation and histone acetylation/methylation, can be modulated by metabolites and other environmental cues in TME. We also discussed the current advances in using metabolic modifiers to epigenetically enhance the efficacy of immune cell therapy. From this review, one can see that immune effector cells in TME are comprehensively reprogramed to be either exhausted effectors, by-standers, or conspirators of cancer cell escape, and metabolites in TME participate in this ugly job. Nevertheless, opportunities coexist with the crisis, targeting TME metabolites could potentially be a valuable supplement to the application of immune cell-based immunotherapy for cancer.

AUTHOR CONTRIBUTIONS

YL, literature research and summary. YW and YH, manuscript writing and revision. All authors contributed to the article and approved the submitted version.

FUNDING

YH was supported by the National Natural Science Foundation of China (NO. 82002787); YW was supported by the Natural Science Foundation of Guangdong Province, China (2020A1515010132).

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frontiers in Immunology

REVIEW
published: 04 May 2021
doi: 10.3389/fimmu.2021.672328

Epigenetic Regulation of NK Cell-Mediated Antitumor Immunity

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Natural killer (NK) cells are critical innate lymphocytes that can directly kill target cells without prior immunization. NK cell activation is controlled by the balance of multiple germline-encoded activating and inhibitory receptors. NK cells are a heterogeneous and plastic population displaying a broad spectrum of functional states (resting, activating, memory, repressed, and exhausted). In this review, we present an overview of the epigenetic regulation of NK cell-mediated antitumor immunity, including DNA methylation, histone modification, transcription factor changes, and microRNA expression. NK cell-based immunotherapy has been recognized as a promising strategy to treat cancer. Since epigenetic alterations are reversible and druggable, these studies will help identify new ways to enhance NK cell-mediated antitumor cytotoxicity by targeting intrinsic epigenetic regulators alone or in combination with other strategies.

Keywords: natural killer (NK) cells, epigenetics, DNA methylation, histone modification, transcription factor, microRNA, antitumor immunity

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

Received: *25 February 2021*
Accepted: *19 April 2021*
Published: *04 May 2021*

Citation:
Xia M, Wang B, Wang Z, Zhang X and Wang X (2021) Epigenetic Regulation of NK Cell-Mediated Antitumor Immunity. Front. Immunol. 12:672328. doi: 10.3389/fimmu.2021.672328

INTRODUCTION

Natural killer (NK) cells are potent effector lymphocytes of the innate immune system. They serve as the first line of defense against infected or transformed cells without prior sensitization. Compared with T and B cells, which recognize targets by their antigen-specific cell surface receptors (TCRs/BCRs), NK cell activation is controlled by the balance between activating and inhibitory signals from multiple germline-encoded receptors. These cells patrol for potential target cells that lack major histocompatibility complex class I (MHC I) or overexpress ligands to activate NK cell receptors (NCRs) (1). NK cells are initially recruited to the tumor microenvironment (TME) during the tumor killing process and then are activated by complex signals arising from multiple ligand-receptor interactions. Activated NK cells release cytotoxic granules containing perforin and granzyme B upon forming an immunological synapse with the target cells (2). Perforin forms pores in the membrane of target cells, thus allowing granzymes to enter the cell and initiate cell death (3, 4). NK cells can also induce cell apoptosis through the engagement of Fas ligands (FasL) or tumor necrosis factor-related apoptosis-inducing ligands (TRAIL) with Fas and TRAIL receptors on tumor cells (5, 6). In a process known as antibody-dependent cell cytotoxicity, NK cells recognize opsonized tumor cells *via* Fc receptors (CD16) and kill them by releasing cytolytic granules. Lysis

leads to an increased release of tumor antigens and further primes adaptive immune responses. In addition to direct cytotoxic activity, NK cells can function as central communicators of innate and adaptive immunity in the TME by secreting multiple chemokines (CCL3, CCL4, CCL5, and XCL1), cytokines (IFN-γ, TGF-β, and IL-10), and growth factors (GM-CSF) (7). In this way, these cells communicate with various immune cells within tumor tissues, including monocytes, granulocytes, dendritic cells, T cells, and stromal cells (8).

NK cells play important roles in cancer immunosurveillance, particularly by eliminating early tumors and metastasis (minimal disease). In 1970s, several groups found non-MHC-restricted antitumor activity of NK cells in mice (9–12). Later, the rapid and potent cytotoxicity of NK cells against target cells was also observed in humans (13). Furthermore, an eleven-year follow-up study found that the impaired NK cell killing capacity in the peripheral blood is correlated with tumor incidence and prognosis (14). Compared with the role of T cells in antitumor immunity and adoptive cellular therapy, NK cells have certain advantages and greater potential "off-the-shelf" utility (7). They are as effective as T cells (15, 16) but less toxic because they cause fewer immune-related adverse events. Mature NK cells are effector cells with a broader reactivity to tumors due to their independent recognition of specific receptors and antigen presentation by MHC molecules. Their lytic responses can be triggered within minutes without clone selection and differentiation (1). The "ready-to-go" state is associated with the unique epigenetic features of NK cells, as shown in the following sections.

NK CELL PLASTICITY

NK cells are a heterogeneous and plastic population. They are classically defined as $CD3^-CD56^+$ cells in humans and divided into two major subsets, $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^{low}$ (17–19). $CD56^{dim}CD16^+$ subsets are highly cytotoxic effector cells that are predominantly found in peripheral blood. $CD56^{bright}CD16^{low}$ subsets are recognized as immature NK cells with immune regulation functions through cytokine secretion. They preferentially reside in secondary lymphoid organs, such as lymph nodes. The surface markers of murine NK cells vary depending on the mouse strain. In C57B/6 and SJL mice, NK cells express NK1.1, NKp46, and CD49b (2). For other strains, such as BALB/c, NK cells express CD49b and NKp46 while possessing allelic variants of NK1.1 (2). Tumor necrosis factor receptor superfamily member CD27 and the integrin CD11b are used to mark NK cell differentiation in mice. The most cytotoxic NK cells are recognized as $CD27^-CD11b^+$, regulatory NK cells are $CD27^+CD11b^+$, and immature NK cells are $CD27^+CD11b^-$ (20, 21).

NK cells belong to the family of innate lymphoid cells (ILCs). NK cells and ILC1s are grouped into group I innate lymphoid cells (22). ILC1s reside in tissues and function as cytokine secretors. Conventional NK (cNK) cells and ILCs arise from distinct progenitors (23). However, many surface markers initially described on NK cells, such as CD122, NK1.1, and NKp46, can be expressed on ILC1s (24). The mixed phenotype can be explained by imprinting the effects of the tissue microenvironment and cell activation state. Therefore, at present, the definition of NK cells based on their phenotype is essentially at a steady state (24). The majority of human mature NK cells can be identified as $CD3^-CD127^-CD7^+CD56^+$ (or $NKp46^+$)T-bet^+Eomes^+ lymphocytes, and mature mouse NK cells can be identified as $CD3^-CD127^-NK1.1^+$ (or $NKp46^+$)T-bet^+Eomes^+ lymphocytes. There are no markers that can unambiguously distinguish NK cells and ILC1s in human or mouse tissues during infection or inflammation (25).

The conversion between NK cells and ILC1s in the TME was recently described (26). Transforming growth factor-β (TGF-β) in the TME could drive NK cells ($CD49a^-CD49b^+Eomes^+$) to convert into intermediate ILC1 (intILC1, $CD49a^+CD49b^+Eomes^+$) populations and ILC1 ($CD49a^+CD49b^-Eomes^{int}$) populations. IntILC1s and ILC1s are less cytotoxic and cannot control local tumor growth and metastasis (27). SMAD4, which is a unique common SMAD, acts as a central mediator that facilitates the canonical TGF-β signaling pathway (28). TGF-β induces salivary gland ILC differentiation by suppressing Eomes through a JNK-dependent, Smad4-independent pathway (29). However, *Smad4* deficiency does not affect ILC1 differentiation but surprisingly alters the phenotype of cNK cells. Cortez et al. reported that *Smad4*-deficient NK cells showed features of ILC1s and lost effector functions to control tumor metastasis. Mechanistically, SMAD4 restrained noncanonical TGF-β signaling mediated by the cytokine receptor TGFβR1 in NK cells (30). A subsequent study by Wang et al. showed that selective deletion of *Smad4* in NK cells led to impaired NK cell maturation, NK cell homeostasis, and NK cell immune surveillance against melanoma metastases and cytomegalovirus. These changes were associated with a downregulation of granzyme B (*Gzmb*), *Kit*, and *Prdm1* in *Smad4*-deficient NK cells and independent of canonical TCF-β signaling (31).

Of note, it has become increasingly clear that various subsets of tissue-resident NK (trNK) cells exist, which differ from cNK cells in their origin, development, and function (reviewed in Ref. 32-34) (32–34). Unlike circulating and widely distributed cNK cells, trNK cells were found to populate multiple tissue sites, including the liver, lung, skin, uterus, salivary gland, adipose tissue, and kidneys (32). trNK cells are distinct from cNK cells in the expression of surface markers and transcription factors. For example, murine liver trNK (LrNK) cells express relatively low levels of NK cell maturation-associated markers, such as CD11b, CD49b (DX5), and Ly49 receptors (35). The development of LrNK is independent of Eomes, while T-bet, Hobit, PLZF, and AhR are more critical for LrNK cell development than cNK cells (34). trNK cells are actively involved in multiple processes, such as antiviral infection, mediating immune tolerance, and promoting fetal growth (34). The accumulation of LrNK cells in hepatocellular carcinoma patients is correlated with poor prognosis (36), suggesting a potential role in tumor development. More comprehensive studies are needed to investigate the role of trNK in antitumor immunity.

Although historically known as innate lymphoid cells, NK cells can also achieve memory characteristics similar to those of adaptive immune cells, such as antigen specificity, longevity, and enhanced recall responses. Memory NK responses were first reported in mouse models of anti-murine cytomegalovirus (MCMV) infection (37) and delayed hypersensitivity reactions to chemical haptens and viral antigens (38, 39). During secondary MCMV infection, memory NK cells bearing the virus-specific Ly49H receptor can rapidly proliferate, degranulate and produce cytokines by recognizing the MCMV-encoded glycoprotein m157 (37). Memory NK cells have also been described in humans expressing NKG2C in HCMV-seropositive individuals (40). Growing evidence suggests that memory-like NK cell responses may occur in response to a broader range of viral, bacterial, and even eukaryotic pathogens (41). The responses of memory-like NK cells against tumors are poorly understood, and two key questions remain to be answered: (1) whether NK cells can acquire memory properties during the antitumor process and (2) whether memory NK cells from infection models can acquire stronger *in vivo* killing capacity targeting tumor cells.

Compared with cNK cells that live less than ten days (42, 43), memory NK cells can persist for years in some individuals and are important for controlling CMV throughout life (44, 45). Similar to $CD8^+$ T cells, NK cells also exhibit an "exhausted" phenotype in individuals with malignancies or chronic viral infections. This phenotype is represented by a loss of activating receptors (e.g., NKG2D) and increased expression of checkpoint receptors (e.g., NKG2A, TIGIT, PD-1, TIM-3, LAG-3), which severely impair their antitumor function (46). Compared with the "suppression" state, which is reversible after the withdrawal of inhibitory signaling, the "exhaustion" state is not transient and undergoes stable epigenetic changes (47). Antagonistic antibodies (Abs) (e.g., anti-PD-1, anti-TIGIT, and anti-NKG2A monoclonal Abs) can recover NK cell antitumor capacity (46, 48). However, epigenetic intervention should be considered to reactivate exhausted NK cells intrinsically in future studies.

EPIGENETIC REGULATORS MODULATING NK CELL-BASED ANTITUMOR IMMUNITY

Epigenetic alterations are reversible and heritable changes that do not alter DNA sequences, including DNA methylation, posttranslational modifications of histone proteins, changes in transcription factors, and noncoding RNA expression. Despite the deep understanding of NK cell biology, research on epigenetic regulation of NK cell function is just beginning. In this review, we provide an overview of the epigenetic regulators that modulate NK cell-based antitumor immunity, and the findings will hopefully help to identify novel approaches and potential targets for tumor immunotherapy.

DNA Methylation

DNA methylation is a heritable epigenetic marker that correlates with gene repression. During the terminal differentiation process, NK cells gradually acquire the ability to produce IFN-γ through demethylation and epigenetic remodeling at the IFNG promoter (**Figure 1**) (49). DNA methylation has been reported to correlate with the gene expression of a variety of NK cell receptors, including killer Ig-like receptors (KIRs) and natural cytotoxic receptors (NCRs). KIRs are polymorphic groups of molecules, and some are expressed while others are silenced in the same cell. Different KIRs can transmit inhibitory or activating signals to NK cells, and effector function is considered to result from the balance of these contributing signals. The expression repertoire of KIRs is critical for NK killing ability. Moderate demethylation of the inhibitory KIR promoter is essential for normal NK recognition and lysis of abnormal cells. Promoter methylation of KIR genes consistently silences KIR expression (50, 51) and chromatin is condensed in early hemopoietic progenitor cells. During NK cell differentiation and maturation, the chromatin structure opens, and KIR genes sequentially become demethylated and transcribed (**Figure 1**) (52). Excessive demethylation of the inhibitory KIR promoter represses NK cytolytic function and results in tumor escape. Some studies demonstrated that acute exercise could cause promoter

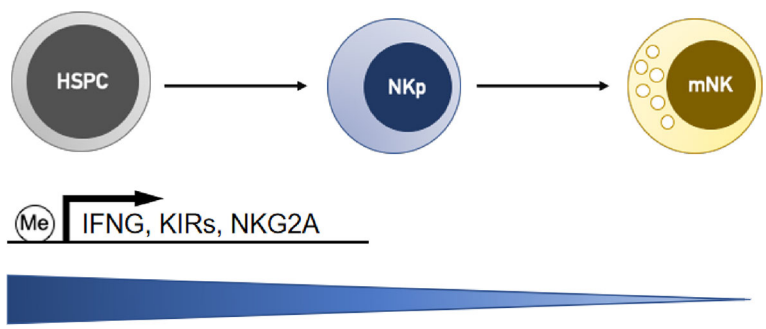

FIGURE 1 | NK cells gradually downregulate DNA methylation levels at the gene promoters of interferon-γ (IFNG) and receptors (KIRs and NKG2A) during the differentiation process, and this activity is correlated with the upregulation of their transcription. HSPC, hemopoietic stem/progenitor cells; NKp, NK cell progenitors; mNK, mature NK cells.

demethylation of the activating NK-cell receptor KIR2DS4 (53) and changed DNA methylation in 33 targets (25 genes) (54). Of the targets, 19 showed decreased methylation and 14 showed increased methylation. Whether these changes lead to functional adaptations needs to be elucidated. In addition, DNA methylation is crucial in maintaining the allele-specific expression of the inhibitory receptor NKG2A. CpGs are methylated in NKG2A-negative stages (hemopoietic stem cells, NK progenitors, and NKG2A-negative NK cells) but hypomethylated specifically in various developmental stages of NKG2A-positive NK cells and NK cell lines (**Figure 1**) (55). Natural killer group 2 member D (NKG2D) is one of the most crucial activating receptors of NK cells for target recognition. The methylation frequency of the NKG2D promoter can be used as a biomarker for detecting hepatitis B virus-associated hepatocellular carcinoma (HCC). NKG2D promoter methylation in HCC patients was higher than that in chronic hepatitis B patients and healthy controls (56).

Hypomethylating agents 5-azacytidine (5-aza) and decitabine (Deci) are approved for the treatment of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). However, the direct effect of demethylating treatment on NK cell function remains controversial (**Table 1**) and should be considered in the application of these drugs. Both 5-aza and Deci can alter the expression of KIRs on NK cells and may thus affect NK reactivity against malignant hematopoietic cells (57–59). Demethylation treatment with 5-aza significantly suppresses the cytolytic activity of the NK-92MI cell line and human polyclonal NK cells, which is related to the overexpression of inhibitory KIRs and impaired granzyme B (GzmB) and perforin (Prf1) release by

TABLE 1 | Epigenetic drugs targeting DNA methylation and histone modification related to NK antitumor cytotoxicity.

Agents		Effects	NK cytotoxicity	References
Hypomethylating agent	5-aza	↑inhibitory KIRs ↓granzyme B and perforin release	↓	(57, 58)
		↑Ki-67$^+$ NK cells ↑IFN-γ production ↑degranulation	↑	(59)
		- inhibitory KIRs ↑NK precursor differentiation	↑	(60)
	Deci	↑inhibitory KIRs ↓NKG2D expression ↑NKp44 expression	U-shaped response (lowest at intermediate dose)	(61)
		↑NKG2DL (ULBP and MICB) on AML cells	↑	(62, 63)
HATi	Curcumin	↓NKG2D transcription ↓NKG2D-dependent NK cell degranulation and IFN-γ secretion	↓	(64)
HDACi	Entinostat (class I HDACi)	↑MIC expression, Death receptors and PD-L1 expression on tumor targets ↑NKG2D expression	↑	(65, 66)
	SAHA (Pan-HDACi)	- degranulation	↓	(67)
	Panobinostat	↓NKG2D, CD16 and NKp46 expression ↓degranulation	↓	(67)
	Romidepsin	- NKG2D, CD16 and NKp46 expression ↓degranulation	↓	(67)
	TSA (Pan-HDACi)	↓NK degranulation ↓IFN-γ production	↓	(68, 69)
	VPA (class I and IIa HDACi)	↓NKG2D and NKp46 expression on resting NK cells		
	NaB (class I and IIa HDACi)	↓NKG2D, NKp44 and NKp46 expression on NK cells stimulated with IL-12, IL-15 and IL-18		
Histone methylase inhibitor	UNC1999	↑NK degranulation	↑	(70)
	EPZ005687 (EZH2 inhibitor)	↑CD122 & NKG2D on NK cells		
	GSK343 GSK126 (EZH2 inhibitor)	↑NKG2D-Ligand on tumor cell surface	↑	(71)
	GSK-J4 (JMJD3/UTX inhibitor)	↓IFN-γ,TNFα,GM-CSF and IL-10 ↓granzyme B, perforin, NCRs, ULBPs in mRNA level	–	(72)
Histone demethylase inhibitor	SP-2509 SP-2577 (scaffolding LSD1 inhibitor)	↓NK cell metabolism	↓	(73, 74)

↑, up-regulated; ↓, down-regulated; -, unchanged.

5-aza, 5-azacytidine; KIRs, killer immunoglobulin-like receptors; IFN-γ; interferon-γ; Deci, decitabine; NKG2DL, NKG2D ligands; ULBP, UL16-binding protein; MICAB, MHC class I chain-related gene B; AML, acute myeloid leukemia; HATi, histone acetyltransferases inhibitor; HDACi, histone deacetylases inhibitor; PD-L1, programmed death ligand-1; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A; VPA, valproic acid; NaB, sodium butyrate; EZH2, enhancer of zeste homolog 2; NCR; natural cytotoxicity receptors; JMJD3, jumonji domain-containing protein D3; TNFα, tumor necrosis factor-alpha; GM-CSF, granulocyte-macrophage colony-stimulating factor; LSD1, lysine-specific histone demethylase 1.

these cells (57, 58). However, another study reported that systemic treatment with 5-aza leads to an increased proportion of Ki-67$^+$ NK cells expressing multiple KIRs in MDS patients. These proliferating NK cells exhibit increased IFN-γ production and degranulation towards tumor target cells (59). However, Kubler et al. found that low-dose and long-term treatment of humanized NSG mice with 5-aza does not induce common inhibitory KIR expression but instead promotes the differentiation of various NK-cell precursor subsets to enhance the antitumor (pediatric BCP-ALL *in vivo*) response (60). The different effects could be determined based on the dose, with high doses of the demethylating agents showing cytotoxicity and lower doses mediating DNA hypomethylation. Deci decreases NK cell cytotoxicity at intermediate concentrations and leads to a U-shaped dose-response curve (0-20 μM). In contrast, increased inhibitory KIRs (KIR3DL1, KIR2DL1, KIR2DL2/DL3), decreased NKG2D, and increased NKp44 expression have been induced by Deci treatment in a linear dose-response manner (61). However, another group reported that low-dose Deci (0.2 mg/kg) reduces the antitumor response of NK cells in tumor-bearing mice (75), and Deci has also been shown to increase the cell surface expression of recombinant UL16 binding protein (ULBP) (62) and MHC class I-related molecule B (MICB) (63), the ligands of NKG2D in AML cells, and the NKG2D-dependent sensitivity of these cells to NK-mediated killing *in vitro*.

Histone Modification

Histone modifications are associated with the opening or closing state of the chromatin structure, which results in the activation or repression of gene transcription (76). Of particular importance are histone acetylation and methylation. The acetylation of lysine residues on histone 3 (AcH3) and 4 (AcH4) is associated with active transcription (77), while methylation contributes to both active and suppressed states of gene expression. The methylation of histone 3 lysine 9 (H3K9) and H3K27 is inhibitory, whereas the methylation of H3K4, H3K36, and H3K79 is activating (78). The level of histone modification is controlled by the interplay between enzymes: e.g., histone acetyltransferases (HATs) vs. deacetylases (HDACs) (79) and histone methyltransferases vs. demethylases. The dynamic histone modification states determine NK cell activation and effector function in antitumor immunity (80).

Histone Acetylation

Histone acetylation precedes the transcription of many genes (e.g., IFNG and NKG2D) involved in regulating NK cell function (81–83). Chang et al. compared long-range histone hyperacetylation patterns across the *Ifng* gene region in T cells and NK cells and found that histone acetylation of the *Ifng* gene depends on stimulation and the transcription factors Stat4 and T-bet in T cells. In contrast, even in resting NK cells, histones along *Ifng* gene region are already acetylated, and additional proximal domains are hyperacetylated after stimulation of transcription (84). These characteristics may partially explain the quick response of NK cells without prior sensitization. The NKL cell line exhibits high levels of AcH3, AcH4, and H3K4me3 in the NKG2D gene. A significantly high level of AcH3, especially H3K9ac, was observed in the NKG2D gene of NK cells from peripheral blood, while a low level of H3K4me3 was present. Repressive histone modifications (H3K27me3 and H3K9me2) to the NKG2D gene in both NKL and peripheral NK cells were hardly detectable (64).

HAT inhibitor (curcumin) incubation reduced H3K9Ac levels of the NKG2D gene, downregulated NKG2D transcription, and led to a marked reduction in NKG2D-dependent NK cell degranulation and IFN-γ secretion by NKL cells (64). HDAC inhibitors (HDACis) have emerged as novel immunomodulatory drugs and have been reported to affect NK cell cytotoxicity against tumors through both receptor and ligand modulation. The expression of activating ligands for NK cell recognition was increased after HDACi treatment on the cell surfaces of neuroblastoma, melanoma, osteosarcoma, colon, and Merkel cell carcinomas (65, 85). However, different HDAC inhibitors were reported to have varying effects on the NK cell phenotype (**Table 1**). There are four subclasses of HDACs (HDAC I, II, III, IV). Treatment with a histone deacetylase inhibitor (trichostatin A, TSA) alone was sufficient to induce inhibitory NKG2A receptor expression in mice (55). Entinostat (a class I HDACi) treatment induced NK activation *via* increased MIC expression in tumor targets as well as enhanced NKG2D expression and ADCC-mediated lysis in primary human NK cells (65, 66). Many HDACis have been reported to negatively regulate the NK antitumor response, including vorinostat (SAHA), panobinostat, romidepsin, TSA, valproic acid (VPA), and sodium butyrate (NaB) (**Table 1**) (67). They affect NK cell activation through cytokine receptors and activating receptors involved in tumor cell recognition (68, 69). The inhibitory effect on nuclear mobilization of p50 and NK-κB activation caused by HDAC inhibitors also resulted in impaired NK cell activation (82).

Histone Methylation

Li et al. screened 4 upregulated (KMT2C, KDM6B, UTY, and JARID2) and 4 downregulated (ASH1L, PRMT2, KDM2B, and KDM4B) histone methyltransferases/demethylases upon activation of human NK cells by gene expression profiling, which was further confirmed by qPCR and western blot in NK92MI cells. These enzymes were mainly associated with H3K4 methylation and H3K27 methylation, and they only affected limited gene loci instead of the global modification state. Bivalent marks with both H3K4me3 and H3K27me3 determined the "poised" chromatin state of many genes associated with NK activation. This state helps the rapid shift in expression above the baseline during the target recognition process. Treatment with UNC1999 could induce NK cell degranulation. In addition, the expression of IFN-γ and TNF-α is increased after treatment with OG-L002 and MM102 (80).

Histone lysine N-methyltransferase Ezh2 (enhancer of zeste homolog 2) contributes to histone repressive marks H3K27me3. Loss of Ezh2 or inhibition of its enzymatic activity with small molecules in both mouse and human hematopoietic stem and progenitor cells enhanced NK cell expansion and cytotoxicity against tumor cells through upregulation of CD122 and NKG2D (**Table 1**) (70). The Ezh2 inhibitor EPZ011989 and combination

treatment with cisplatin in HT1376 (bladder cancer cell line) xenografts led to increased expression of CD86, MIP-1α, and CD3d at the transcript level as well as CD56 and NCR1 at the protein level, indicating an active state of NK cells (86). Ezh2 was also found to be a transcriptional repressor of NKG2D ligands. Ezh2 inhibition enhanced NK cell eradication of tumor cells in hepatocellular carcinoma (**Table 1**) (71). Jumonji-type histone H3K27 demethylases (e.g., JMJD3/UTX) have been identified as key regulators of cytokine production in human NK cell subsets. The JMJD3/UTX inhibitor GSK-J4 increased global levels of the repressive H3K27me3 mark around the transcription starting site (TSS) of effector cytokine genes. However, NK cell cytotoxic killing activity against tumor cells was unaffected after treatment with GSK-J4 (**Table 1**) (72).

Methylation of H3K4 is an activating mark for gene transcription. An H3K4me1-marked latent enhancer at the *Ifng* locus was essential for NK memory in a systemic endotoxemia model (87). The H3K4me3 demethylase Kdm5a associates with p50 and binds to the suppressor of cytokine signaling 1 (Socs1) promoter region in resting NK cells, thus leading to a repressive chromatin configuration. Kdm5a deficiency impairs the activation of NK cells, leading to decreased IFN-γ production and impaired phosphorylation and nuclear localization of STAT4 (88). LSD1 is a histone demethylase of H3K4me1/2 and H3K9me1/2. Catalytic LSD1 inhibitors blocking demethylase activity are unaffected on NK cells, while scaffolding inhibitors disrupting epigenetic complexes, including LSD1, impair NK cell metabolism and cytotoxicity through depletion of glutathione (**Table 1**) (73, 74).

Transcription Factors

Transcription factors (TFs) are specific kinds of proteins that can activate or suppress the transcriptional activity of target DNA sequences by specifically recognizing and binding them. Many TFs have been shown to highly modulate the function of human or murine NK cells and affect the eradication of tumor cells (**Figure 2A**) (reviewed in Ref. 89-91) (89–91). Kwon HJ et al. reported that silencing the expression of the NF-κB p65 subunit caused a significant reduction in the mRNA levels of IFN-γ, TNF-α, MIP-1α/β, GramB, and IκBα induced by NKG2D and 2B4 coengagement (92). The T-box transcription factors T-bet and Eomes are both critical in driving the differentiation and function of NK cells (93). T-bet deficiency impairs the longevity and function of NK cells in inhibiting cancer metastasis, which further precludes the initiation of a potent adaptive response to tumors in mice. Adoptive transfer of wild-type activated NK cells (but not T-bet$^{-/-}$ NK cells) protects T-bet$^{-/-}$ animals after melanoma challenge (94). Aiolos is required for the maturation of CD11b^{+}CD27^{-} NK cells. However, NK cells lacking Aiolos are strongly hyperreactive to various NK cell-mediated tumor models but impaired in controlling viral infection (95). Foxo1 was identified as a negative intrinsic regulator of NK cell homing, late-stage maturation, and effector functions, and it can directly target IFN-γ expression; moreover, Foxo1 deficiency increases the NK cell killing capacity of tumor cells *ex vivo* and the antimetastatic activity *in vivo*. Foxo1 suppresses Tbx21 expression through direct binding to its promoter in human

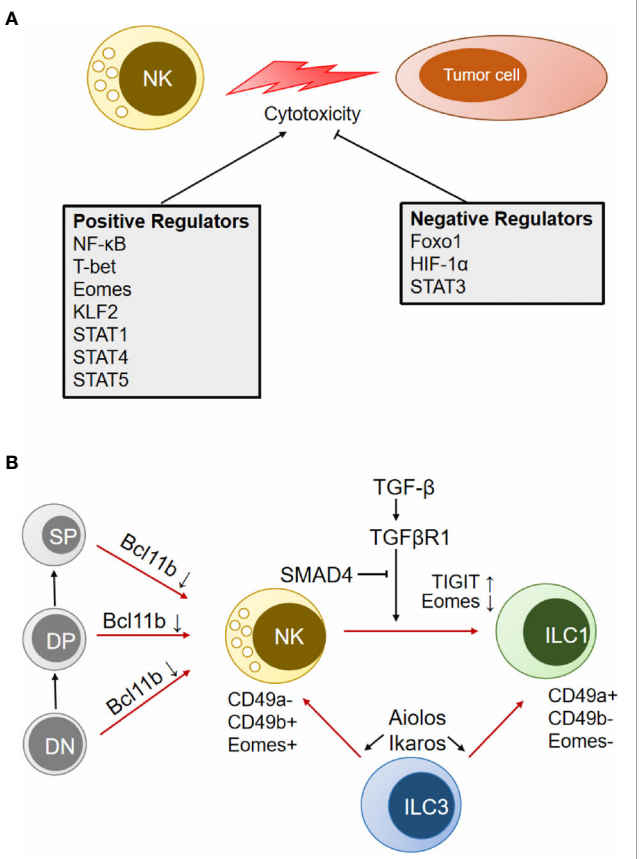

FIGURE 2 | Transcription factors (TFs) that modulate NK cell cytotoxicity and transdifferentiation. **(A)** TFs that positively and negatively regulate NK antitumor cytotoxicity are indicated separately. **(B)** Schematic representation of multiple TFs involved in the transdifferentiation between NK cells and other immune cells. DN, double-negative cells in the thymus; DP, double-positive cells in the thymus; SP, single-positive cells in the thymus; ILC, innate lymphoid cells.

NK cells and through association with the promoter *via* recruitment by Sp1 in murine NK cells (96). Phosphorylation-mediated inactivation of Foxo1 facilitates the activating receptor CD226 regulation of NK cell antitumor responses (97). Krupple-like factor 2 (KLF2) is a key TF responsible for expanding transferred NK cells and prolonging their functionality within the tumor. KLF2 imprints a homeostatic pattern on mature NK cells that allows them to migrate to IL-15-rich microenvironments (98). Cells adapt to hypoxia in solid tumors by upregulating HIF-1α. Inhibition of HIF-1α unleashes the antitumor activity of human tumor-infiltrating NK cells associated with high expression of IFN-γ in an IL-18-dependent manner (99).

It has been reported that the signal transducer and activator of transcription (STAT) family (STAT1, STAT3, STAT4, STAT5) positively or negatively regulates NK cell activity (**Figure 2A**) (100). STAT1 dysfunction in humans and genetic deletion in mice leads to impaired NK cell antitumor cytotoxicity (101). Mutation of the S727 phosphorylation site of STAT1 (Stat1-S727A) increases the expression of perforin and

granzyme B and enhances NK cell cytotoxicity in various tumor models, including for melanoma, leukemia, and metastasizing breast cancer. Inhibition of upstream cyclin-dependent kinase 8 (CDK8) may be a therapeutic strategy for stimulating NK cell-mediated tumor surveillance (102). Full-length STAT1α is efficient for NK cell maturation and tumor control in mice, while NK cells from the C-terminally truncated STAT1β isoform show impaired maturation and effector functions (103). STAT-3 regulates all aspects of NK biology, including almost all of the pathways for target cell killing and the reciprocal regulatory interaction between NK cells and other components of the immune system, which has been presented in detail by Nicholas A. Cacalono (104). STAT4 signaling in NK cells could be activated by IL-2 (105) and IL-12 (106), which specifically bind to the human perforin gene and induce activation of NK antitumor activity. Eckelhart et al. found that $STAT5^{fl/fl}$ Ncr1-iCreTag mice show a marked reduction in NK cells in the spleen and lymph nodes and severely impaired NK-dependent antitumor activity (107). There are two homologs of STAT5, STAT5A and STAT5B, which can form homos, heterodimers, and tetramers. It was reported that the loss of STAT5B (but not STAT5A) reduces NK cell numbers and cytotoxicity (108). However, recent studies have shown that STAT5A deficiency is sufficient to compromise NK cell homeostasis, responsiveness, and tumoricidal function (109, 110).

In addition, several TFs have been shown to control the transdifferentiation between NK cells and other immune cells (T cells, ILCs) (**Figure 2B**). Downregulation of Eomes by TGF-β signaling in the TME could induce the conversion of mouse NK cells to an NK-ILC1 intermediate cell type (intILC1s) and, finally, to ILC1s, which are less cytotoxic and cannot control local tumor growth and metastasis (27). Cortez et al. found that SMAD4 is a negative regulator of NK-ILC1s conversion in a noncanonical TGF-β signaling pathway (30). SMAD4 is the only common SMAD in TGF-β signaling that usually impedes immune cell activation in the tumor microenvironment. Selective deletion of Smad4 in NK cells impairs tumor cell rejection, promotes tumor cell metastases, and impedes NK cell homeostasis and maturation. GzmB was identified as a direct target of a transcriptional complex formed by SMAD4 and JUNB (31). It was also found that ILC3 could transdifferentiate into IFN-γ-producing ILC1 and NK cells by IL-1β plus IL-12 stimulation, which is associated with the upregulation of T-bet and Aiolos. Degradation of Aiolos and Ikaros proteins by lenalidomide inhibits ILC1/NK cell transdifferentiation and ILC1/NK cell function (111). Bcl11b, a zinc finger transcription factor, is essential for the maintenance of T-cell identity. Upon Bcl11b deletion, immature thymic T cells could convert to NK cells and acquire NK cell properties (112, 113). The converted NK cells were called T-to-natural killer (ITNK) cells and exhibited enhanced antitumor activity. They are considered an attractive cell source for cancer immunotherapy (114).

miRNA

MicroRNAs (miRNAs) are small single-stranded noncoding RNAs that target mRNA and promote degradation by binding to the 3' untranslated region (UTR) (115). miRNAs can modulate gene expression involved in the development, maturation, and effector functions of NK cells (**Figure 3**) (reviewed in Ref. 116) (116).

Prf1 and GzmB are the main effector molecules of NK cells. Prf1 could be targeted by miR-30e (117) and miR-150 (118), GzmB could be targeted by miR-378 (117), while both could be targeted directly by miR-27a* (119) in resting and activated states and indirectly by miR-27a-5p (120) by downregulating the expression of C-X3-C motif chemokine receptor 1 (CX3CR1) under TGF-β1 signaling. Tumor cells upregulate miR-561-5p, which in turn inhibits the production of CX3CL1 and subsequently reduces NK cell recruitment to the tumor (**Figure 3A**) (121). Wang et al. reported that miR-146a negatively regulates IFN-γ production in human NK cells by targeting the NK-κB signaling pathway (**Figure 3A**) (122). MiR-146a overexpression significantly suppresses the cytotoxic activity of NK92 cells by targeting STAT1 signal transduction (123). In contrast, miR-181 was found to promote IFN-γ production in primary NK cells in response to cytokine stimulation by targeting nemo-like kinase (NLK), an inhibitor of Notch signaling (124). MiR-362-5p overexpression upregulated Prf1, GzmB, IFN-γ, and CD107a in human NK cells (125). Several reports have shown that miR-155 can enhance NK cell functions by regulating molecules involved in NK cell activation and IFN-γ release (126–128).

Moreover, miRNAs can control the expression of activating and inhibitory receptors on the surface of NK cells or that of their ligands on tumor cells (**Figure 3B**). Human miR-1245 could downregulate NKG2D on NK cells and, therefore, impair NKG2D-mediated functions of NK cells (129). NKG2D ligands (MICA/B) could also be repressed by miR-20a, miR-93, miR-106b, miR-373, and miR-520d in human cancer cells (HeLa, 293T, DU145, and glioma cells) (130, 131). In breast cancer cells, the miR-17-92 cluster (miR-20a, miR-20b, miR-93, and miR-106b), which could be inhibited by the HDAC inhibitors SAHA and VPA, downregulates the expression of MICA/B by targeting the mRNA 3'-UTR and downregulates ULBP2 by inhibiting the MAPK/ERK signaling pathway (132). The transcription and translation of DNAX-activating protein 12 kDa (DAP12), an exclusive signaling adaptor of many NK cell receptors, could be repressed by human miR-183, thus leading to the abrogation of NK cell antitumor function (133). In contrast, miR-30c-1* (134) promotes NK cell cytotoxicity against hepatoma cells by targeting the transcription factor HMBOX1 and miR-30c (135) could promote the cytotoxicity of NKL cells *in vitro* by upregulating the expression levels of NKG2D, CD107a, and FasL. Inhibitory receptors (e.g., KIRs, NKG2A, PD-1, TIGIT, TIM-3) function as immune checkpoints associated with NK cell exhaustion and the immune escape of tumor cells. MiR-146a-5p can downregulate the expression of both KIR2DL1 and KIR2DL2 (136). Three miRNAs, miR-26a-5p, miR-26b-5p, and miR-185-5p, were identified as inhibitors of the expression of inhibitory KIR3DL3, whose function has not yet been demonstrated (137). MiR-182 mediates a complex modulation of NKG2D and NKG2A levels at different stages of human

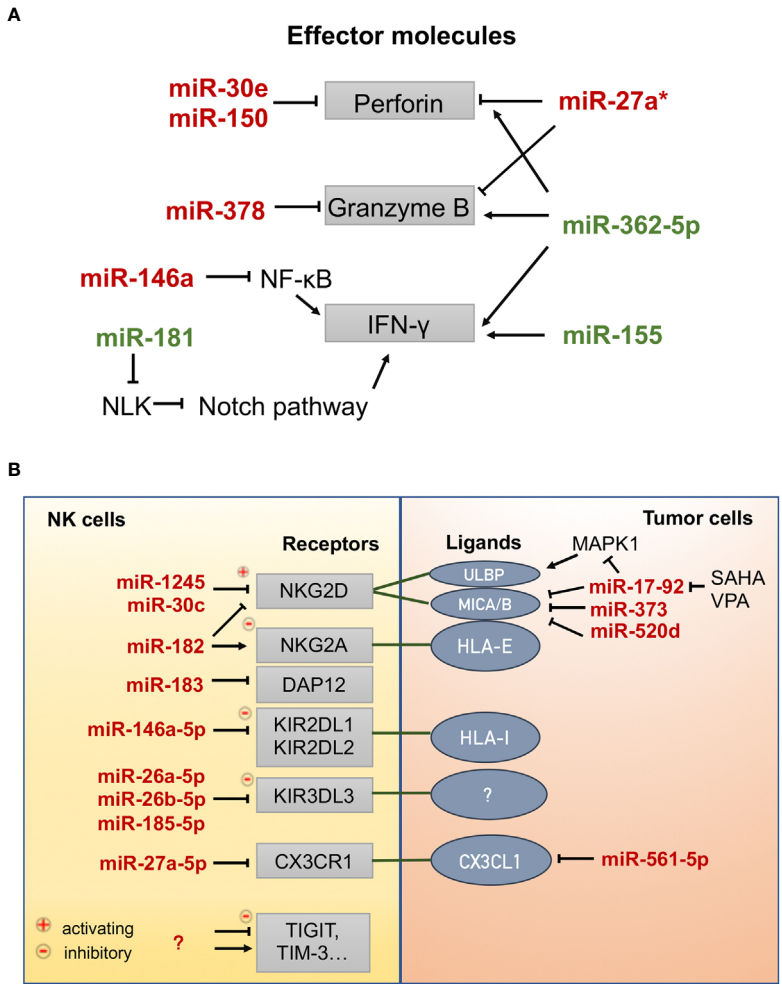

FIGURE 3 | MicroRNAs involved in the effector functions of NK cells. **(A)** MicroRNAs that positively (green) or negatively (red) regulate the expression of effector molecules (perforin, granzyme B, and interferon-γ). NLK: nemo-like kinase, Notch signaling inhibitor. **(B)** MicroRNAs that regulate the expression of receptors on NK cells and ligands on tumor cells. CX3CR1, C-X3-C motif chemokine receptor 1; CX3CL1, C-X3-C motif chemokine ligand 1; DAP12, DNAX-activating protein 12 kDa, an exclusive signaling adaptor of many NK cell receptors; HLA-I, human leukocyte antigen, class I; HLA-E, human leukocyte antigen, Class I, E; KIR2DL1, killer cell immunoglobulin-like receptor, two Ig domains and long cytoplasmic tail 1; KIR2DL2, killer cell immunoglobulin-like receptor, two Ig domains and long cytoplasmic tail 2; KIR3DL3, killer cell immunoglobulin-like receptor, three Ig domains and long cytoplasmic tail 3; MICA/B, MHC class I-related molecule A/B; NKG2A, natural-killer group 2 member A; NKG2D, natural-killer group 2 member D; SAHA, suberoylanilide hydroxamic acid (vorinostat), histone deacetylase inhibitor; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM-3, T cell immunoglobulin and mucin domain-containing protein 3; ULBP, UL16 binding protein; VPA, valproic acid, histone deacetylase inhibitor.

hepatocellular carcinoma, resulting in increased Prf1 expression (138). Some miRNAs have been found to target PD-1 [miR-28 (139), miR-138 (140), miR-4717 (141)] and TIM-3 [miR-28 (139)] in T cells and cause T cell exhaustion. Thus, these miRNAs may also play a regulatory role in NK cells; however, experimental evidence has not been presented.

PERSPECTIVES

NK cells play a crucial role in preventing tumor initiation and metastasis. Many studies have illustrated the epigenetic regulatory mechanism of NK cell antitumor cytotoxicity, and they mainly focused on the expression of NK cell receptors and effector molecules, as we reviewed above. Multiple modulators always participate in epigenetic regulation. For example, histone modifications determine the open/closed state of chromatin, which affects the binding of transcription factors to specific regulatory sites. Additional research should focus on the interactions between different epigenetic modulators rather than just studying individual molecules. Recent technological advances have allowed us to gain a deeper understanding of NK cells. For example, single-cell RNA sequencing helps decipher the similarities and differences between humans and mice and between blood and splenic NK cells (142). Very recently, Li et al. applied the transposase accessible chromatin with sequencing

(ATAC-seq) technique to define two distinct TF clusters that dynamically regulate NK cell differentiation in a homemade *in vitro* NK cell differentiation system (143). NK cells are a heterogeneous population that consists of multiple subsets and various states. The tissue site shapes the functional potential of NK cell subsets. Whole transcriptome profiling reveals the site-specific variations of NK cells in the lymph node, lung, blood, bone marrow, and spleen (33). However, the epigenetic features of these subsets are still a mystery.

The "states" (resting, activating, memory, repressed, and exhausted) of NK cells are controlled epigenetically, although insights into the underlying mechanism are very limited. Adaptive NK cells exhibit a unique whole-genome epigenetic signature similar to that of effector memory $CD8^+$ T cells but not conventional NK cells (144). Chronic stimulation (NKG2C Abs with IL-15) could induce exhaustion in primary adaptive NK cells, thereby upregulating the expression of checkpoint receptors LAG-3 and PD-1. These NK cells are dysfunctional when challenged with tumor targets and exhibit a whole genome-DNA methylation profile similar to the epigenetically remodeled profiles of exhausted $CD8^+$ T cells (145). It is reasonable to presume that NK cells are similar to T cells and show susceptibility to exhaustion during the antitumor war. However, there is a lack of consensus on the defining features of NK cell dysfunctional states, such as senescence, suppression, and exhaustion (47). Further consideration is needed to determine the state of NK cells in the antitumor response and how their epigenetic landscape changes during the process.

NK cell-based immunotherapy is an effective supplement to T cell-based therapy. Various approaches have been introduced to activate NK cells in adoptive cell therapy for better clinical outcomes, including generating CAR-NKs and inducing ADCC by mAbs, immune checkpoint blockade, engineered cytokine stimulatory, and so on (146). Even so, NK cell-based therapies are still in the early stages of development. Other than these "extrinsic" strategies, approaches that target "intrinsic" epigenetic regulators should be taken into consideration. Research on the epigenetic control of NK cell functions will provide new evidence for developing drugs and effective cancer prevention approaches. For example, demethylating agents can restore the absence of transcription of NKG2DL associated with high levels of DNA methylation in tumor cells. Some histone modification regulators (e.g., EZH2 and LSD1) have been found to be aberrantly overexpressed in various malignant tumors. Small molecular inhibitors are in clinical or preclinical development. From our perspective, these inhibitors also have potential applications in improving the *in vitro* expansion of NK cell cytotoxicity. More studies are needed to further elucidate the application of epigenetic drugs in NK cell-based immunotherapy, alone or in combination with other strategies.

AUTHOR CONTRIBUTIONS

MX and XW conceived and designed the manuscript. MX did literature searching, drafted the manuscript, and drew the figures. BW and ZW did literature searching and drafted several sections. XZ and XW reviewed and revised the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Scientific Research Common Program of Beijing Municipal Commission of Education (KM201910025026 to MX), the Support Project of High-level Teachers in Beijing Municipal Universities in the Period of 13th Five–year Plan (IDHT20190510 to XW and XZ), and the National Natural Science Foundation of China (81972652 to XW).

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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: 07 May 2021
doi: 10.3389/fimmu.2021.643298

Immunomodulation of T Helper Cells by Tumor Microenvironment in Oral Cancer Is Associated With CCR8 Expression and Rapid Membrane Vitamin D Signaling Pathway

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The immune system plays a key role in the protective response against oral cancer; however, the tumor microenvironment (TME) impairs this anti-cancer response by modulating T helper (Th) responses and promoting an anti-inflammatory environment. Regulatory T cells (Tregs) and Th2 effector cells (Teff) are associated with poor prognosis in oral squamous cell carcinoma (OSCC). However, the main immunomodulatory mechanisms associated with the enrichment of these subsets in OSCC remain unknown. We characterized Th-like lineages in Tregs and Teff and evaluated immunomodulatory changes induced by the TME in OSCC. Our phenotypic data revealed a higher distribution of tumour-infiltrating CCR8^{+} and Th2-like Treg in OSCC compared with non-malignant samples, whereas the percentages of Th1 cells were reduced in cancer. We then analyzed the direct effect of the TME by exposing T cell subsets to cancer secretomes and observed the OSCC secretome induced CCR8 expression and reduced cytokine production from both subsets. Transcriptomic

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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 Immunology

Received: *17 December 2020*
Accepted: *22 April 2021*
Published: *07 May 2021*

Citation:
Fraga M, Yáñez M, Sherman M, Llerena F, Hernandez M, Nourdin G, Álvarez F, Urrizola J, Rivera C, Lamperti L, Nova L, Castro S, Zambrano O, Cifuentes A, Campos L, Moya S, Pastor J, Nuñez M, Gatica J, Figueroa J, Zúñiga F, Salomón C, Cerda G, Puentes R, Labarca G, Vidal M, McGregor R and Nova-Lamperti E (2021) Immunomodulation of T Helper Cells by Tumor Microenvironment in Oral Cancer Is Associated With CCR8 Expression and Rapid Membrane Vitamin D Signaling Pathway. Front. Immunol. 12:643298. doi: 10.3389/fimmu.2021.643298

analysis showed that the co-culture with OSCC secretome induced several gene changes associated with the vitamin D (VitD) signaling pathway in T cells. In addition, proteomic analysis identified the presence of several proteins associated with prostaglandin E2 (PGE2) production by rapid membrane VitD signaling and a reduced presence of the VitD binding protein. Thus, we analyzed the effect of VitD and PGE2 and observed that VitD promotes a regulatory Th2-like response with CCR8 expression whilst PGE2 also modulated CCR8 but inhibited cytokine production in combination with VitD. Finally, we evaluated the presence of CCR8 ligand in OSCC and observed increased chemokine CCL18, which was also able to upregulate CCR8 in activated Th cells. Overall, our data showed the immunomodulatory changes induced by the TME involving CCR8 expression and regulatory Th2 phenotypes, which are associated with PGE2 mediated VitD signaling pathway and CCL18 expression in OSCC.

Keywords: oral cancer, immunomodulation, cancer immunology, Th-like Tregs, CCR8

INTRODUCTION

Oral cancer is a malignant neoplasm developed in the oral cavity with high mortality and morbidity due to late-stage diagnosis and high incidence of metastasis (1). Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, representing more than 90% of the cases, and it has been linked with uncontrolled proliferation of squamous epithelial cells due to environmental-mediated genetic mutations. Risk factors such as long-term use of tobacco, alcohol abuse, excessive sun exposure, human papillomavirus (HPV) infection and a weakened immune system have been associated with OSCC (2, 3). In fact, it has been proposed that the origin of oral cancer is associated with DNA alteration mediated by environmental carcinogens, since 3 to 6 mutations are required to transform a healthy cell to a malignant cell (4). It is the impaired or overwhelmed anti-tumor immune response in the patient is the main factor that favors subsequent tumor progression (5). This altered response is not only associated with cancer cells escaping the immune control, but also to the immunomodulatory effects of the tumor microenvironment by contact dependent and soluble mechanisms, promoting a regulatory immune repertoire and inducing an anti-inflammatory environment.

The immunomodulatory mechanisms exerted by the tumor microenvironment include the contribution of cancer-associated immune cells, the expression of inhibitory checkpoints (6) and the production of soluble factors such as proteins, metabolites, chemical factors (7–9) and extracellular vesicles (10). In OSCC, the presence of cytokines such as IL-4, IL-5, IL-10, TGF-β, IL-17, IL-1α and immune-checkpoint inhibitors such as PD-L1 and Indoleamine 2,3-Dioxygenase 1 (IDO1) have been associated with poor prognosis (11). Several chemokines have also been associated with immunomodulation in OSCC such as CCL18, CXCL13 and CCL4. It is however not clear whether the chemokines exert direct changes in the repertoire or phenotype of immune cells. Moreover, high PD-L1 expression has been associated with good overall survival since its expression is higher in low-grade invasive OSCC cell lines than high-grade invasive OSCC cell lines (12, 13). Therefore, novel mechanisms need to be addressed to understand how this cancer modulates the immune system. In terms of metabolic changes, glycolysis-related proteins and mitochondrial enzymes (14), are also significantly increased in the carcinogenesis of OSCC making it is possible that the active glycolytic activity of cancer cells also affects the function of the immune cells. In terms of the immune repertoire, it has been shown that OSCC includes cells with a pro-tumoral role such as tumor associated macrophages (TAMs), cancer-associated fibroblasts (CAF) and regulatory T cells (Tregs) (11).

Previous data from our lab characterized the Th-like Tregs based on the expression of three chemokine receptors, immune transcriptomic profiles and specific lineage cytokine production, defining Th1 as $CXCR3^+CCR6^-CCR4^+$, Th2 as $CXCR3^-CCR6^-CCR4^+$, Th17 as $CXCR3^-CCR6^+CCR4^+$ and Th1/17 $CXCR3^+CCR6^+CCR4^+$ (15). We found that Th2-like Tregs expressed CCR8 and exhibited higher viability than other Th-like Tregs subsets, however suppression capacity was similar between subsets. However, Th2-like Tregs and Th2 Teff migrated more than other Th-like subsets a phenomenon not mediated by CCR4 expression. Finally, we analyzed the presence of Th-like Tregs in blood, thymus, spleen, liver, skin, colon and tissues and blood from patients with melanoma and colon cancer. We observed a high presence of Th2-like Tregs and Th2 effector cells (Teff) in melanoma and colorectal cancer at late-stage. Here we progress these findings by investigating the distribution of these subsets in a cancer that has been traditionally associated with late-stage detection to evaluate if there is a specific subset enriched in well-established tumors and the main mechanism associated with the enrichment of Th2 subsets in cancer areas.

In this study we analyzed the distribution of tissue resident Th-like Tregs and Teff in OSCC compared to non-malignant biopsies allowing us to investigate mechanisms associated with the presence of Th2-like Tregs in the tumor environment. Our results revealed that the Treg/Teff ratio and the percentages of Th2-like and $CCR8^+$ T cell subsets were higher in OSCC biopsies

compared to non-malignant biopsies. We then analyzed whether the OSCC tumor secreted-factors defined as secretome, were promoting these phenotypes and we observed that the OSCC secretome induced CCR8 expression and reduced cytokine production on both subsets. We then performed a proteomic and transcriptomic analysis of the secretome and the Th subsets after co-culture, and observed several proteins associated with prostaglandin E (PGE2) production by rapid membrane vitamin D (VitD) signaling and VitD transport in OSCC. In addition, several genes modulated by the OSCC secretome were associated with the VitD signaling pathway in both Th subsets. Since PGE2 and VitD have previously been related to CCR8 expression we analyzed their presence in the TME and their effect on T cell phenotype. The data revealed that cancer areas had higher PGE2 and the combination of the active form of VitD and PGE2 induced CCR8 in T cells and reduced cytokine production. In addition, Vitamin D promoted Th2-like Treg responses by regulating transcription factors and cytokine production. Finally, we evaluated the presence of CCR8 ligand in OSCC and observed higher chemokine CCL18, which was not promoting migration of $CCR8^+$ cells but induced CCR8 expression by direct contact. Overall, our data suggest that the secretome from oral cancer induces CCR8 and promotes a Th2 lineage in the T cell repertoire by several mechanisms; rapid membrane VitD mediated PGE2 production, accumulation of Vitamin D in cancer areas and increasing CCL18 levels.

MATERIALS AND METHODS

Patient

Peripheral blood and biopsies were obtained from healthy volunteers and patients, after informed consent was approved. Patients with and without OSCC were consented in accordance with the Talcahuano Health Service Research Ethics Committee, reference number 19-06-11 and Concepcion Health Service Research Ethics Committee, reference number 19-03-07 and in accordance with the Declaration of Helsinki. Patient data are described in **Table 1**.

The Isolation of Th Subsets From Biopsies

Tissues from OSCC and control group were subjected to mechanical tissue disruption with sharps elements to reach small piece (< 0.1 cm). These pieces were then transferred to a

TABLE 1 | Patient data.

OSCC				
Patient ID	**Gender**	**Age**	**Diagnosis**	**Stage**
CO-01	Male	73	Well-differentiated squamous cell carcinoma	T2N1M0/II B
CO-02	Male	88	Well-differentiated squamous cell carcinoma	T2NOM0/II A
CO-03	Male	76	Moderately differentiated squamous cell carcinoma	T4N2M0/III A
CO-04	Male	56	Well-differentiated squamous cell carcinoma	T3N1M0/III A
CO-05	Male	70	Moderately differentiated squamous cell carcinoma	T2N2M0/III A
CO-011	Male	66	Moderately differentiated squamous cell carcinoma	T3 N2b MX/III A
CO-012	Male	73	Moderately differentiated squamous cell carcinoma	Unknown
CO-017	Male	74	Moderately differentiated squamous cell carcinoma	Unknown
CO-018	Male	66	Moderately differentiated squamous cell carcinoma	T1N0M0
CO-021	Male	58	Moderately differentiated squamous cell carcinoma	T4aN0M0/IIIB
CO-024	Female	76	Well-differentiated squamous cell carcinoma	T1N0M0/I
CO-025	Male	67	Well-differentiated squamous cell carcinoma	T2N0M0/II A
IHC-01	Male	76	Moderately differentiated squamous cell carcinoma	T3N2M0/III C
IHC-02	Male	70	Well-differentiated squamous cell carcinoma	T1N0M0/I
IHC-03	Male	74	Moderately differentiated squamous cell carcinoma	Unknown
Control				
Patient ID	**Gender**	**Age**	**Diagnosis**	
CO-06	Female	71	Conjunctival epithelial hyperplasia	
CO-07	Male	55	Conjunctival epithelial hyperplasia	
CO-08	Female	65	Conjunctival epithelial hyperplasia	
CO-09	Female	54	Conjunctival epithelial hyperplasia	
CO-010	Female	61	Conjunctival epithelial hyperplasia	
CO-013	Male	43	Healthy gum	
CO-015	Male	33	Healthy gum	
CO-016	Male	42	Fibrous hyperplasia	
CO-019	Female	67	Conjunctival epithelial hyperplasia	
CO-020	Female	50	Conjunctival epithelial hyperplasia	
CO-023	Female	30	Fibrous hyperplasia	
CO-026	Male	25	Healthy gum	
CO-027	Female	25	Healthy gum	
IHC-04	Female	72	Conjunctival epithelial hyperplasia	
IHC-05	Male	58	Conjunctival epithelial hyperplasia	
IHC-06	Male	72	Conjunctival epithelial hyperplasia	

recipient with serum-free medium X-VIVO15 (LONZA) with 1 mg/mL of collagenase (GIBCO) and 10 U/mL of DNase (Worthington) for an enzymatic digestion for 1 h at 37 °C under constant agitation. The digested sample was filtered (70um) to obtain cells from the biopsies. To obtain the mononuclear cell fraction, cells were isolated by density-gradient centrifugation at 400 x g for 20 min at room temperature using Lymphoprep (Axis Shield). Cells were washed with PBS at 300 x g for 10 min and live cells were counted using the viability Trypan Blue staining.

Flow Cytometry

PBMCs and mononuclear cells obtained from tissues were stained with anti-CD4, anti-CD25, anti-CD127, anti-CXCR3, anti-CCR4, anti-CCR6, anti-CD45RA and anti-CCR8 for 30 min at 4°C in the dark. Samples were acquired on LSR Fortessa (BD) and files analyzed using FlowJo (Tree Star). Gates were set based on biological controls and fluorescence minus one control (FMO).

Teff and Treg Cell Isolation From Peripheral Blood for Functional Assays

PBMCs were isolated as previously described and negative isolation of memory $CD4^+$ T cells was performed with magnetic bead separation with the Memory $CD4^+$ T Cell Isolation Kit, human (Miltenyi Biotec). Memory Teff and Tregs were then sorted on a BD FACSAria II (BD) based on CD4, CD25, CD127 and CD45RA expression.

Secretome Collection

A standardized piece of tissue (weight about 0.1 g) from the oral cancer and control biopsies was cut and incubated in X-VIVO15 (LONZA) serum-free medium for 48 h at 37°C. After the incubation the medium was collected, debris was eliminated by centrifugation and filtration (0.22um), and the medium with all proteins and factors secreted from the tissue (Secretome) was stored by -80 °C until use.

Cell Culture With Secretomes

Sorted Teff and Tregs from healthy donors were activated with anti-CD3/CD28 beads (1:5 ratio) (Life Technologies) and 1000 UI IL-2 for 5 days a 37°C. Then, 100 uL of OSCC and control secretomes were added to $2x10^5$ Teff or $2x10^5$ Tregs (in 100uL) in XVIVO-15 serum-free medium 48h a 37°C. After the incubation, the supernatants were stored for further cytokine production measurement using the Cytokine Bead Array Th1/2/17 Kit (BD) and the cells were counted (CountBright Absolute Counting Beads), stained with Live/Dead dye (Life Technologies), anti-CXCR3, anti-CCR4, anti-CCR6, anti-CCR8, anti-PD-1 and anti-TIGIT (all BioLegend) and analyzed by flow cytometry. For the analysis of cells after secretome co-culture, cells were washed after co-culture with secretome and cultured in new media X-VIVO15 (LONZA) serum-free medium for 48 h at 37°C with anti-CD3/CD28 beads (1:5 ratio) (Life Technologies) and 1000 UI IL-2. After the incubation, the supernatants were stored for further cytokine production measurement and the cells were stained with Live/Dead dye (Life Technologies), anti-CCR6, anti-CCR8, anti-PD-1 and anti-TIGIT (all BioLegend).

RNA-Seq Targeted Panel

Sorted Teff and Tregs from healthy donors were activated with anti-CD3/CD28 beads (1:5 ratio) (Life Technologies) and 1000 UI IL-2 for 5 days a 37°C. Then, 100 uL of OSCC and control secretomes were added to $2x10^5$ Teff or $2x10^5$ Tregs (in 100uL) in XVIVO-15 serum-free medium 48 h a 37°C. Cells were lysed in TRIzol, and RNA was isolated with Direct-Zol RNA MicroPrep w/Zymo-Spin columns. RNA-seq was performed using the QIAGEN Human Inflammation and Immunity Transcriptome RNA targeted panel (QIAGEN). Samples were sequenced with the Illumina NextSeq using NextSeq 500/550 Mild Output Kit v2.5 (150 Cycles) (Illumina). Volcano plots and pathway analysis were performed initially using QIAseq targeted RNA data analysis tools (QIAGEN). In addition, the quality of each sequencing library was verified using FastQC software package and summarized using MultiQC software package (16). The reads were aligned to the human reference genome (hg38) using STAR (17), a high-performance community-standard aligner. The expected RSEM counts were rounded to the nearest integer value and the transcripts with zero counts across all the samples are filtered out. Differential expression analysis was performed using DESeq2 package (18) between the cohorts (OSCC versus Control, Teff OSCC versus Control and Treg OSCC versus Control). A pathway enrichment analysis was performed using the Gene Ontology Consortium database (data-version Released 2021-02-01) including biological processes. Cytoscape v.3.8.2 with the ClueGO plugin v.2.5.7 was used with a ($p<0.01$) and a kappa statistics score = 0.4 to calculate the relationships between the terms based on the similarity of their associated genes. P-value is the probability of seeing at least x number of genes out of the total n genes in the list annotated to a particular GO term, given the proportion of genes in the whole genome that are annotated to that GO Term.

Proteomic Analysis

Secretome Protein Depletion

The secretome proteins were depleted with Top 2 Abundant Protein Depletion Spin Columns (Thermo Scientific), 200 ug of secretome proteins were added per column and the protocol suggested by the manufacturer was followed.

Protein Extraction and Digestion for nLC-MS/MS

The previously depleted proteins were subjected to precipitation using 5: 1 v/v cold acetone 100% v/v and incubated overnight at -20° C, then they were centrifuged at 15,000 g for 10 min, the supernatant was discarded, and the pellet was washed 3 times with acetone at 90% v/v, later the proteins were dried in a rotary concentrator at 4°C, and finally they were resuspended in 8 M urea with 25 mM of ammonium bicarbonate pH 8. The proteins were reduced using a final concentration of 20 mM DTT for 1 h, then they were alkylated incubating for 1 h with 20 mM iodoacetamide in the dark, then the proteins were quantified using the Qubit protein quantification kit. 10 ug of total proteins were diluted to 1 M urea using 25 mM ammonium bicarbonate pH 8, then the proteins were digested with trypsin/LyC (Promega) in a 1:50 ratio overnight at 37° C. The peptides were cleaned using Pierce C-18 Spin Columns (Thermo Scientific) using the protocol suggested by the

manufacturer, the eluted peptides were dried using a rotary concentrator at 4°C and resuspended in 2% ACN with 0.1% v/v Formic Acid (MERCK), and quantified using Direct detect (MERCK Millipore).

Liquid Chromatography

200 ng of secretome tryptic peptides were injected in nanoELUTE (Bruker Daltonics, Bremen, Germany) ultra-high-pressure nano-flow chromatography system was coupled online to a hybrid trapped ion mobility spectrometry - quadrupole time of flight mass spectrometer (timsTOF Pro, Bruker Daltonics, Bremen, Germany) with a modified nano-electrospray ion source (CaptiveSpray, Bruker Daltonics). Liquid chromatography was performed at 50°C and with a constant flow of 400 nL/min on a reversed-phase column Aurora Series CSI (25 cm x 75µm i.d. C18 1.6 µm) (*ionopticks* Australia). Mobile phases A and B were watered with 0.1% formic acid (v/v) and 99.9/0.1% ACN/formic acid (v/vol), respectively. In 90-min experiments, peptides were separated with a linear gradient from 2 to 17% B within 57 min, followed by an increase to 25% B within 21 min and further to 35% within 13 min, followed by a washing step at 85% B and re-equilibration.

The timsTOF Pro Mass Spectrometer-The timsTOF Pro

All further experiments were acquired with a 100 ms ramp and 10 PASEF MS/MS scans per topN acquisition cycle. In TOF mass spectrometry, signal-to-noise ratios can conveniently be increased by summation of individual TOF scans. Thus, low-abundance precursors with an intensity below a 'target value' were repeatedly scheduled for PASEF-MS/MS scans until the summed ion count reached the target value (e.g. four times for a precursor with the intensity 5000 arbitrary units (a.u.) and a target value of 20,000 a.u.). The target value to 20,000 a.u was set. MS and MS/MS spectra were recorded from m/z 100 to 1700. Suitable precursor ions for PASEF-MS/MS were selected in real time from TIMS-MS survey scans by a sophisticated PASEF scheduling algorithm. A polygon filter was applied to the m/z and ion mobility plane to select features most likely representing peptide precursors rather than singly charged background ions. quadrupole isolation width was set to 2 Th for m/z < 700 and 3 Th for m/z > 700, and the collision energy was ramped stepwise as a function of increasing ion mobility: 52 eV for 0 –19% of the ramp time; 47 eV from 19 –38%; 42 eV from 38 –57%; 37 eV from 57–76%; and 32 eV for the remainder (19). The TIMS elution voltage was calibrated linearly to obtain reduced ion mobility coefficients (1/K0) using three selected ions of the Agilent ESI-L Tuning Mix (m/z 622, 922, 1222) (20). Collisional cross sections were calculated from the Mason Schamp equation.

Database Searching

Tandem mass spectra were extracted by Tims Control version 2.0. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using PEAKS Studio (Bioinformatics Solutions, Waterloo, ON Canada; version 10.5 (2019-11-20). PEAKS Studio was set up to search the [UniProt_SwissProt] database (unknown version, 21040 entries) assuming the digestion enzyme trypsin. PEAKS Studio was searched with a fragment ion mass tolerance of 0,050 Da and a parent ion tolerance of 50 PPM. Carbamidomethyl of cysteine was specified in PEAKS Studio as a fixed modification. Deamidated of asparagine and glutamine, oxidation of methionine, acetyl of the n-terminus and carbamyl of lysine and the n-terminus were specified in PEAKS Studio as variable modifications.

Criteria for Protein Identification

Scaffold (version Scaffold_4.8.9, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95,0% probability by the Peptide Prophet algorithm (21) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99,0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (22). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Ingenuity Pathway Analysis (IPA) of Identified Proteins

Pathway enrichment analyses were performed with Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) as previously described (23, 24). IPA was performed to identify canonical pathways, diseases and functions, and protein networks. Significantly enriched pathways for the proteins and pathways were identified with the criterion p-value < 0.05.

Vitamin D in Secretomes

Levels of 25(OH)VitD in cancer and control secretomes were determined using the competitive imunoluminometric assay Maglumi 25-OH Vitamin D kit (Snibe) performed on the Maglumi fully auto analyzer according to manufacturer's instructions.

PGE2 ELISA

Levels of Prostaglandin E2 in cancer and control secretomes were determined by PGE2 high sensitivity ELISA kit (Enzo) according to manufacturer's instructions.

Vitamin D Effect on Th Differentiation

$2x10^5$ sorted Teff (Treg-depleted) from healthy donors were activated with anti-CD3/CD28 beads (1:5 ratio) (Life Technologies) in XVIVO-15 media for 5 days a 37°C in the presence or absence of 1,25(OH)VitD (10nM in ethanol) or carrier (ethanol). The supernatants were stored for cytokine measurement using the Cytokine Bead Array Th1/2/17 Kit (BD) and the cells were counted (CountBright Absolute Counting Beads) and stained with Live/Dead dye (Life Technologies), anti-FOXP3, anti-GATA3, anti-Tbet and anti-RORγt and analyzed by flow cytometry.

CCR8 Upregulation in Vitamin D and Prostaglandin E2 Culture

Sorted Th cells from healthy donors were activated with anti-CD3/CD28 beads (1:5 ratio)(Life Technologies) in XVIVO-15 media for 5 days a 37°C. Then, prostaglandin E2 (10 uM), 1,25 (OH)VitD (10nM) and recombinants chemokines CCL1 and CCL18 (0.5ug/mL) were added to $1x10^5$ Th in XVIVO-15 serum-free medium for 72h a 37°C. After the incubation, the supernatants were stored for further cytokine production measurement using the Cytokine Bead Array Th1/2/17 Kit (BD) and live cells were counted (CountBright Absolute Counting Beads), stained with anti-CCR8 and analyzed by flow cytometry.

Immunohistochemistry

Control and OSCC tissue embedded in paraffin were cut into 10 um slides. Paraffin was then removed with alcohols in ascendant concentrations. Slides were incubated with primary antibody, rabbit pAb anti-CCL1 and anti-CCL18 (all Biorbyt), overnight at 4°C. After wash with PBS to eliminate the excess of primary antibody, the slides were incubated with secondary antibody (Donkey HRP anti-rabbit IgG) (Abcam) for 1h at room temperature. The excess of secondary antibody was removed with PBS, and the slides were revealed with diaminobenzidine and observed with an optical microscope. The semi quantification of CCL1 and CCL18 was performed using ImageJ as follows. Images were open and transform as RGB Stack (Image → Type), then stack montage were performed (Image → Stacks) and finally threshold was set up to identify the positive staining (Image → Adjust → Threshold). Finally, we set up measurements: Area, area fraction, limit to threshold and display label (Analyze → Set measurements) and measured the positive staining (Analyze → Measure).

Chemotaxis Assays

T cell migration was assessed using a 96 well 5-µm-pore Transwell filter system (Corning). The top chambers were incubated with Cell Trace Violet$^+$ memory Teffs and unstained memory Tregs, sorted and rested prior experiment. After resting, 5×10^4 Teffs + 5×10^4 Tregs in 50 uL X-VIVO15 serum-free medium were placed in the top chamber. The bottom chambers were filled with 100 uL X-VIVO15 serum-free only or 100 uL of X-VIVO15 with CCL18 (0.5 ug/mL, Novus Biologicals) or CCL1 (0.5 ug/mL, BioLegend). After 1h at 37°C, cells were harvested from bottom compartments and counted (CountBright Absolute Counting Beads) with flow cytometry. The percentage of migration for each subset was calculated as (number of Th cells in the bottom chamber after 60 min × 100)/initial number of Th cells in the top chamber.

Statistical analysis

Statistical tests were performed using Prism 9 software (GraphPad). Data are expressed as mean ± SEM where applicable using individual values, column bar charts, box and whiskers plots. Unpaired t test was used to compare one variable between unpaired samples (control *vs* OSCC). Paired t test was used to compare one variable between paired samples (close *vs* distant). Two-way ANOVA was used to compare two related variables between subsets from the same donor (Th subsets). Ordinary One-way ANOVA was used to compare one related variable (CCL18 levels). Post hoc tests were used as indicated in the figure legends. p values are reported as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

Th2-Like Tregs and CCR8$^+$ Tregs Are Enriched in Biopsies From Patients With OSCC

Peripheral blood derived Th-like Tregs and Teff have previously been characterized based on the expression of three chemokine receptors by our research group in several tissues including thymus, spleen, skin, colon and peripheral blood (15). In addition, we analyzed their distribution in malignant biopsies and observed a higher distribution of tissue-resident Th2-like subsets in melanoma and colorectal cancer compared to healthy skin and colon. In this study we analyzed the repertoire of infiltrated Th cells in oral cancer as this cancer is normally diagnosed at late stage. Tregs and Teff were identified by flow cytometry in tissues samples from patients with OSCC or patients without malignant oral lesions (**Table 1**) based on CD4, CD25, CD127 and CD45RA expression and chemokine receptors CCR4, CXCR3 and CCR6 expression (**Figure 1A**) as previously reported (15). FoxP3 staining was used to confirm Treg selection (**Supplementary Figure 1**). The Treg/Teff ratio between tissue resident T cells from patients with OSCC and their counterparts from donors without oral cancer was higher in the cancer, mostly due to an increase in Tregs (**Figure 1B**). Both Tregs and Teffs were mainly memory in the oral cavity with no difference observed between cancer and control (**Figure 1C**). From the memory population, we analyzed the expression of CCR4 and observed that Tregs in OSCC expressed lower CCR4 levels than tissue resident Tregs from controls, whereas no difference was observed in Teffs (**Figure 1D**). After analyzing the presence of CCR4 expression to define Th-like subsets, we analyzed the distribution of Th-like Treg and Teff subsets in both conditions. We observed increased percentages of Th2 and reduced percentages of Th1 subsets in Tregs and Teffs obtained from malignant tissues (**Figure 1E**). We have previously shown that Th2-like Tregs are the main CCR8$^+$ population within Tregs, therefore we analyze the expression of this chemokine receptors in Tregs and Teff (**Figure 1F**). The analysis showed an increased expression of CCR8 in Tregs from OSCC samples in comparison with control samples and the presence of CCR8$^+$ Tregs was independent of the presence of Th2-like Tregs. Our results were consistent with previous data in other malignancies, showing an imbalance between Th2/Th1 subsets in cancer with more than half of the Tregs found in oral cancer being either Th2-like or CCR8$^+$ Tregs. The origin of these subsets is unknown so we next studied whether the local OSCC environment could induce this phenotype.

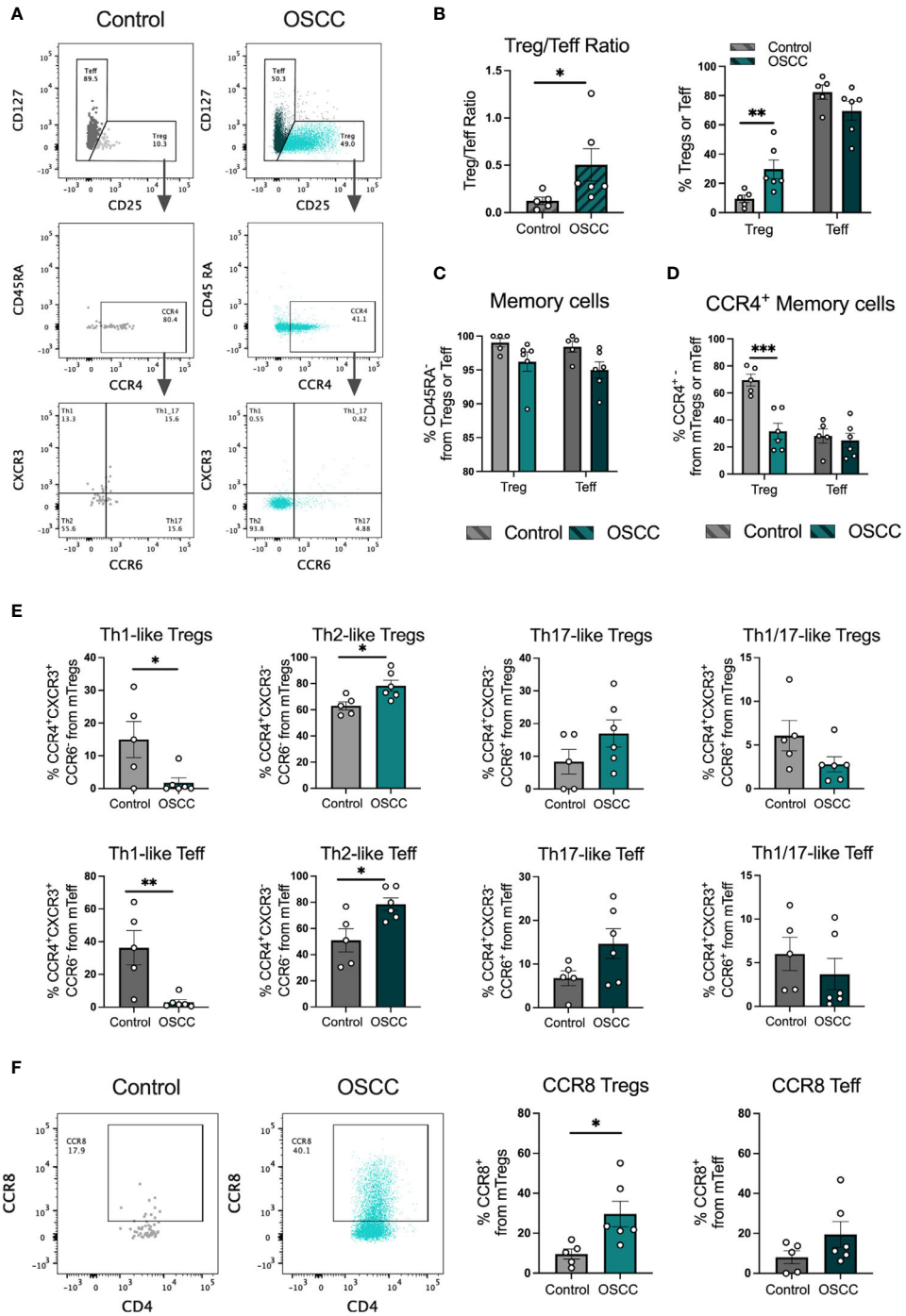

FIGURE 1 | Th2-like T cell subsets and CCR8$^+$ Tregs are the main tumor infiltrating Th subsets in OSCC. **(A)** Representative dot plots of tissue-resident cells obtained from a biopsy from a patient with OSCC and a control patient without malignancy. CD4$^+$ T cells were divided into Teff and Tregs using CD25 and CD127 staining. Then, memory cells were selected as CD45RA$^-$ and CCR4 and CCR8 expression was evaluated within the memory population. Within the CCR4$^+$ subsets, Th1 were defined as CXCR3$^+$CCR6$^-$, Th2 as CXCR3$^-$CCR6$^-$, Th17 as CXCR3$^-$CCR6$^+$ and Th1/17 CXCR3$^+$CCR6$^+$. **(B)** Comparison of the Treg/Teff ratio and percentages of Tregs and Teff between OSCC patients and patients without malignancy. **(C)** Comparison of memory Tregs and Teff between OSCC patients and patients without malignancy. **(D)** Comparison of CCR4 expression within the memory Treg and Teff population between OSCC patients and patients without malignancy. **(E)** Comparison of tissue resident memory CCR4$^+$ Th-like Tregs and Th-like Teff between OSCC patients and patients without malignancy. **(F)** Representative dot plots and comparison of CCR8 expression within the memory Treg and Teff population OSCC patients and patients without malignancy. Data are presented as mean ± SEM using bars with scatter dot plots (Unpaired t test). For all statistical tests, $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ were considered significant.

Secretome From Oral Cancer Promotes the Expression of CCR8, PD-1 and TIGIT But Suppress Cytokine Production in Th Cells in OSCC

In order to identify whether the malignant environment was able to regulate the expression of CXCR3, CCR4, CCR6 and CCR8, we analyzed the direct effect of malignant and non-malignant secretome on viability and chemokine receptor expression in peripheral blood Tregs and Teff from healthy donors. The secretome has previously been defined as the proteins and metabolites secreted by a cell or tissue (25), thus we used a standard tissue piece of 0.1 g from a malignant or non-malignant biopsy to collect secretome in X-VIVO media without serum for 48h. Memory Tregs and Teff were activated and expanded for 5 days in the presence of IL-2 and anti-CD3CD28 beads. After expansion, cells were washed, co-cultured with malignant or control secretomes for 48h and expression of chemokine receptors was measured by flow cytometry (**Figure 2A**). First, we analyzed the cell count of live cells to see whether the co-culture with the secretome was affecting viability, however we observed a difference but it did not reach significance (**Figure 2B**). When expression of chemokine receptors was analyzed no difference in CXCR3 and CCR4 levels was observed for either subset, however in Tregs we observed a significant up regulation of CCR6. A significant increment was also observed in CCR8 expression within the Tregs and Teffs cultured with cancer secretome compared to control samples (**Figure 2C**), suggesting the tumor environment was regulating CCR8 expression in both subsets. Since the data showed a direct effect of the malignant environment on the T cell phenotype, we analyzed whether the secretome could also modulate the suppressive molecules PD-1 and TIGIT as well as cytokine secretion. PD-1 has been found expressed in cells with an exhausted phenotype (26) whereas TIGIT has been associated with selective Th1 and Th17, but not Th2 suppression (27), thus both molecules are relevant to cancer-related Th responses. Regarding the expression of PD-1 and TIGIT (**Figure 3A**), we observed that the co-culture between the T cell subsets and the malignant secretome induced PD-1 upregulation in Tregs and Teff in comparison with the control secretome (**Figure 3B**). Similar upregulation by malignant OSCC secretome was observed for TIGIT in both subsets (**Figure 3C**). Finally, when cytokines were analyzed, we observed that all cytokines were significantly inhibited in the presence of OSCC secretome except for IL-4 in Teffs (**Figure 3D**). Since CCR8 has been associated with a Th2 phenotype, we sorted $CCR8^-$ and $CCR8^+$ Tregs and Teff to evaluate the main cytokines produced by both subsets. Interestingly and similar to the data obtained from cancer secretomes, $CCR8^+$ Tregs secreted less cytokines than $CCR8^-$ Tregs, whereas $CCR8^+$ Teff secrete IL-4, but not IFN-γ and IL-17 (**Supplementary Figure 2**). Overall, our data showed that the secretome was able to impair the capacity to secrete Th-like cytokines, promote CCR8 expression and induce regulatory molecules. In order to evaluate whether the effect of the secretome was sustained over time after removing the cells from the malignant environment, we washed the cells after co-culture with secretomes, cultured them again in new media for 48h and analyzed phenotype and cytokine secretion. The results showed a significant reduction of Teff, but not Tregs after previous co-culture with malignant secretome (**Figure 4A**). CCR6 was upregulated in Teff previously co-cultured with OSCC secretome, whereas CCR8 maintained its up regulation in both subsets (**Figure 4B**). PD-1 and TIGIT also maintained their significant upregulation after previous co-culture with malignant secretome in both subsets (**Figure 4C**). No differences were observed in cytokine secretion between Tregs, however for Teff, IL-17 and IL-10 maintained its downregulation after removing the secretome (**Figure 4D**) but is difficult to interpret these results since Teff viability was compromised. Our results indicate that the OSCC secretome affects the viability of Teff after exposure, induces and sustained the up regulation of CCR8, PD-1 and TIGIT expression even after removing the secretome and suppresses cytokine production during direct contact.

Transcriptomic Immune Characterization Revealed That Secretome From OSCC Potentiate the Vitamin D and Prostaglandin E Signaling in Tregs and Teff

After demonstrating that the secretome is capable of affecting both Tregs and Teffs phenotypically and functionally, we analyzed the transcriptomic immune profile in 3 paired-donor peripheral blood Tregs and Teff from healthy volunteers after 48h of co-culture with OSCC or control secretome using the same protocol previously for **Figure 2**. After co-culture, cells were washed, stored in Trizol and 491 immune related genes were analyzed using the Human Inflammation and Immunity Transcriptome RNA targeted panel. We aim to identify relevant genes and potential pathways promoted or inhibited by the OSCC secretome in Th cells. We compared the transcriptome from Tregs and Teffs co-cultured with OSCC versus control secretome using volcano plots (**Figure 5A**). We then identified the top up regulated genes (positive value) and down regulated genes (negative value) according to their p value, normalized as Log(1/pvalue) in both subsets (**Figure 5B**). Results revealed that several transcripts were commonly upregulated in Tregs and Teff such as *ISG20, CXCR4, IL1RL1, PTGER2, MYC, CASP8, CD86, FOXP1, TLR2, CXCL2 and MAF*. Additionally, similar transcripts were commonly downregulated in Tregs and Teff such as *CD74, IL-9, TBX21* (Tbet), CXCL16, CD70 and GZMA (**Figure 5B**) (**Supplementary Table**). Interestingly, we did not observe significant differences regarding CCR8 expression, however we observed higher expression of its ligand CCL18 in Th cell co-cultured with OSCC secretome. After analyzing gene expression, we investigated significant signaling pathways found in Tregs and Teff co-cultured with OSCC by performing a pathway enrichment analysis using the Gene Ontology Consortium database (**Figure 5C**). The analysis revealed 11 significant pathways, from which the most related to T cells responses were associated with VitD signaling, wound healing regulation, prostaglandin E response, angiogenesis, negative regulation of epithelial cell migration, sterol transport and response to ketone. Other pathways identified were positive regulation of odontogenesis and female gonad development. VitD and PGE2 have been previously associated with CCR8 expression and Th1 inhibition, thus we evaluate the content of the secretome to see whether these metabolites were present.

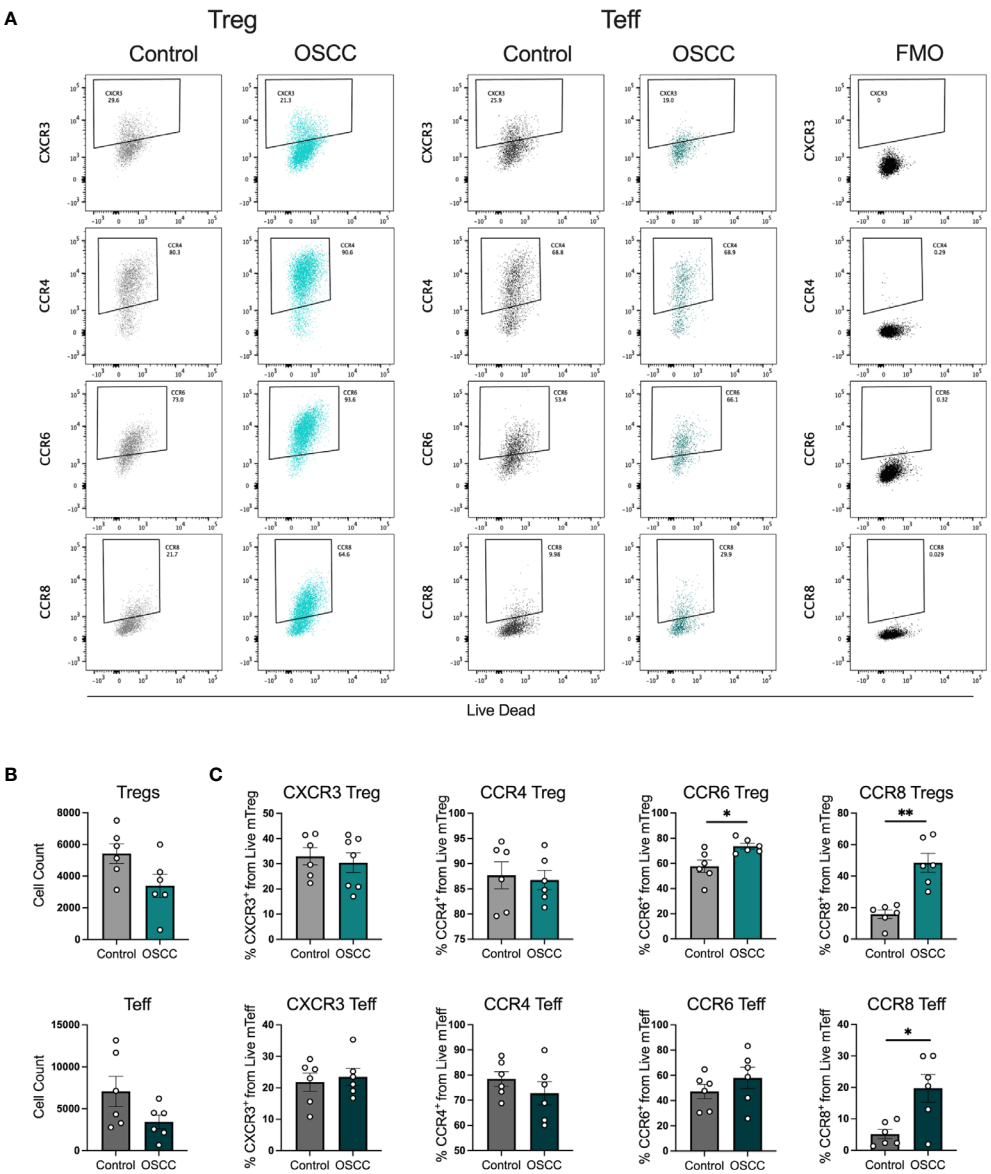

FIGURE 2 | OSCC secretome up regulates CCR8 expression in Treg and Teff. **(A)** Representative dot plots of chemokine receptor expression CXCR3, CCR4, CCR6 and CCR8 in Tregs and Teff after co-culture with control or OSCC secretome. Briefly, sorted memory Tregs and Teff obtained from peripheral blood from 3 healthy donors were pre-activated with anti-CD3/CD28 beads (1:5) in the presence of IL-2 (1000U). After activation, $2x10^5$ Tregs and Teff were co-cultured with secretomes from OSCC or control samples for 48h. After co-culture, cells were stained with Live/Dead dye, chemokine receptor expression and counted with counting beads. **(B)** Comparison of cell counts between Tregs and Teff co-cultured with OSCC or control secretome. **(C)** Comparison of CXCR3, CCR4, CCR6 and CCR8 expression between Tregs and Teff co-cultured with OSCC or control secretome. Data are presented as mean ± SEM using bars with scatter dot plots (Unpaired t test). For all statistical tests, $^{**}p < 0.01$ and $^{*}p < 0.05$ were considered significant.

Proteomic Analysis of Secretome From Oral Cancer Revealed a Significant Pathway Associated With Prostaglandin E Production by the Vitamin D Membrane Cascade in OSCC

The protein content of OSCC and control secretomes was evaluated in order to delineate the potential mechanisms associated with CCR8 expression and the pathways observed in the transcriptomic analysis. A qualitative and quantitative proteomic analysis was performed in 5 OSCC and 5 non-malignant pooled secretomes. The data revealed that 976 proteins were found exclusively in cancer secretome, 933 proteins were found exclusively in control secretome and 1722 proteins were found in both conditions (**Figure 6A**) (**Supplementary Table**). Scaffold4.0 and intuitive pathway analysis (IPA) were used to analyze the data set in a quantitative manner. The analysis revealed amongst diseases

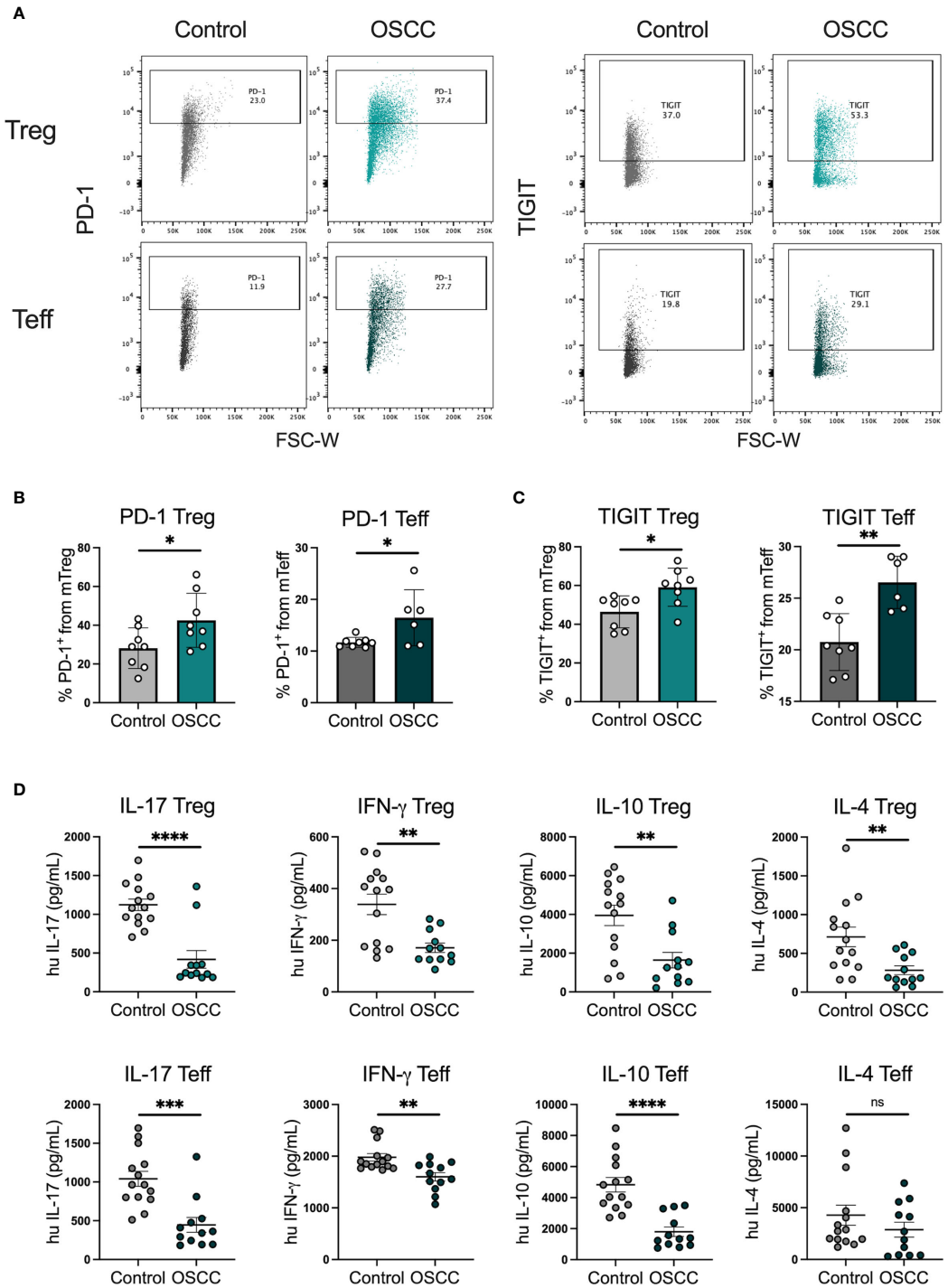

FIGURE 3 | OSCC secretome promote PD-1 and TIGIT expression and inhibit cytokine production in comparison with control secretome. **(A)** Representative dot plots of PD-1 and TIGIT expression in Tregs and Teff after co-culture with control or OSCC secretome. Briefly, sorted memory Tregs and Teff obtained from peripheral blood from 4 healthy donors were pre-activated with anti-CD3/CD28 beads (1:5) in the presence of IL-2 (1000U). After activation, $2x10^5$ Tregs and Teff were co-cultured with secretomes from OSCC or control samples for 48h and cells were stained with PD-1 and TIGIT, whereas the supernatants were used to measure cytokines using cytokine bead array. Expression of both suppressive molecules was measured by flow cytometry. **(B)** Comparison of PD-1 expression between memory Tregs and Teff co-cultured with OSCC or control secretome. **(C)** Comparison of TIGIT expression between memory Tregs and Teff co-cultured with OSCC or control secretome. **(D)** Comparison of secreted Th cytokines from Tregs and Teff co-cultured with OSCC or control secretome. Data are presented as mean ± SEM using bars with scatter dot plots for phenotype and scatter dot plots for cytokine secretion (Unpaired t test). For all statistical tests, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ were considered significant. ns, not significant.

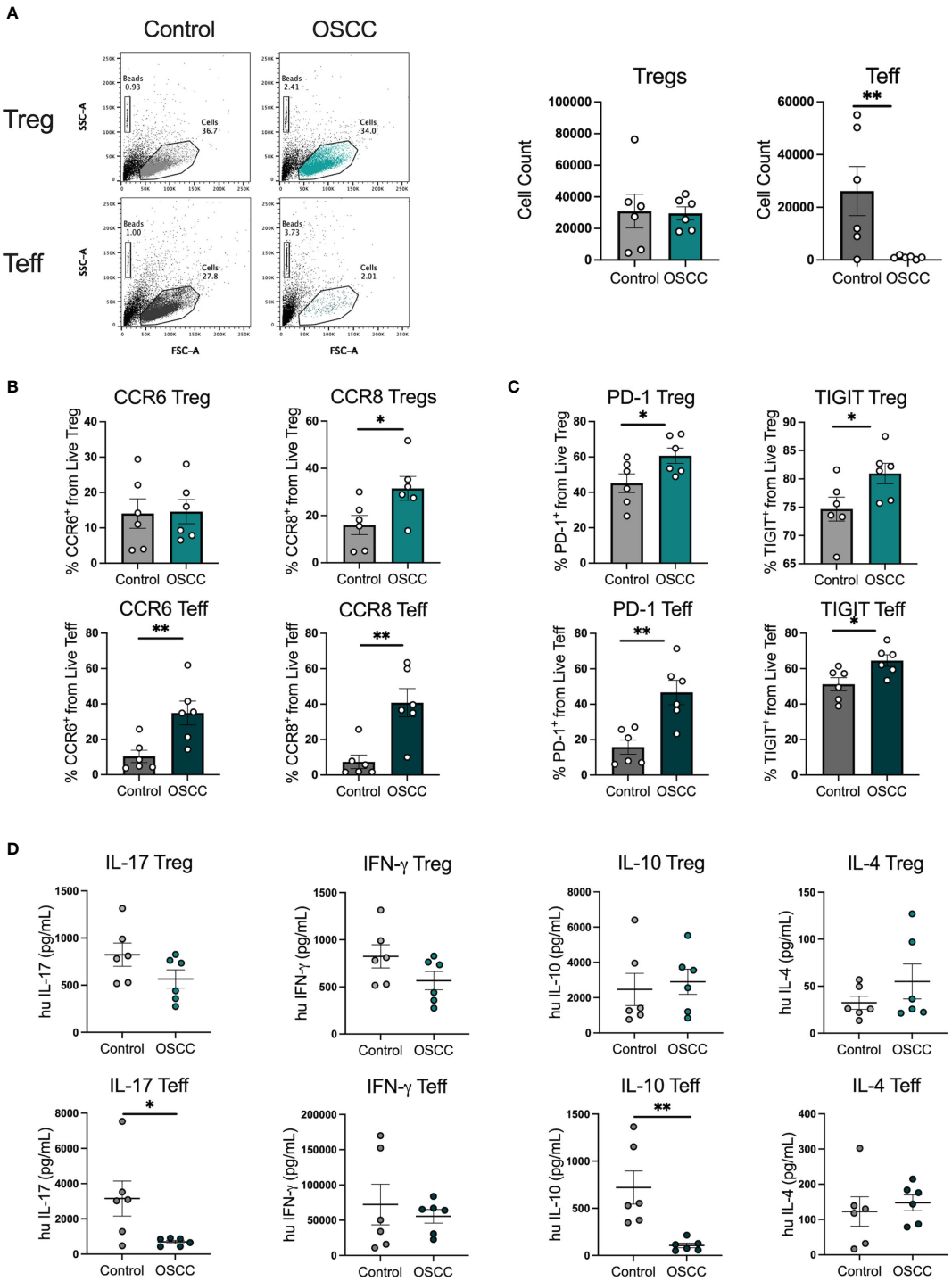

FIGURE 4 | Pre-treatment with OSCC secretome affects Teff cell counts but CCR8, PD-1 and TIGIT expression is maintained after secretome removal in Treg and Teff. **(A)** Representative dot plots and cumulative data of Tregs and Teff cell counts after 48h of culture after removal of control or OSCC secretome. Briefly, sorted Tregs and Teff were pre-activated with anti-CD3/CD28 beads (1:5) in the presence of IL-2 (1000U). After activation, $1x10^5$ Tregs and Teff were co-cultured with secretomes from OSCC or control samples for 48h. Then, cells were washed and Tregs and Teff were cultured with anti-CD3/CD28 beads (1:5) in the presence of IL-2 (1000U) for 48h. **(B)** CCR6 and CCR8 expression was measured by flow cytometry in live Tregs and Teff. **(C)** PD-1 and TIGIT expression was measured by flow cytometry in live Tregs and Teff. **(D)** Supernatants of Tregs and Teff were collected and cytokines were measured with cytokine bead array. Data are presented as mean ± SEM using bars with scatter dot plots for phenotype and scatter dot plots for cytokine secretion (Unpaired t test). For all statistical tests, $^{**}p < 0.01$ and $^{*}p < 0.05$ were considered significant.

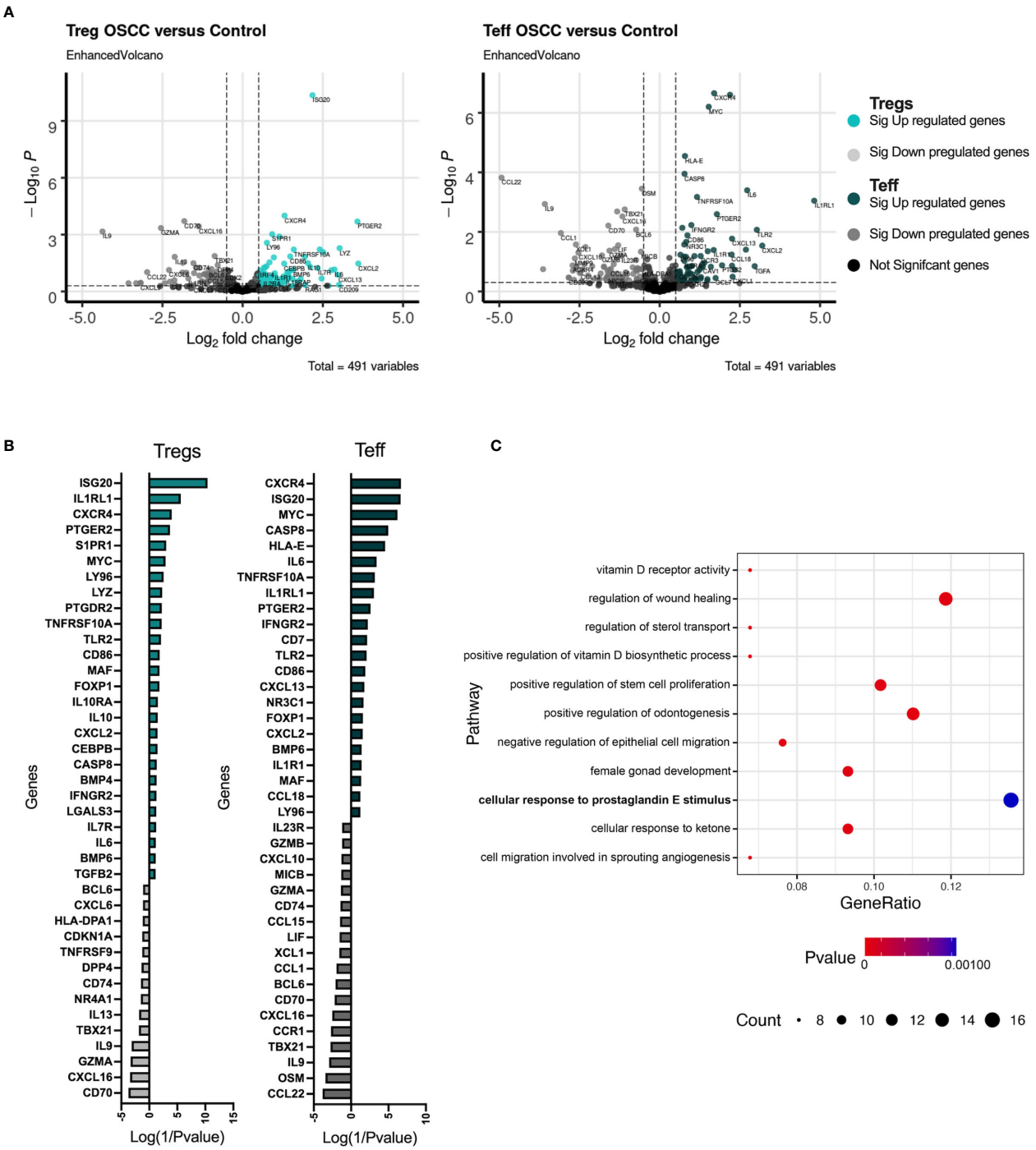

FIGURE 5 | Transcriptomic analysis of Tregs and Teff after co-culture with control or OSCC secretome revealed pathways associated with the VitD and PGE2 signaling. **(A)** Volcano plots showing RNA-seq data obtained from 3 paired Tregs and Teff after co-culture with control or OSCC secretome. Vertical dotted lines indicate 1.5-fold change threshold and horizontal dotted line indicate P value 0.05. Colored dots show significant up regulated genes, whereas grey dots show significant down regulated genes in Th subsets when comparing cells co-cultured with OSCC secretome versus control secretomes. **(B)** Heatmap showing upregulated (colored with positive values) and downregulated (grey with negative values) genes in Tregs and Teff after co-culture with control or OSCC secretome. Log(1/Pvalue) was used to normalize the p values obtained when comparing each gene between control and OSCC secretome in Treg or Teff. **(C)** A pathway enrichment analysis was performed using the Gene Ontology Consortium database (data-version Released 2021-02-01) including biological processes. Cytoscape v.3.8.2 with the ClueGO plugin v.2.5.7 was used with a ($p<0.01$) and a kappa statistics score = 0.4 to calculate the relationships between the terms based on the similarity of their associated genes. Circles represent gene counts found in each pathway and p value is the probability of seeing at least x number of genes out of the total n genes in the list annotated to a particular GO term.

associated with OSCC secretomes were; cancer, connective tissue disorders and infectious diseases, (**Supplementary Table**). Looking at relevant groups of proteins differentially expressed between samples, we observed enrichment of proteins from the PGE2 production by rapid membrane VitD signaling pathway (**Figures 6B, C**), including Pdia3, Caveolin-1, PLAA, CAMKII and PTGS2 (**Figure 6D**). Interestingly, Pdia3 has been previously reported as one of the key hub genes in OSCC, validated by gene expression and immunohistochemistry (28). Within the VitD pathway, the VitD binding protein (VDBP also known as GC) was significantly reduced in OSCC samples (**Figure 6E**), suggesting an impairment in the transport of VitD from the skin to circulation as previously reported (29), which suggest that this metabolite is more concentrated in cancer samples. In order to understand whether the VitD rapid signaling pathways was associated with the Th phenotypic and functional changes, the levels of 25-hydroxyvitamin (25(OH)) VitD and PGE2 were measured in the secretomes. First, we observed similar levels of VDBP-unbound 25(OH) VitD (**Figure 6F**) in both conditions, however PGE2 was higher in OSCC samples than control samples (**Figure 6G**) and in samples obtained from cancer areas compared with samples obtained from distal cancer areas from the same cancer patient (**Figure 6H**). This data suggested that the production of VitD *in vitro* is not different as the same amount of tissue was used in culture. Despite this, the induction of PGE2 was augmented in cancer secretomes suggesting that this signaling pathways is activated in OSCC. In addition, the GC (VitD binging protein) was one of the top ten significantly reduced proteins in the OSCC proteomic analysis, suggesting that the transport of VitD from the tissue to peripheral circulation may be impaired, inducing an accumulation of VitD in the malignant environment. Overall, the characterization of the OSCC secretome revealed several proteins associated with the prostaglandin E production by rapid membrane VitD signaling and potential accumulation of VitD by reduced presence of the VitD binding protein.

VitD Promote a Th2-like Treg Phenotype and Combination of PGE2 and VitD Modulate CCR8 Expression and Cytokine Production in Th Cells

Since VitD and PGE2 were within the pathways identified in the transcriptomic and proteomic analysis, we evaluated whether these metabolites were associated with the changes induced by OSCC secretome. First, we analyzed cell counts of live sorted memory Teff from peripheral blood after anti-CD3/CD28 activation in the presence or absence of VitD (10nM) at 24h, 72h and 120h post-activation, as it has been shown that VitD has antiproliferative properties (30). Our data showed that cell counts (**Figure 7A**) and division index (**Figure 7B**) were significantly higher in the presence of VitD after 5 days. We then analyze whether VitD also modulates Th transcription factors at 120h post activation in the presence or absence of VitD (10nM) and observed significant inhibition of Tbet and induction of FoxP3 in the presence of VitD (**Figure 7C**). We next characterized the secretion of Th cytokines on sorted memory Teff from peripheral blood following anti-CD3/CD28 activation in the presence or absence of VitD (10nM) at 6h, 12h, 24h, 72h and 120h post-activation. The VitD receptor is induced after TCR activation (31), thus, we observed significant differences at 72h and 120h post-activation in response to VitD (**Figure 7D**). The data showed that VitD inhibits Th1 responses by significantly reducing IFN-γ and TNF-α production, limits IL-17 secretion and promotes IL-10 and Th2 cytokines such as IL-4, IL-5, IL-6 and IL-13. We then analyzed the effect of PGE2 in combination with VitD in pre-activated Teff for 72h and we observed no difference in cell counts (**Figure 7E**), however both VitD and PGE2 induced CCR8 expression (**Figure 7F**). When cytokine secretion was analyzed, we observed that PGE2 inhibited secretion of IFN-γ, IL-17, IL-10 and IL-4 (**Figure 7G**). Altogether these results demonstrated that VitD modulates Th responses by causing an imbalance in the Th1/Th2 responses and by inducing regulatory cells by promoting FoxP3 expression. In addition, VitD and PGE induce CCR8 expression and inhibit cytokine secretion.

CCR8 Ligand CCL18 Is Increased in Histological Samples From Malignant Oral Mucosa and Promote CCR8 Upregulation by Direct Contact

Beside the role of skin mediations (32) in the induction of CCR8 expression, the effect of their ligands CCL1 and CCL18 (33) has also been associated with the upregulation of its receptor and chemotaxis of $CCR8^+$ cells. Thus, we analyzed the expression CCL1 and CCL18 in OSCC and control histological samples. The analysis revealed that CCR8 ligands, CCL1 and CCL18, were highly expressed in the oral cavity, however only CCL18 reach significance when comparing OSCC tissues with non-malignant oral mucosa (**Figure 8A**) (**Supplementary Figure 3**). Interestingly, the expression of CCL18 was mainly observed in the basal stratified squamous epithelium in non-malignant samples, whereas its expression in cancer samples was within the squamous cell carcinoma. CCL1 and CCL18 may either play a role in $CCR8^+$ Treg migration to the malignant zone of oral cancer or they might induce its expression directly, thus, we measure chemotaxis and CCR8 induction in response to recombinant chemokines CCL1 and CCL18. Peripheral blood Tregs and Teff were isolated from the same donor, Teff were stained with Cell trace violet and both subsets were combined in a 1:1 ratio and seeded in the top chamber of a 5um Transwell. In the bottom chamber recombinant chemokines CCL1 or CCL18 were added and media without chemokines was used as a control. After 1h, migrated cells were recovered and counted (**Supplementary Figure 4**). When T cell migration was analyzed, we observed that CCL1 and CCL18 induce preferential migration of Tregs over Teff, however only migration to CCL1 induce significant chemotaxis in comparison with media without chemokines (**Figure 8B**). When the effect of direct contact was analyzed, we observed that only CCL18 induced CCR8 expression in pre-activated Teff (**Figure 8C**). This data showed that CCL18 is increased in OSCC and it can also induce CCR8 expression independently of the VitD signaling pathway.

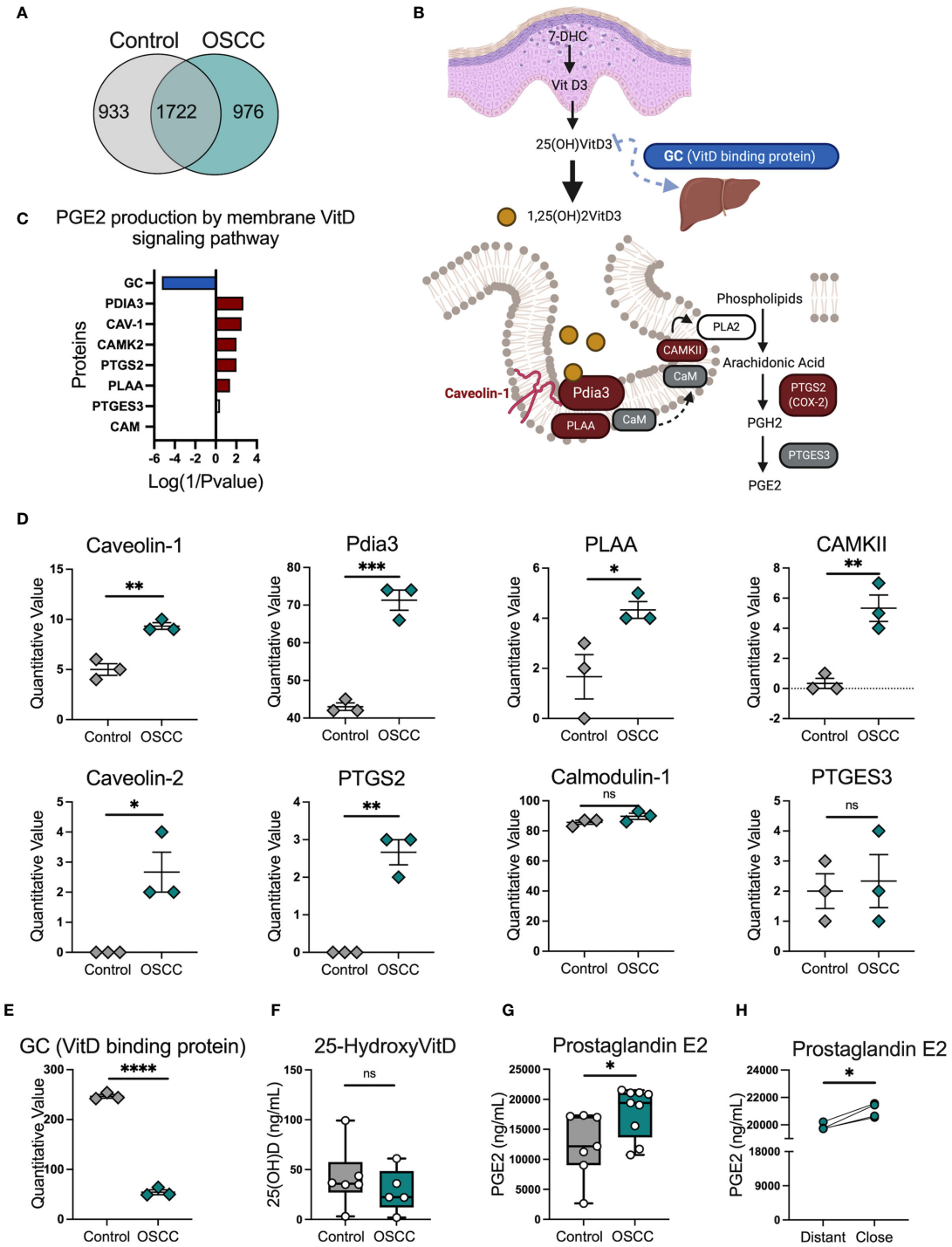

FIGURE 6 | Proteomic analysis identified several proteins related with the PGE2 production by membrane vitamin D signaling pathway in OSCC secretomes. **(A)** Venn diagram of unique and common proteins identified in secretome obtained from biopsies from 5 OSCC and 5 control samples using timsTOF Pro. **(B)** Proteins and **(C)** diagram of the PGE2 production by membrane vitamin D signaling pathway. Briefly, overexpressed proteins in OSCC were colored in red, reduced proteins in OSCC were colored in blue and proteins present in the secretomes but with no statistical difference between control and OSCC were colored in grey. **(D)** Quantitative values of proteins from the PGE2 production by membrane vitamin D signaling pathway, data are presented as mean ± SEM using scatter dot plots (Unpaired t test). **(E)** Quantitative values of vitamin D binding protein or GC, data are presented as mean ± SEM using scatter dot plots (Unpaired t test). **(F)** Levels of 25(OH)VitD and **(G)** PGE2 were compared between cancer and control secretomes, data are presented as mean ± SEM using bars with scatter dot plots (Unpaired t test). **(H)** Levels of PGE2 were measured in secretomes from distant and close OSCC biopsies to the tumor site, data is presented with individual symbols with paired lines (Paired t test). For all statistical tests, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ were considered significant. ns, not significant.

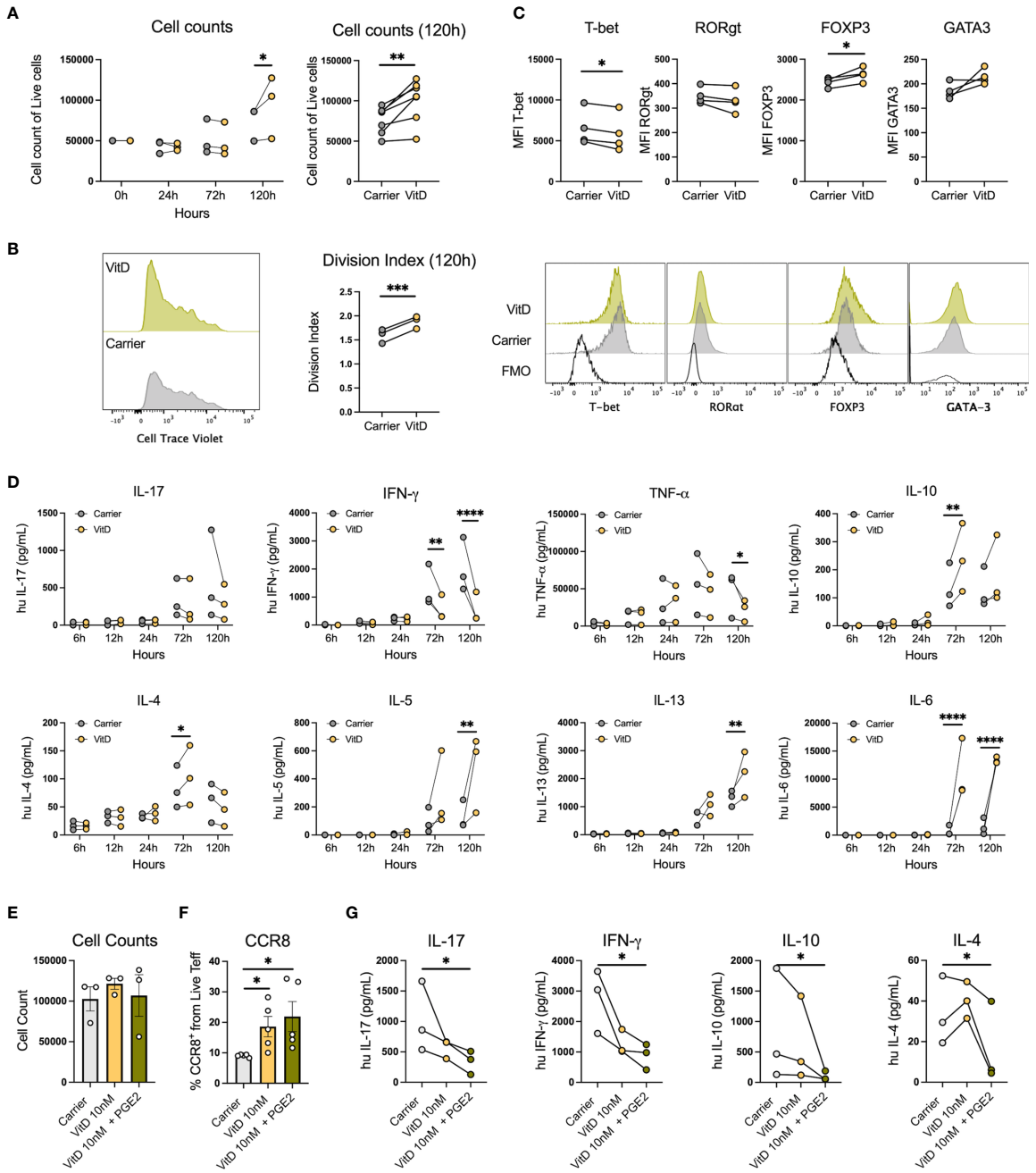

FIGURE 7 | PGE2 with VitD induce CCR8 expression and inhibit cytokine production in Th cells. **(A)** Representative histograms and cumulative data of cell counts and **(B)** division index of sorted memory Teffs (2×10^5) activated with anti-CD3/CD28 beads (1:5) in the presence or absence of 1,25(OH)VitD3 (10nM in ethanol) or Carrier (ethanol) at 24h, 72h and 120h post activation. Data are presented as individual symbols with paired lines (Two-way Repeated Measure ANOVA and Paired t test). **(C)** Representative histograms and cumulative data of transcription factor expression of sorted memory Teffs (2×10^5) activated with anti-CD3/CD28 beads (1:5) in the presence or absence of VitD (10nM in ethanol) or Carrier (ethanol) at 120h post activation. Data are presented as individual symbols with paired lines (Paired t test). **(D)** Cytokines were measured in supernatants obtained from sorted memory Teffs (2×10^5) activated with anti-CD3/CD28 beads (1:5) in the presence or absence of 1,25(OH)VitD3 (10nM in ethanol) or Carrier (ethanol) at 6h, 12h, 24h, 72h and 120h post activation. Data are presented as individual symbols with paired lines (Two-way Repeated Measure ANOVA). **(E)** Cell counts, **(F)** CCR8 expression and **(G)** cytokine production were measured in anti-CD3/CD28 pre-activated Teff cells ($1x10^5$) cocultured with carrier (ethanol), 1,25(OH)VitD3 (10nM in ethanol) or 1,25(OH)VitD3 (10nM in ethanol) in combination with PGE2 (5uM) for 72h with flow cytometry. Data is presented as mean ± SEM using column bars plots with bars with scatter dot plots for phenotype and individual symbols with paired lines values for cytokine production (Paired t test). For all statistical tests, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ were considered significant.

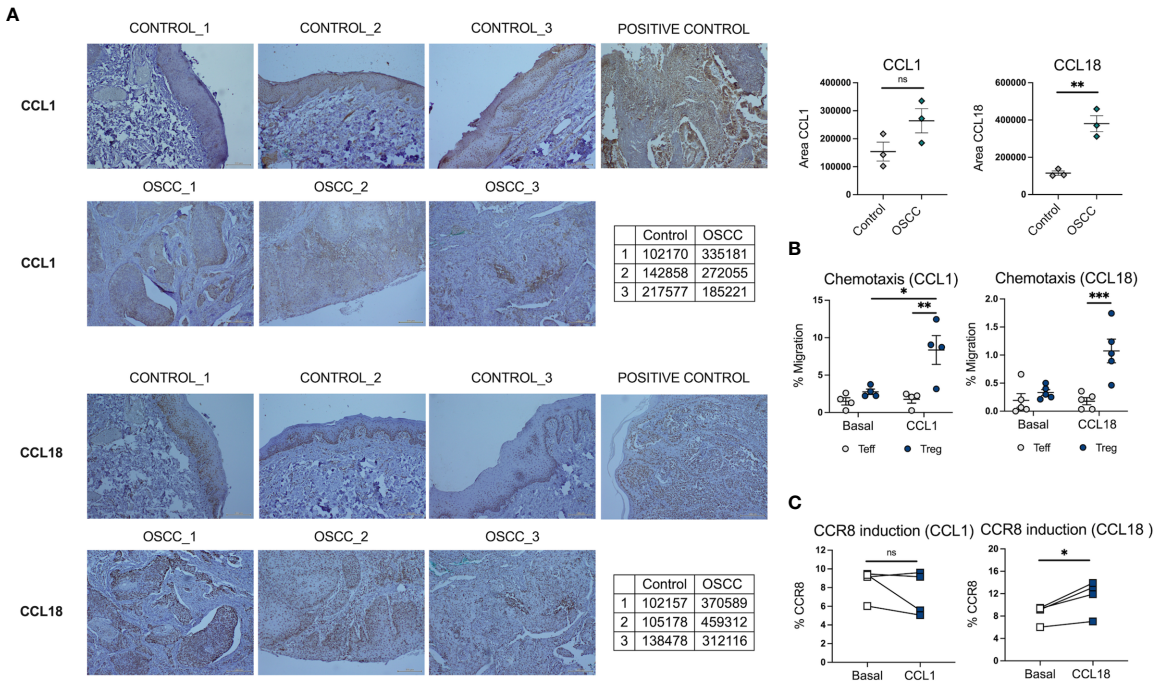

FIGURE 8 | CCL18 is augmented in histological samples of OSCC patients and also induce CCR8 expression in Teff. **(A)** Representative histological staining of CCL1 and CCL18 in a biopsy from a patient with OSCC and a patient without malignancy, using colon carcinoma as a positive control for CCL1 and melanoma as a positive control for CCL18. **(B)** Semi-quantification of area for CCL1 and CCL18 staining by ImageJ, data is presented as mean ± SEM using individual values described in the tables (Unpaired t test). **(C)** Percentage of migrated memory Teffs and Tregs to recombinant chemokines CCL1 and CCL18. Sorted Cell trace violet^{+} Memory Teffs (5×10^{4}) and unstained memory Tregs (5×10^{4}) were placed in the top chamber of a 5-μm-pore Transwell filter system. Bottom chambers were filled with media only, CCL1 or CCL18, (all 0.5 ug/mL). The percentage of migration for each subset was calculated as (number of cells in the bottom chamber after 1 h × 100)/initial number of cells in the top chamber. Data are presented as mean ± SEM using scatter dot plots (Paired t test). **(C)** CCR8 expression was measured in pre-activated memory Teffs (1×10^{5}) cultured with media only, or media with CCL1 or CCL18, (all 0.5 ug/mL) for 72h, data are presented using individual symbols with paired lines (Paired t test). For all statistical tests, $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ were considered significant. ns, not significant.

DISCUSSION

T lymphocytes have been the most representative and well-studied tumor-infiltrating subset in oral cancer. The presence of infiltrated Th cells in tumors has been correlated with rapid cancer progression (34) and poor prognosis (35). Several studies have identified the phenotype of Th cells in tumors and some authors have observed an imbalance in the different Th lineages in oral cancer, being Th2 cells augmented and Th1 cells reduced in comparison with samples from healthy donors (36). In general, pro-inflammatory Th1 responses have been associated with good prognosis in cancer, as these responses increase macrophage mediated phagocytosis, activates B cells to promote the production of opsonizing antibodies, activates complement and activates CD8^{+} T cells to promote cytotoxic mechanisms (37). Th2 cytokines,such as IL-4 and IL-10, are increased in late-stage cancers in comparison to Th1 cytokines that are more prevalent in the early-stage (38). This indicates that the immune responses are associated with cancer progression, and changes in the repertoire of cells directed by the tumor could be detrimental. Th17 cells have also been associated with tumor progression in oral cancer (39), as well as Tregs, which have been found increased not only in the oral tissue, but also in peripheral blood (40, 41), expressing CTLA-4^{+}, HLA-DR^{+} and granzyme B^{+} (42) and inhibiting IFN-γ, and promoting IL-10 and TGF-β secretion (43, 44). In addition, a positive correlation between Treg infiltration and the TNM score has been observed in this cancer (45, 46). Furthermore, T cells can modulate other immune cells such as macrophages, which can also potentiate cancer progression, specially M2 macrophages, as previously reported (47).

Our previous data reveled a significant association between Th2-like Tregs with colorectal cancer and melanoma, however despite the fact we observed higher percentages of Th2-like Tregs in OSCC in comparison with control samples, Th-like subsets overall did not cover the majority of the memory Treg population as it occurs in peripheral blood due to high CCR4 expression in circulation (15). This was an interesting observation as CCR4 has been previously used to identify cutaneous Th subsets (48). On the other hand, CCR8 was the main chemokine receptor expressed in Tregs from breast cancer (49, 50), and in Tregs of lung adenocarcinoma, melanoma and

colorectal adenocarcinoma in comparison with their counterpart (51) effector population (49). CCR8 is also increased in Tregs from colorectal cancer (51). In term of the role of CCR8 in Tregs, Coghill et al. demonstrated in a graft-versus-host disease (GVHD) mouse model wherein CCR8 was required for Treg survival *in vivo*. Interestingly, this study showed no effect in terms of activation and proliferation and the addition of CCL1 and CCL18 showed no effect on Treg viability *in vitro*. However they suggest that the interaction between Tregs and DCs was required to induce CCR8-mediated survival (52). Other studies analyzed CCR8 Tregs from human blood analyzed their suppressive capacity in the presence of four CCR8 ligands CCL1, CCL18, CCL16 and CCL18. Their results demonstrated that CCL1 was the only ligand able to promote Treg suppressive function and $Ca2^{+}$ flux post activation (33). However, previous data from other authors demonstrated that CCL18 was also able to induce $Ca2^{+}$ flux in CCR8 transfected cells (53). With regards to CCR8 expression, Barsheshet et al. (33), showed increased CCL1-mediated CCR8 expression in Tregs, however they did not analyze the effect of other ligands in order to understand whether this effect was specific to CCL1. Our data showed that CCL18 was the main chemokine increased in OSCC, associated to cancer cells mainly by immunohistochemical analysis. This could be explained by the important role of CCL18 in oral cancer where it promotes hyperplasia and metastasis by JAK2/STAT3 signaling pathways (54). In fact, in a study focused on the alterations of chemokine and chemokine receptors in premalignant stages of OSCC, CCL18 was the top one gene significantly upregulated in oral leukoplakia samples in comparison with normal epithelia (55). In this context, another study demonstrated that CCL18 induced cell epithelial-mesenchymal transition and promoted cell migration and invasion (56), therefore it would be interesting to investigate factors that promote CCL18 expression in oral epithelia, and how CCL18 upregulation affects cancer cells. We observed that the OSCC secretome induced *CCL18* gene expression in Teff and CCL18 was able to promote CCR8 expression, therefore it would be interesting to observe how the tumor environment is able to regulate this chemokine to promote $CCR8^{+}$ cells. We demonstrated that $CCR8^{+}$ Teff were reducing Th1 responses and promoting Th2 responses, whereas $CCR8^{+}$ Tregs produce less cytokines than $CCR8^{-}$ Tregs. This result suggests that CCR8 expression in Tregs identifies a population with a reduced capacity to secrete cytokines, both anti and pro inflammatory. Since the transcriptomic data showed induction of IL-10 in Th cells by OSCC secretome, it is possible that other post transcriptional mechanisms may play a role in the regulation of surface markers and cytokines.

VitD signaling responses can be triggered by gene transcription after VitD-VitD Receptor (VDR) binding to response elements and by Pdia3-mediated rapid membrane response (57). The latter is a rapid response that requires the presence of Pdia3 and Cav-1, where Cav-1 acts as a scaffolding protein, and Pdia3-Cav-1 form a membrane receptor complex in caveolae, triggering the binding of PLAA to Pdia3 and activating PLA2 *via* PLAA (57, 58). Subsequently the activation of PLA2 by PLAA, results in the production of PGE2 *via* arachidonic acid (58). A largescale transcriptomics analysis of differentially expressed genes from 326 OSCC and 165 normal controls revealed that the main enriched pathway regulated were extracellular matrix (ECM)-receptor interaction and focal adhesion according to several genes related to ECM structure such as laminins, collagen and integrins (28). The authors also revealed three upregulated hubs (defined as genes with significant interaction partners regulating the differentially expressed genes), named BGH3 (Transforming growth factor-beta-induced protein ig-h3), MMP9 (Matrix metalloproteinase-9) and Pdia3. The hub genes were then validated by immunohistochemistry and Pdia3 was absent in normal oral mucosa, while a high percentage of positive expression was found in OSCC (28). In addition, Pdia3 in combination with caveolin and PLAA, have been associated with the production of PGE2 by VitD signaling (57, 58), which in turn is associated with CCR8 expression on T cells (32). Our proteomic analysis showed MMP9 and Pdia3 as proteins significantly increased in OSCC samples. In addition, several proteins related with the rapid membrane VitD pathway were upregulated. The data also revealed reduced VDBP in OSCC proteomic samples, suggesting a potential imbalance in the transport of VitD, promoting its accumulation in the cancer area,as previously shown in a VDBP knock out mouse model (29). Our transcriptomic data showed several pathways associated with VitD and PGE2 responses, possibly due to the accumulation of VitD in cancer areas and the production of PGE2 *via* VitD membrane signaling pathway. In addition, one of the top genes up regulated by the secretomes in both T cell subsets was PTGER2, the prostaglandin E receptor 2, suggesting that the secretome not only contained more PGE2, but also induce the transcription of the receptor, possibly due to the effect of PGE2 (59). PGE2 as well as VitD has been shown to inhibit Th1 responses (60, 61), which was one of the main effects of the OSCC secretome by downregulating Tbet (*TBX21*) and IFN-γ production. These results suggest that the cancer impairs the VitD transport, promoting VitD accumulation and the activation of the production of PGE2 *via* the VitD membrane signaling pathway. In this environment, activated T cells expressing VitD receptor respond to these metabolites by reducing antitumor responses and promoting a regulatory phenotype.

It is well known that the TME can support angiogenesis, tumor progression, and immune evasion from T lymphocyte recognition (62). In this context, the immune checkpoint (e.g., PD-1, PD-L1, or TIGIT), can be modified by the TME to impair the endogenous antitumor T cell responses (62). Interestingly, high PD-L1 expression has been associated with good overall survival in oral squamous cell carcinoma (12), however other authors have shown increased PD-1-PDL-1 expression by conventional and fluorescent immunohistochemistry in OSCC, even before malignant transformation in early premalignant lesions (63). Other studies found an association between PD-L1 and PD-1 immunoreactivity and malignant clinicopathological features and a poor prognosis (64, 65). We did not check PD-1 or PDL-1 expression in tissues, but we found

that OSCC secretomes were able to upregulate PD-1 expression on Teff and Tregs. The induction of PD-1 in T cells can promote PD-1-PDL-1 binding, which in turn inhibits the lymphocytes activation and cytokine secretion (66). TIGIT is another inhibitory molecule that has been found in several studies aimed at identifying genetic profile of tumor infiltrating T cells. This marker is associated with inhibition of Th1 and Th17 responses, but not Th2 responses (27). In cancer, co-expression of TIGIT and PD-1 has been observed in tumor infiltrated $CD8^+$ T cells (67) and its expression is increased in Tregs within Th subsets (68). CD155, expressed in cancer cells, binds to TIGIT on T cells to induce direct inhibitory signals and disrupt CD226-mediated T cell activation (69). Interestingly, we observed no induction of PD-1 and TIGIT by VitD, thus these markers were induced by other unknown mechanisms.

Traditionally, OSCC has been associated with late-stage diagnosis and poor prognosis. Palliative care is the only treatment in some cases, and when surgery is possible, it can prolong survival, but it also affects the quality of life of the patients and their relatives. It is thus crucial to understand the molecular aspects of this cancer in order to identify potential mechanism to improve the anti-tumor response. This study revealed novel information regarding the immunoregulatory effect of tumor environment from OSCC affecting Th subsets. The understanding of these responses could help to identify potential treatments in order to improve survival in patients with late-stage OSCC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee from the Health Service Talcahuano number 19-06-11 and Ethical Committee from the Health Service Concepcion number 19-03-07. The patients/ participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MF, FL, and EN-L performed experiments. MY and MS recruited patients, analyzed histological samples, defined OSCC pathological state, participated in the OSCC diagnosis and collected the clinical data. MH designed, performed, and analyzed the proteomic data. GN analyzed the proteomic data. EN-L, MF, GN, FZ, GL, and FA performed the RNA sequencing experiment. LL analyzed vitamin D levels. MY, MS, JU, LN, SC, OZ, AC, LC, SM, JP, MN, JG, JF, and RP performed surgery or dental procedures resulting in sample collection. CR and CS contributed with pathway analysis from proteomic data. GC performed cell sorting. MV analyzed the transcriptomic data. MF and EN-L wrote the first draft of the manuscript. EN-L and RM wrote the second draft of the manuscript. MF and EN-L organized the database, analyzed the data, performed pathway analysis, transported the samples, created the figures and performed the statistical analysis. EN-L designed the study and directed the project. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the Chilean Agency of Investigation and Development (ANID) grant FONDECYT 11170610 and PAI79170073. MF was funded by FONDECYT 11170610, Sindicato-2 Postgraduate Scholarship and University of Concepcion Postgraduate Scholarship. FL was funded by University of Concepcion Postgraduate Scholarship. EN-L was funded by FONDECYT 11170610, PAI79170073 and FONDECYT 1211480. CS is supported by Lions Medical Research Foundation, Diabetes Australia, National Health and Medical Research Council (NHMRC) and FONDECYT 1170809.

ACKNOWLEDGMENTS

We acknowledge ¨Las Higueras¨ Hospital, Guillermo Grant Benavente´s Hospital, Family Heath Center ¨CESFAM Penco-Lirquén¨ and the Pathological Anatomy Unit at University of Concepcion to provide the infrastructure to recruit patient and collect samples. We acknowledge Dr. Luis Urra, Dr. Camila Champin, Dr. Álvaro Compán, Dr. Joaquin Ulloa, Dr. Juan Munzenmayer, Dr. Jorge Beltrán, Dr. Mario Gutiérrez from Guillermo Grant Benavente´s Hospital for performing surgery or dental procedures resulting in sample collection. We acknowledge the Biotechnology Center (CB-FONDEQUIP EQM120148) and the Advance Microscopy Centre (CMA BIO-BIO ANID PIA ECM-12) at University of Concepcion. We acknowledge Melisa Institute, EMSA laboratory and PreveGen laboratory for providing the infrastructure and human resources to perform crucial experiments. We acknowledge reactome.org, scaffold - Proteome software and BioRender software. We thank with gratitude to all our participants for their contribution with their samples and clinical data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.643298/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: 07 May 2021
doi: 10.3389/fimmu.2021.642285

Mechanisms of Macrophage Plasticity in the Tumor Environment: Manipulating Activation State to Improve Outcomes

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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 December 2020*
Accepted: *16 April 2021*
Published: *07 May 2021*

Citation:
Ricketts TD, Prieto-Dominguez N, Gowda PS and Ubil E (2021) Mechanisms of Macrophage Plasticity in the Tumor Environment: Manipulating Activation State to Improve Outcomes. Front. Immunol. 12:642285. doi: 10.3389/fimmu.2021.642285

Macrophages are a specialized class of innate immune cells with multifaceted roles in modulation of the inflammatory response, homeostasis, and wound healing. While developmentally derived or originating from circulating monocytes, naïve macrophages can adopt a spectrum of context-dependent activation states ranging from pro-inflammatory (classically activated, M1) to pro-wound healing (alternatively activated, M2). Tumors are known to exploit macrophage polarization states to foster a tumor-permissive milieu, particularly by skewing macrophages toward a pro-tumor (M2) phenotype. These pro-tumoral macrophages can support cancer progression by several mechanisms including immune suppression, growth factor production, promotion of angiogenesis and tissue remodeling. By preventing the adoption of this pro-tumor phenotype or reprogramming these macrophages to a more pro-inflammatory state, it may be possible to inhibit tumor growth. Here, we describe types of tumor-derived signaling that facilitate macrophage reprogramming, including paracrine signaling and activation of innate immune checkpoints. We also describe intervention strategies targeting macrophage plasticity to limit disease progression and address their implications in cancer chemo- and immunotherapy.

Keywords: cancer, macrophage, plasticity, therapy, tumor, inflammation

INTRODUCTION

Macrophages represent one of the most phenotypically diverse innate immune cell populations. They are key homeostatic regulators that activate and modulate the innate and, subsequent adaptive immune response to infectious agents and host-derived components. Much like other innate immune cells, they are hard-wired to respond to cues rather than being "educated" to elicit a response, as is the case of adaptive immune cells (1). Macrophages are equipped with a variety of Pattern Recognition Receptors (PRRs) that, once activated, trigger pre-determined programs in response to environmental stimuli. Some pro-inflammatory stimuli include Pathogen-Associated Molecular Patterns (PAMPs), cellular or chemical moieties derived from pathogens, or Damage-Associated Molecular Patterns (DAMPs) which are released by damaged cells and malignancies.

These signatures permit macrophage adoption of the appropriate functional phenotype to restore physiological equilibrium.

During infections, macrophage polarization to the proinflammatory state is crucial for the production of type 1 cytokines such as interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) and interleukin 12 (IL-12) for host resistance (2–4). This is similar to the response following injury. Cells in damaged tissues undergo necrosis and release their contents in an uncontrolled manner (5–7). Contrary to apoptosis, which is a highly organized program for cell death, necrosis is more immunogenic and induces a macrophage pro-inflammatory response. Cellular components released during necrosis act as DAMPs that, when bound to PRRs like Toll-like Receptors (TLRs), initiate pro-inflammatory signaling in resident and extravasated monocyte-derived macrophages. Activation of PRRs, and other sensors, facilitate the adoption of a pre-programmed pro-inflammatory state, also termed M1 or "classically activated" (**Figure 1**). This occurs through increased activation of signaling pathways involving NFκB, p38, MAPK, and others, which regulate the expression of pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-12 (8, 9)) (**Figure 2**). These macrophage-secreted signals recruit a variety of other immune cells that pioneer the clearance of infected and damaged material.

A hallmark of the pro-inflammatory response is the destruction of damaged cells and those in the immediate vicinity. This creates a need for wound healing to restore tissue integrity. Upon removal of damaged tissue, the aggregate population of macrophages at the site of injury transitions to a pro-wound healing phenotype, also referred to as M2 (**Figure 1**). This transition is triggered by anti-inflammatory mediators following the loss of pro-inflammatory signals, like DAMPs. These pro-wound healing macrophages coordinate the proliferation of key cell types including vascular endothelial cells, which promote recellularization by delivering oxygen and nutrients to the site of repair, and fibroblasts which drive scar formation (10–12). Macrophages also dampen the local inflammatory response, fostering a more hospitable environment for continued repair, cellular proliferation and the prevention of extensive or persistent inflammation that might contribute to further tissue damage (13–16).

While macrophage plasticity is beneficial during the wound healing process, the macrophage response is subverted during cancer. Often termed "a wound that does not heal" (17), tumors

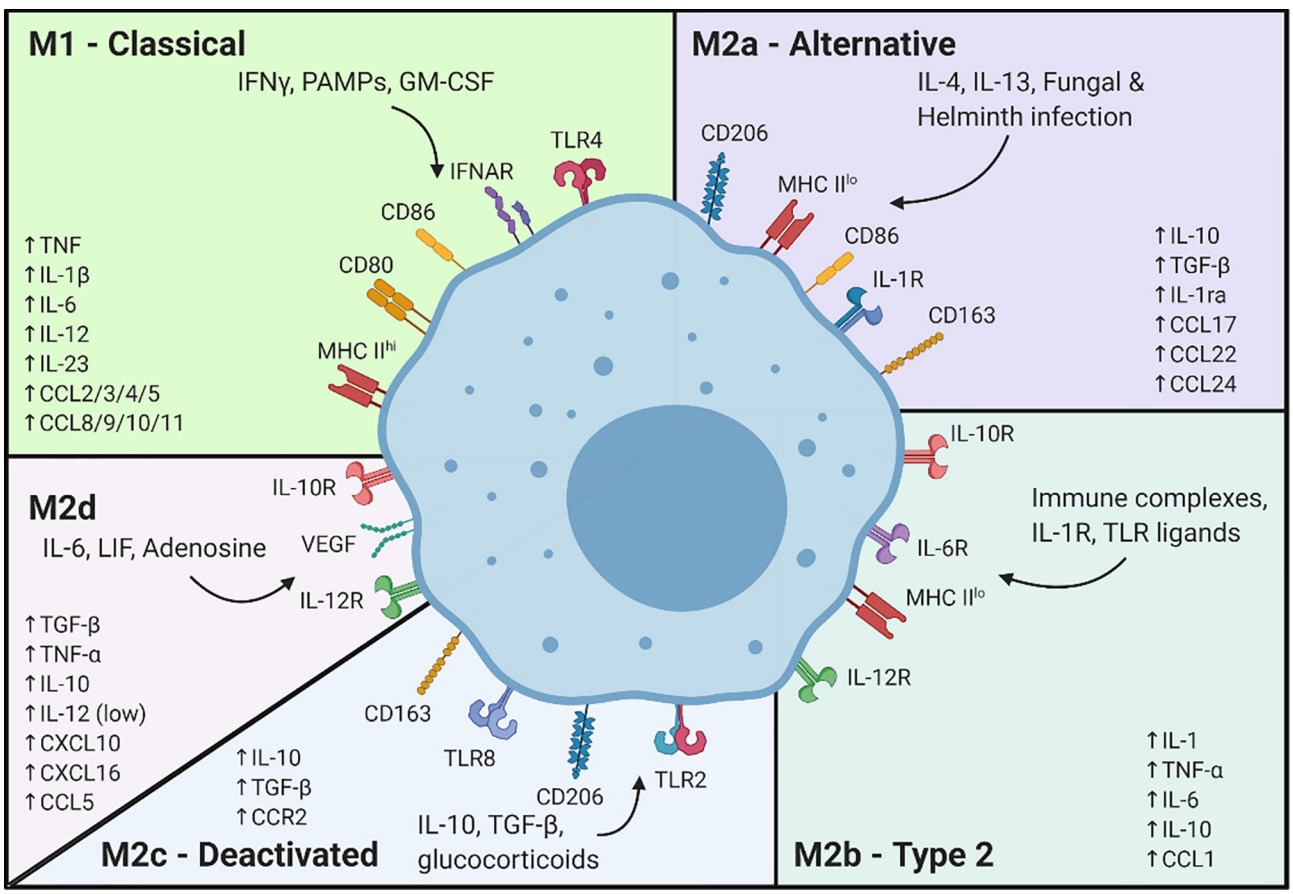

FIGURE 1 | Signals associated with macrophage differentiation to the classically and alternatively activated subsets. Created with BioRender.

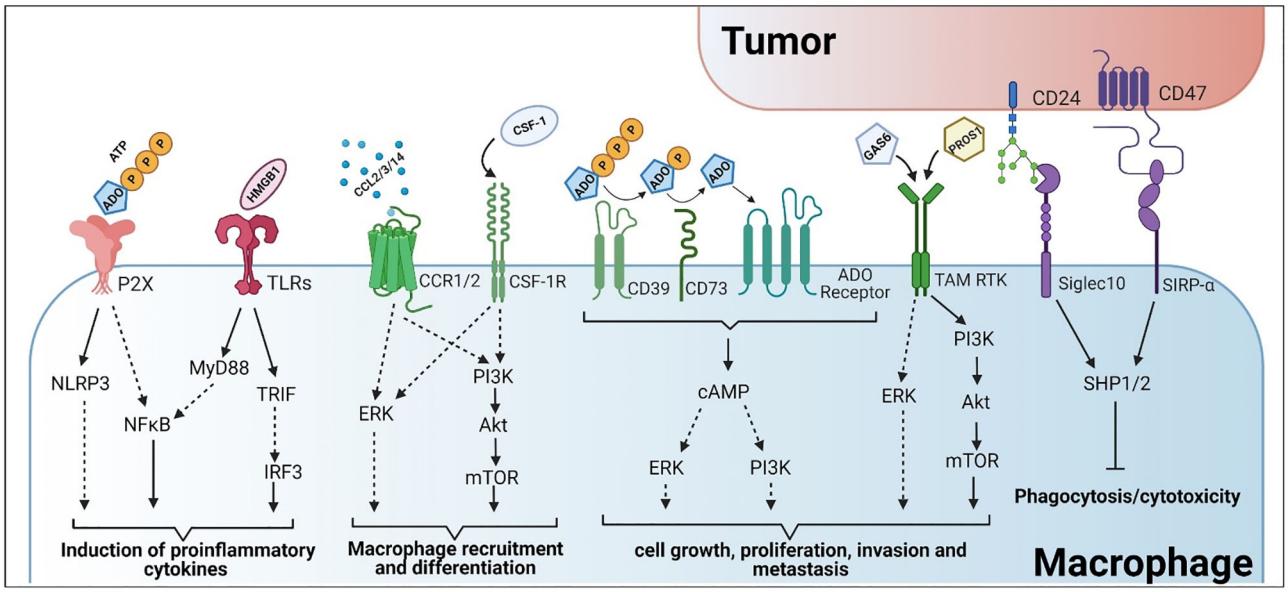

FIGURE 2 | Tumor-macrophage interactions and their subsequent roles in immune evasion and activation. Created with BioRender.

manipulate and reshape the immune response to promote and sustain tumor growth. Presumably, due to the inhospitable nature of the tumor microenvironment (e.g., hypoxia, nutrient starvation), cancer cells undergo necrotic death which should induce the macrophage pro-inflammatory response, ultimately leading to further immune activation and reduced tumor growth. However, in many tumors, the pro-wound healing phenotype is predominant, which actually supports cancer progression. This review outlines strategies employed by tumors to mitigate macrophage pro-inflammatory activation or engage the pro-wound healing response. Current therapeutic interventions that alter the intra-tumoral M1/M2 balance and shift it towards a more pro-inflammatory/anti-tumor response are also described. We also explore potential conceptual flaws in the current pro-inflammatory/pro-wound healing paradigm in cancer, based on recent single-cell RNA-seq findings, and implications these could have in the manipulation of macrophage activation state to reduce tumor growth.

THE ROLE OF MACROPHAGES IN THE ANTI-TUMOR RESPONSE

During tumorigenesis, genetic mutations can be acquired through exposure to chemical carcinogens (18), radiation (19) or viral infections (20, 21). Alternatively, inherited mutations (22, 23) or those accumulated during chronic inflammation (24–26) may also drive carcinogenesis. Cell intrinsic tumor suppressive mechanisms, like DNA repair, senescence or apoptosis (27), often fail to contain tumor cell proliferation, promoting the need for immune-mediated elimination of the aberrant cells. Ideally, early responding immune cells, like macrophages, will detect and eliminate tumor cells. Much like during wound healing, macrophages may detect DAMPs, possibly from hypoxia-induced tumor cell death or dysregulated cellular processes (28), to trigger a pro-inflammatory response and pave the way for true wound healing or a return to homeostasis. Alternatively, macrophages or dendritic cells, as antigen presenting cells, may engulf tumor neo-antigens, process them and present antigenic peptides to tissue resident CD8+ or CD4+ T cells, or in the case of dendritic cells, transit to the draining lymph node to activate T cells (29–31). Whether for tissue resident or T cells transiting from the lymph nodes, pro-inflammatory macrophages provide co-stimulatory signals such as CD40 (32) or CD80/86 (33), secrete activating cytokines (34), and generate nitric oxide to increase vascular permeability and immune cell infiltrate. T cells with the cognate receptor matching the tumor neo-antigen, in the presence of co-stimulation, should eradicate tumor cells unless they encounter other immuno-suppressive signals.

While many early-stage tumors are presumably destroyed through these mechanisms, the immune response to cancer is clearly not effective. Rather, based on the immune-editing hypothesis (35), the pro-inflammatory response applies a selective pressure, forcing tumors to "evolve" to avoid detection (e.g., through reduced antigenic protein expression, reduction in antigen presentation (35) or suppression of the local immune response (36)). Alternatively, nascent tumors may undergo a period of dormancy, and may later be reactivated by acquired secondary or tertiary mutations that allow for reduced immunogenicity or increased immune suppression. Collectively, this evolution is thought to allow tumor cells to reach an equilibrium with the immune response. Following this

equilibrium state, tumors may effectively "escape" the immune response by utilizing mechanisms to prevent immune activation, allowing them to grow largely unchecked.

Consequently, these immuno-editing processes may limit macrophage responsiveness to DAMPs and tumor neo-antigens, effectively abrogating their ability to transition to an M1 phenotype (37) and promote T cell activation. In many tumors, there is a promotion of the M2 phenotype which fosters tumor growth. Presumably, either acquired through the equilibrium/escape processes of immuno-editing or because tumors provide contextual cues similar to those that promote the pro-wound healing response. These M2 macrophages are pro-tumorigenic and are often denoted as tumor-associated macrophages (TAMs). Akin to the wound healing response, macrophages facilitate cellular proliferation through production of growth factors like Wnts (38), CXCL8 (39) or IL-6 (40, 41). However, instead of promoting the re-growth of tissue resident cells, these factors drive tumor growth. Likewise, macrophages also secrete key effectors of vascularization, like the vascular endothelial growth factor (VEGF) (42, 43), platelet-derived growth factor (PDGF) (44) and transforming growth factor β (TGFβ) (45) to promote angiogenesis (**Figure 1**). These physiologic processes are hijacked to increase blood flow to the tumor, increasing tumor cell access to oxygen and nutrients for continued cell proliferation. M2 macrophages may also maintain tumor growth through the remodeling of the extracellular matrix (ECM) through secretion of matrix metalloproteases (MMPs) and other factors (45, 46) (**Figure 1**).

In the tumor context, pro-inflammatory macrophages are considered a positive prognostic marker (47–49). Pro-inflammatory macrophages are thought to positively regulate the immune response and kill tumor cells directly. These polarized macrophages prevent tumor growth by generating factors such as reactive oxygen and nitrogen species, or other secreted factors like TNFα, that lead to tumor cell death (50–53). Macrophages can be induced to a pro-inflammatory state by other immune cells, such as through the secretion of IFNγ by T cells, or directly by tumor cells. Alternatively, DAMPs can be released by necrotic or necroptotic tumor cell death due to hypoxia or nutrient deprivation within the tumor microenvironment (54, 55). These DAMPs, whether they be nucleic acids, ATP, stress-related proteins such as heat shock proteins (HSPs) (56–58), or transcription factors such as HMGB1, HMGN1 (59–65), bind to and activate two major classes of PRRs including the TLRs or the NOD-like receptor (NLR) family. Interestingly, several TLRs that recognize pathogenic signatures also recognize DAMPs. For instance, TLR4, which is activated by the binding of bacterial lipopolysaccharide (LPS) also recognizes HSPs and transcription factors (66).

Conversely, the presence of M2 pro-wound healing macrophages in tumors is generally a negative prognostic marker, with patients with high numbers of intra-tumoral M2 macrophages showing decreased survival (67). Tumor cells are known to secrete, or induce the secretion of, factors like IL-4, IL-10 or IL-13 that polarize macrophages toward an M2 phenotype (44, 68). Some pro-wound healing properties of M2 macrophages foster tumor growth and prepare a tumor-friendly milieu (**Figure 1**). M2 macrophages can act to directly increase tumor growth by secretion of growth factors like endothelial growth factor (EGF), VEGF and TGFβ (69–73), and can reduce the hypoxia inherent in most tumors while allowing the delivery of nutrients to sustain tumor growth. M2 macrophages also assist in the remodeling of the tumor microenvironment. Regulation of fibroblast ECM placement, degradation of existing ECMs through MMPs and chemotactic migration signals, allow continued tumor growth and metastasis. In some cases, live cell imaging has shown tumor cells utilizing accessory macrophages to travel to blood vessels and allow entry into the vasculature (74–76).

MACROPHAGE-DIRECTED THERAPEUTIC STRATEGIES FOR CANCER TREATMENT

Based on knowledge garnered from the study of macrophage activation states in tumors, as well as associated signaling affecting polarization, several strategies have been developed to mitigate tumor progression by altering macrophage infiltration or by activating/re-activating them to a pro-inflammatory state. While a limited number of macrophage-directed therapeutics are currently in use in clinical trials, continued identification and pharmacological targeting of macrophages is expected to bolster the use of macrophage targeted agents.

Macrophage Depletion to Reduce Pro-Tumoral Activity

Since higher numbers of TAMs are associated with worse cancer prognosis, research has focused on reducing their numbers by targeting their tumor recruitment and differentiation (77–79). As a result, some of the subsequent strategies are being tested for clinical use and may be broadly available soon.

Macrophages, similar to other phagocytes, can be selectively targeted by complexing cellular pro-apoptotic substances, such as bisphosphonates, into nanoparticles (80) (**Table 1**). The deletion of TAMs by using clodronate encapsulated in liposomes (clodrolip) leads to reduced teratocarcinoma and rhabdomyosarcoma tumor growth in pre-clinical murine studies (144). This inhibition was coupled with a decrease in tumor microvascular density, suggesting its potential combination with VEGF-neutralizing agents to maximize its effect (144).

Alternatively, inhibition of the chemotactic axis CCL2-CCR2 may prevent the accumulation of circulating macrophages within the tumor microenvironment. Indeed, several monotherapy or combinational clinical trials are currently underway with positive results (81). However, CCL2-CCR2 inhibitors should be carefully administered since the sudden interruption of therapeutic regimens could dramatically increase tumor progression and metastasis (145).

Additionally, targeting the monocyte/macrophage colony stimulating factor (CSF-1) and its receptor (CSF-1R) is a

TABLE 1 | Summary of preclinical, clinical and current therapeutic approaches targeting macrophages for the treatment of various malignancies.

Therapeutic Agent	Therapeutic Modality	Indication	Target	Effect	Development Status	References
Anti-CCR2	Monoclonal antibodies (mAbs), small molecule inhibitor	Metastatic solid tumors	CCL2/CCR2	CCR2 antagonist blocks the adaptation of TAM features	Phase I/II clinical trials	(81–84)
Anti-CD24	mAbs	Advanced solid tumors	CD24/Siglec10	Increases expression of M1 macrophages and phagocytosis	Preclinical	(85, 86)
Anti-CD39	mAbs	Advanced solid tumors	CD39	Increases extracellular ATP, promotes M1 phenotype	Phase I clinical trials	(87–89)
Anti-CD40	Vaccine, mAbs	Lung cancer, metastatic melanoma, solid cancers	CD40	CD40 agonism promotes proinflammatory activity and increases antigen presentation	Phase I/II clinical trials	(90–93)
Anti-CD47	mAbs	Advanced solid tumors, hematologic malignancies	CD47/SIRPa	Increases macrophage phagocytosis and M1 activation	Phase I/II clinical trials	(94–96)
Anti-CD73	mAbs	Advanced or metastatic cancer	CD73	Promotes anti-tumorigenic macrophage activation	Phase I/II clinical trials	(87, 88, 97)
Anti-CSFR1	Blocking antibodies, small molecule inhibitor (BLZ945)	Advanced solid tumors	CSF1/CSFR1	Increases proinflammatory and tumoricidal activity, inhibits recruitment of immunosuppressive populations	Phase I/II clinical trials	(98–101)
Bemcentinib	Small molecule inhibitor	Advanced or Metastatic Solid Tumors	Axl RTK	Inhibits polarization to the anti-inflammatory macrophage phenotype	Phase I/II clinical trials	(102–104)
BMS-777607	Small molecule inhibitor	Advanced solid tumors	TAM RTKs	Restores proinflammatory immune activation, decreases immune suppressive cytokines and efferocytosis	Phase I/II clinical trials	(105, 106)
Clodronate	Bisphosphonate	Breast, prostate and bone neoplasms	Complement receptors	Depletes TAMs	Phase III	(107–111)
CpG ODN	Single stranded DNA, vaccine adjuvant	Breast cancer, malignant melanoma, glioblastoma, leukemia	TLR9	TLR9 agonist to switch macrophage polarization to proinflammatory	Phase I/II clinical trials	(112–114)
Dasatinib	Small molecule inhibitor	Chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) advanced cancer	Src family tyrosine kinases	TAM depletion	Phase IV clinical trials, FDA approved for CML and ALL	(115–117)
Ferumoxytol	Metallic nanoparticles	Breast cancer, small cell lung cancer	Varies based on surface conjugates of nanoparticles	Reprograming of TAMs to tumoricidal, proinflammatory macrophages	Pre-clinical	(118–120)
IL-12	Polymeric nanoparticles, vaccine, gene therapy	Metastatic cancer, solid tumors	IL-12R	Re-education of TAMs	Phase I/II clinical trials	(121. 122, 123)
Imatinib	Small molecule inhibitor	Metastatic, advanced solid tumors, refractory malignancies	STAT6	Inhibits macrophage polarization to anti-inflammatory subset	Phase IV clinical trials FDA approved for CML	(80, 124, 125)
Imiquimod	Topical, vaccine, small molecule inhibitor	Basal cell carcinoma (BCC), skin cancer, solid tumors	TLR7	Reprogramming TAMs toward proinflammatory phenotype	Phase IV clinical trials	(126–128)
Nilotinib	Small molecule inhibitor	Solid tumors, neoplasms, gastrointestinal stromal tumors	BCR-ABL	Inhibits macrophage polarization to anti-inflammatory subset	Phase IV clinical trials FDA approved for CML	(80, 125)
P2X7 antagonism	Topical	BCC	ATP/purinergic receptor	Promotes M1 activation and phagocytosis	Phase I	(129–131)
STAT3 Inhibitors	Small molecular inhibitor	Advanced solid tumors	STAT3	Inhibits polarization to anti-inflammatory phenotype	Phase I/II clinical trials	(132–134)
STAT6 inhibitors	Small molecular inhibitor	–	STAT6	Inhibits polarization to anti-inflammatory phenotype	–	(135–137)
Sunitinib	Small molecular inhibitor	Refractory solid tumors, renal cell carcinoma (RCC), gastrointestinal stromal tumors (GIST)	Multi-targeted RTKs	Blockade of anti-inflammatory phenotype	Phase IV clinical trials, FDA approved for RCC and GIST	(80, 138)
Zoledronic acid	Bisphosphonate	Breast cancer, prostate cancer, metastatic neoplasms	TLR4	Phenotype switch to proinflammatory	Phase IV clinical trials	(139–143)

tractable strategy for macrophage depletion. In the absence of this signal, bloodborne monocytes are unable to differentiate into macrophages, preventing macrophage tumoral accumulation (146). Accordingly, several CSF-1R/CSF-1 targeted therapies, such as PLX3397, JNJ-40346527 and BLZ945, are currently being tested in clinical trials either alone or in combination for the treatment of several cancers (98, 147–149). However, these inhibitors can also stimulate the recruitment of tumor-promoting granulocytes to the site of the tumor, resulting in therapy failure (150). Therefore, combination of CSF-1R repressor with adaptive immune checkpoint inhibitors may be an interesting strategy to mitigate this unexpected effect (150).

Finally, the antineoplastic agent, trabectedin, also depletes TAMs to induce pro-inflammatory T cell recruitment in pancreatic ductal adenocarcinoma preclinical models (151). Therefore, it could also be a potential new strategy for TAM depletion during cancer treatment.

Manipulating Macrophage Activation State to Improve the Anti-Tumor Response

Using *in vitro* models of macrophage polarization, it has been shown that responses to respective M1/M2 stimuli are transient. Treatment with M1 inducing agents, like LPS and IFNγ, induce a pro-inflammatory response within 2-4 hours, which may subside within 24-48 hours (51, 152). After this transient activation, macrophages return to a "resting" state akin to the naïve (M0) polarization. Likewise, activation with one stimulus does not preclude the ability to adopt a subsequent, alternative polarization. A notable example is when stimulating conditions are switched from IFNγ to IL-4 or vice versa, macrophages adopt the profile of the most current cytokine microenvironment (153). Gao and colleagues utilized M-CSF and IL-4 to induce human monocyte differentiation to the M2 phenotype. Following M2 polarization, macrophages were treated with lactoferrin-containing IgG immunocomplex (LTF-IC), which promotes M1-like activation and is an immune activator in rheumatoid arthritis (154). After M1 stimulation, M2 marker expression was reduced while M1 markers were increased. In a similar experiment, Cheng et al. induced M2 polarization in murine RAW264.7 cells using IL-4 and IL-13. Subsequent treatment of M2 macrophages with a β-1,6-glucan (AAMP-A70) caused a reduction of M2 polarization concurrently with increased M1 marker expression (155). These findings are particularly important in the context of cancer treatment, as they clearly demonstrate the plasticity of macrophages depend on the environmental stimuli.

Considering the transient and plastic nature of macrophages, paired with the negative prognosis of intra-tumoral M2 macrophage accumulation, several approaches have been developed to repolarize M2 macrophages to an M1 phenotype. Macrophages, much like T cells, also have immune checkpoints. The prevention of tumors from activating innate immune checkpoints, is another approach in preventing the suppression of macrophage anti-tumor responses. Alternative approaches that manipulate the plasticity of macrophages are being heavily explored. Several of these strategies are described in the following sections.

Pro-Inflammatory Stimulation *via* TLR Agonism

The activation of TLRs, surface or endosomal proteins able to detect cellular damage and induce a proinflammatory immune response, have been broadly used therapeutically to alter macrophage activation in several diseases, including cancer (156–158) (**Figure 2**). The rationale is that the stimulation of these receptors, particularly within the tumor environment, may activate the pro-inflammatory response seen during the early stages of wound healing and infection, leading to the eradication of tumor cells (159, 160). Moreover, the release of tumor-derived DAMPs and neo-antigens during this process should generate a positive feedback loop to further increase the anti-tumor response (75, 159). A potential drawback of this form of therapy is tolerization, a state of unresponsiveness that appears after repetitive exposure to the same inductor, characterized by the release of anti-inflammatory factors that mask TLR activation (161).

Components of pathogenic organisms, such as LPS, derived mainly from *Eschericia coli*, are commonly used tools to activate macrophages and induce a pro-inflammatory state, often in combination with IFNγ to maximize the effects (162). However, LPS administration in humans produces severe toxicity and multiple exposures rapidly lead to tolerance, thus new strategies to improve its clinical use are currently being investigated (162). More recently, TLR3, TLR7/8 and TLR9 agonists have risen as new therapeutic alternatives to induce a TLR-dependent, tumor-localized pro-inflammatory response (163). For instance, the TLR7 agonist, Imiquimod, induces a robust rejection of skin primary malignancies and metastases by generating a pro-inflammatory tumor microenvironment in human patients (164) (**Table 1**). Similarly, polyinosinic-polycytidylic acid (poly-IC), a TLR3 agonist, triggers T cell tumor infiltration and Th1 responses, which should in turn activate macrophages through IFNγ signaling, to reduce malignant growth (165). Finally, the TLR9 agonist family CpG oligodeoxynucleotides (CpG ODN) have also shown strong cancer cytotoxic effects by exerting a potent tumor-localized immunostimulatory action (166) (**Table 1**). Based on early successes, these TLR agonists are currently in Phase 1/2/3 clinical trials (162, 163).

To target macrophages more specifically, nanoparticles that take advantage of the phagocytic properties of macrophages are being developed. After injection, nanoparticles are trafficked to the tumor where they are engulfed by macrophages. Techniques are being developed to package TLR agonists into nanoparticles for more specific activation of these immune cells (167). This novel approach would reduce the off-target effects of TLR agonists on other immune cells, such as lymphocytes, as well as to reduce their tolerizing effects (168). Furthermore, injected nanoparticles tend to accumulate in the tumor because of often ill-formed and leaky tumor vasculature, leading to a therapy more targeted to intra-tumoral macrophages (169). Loading β-cyclodextrin nanoparticles with the TLR7/8 agonist R484 has surfaced as one of the most promising techniques to restrain tumor growth by shifting TAM behavior to the M1 state (170).

Activating ATP NOD-Like Receptors to Promote M1 Polarization

Purinergic activation of macrophages plays a crucial role for the secretion of the pro-inflammatory cytokines, IL-1β and IL-18, and can be mediated through the activation of the NLRP3 inflammasome (171–173) (**Figure 2**). Cellular stress (e.g., exposure to chemotherapeutics, toxins, and radiation) and tissue damage are key contributors to ATP release into the extracellular environment (174). Release of ATP is one of the most potent DAMPs for immune activation, promoting M1 macrophage polarization and increasing macrophage tumoricidal potential (87, 129, 175), (**Figure 2**). However, to maintain the cellular ATP equilibrium, tumor cells, macrophages, and other immune cells, express ectonucleotidases to maintain the concentration gradient. CD39 and CD73 are ectonucleotidases that are involved in the formation of the metabolite adenosine (ADO). CD39 sequentially hydrolyzes ATP and ADP to form AMP, whereas CD73 hydrolyzes AMP to form ADO (**Figure 2**). This shift in the concentration gradient also acts as a switch to a more M2-like functional program and attenuates the anti-tumor response. Adenosine activates ADO/purinergic G-coupled protein receptors on tumor and immune cells, such as macrophages, to induce immunosuppression (176). Likewise, ADO also functions to inhibit TLR signaling and the secretion of proinflammatory cytokines such as TNFα, IL-6, and IL-8 from activated human monocytes (177). Given the contrasting nature of ATP versus ADO signaling for macrophage activation in tumor immunity, this interface serves as a potential target for the clearance of tumor cells. Inhibition of CD39 in preclinical models have shown significant promise in diminishing the immunosuppressive activity of TAMs, whereas inhibition of CD73 proved effective in controlling metastatic growth (178) (**Table 1**). Furthermore, combinational therapeutic strategies employing innate immune checkpoint inhibitors and anti-CD39 or anti-CD73 promoted antitumor immunity (88). Lastly, antagonism of the ATP receptors (P2X7) increases tumor infiltrating immune effector populations and decreases tumor burden (130) (**Table 1**).

Macrophage Polarization by Targeting Intracellular Signaling Mechanisms

In addition to mimicking extracellular pro-inflammatory stimuli, intracellular signaling pathways are also being targeted to reduce the prevalence of M2 signaling in tumors. This has been observed in the tumor-mediated manipulation of macrophage PI3Kγ signaling to reduce the pro-inflammatory response (179). Actually, targeting PI3Kγ pharmacologically has effectively "flipped the switch" from M2 to M1 in preclinical models (179, 180). PI3K is a family of phosphorylation enzymes that act on the 3' end of phosphatidylinositol (PI) and work in conjunction with the Akt family of serine/threonine kinases and the mechanistic target of rapamycin complex (mTORC) 2 to switch the activation status of TLR-stimulated macrophages to a less pro-inflammatory program (181, 182) (**Figure 2**). PI3K/Akt signaling is involved in migration and diapedesis of innate immune effectors such as neutrophils and monocytes/macrophages and is associated with the upregulation and stabilization of hypoxia-induced transcription factors in macrophages (183). Induction of these transcription factors is associated with the hypoxic tumor microenvironment and stimulates M2-like characteristics in macrophages, thus supporting tumorigenesis and metastasis (184–186). Moreover, the PI3K/Akt pathway also promotes macrophage-mediated remodeling of the ECM, angiogenesis and immunosuppression of the adaptive immune response. Inhibition of PI3K signaling has shown considerable effects in regulating VEGF expression, a known factor that stimulates the adoption of the M2 functional program (183). There are several preclinical and clinical studies aimed at manipulating PI3K signaling to improve tumor outcomes. Inhibition of this pathway has been shown to increase macrophage infiltration and production of proinflammatory cytokines and chemokines (187). Akt signaling has differential downstream effects and deficiencies in Akt1 induced M1 activation (188). Consequently, inhibition of Akt signaling disrupts mTORC2 aggregation which diminished macrophage viability and proliferation (189).

The signal transducer and activator of transcription (STAT) signaling pathway is also of clinical interest. Downstream of several receptor tyrosine kinases, the STAT family communicates signals from the cytosolic face of the plasma membrane to the nucleus, where STAT dimers act as transcription factors and transcriptional modulators. STAT1 is recognized as a pro-inflammatory mediator and signaling can be initiated by type I and II interferons, growth factors, TLR activity and cytokine release. STAT1 signaling has broad effects on cancer and can either be antitumoral or pro-tumoral. Antitumoral STAT1 signaling is usually attributed to the tumoricidal activity of M1 macrophages while the pro-tumoral action is affiliated with the enrichment of STAT1-dependent genes that protect against genotoxic damage or promote tumor growth (190). Conversely, STAT3 is broadly recognized as an anti-inflammatory regulator, stimulating M2-like macrophage polarization. STAT3 phosphorylation can be triggered by interleukins such as IL-8, IL-10, IL-35 and growth factors such as EGF. Following activation, STAT3 signaling promotes a myriad of pro-tumoral outcomes such as the inhibition of apoptosis, cell proliferation, metastasis, angiogenesis and therapeutic resistance (41, 191). Studies targeting the activation of STAT1 or the suppression of STAT3 may be crucial for manipulating the balance of M1/M2 signaling.

Other transcription factors are also under study for potential roles in M1/M2 plasticity. These include KLF6, Zeb1 and NFAT1. KLF6 is a transcriptional regulator of macrophage polarization that serves as a phenotypic switch to transform M2-polarized TAMs to M1, effectively inhibiting tumor proliferation and migration (192). Contrariwise, ZEB1 is associated with TAM pro-tumoral activity, indicated by its ability to pioneer epithelial to mesenchymal transition to maintain tumor progression and initiate metastasis (8). Nuclear factor of activated T cell (NFAT) also supports the M2-like phenotype of TAMs through the regulation of

interleukins (IL-6, IL-10, IL-12) and multiple TLR-induced genes such as iNOS (193). NFAT1 is overexpressed in TAMs and promotes tumor cell proliferation, invasion and metastasis and facilitates the recruitment of macrophage populations that are associated with poorer outcomes (194, 195). Given the role of NFAT signaling in regulating immune homeostasis, NFAT inhibition may effectively suppress anti-inflammatory cytokine production while subsequently initiating pro-inflammatory and tumoricidal programs within these tumor-associated macrophage populations.

Unfortunately, because individual transcription factors tend to be involved in transcriptional regulation throughout the genome, specifically targeting them to selectively target individual regulatory programs remains elusive. However, as time goes on, it may be possible to more selectively target individual immune cell types or add co-factors to increase specificity, yielding more robust anti-tumor efficacy.

Manipulating Macrophage Metabolism to Increase M1 Polarization

The metabolic changes associated with M1/M2 polarization may also regulate activation state (196, 197). Much like the distinct glutaminase-dependent differentiations of Th17 and Th1 T cells to regulate the immune response (198), direct metabolic changes in macrophages, or the output of altered metabolism, can affect M1/M2 polarization.

Arginase is essential for amino acid metabolism and has potent immunomodulatory effects through the catalysis of L-arginine. L-arginine is involved in nitric oxide synthesis which contributes to the tumoricidal activity of macrophages (199). However, the catabolism of L-arginine by arginase results in the formation of L-ornithine and its decomposition product, putrescine, which are known to support the cell growth and proliferation of tumor cells (199–202). Furthermore, increased production by TAMs impairs the antitumor immune response (203). Likewise, putrescine induces macrophage efferocytosis to prevent inflammation and promote tissue repair (204), a hallmark of tumor progression. Catabolism of L-arginine also has devastating consequences for other immune effectors, such as cell cycle arrest and anergy (203). Inhibition of arginase I expression reduces tumor burden and subsequently increases lymphocyte infiltration within the tumor microenvironment (205, 206) indicating significant potential for clinical testing.

Like arginase, indoleamine 2,3-dioxygenase (IDO1) is an immunosuppressive molecule secreted by TAMs. IDO1 catabolizes tryptophan to kynurenine which binds to the aryl hydrocarbon receptor to trigger a myriad of immunoregulatory mechanisms in immune cells (207). The signaling cascade triggered by IDO1 enzymatic activity facilitates immune evasion by diminishing lymphocyte responsiveness and anticancer immunosurveillance (208–210). IDO1 activity is also suggested to increase tolerance in macrophages, downregulate antigen presentation molecules (HLA-DR) and decreased macrophage phagocytic activity (211). Furthermore, IDO has also been shown to increase M2 polarization and recruitment while inhibition of IDO activity increases M1 populations (212). IDO1 inhibition prevents tryptophan depletion and subsequently blocks the associated downstream immunosuppressive signals (213, 214). This suggests that targeting IDO enzymatic activity in tumors that overexpress this enzyme may improve macrophage polarization to M1, immune activation and immunotherapeutic efficacy.

Targeting Innate Immune Checkpoints to Improve Therapeutic Outcomes

Much like the adaptive immune response, immune checkpoints have been discovered and characterized for innate immune cells. One example is the Tyro3/Axl/Mer (TAM) family of receptor tyrosine kinases, (**Figure 2**). During normal physiological processes, this family of receptors is instrumental in apoptotic cell engulfment and degradation (efferocytosis). The TAM family of receptors has 5 known ligands, Gas6 (215), Pros1 (216), Gal3 (217), Tubby and Tulp1 (218). As cells undergo apoptosis, phosphatidylserine that has flipped from the cytosolic face of the plasma membrane to the extracellular region is recognized by these ligands to form a bridge to the TAM receptors. However, these ligands can also activate the TAM receptors in the absence of phosphatidylserine (219), though activation is reduced. Lastly, kinase inhibition or genetic loss of Mer prevents internalization of apoptotic material (220, 221).

In addition to its role in efferocytosis, genetic lack of Mer is associated with hypersensitivity to TLR activation (222, 223), suggesting its role in limiting the innate immune response and preventing autoimmunity. More recently, it was shown by Lemke and Rothlin, in dendritic cells, that activation of Mer initiates an anti-inflammatory program involving upregulation of Socs1/2 (224). Later, Cook et al., demonstrated, in the context of cancer, that genetic deletion of Mer was associated with reduced M2 macrophage polarization with increased M1 (225). Ubil et al. later showed that tumor-secreted Pros1, acting on Mer and Tyro3 induces the downregulation of pro-inflammatory gene expression (51). Mice bearing tumors with genetic deletion of Pros1 showed increased intra-tumoral macrophages that were skewed towards the M1 phenotype. This was associated with increased adaptive immune infiltrate with approximately 5-fold more CD4+ and CD8+ T cells as well as a ~50% reduction in Tregs. Mice with Pros1 deficient tumors lived ~30% longer than mice with parental tumors. Furthermore, addition of the TLR7/8 agonist, Resiquimod, did not improve survival in mice bearing Pros1 replete tumors whereas survival duration was doubled for mice whose tumors lacked Pros1. Taken together, these findings demonstrate that tumor secretions can dampen the innate, macrophage, response and subsequently the adaptive immune response. TAM kinase inhibitors are currently in Phase I clinical trials for the treatment of human cancers.

Another marker involved in immune checkpoints and expressed by intra-tumoral macrophages is PD-L1. PD-L1 is generally associated with expression by tumors, particularly in response to IFNγ. When tumor expressed PD-L1 binds to PD-1 on T cells, it leads to T cell inactivation and facilitates tumor immune evasion. Tumors are also able to induce expression of PD-L1 in macrophages to similarly limit the

action of effector T cells (226). Macrophage PD-L1 - T cell PD-1 interactions are, therefore, at the interface of innate and adaptive immune responses.

Several PD-1 and PD-L1 targeted therapeutics are currently in the clinic for treatment of various forms of cancer (227). In addition to the direct effects of blocking PD-1/PD-L1 interactions, PD-1 targeted treatments also induce secondary effects, such as the increased polarization of macrophages from a pro-wound healing phenotype to a more anti-tumor, pro-inflammatory, state. Xiong et al. characterized intra-tumoral macrophage polarization states of MC38 tumor bearing mice after anti-PD-1 treatment. They observed an increase in the numbers of M1-like and M1/M2 intermediate macrophages with a decrease in M2-like phenotypes. Using IFNγ depletion of supernatants from tumors which had either been treated with vehicle or anti-PD-1 antibody, they determined that IFNγ was a primary driver of macrophage polarization (228). Presumably, anti-PD-1 treatment of tumor bearing mice led to increased T cell activation, including IFNγ secretion. In turn, polarization of intra-tumoral macrophages were skewed towards an M1 state, including increased antigen presentation and expression of pro-inflammatory cytokines. Activated M1 macrophages increased T cell activation in a self-reinforcing cycle, ultimately leading to reduced tumor growth. This study succinctly demonstrates the importance and inter-relatedness of the innate and adaptive immune functions in limiting tumor progression.

Targeting "Don't Eat Me" Signaling to Improve Macrophage Activation and Antitumor Immunity

A crucial aspect of macrophage activity is phagocytosis, the internalization of cells, pathogens, and other particles for tissue homeostasis. As key endocytosing immune cells, macrophages are the primary phagocytic population and should be able to recognize aberrant cells and clear them using this process. However, tumor cells express anti-phagocytic ligands or "don't eat me" signals similar to healthy cells in order to avoid elimination.

CD47 is an immunoglobulin that is crucial in self recognition for the maintenance of immune tolerance and homeostasis. It complexes with the signal regulatory protein α (SIRPα) on phagocytic cells to inhibit uptake and subsequent immune activation (229). However, this molecule is also expressed on the surface of many tumor cells and plays a key role in immune evasion (**Figure 2**). CD47/SIRPα signaling leads to the phosphorylation of the SIRPα cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) resulting in the recruitment of the tyrosine phosphatases SHP1/2. This signaling mechanism prevents the accumulation of myosin at the phagocytic synapse, effectively inhibiting phagocytosis (230–232). This process is crucial in preventing uncontrolled clearance of healthy cells but becomes a detriment based on its role in facilitating immune evasion in cancer. As such, these signals are also targeted to improve the antitumor response. CD47 blockade has shown significant efficacy in the treatment of several hematological cancers and solid tumors which may be mediated by innate immune effector populations such as macrophages (94, 95, 233, 234) (**Table 1**). Furthermore, preclinical models of the CD47/SIRPα signaling axis are highly efficacious for treating multiple cancer types and are currently being probed in clinical trials.

CD24 is another "don't eat me" signal that is expressed by many tumor types (**Figure 2**). CD24 is a glycosylphosphatidylinositol anchored protein that is known to complex with Siglec10 on macrophages and other innate immune cells for the suppression of the inflammatory response in many conditions including sepsis, liver damage and infection (85, 235, 236). Like CD47 signaling, the CD24/Siglec10 signaling axis results in the recruitment of SHP1/2 at the ITIMs of Siglec10, inhibiting the TLR-mediated inflammatory response and the cytoskeleton rearrangement required for phagocytosis (85). As such, the CD24/Siglec10 complex is a potent inhibitor of macrophage phagocytic activity and is protective of cancer cells. Inhibition of the CD24/Siglec10 signaling axis restores the macrophage-mediated antitumor response by enhancing phagocytic clearance of tumor cells (85, 86). Moreover, increased uptake of antigenic materials is also associated with increased immune activation and infiltration within the tumor microenvironment (85).

The importance of these signaling cascades in regulating macrophage plasticity are extensively studied and new models are currently being probed to increase innate immune activation and improve current immunotherapeutic approaches. A summary of these targets and their effect on macrophage activity within the tumor microenvironment, along with their development status, are described in **Table 1**.

CURRENT EXPERIMENTAL MODELING OF M1/M2 PHENOTYPES MAY NOT ACCURATELY REPRESENT INTRA-TUMORAL MACROPHAGE POLARIZATION STATES

To model macrophage responses, the M1/M2 paradigm was developed and dates back more than 20 years (237). In early models, naïve macrophages were induced to adopt two known polarization states (238). Since then, through decades of research, multiple *in vitro* models of M1 and M2 polarization have been developed in which various exogenous stimuli can induce activation states that mimic physiological conditions (e.g., pathogenic infection (239–241), pro-inflammatory activation by T cells (242, 243), etc.). At present, experimental macrophage models have been delineated into 5 core subsets: M1, M2a, M2b, M2c and M2d (244), (**Figure 1**).

Historically, activation of the M1 state has been modeled using stimuli such as LPS, IFNγ (a pro-inflammatory signal derived from activated T cells) or both in combination. While LPS induces TLR4 activation and downstream NFκB signaling, IFNγ binds the IFNgR1/2 complex, leading to STAT1 phosphorylation and nuclear translocation to mediate pro-inflammatory gene expression (245, 246). Alternatively, addition of TNFα (247) to naïve macrophages yields a similar

activation state. TNFα binds to TNFR1 and TNFR2, leading to activation of downstream signaling cascades including p38 (248, 249) and others (250–253). The pro-inflammatory signaling pathways tend to converge on NFκB, STAT1 and MAPK pathways, with significant crosstalk effectively leading to similar outcomes in terms of gene expression changes and activation states.

M2 activation states are comparatively more complicated with at least 4 different subsets being identified, including M2a, M2b, M2c and the relatively newer M2d phenotype (152, 254, 255) (**Figure 1**). Induced by IL-4, IL-13 or the combination thereof, M2a has been described as an anti-inflammatory and pro-wound healing subset (256–258). M2b, which is induced by addition of IL-1β, has shown immuno-regulatory properties and associated gene expression (244, 259). M2c macrophages, induced by treatment with IL-10, show increased expression of immune suppressive and tissue remodeling markers (260). Some indications also suggest efferocytosis is increased in M2c macrophages (261). Finally, in an attempt to create a model of TAMs (M2d), it was discovered that treatment with IL-6 could cause upregulation of tumor growth and angiogenesis markers (262).

At this point, there is not one clearly prevailing macrophage M2 subset that best represents tumor associated macrophages. Instead, researchers often combine multiple stimuli, such as IL-4 (M2a), IL-13 (M2a) and IL-10 (M2c), which are present in the tumor microenvironment, to mimic tumor associated macrophages (263, 264).

While continually improving, our understanding of intra-tumoral macrophage activation states have led to an iterative improvement in models. However, newer and better methodologies are currently being utilized to disaggregate our current population-level understanding. Specifically, single cell RNA-seq (sc-RNA-seq) has refined our understanding of intra-tumoral macrophage heterogeneity and called into question some of our existing paradigms on "either/or" M1/M2 polarization.

SINGLE-CELL RNA-SEQ DATA SHEDS NEW LIGHT ON INTRA-TUMORAL MACROPHAGE POLARIZATION

Based on established *in vitro* models of macrophage polarization (M1/M2), early characterization of intra-tumoral macrophages focused on a few pro-inflammatory or pro-wound healing markers (e.g., iNOS, IL-1, CD206, etc.) to identify activation states. As more nuanced models of polarization have been developed, additional markers have been identified, demonstrating that rather than adhering to distinct polarized types, macrophages exhibit a spectrum of overlapping activation states. Further complicating the ability to describe tumor associated macrophages is that spatial location and microenvironmental factors can have major impacts on polarity, causing macrophages in one part of the tumor to have very different activation states than those in adjacent locations. The advent of single cell RNA-seq has opened new venues for understanding intra-tumoral macrophage activation and may identify misconceptions about how macrophages behave in the tumor microenvironment. This new technique allows for the characterization of individual cells within the tumor resident immune cell subset. Depending on the process flow, immune cell subtypes may be enriched prior to single-cell RNA-seq analysis (265, 266) or bioinformatically identified based on expression patterns (267). Several variations of single-cell RNA-seq exist, some of which also incorporate locational data.

Characterization of Macrophage Activation State in Tumors

Using single-cell RNA-seq to characterize immune subset in primary breast cancer samples, Chung et al. found that macrophages tend toward the M2 phenotype (265), confirming previous findings that breast cancer tends to foster M2 polarization (46, 268). Of the 515 cells from 11 patients characterized, most non-carcinoma cells in the cancer samples were identified as immune cells based on their gene expression signatures. TAMs were primarily found to have pro-wound healing M2-associated profiles (269, 270). A key finding of this paper is that it supports the notion that in breast cancer, many macrophages and other innate and adaptive cell populations have an immune suppressive phenotype.

Recognizing that there is robust heterogeneity of intra-tumoral macrophage polarization states, single cell RNA-seq is also being used to determine whether there are discrete activation states or whether there is a contiguous spectrum driven by local microenvironmental conditions. Azizi et al., employed a large-scale, high-dimensional analysis platform to characterize the immune profiles of more than 45,000 cells from eight breast carcinomas, matched with normal breast tissue, blood and lymph nodes using single-cell RNA-seq (271). To do so, they collected CD45 positive cells from treatment-naïve breast cancer patients including estrogen receptor (ER+) and progesterone receptor (PR+) positive, human epidermal growth factor receptor 2 amplified (HER2+) and triple negative (TNBC) tumors. These CD45+ cells were isolated by fluorescence-activated cell sorting (FACS) and subjected to single-cell RNA-seq using the inDrop platform (272, 273). Data was preprocessed using the SEQC pipeline with the Bayesian clustering and normalization method, Biscuit, utilized for data analysis. One of the key findings of the study is that intra-tumoral macrophages have higher numbers, diversity and activation relative to those derived from normal tissues or lymph nodes. Somewhat surprisingly, the authors of this study found a positive correlation between M1 and M2 gene expression, with simultaneous co-expression of markers associated with both activation states. This is in direct contrast to previous results from *in vitro* model studies, in which one or more agents used to activate macrophages led to one aggregate activation state, either M1 or M2.

A different study, characterizing the heterogeneity of macrophages activation states in gliomas using single-cell RNA-seq made a similar observation on the simultaneous co-expression of M1 and M2 markers in TAMs. This study, conducted by Muller et al. (274), compared marker expression

of two macrophage populations – brain-resident microglia, derived from progenitors that migrated to the central nervous system (CNS) and bone marrow-derived monocytes that extravasate through the blood brain barrier and differentiate into macrophages. Similar to Azizi et al., Muller et al., found that macrophages could co-express M1 and M2 markers simultaneously with 66% of tumor associated macrophages co-expressing the canonical M2 marker, IL-10, while also expressing the M1 marker, TNFα. They confirmed their results by using flow cytometry of tumor derived macrophages to show that CD11b+ cells could co-express the M1 co-stimulatory marker, CD86, while also expressing CD206.

Taken together, these studies call the M1/M2 polarization paradigm into question. While, to some extent, supporting the notion that a spectrum of intra-tumoral macrophage activation states exist (275, 276), the finding of simultaneous M1 and M2 associated markers by macrophages is quite novel. Perhaps historical use of conventional models coupled with aggregate analyses of pooled macrophage populations fail to detect a more widespread phenomenon of M1/M2 marker co-expression in tumors. Further experiments and analysis will be required to confirm these finding. Also, development of model systems that better recapitulate the dual activation states observed *in vivo* may yield better understanding of how intra-tumoral macrophages will respond to targeted therapeutics. Perhaps most importantly, these findings suggest that activating, or re-activating, the M1 phenotype in tumors may consequently lead to concurrent increased M2 polarization, thereby confounding outcomes.

Using Single Cell RNA-Seq Based Methods to Characterize Macrophage Activation While Incorporating Spatial Localization Within the Tumor

Conventional large-scale characterization of macrophage polarization loses spatial resolution. As such, novel single-cell RNA-seq/bioinformatic approaches are being developed that provide contextual identity. One such technique involves the use of spatial transcriptomics (277). This method performs unbiased mapping of transcripts over entire tissue sections using spatially barcoded oligo-deoxythymidine microarrays. Individual microarray spots capture transcriptome information from between 10-200 cells and the data is integrated with single cell RNA-seq data to provide both cellular context and transcription data at the single cell level. Using this approach, Moncada and colleagues performed multimodal intersection analysis on patient pancreatic ductal adenocarcinoma (PDAC) tumors (278). One of their key findings was that macrophages seem to adhere to the M1/M2 paradigm and exist in two main subpopulations. The first was a pro-inflammatory M1 subset, which expressed IL-1β, and a second subset, which expressed M2 associated genes like CD163 (278). Likewise, the two subpopulations were differentially localized, with M1 macrophages enriched in the cancerous regions or the stroma, while M2-like macrophages were enriched in the ducts. This data demonstrates that two opposing macrophage polarizations can exist in the same tumor, though their activation state is driven by local micro-environmental conditions. These findings suggest that, fundamentally, treatments may be more effective if they can be selectively targeted to regions where they will make the biggest change. Conversely, systemic treatment with an M1 inducing agent could disrupt essential processes and induce off-target effects.

Derivation of M2 Macrophage Subpopulations

Circulating monocytes are recruited to tumors by the expression of chemoattractants such as CCL2 (279–281), S100A8 and S100A9 (282, 283). Once monocytes extravasate, they are thought to differentiate into M1 or M2 macrophages based on signals from the tumor microenvironment. In a recent study, Song et al. used single-cell RNA-seq to characterize the differentiation process of extravasating monocytes. 11,485 cells from Non-Small Cell Lung Cancer (NSCLC) patients were used to develop a model of divergent monocyte differentiation into M1 or M2 macrophages. While there were differences between patients, on average, a substantially larger proportion of the recruited monocytes adopted the M2 phenotype (283). In CD14+ cells derived from in NSCLC samples, expression of polarization markers was stratified along a continuum effectively providing a snapshot of macrophage differentiation states. Work by Song et al., may enable the identification of specific lineage markers that will allow prediction of future differentiation states. They also identified signals from tumor-derived epithelial cells that skew differentiation to the M2 phenotype. By better understanding the process through which tumor resident M2 macrophages are derived, it may be possible to develop specific interventions that prevent accumulation of M2 macrophages.

OPEN QUESTIONS IN MACROPHAGE PLASTICITY DURING CANCER

Macrophages are a highly plastic innate immune cell subset. Depending on contextual cues from their local environment, they adopt phenotypes across a spectrum of activation states, ranging from pro-inflammatory (M1) to pro-wound healing (M2). Further, macrophages, both individually and in aggregate, can readily transition from one polarization state to the next depending on the most recent signals prevailing in their environment. This plasticity allows them to effectively adapt to the changing environments associated with infection and wound healing and facilitate the return to immune homeostasis. Unfortunately, in the context of cancer, macrophage plasticity is subverted to benefit continued tumor progression. Either by tumor-mediated suppression of M1 polarization or through the evolved lack of pro-inflammatory cues associated with cancer, intra-tumoral macrophages are generally of the pro-wound healing (M2) phenotype. The pro-wound healing properties which would be beneficial during injury repair, such as production of growth factors or promotion of angiogenesis, support continued tumor cell proliferation and tumor expansion.

Recognizing the inherent plasticity of macrophages, several therapeutics have been developed to either reduce the number of intra-tumoral macrophages, thereby reducing the M2 pool, or alter the M1/M2 balance to favor a more pro-inflammatory/anti-tumor response. Numerous clinical trials have demonstrated that increasing M1-associated polarization or effector functions can improve clinical outcomes. This is, perhaps, not surprising since a pro-inflammatory milieu is associated with better patient outcomes for many cancer types. However, to realize the promise of these new treatment modalities, several factors still need to be considered. As we have learned from adaptive immune targeted treatments, activation or checkpoint blockade alone are not likely to be sufficient to generate durable responses in several cancer types. Rather, macrophage targeted therapies will likely require co-treatments targeting the cancer directly (e.g., chemotherapy) or the adaptive immune response (e.g., checkpoint directed therapeutics) or both. Also, for the most part, M1 polarization is thought to reduce tumor growth. However, chronic and persistent local inflammatory conditions are also known to induce tumor formation (284–287). A prime example is that increased inflammation associated with obesity can actually increase the likelihood of tumor progression (288). Several other preclinical models of inflammation, such as colitis-induced colon cancer (72–76), have shown that persistent inflammation exacerbates tumor progression. As an illustration, in a high-fat diet induced inflammation model, prostate cancer progression was substantially increased (289). The rationale is that persistent cell damaging conditions may elicit genetic mutation or cell signaling alterations that foster tumor growth. While the current paradigm is that "more inflammation is better", there is likely to be an optimal amount of inflammation so as not to induce secondary tumor formation.

Another key question to be addressed, in addition to finding optimal combinations, is how to limit potential engagement of the autoimmune response. Even if a macrophage targeted therapy is successful in generating an anti-tumor response, what are the best ways to ensure it is targeted strictly to the tumor and not surrounding healthy tissues or organ systems? While some delivery systems, like nanoparticles, favor intra-tumoral macrophages, many require systemic delivery, increasing the potential for off-target effects. Potentially compounding the likelihood of off-target effects is reliance upon the bystander effect to generate an anti-tumor response. For example, TLR agonists mimic PAMPs and DAMPs that would be released during infection or injury. However, the resulting immune activation does not target tumor-intrinsic moieties, but rather utilize the destructive potential of pro-inflammatory macrophages to either kill neighboring tumor cells or activate other local immune cells. This lack of tumor specificity opens the greater possibility of non-specific cellular damage or even autoimmunity based on the release of cryptic epitopes.

In addition to questions of developing targeted therapeutics, some basic scientific questions also remain unanswered about macrophages in the tumor environment. While several models have shown, *in vitro*, that macrophages can move from one polarization state to the next, it is unclear whether this is also true in tumors. For instance, lack of lineage tracing prevents the accurate monitoring of individual intra-tumoral macrophages to determine what happens after treatment. Are macrophages that are present in the tumor prior to treatment adopting another phenotype or is macrophage turnover the cause for an aggregate shift in polarization? Development and use of lineage tracing models would provide a more expansive knowledge of macrophage activation during treatment.

Other questions that have arisen with the advent of single-cell RNA-seq include whether there is a previously unknown macrophage state the possesses elements of both the diametrically opposed M1 and M2 phenotypes. Can both activation states co-exist in one cell or group of cells? What environmental or cell intrinsic factors would allow for dual expression of pro- and anti-inflammatory markers? Do these dual activation macrophages also exist during wound healing or response to pathogenic infection or are they a cancer-specific phenomenon? Are there ways in which these specialized cells can be modeled *in vitro*? Perhaps most importantly, how do pro-inflammatory inducing treatments affect dual M1/M2 macrophages? Does their presence confound treatments focusing on M1 induction? For instance, if a TLR agonist is utilized for treatment, does it also increase the expression of M2 associated markers, simultaneously activating and inactivating the immune response? Further analysis of single-cell RNA-seq data may answer these questions. However, it may be possible, using flow cytometry or other techniques, to isolate these cells and characterize them using more traditional biochemical methods.

While there is a more comprehensive understanding of macrophage biology now than in the past, development of macrophage targeted therapeutics has trailed behind those promoting the adaptive immune response. Continuing to address the unanswered questions presented here, as well continued testing, both alone and in combination with other therapeutics, may bridge the gap, providing new hope for improved survival of cancer patients.

AUTHOR CONTRIBUTIONS

TR - wrote the manuscript, prepared figures, and edited final work. NP-D - wrote manuscript and edited final work. PG - wrote manuscript. EU - conceptualized the work, wrote manuscript, and edited final work. All authors contributed to the article and approved the submitted version.

FUNDING

NIH/NCI K22 Transition Career Development Award (1 K22 CA237742-01) - Funding for EU, and University of Alabama at Birmingham Development Funds - Funding for EU.

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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: 02 June 2021
doi: 10.3389/fimmu.2021.689132

The Combination of Immune Checkpoint Blockade and Angiogenesis Inhibitors in the Treatment of Advanced Non-Small Cell Lung Cancer

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Immune checkpoint blockade (ICB) has become a standard treatment for non-small cell lung cancer (NSCLC). However, most patients with NSCLC do not benefit from these treatments. Abnormal vasculature is a hallmark of solid tumors and is involved in tumor immune escape. These abnormalities stem from the increase in the expression of pro-angiogenic factors, which is involved in the regulation of the function and migration of immune cells. Anti-angiogenic agents can normalize blood vessels, and thus transforming the tumor microenvironment from immunosuppressive to immune-supportive by increasing the infiltration and activation of immune cells. Therefore, the combination of immunotherapy with anti-angiogenesis is a promising strategy for cancer treatment. Here, we outline the current understanding of the mechanisms of vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) signaling in tumor immune escape and progression, and summarize the preclinical studies and current clinical data of the combination of ICB and anti-angiogenic drugs in the treatment of advanced NSCLC.

Keywords: NSCLC, immunotherapy, immune checkpoint blockade, angiogenesis inhibitors, combination therapy, tumor microenvironment

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Edited by:
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Capital Medical University, China

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

Received: *31 March 2021*
Accepted: *18 May 2021*
Published: *02 June 2021*

Citation:
Ren S, Xiong X, You H, Shen J and Zhou P (2021) The Combination of Immune Checkpoint Blockade and Angiogenesis Inhibitors in the Treatment of Advanced Non-Small Cell Lung Cancer. Front. Immunol. 12:689132. doi: 10.3389/fimmu.2021.689132

INTRODUCTION

Lung cancer is one of the most common cancer types with high mortality in the world (1). Adenocarcinoma, squamous cell carcinoma and large cell carcinoma are the three major kinds of NSCLC comprising 85% of all lung cancers (2). Because of the lack of early diagnosis indicators, more than 70% of cancer patients have experienced local invasion, lymph node and distant metastasis at the first diagnosis (3). These patients have extremely poor prognoses. The five-year survival rate of patients at this stage is only 4% (4).

In the past decade, immunotherapy has made significant progress for the treatment of NSCLC. Improving the therapeutic effect *via* combination strategy has become the main direction in the field. A number of clinical trials testing the combination of immunotherapy and anti-angiogenesis have shown promising results in different tumor types including NSCLC. However, due to the complicated regulatory mechanisms of these two kinds of therapies, how to collaboratively use them to obtain the maximal therapeutic effect remains to be answered. Understanding the potential mechanisms of combination might help to select appropriate patients and treat them at right timing with optimized dosages of drugs.

IMMUNE CHECKPOINTS AND INHIBITORS

Immune checkpoint inhibitors (ICIs) are widely used in the treatment of NSCLC. A series of receptor/ligand pairs such as CD28-CTLA4/B7 and programmed cell death-1/programmed death ligand 1 (PD-1/PD-L1) are involved in the antitumor immune response at different stages (5, 6). These costimulatory and coinhibitory receptor/ligand pairs are collectively referred to as immune checkpoints (7). PD-1 is expressed on a variety of immune cells, such as T cells, NK cells, B cells, and monocytes (8). The PD-1 pathway mediates inhibitory signaling triggered by the binding to PD-L1. PD-L1 expressed on cancer cells could suppress effector T cells and thus prevent T cell-mediated tumor destruction (9). Therefore, blocking the PD-1/PD-L1 inhibitory pathway can reactivate the immune attack on tumor cells, thereby treating cancer (10).

A number of PD-1, PD-L1 and CTLA-4 inhibitors, including Pembrolizumab (11), nivolumab (12), atezolizumab (13), durvalumab (14), avelumab (15) and ipilimumab (16), have been approved for the treatment of advanced NSCLC. Pembrolizumab and nivolumab have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of non-small cell lung cancer with positive PD-L1 expression. The PACIFIC (17) Phase III clinical trial (NCT02125461) in Europe makes durvalumab the only phase III immunotherapy drug recommended by the current guidelines. Japan is also conducting trails of atezolizumab, such as J-TAIL (NCT03645330) (https://clinicaltrials.gov/ct2/show/NCT03645330), J-TAIL-2 (NCT04501497) (https://clinicaltrials.gov/ct2/show/NCT04501497), and durvalumab, AYAME (NCT03995875) (https://clinicaltrials.gov/ct2/show/NCT03995875). In China, according to the ORIENT-11 study (NCT03607539), sintilimab has been approved as the first-line treatment for non-squamous NSCLC combined with pemetrexed and platinum chemotherapy. The Phase III trial (NCT03134872) (18) of SHR-1210 combined with pemetrexed and carboplatin in the treatment of non-squamous non-small cell lung cancer is also ongoing. Nevertheless, due to the tumor heterogeneity and the complexity of the tumor microenvironment (TME), the overall response rates to ICI therapy keep at low levels (19). To increase the therapeutic efficacy, combination strategies have become the major focus of cancer immunotherapy (20). A large number of clinical trials are testing the combination of immunotherapy with traditional therapies such as surgery, chemotherapy, radiotherapy, targeted therapy and other treatment methods.

ICIs obtain therapeutic effect by inducing a durable antitumor immune response (21). However, high levels of immunosuppressive cells in the TME and insufficient infiltration of effector cells into tumor severely impair the antitumor immunity, and thus decreasing the efficacy of ICIs. Recent studies have shown that pro-angiogenic factors in tumor promote the development of immunosuppressive cells, and neovessels reduce the infiltration of effector cells (22). The combination with anti-angiogenic agents is thought to be a promising strategy to enhance the therapeutic efficacy of ICIs.

TUMOR ANGIOGENESIS AND INHIBITORS

Angiogenesis is a hallmark of cancer associated with occurrence, proliferation and metastasis of tumors (23). Targeting the angiogenesis pathway has been found to be effective in the treatment of a variety of cancers including NSCLC. The abnormal structure and function of tumor angiogenesis facilitate the development of a hostile tumor microenvironment characterized by increased interstitial pressure, hypoxia and acidosis (24). Hypoxia further induces the expression of genes involved in blood vessel formation and cell proliferation, and thus exacerbating the TME (25). VEGFs, a family of secreted glycoproteins, play an essential role in the angiogenesis of tumor, which include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, placental growth factor (PIGF) (26). There are three VEGF receptors, VEGFR-1, -2 and -3. The effect of VEGF in promoting angiogenesis is mainly mediated by VEGFR-2. Signaling pathways downstream VEGFR-2, such as phospholipase C gamma (PLCγ), Raf and phosphoinositide-3-kinase (PI3K) (22), promote angiogenesis and vascular permeability by regulating the differentiation, migration, proliferation and survival of microvascular endothelial cells (27). Both monoclonal antibodies blocking the interaction between VEGF and VEGFR or small molecules targeting downstream signaling could inhibit tumor angiogenesis (28). As listed in **Figure 1**, both monoclonal antibodies and small molecule inhibitors interfering angiogenesis have been approved for the treatment in various cancer types.

Bevacizumab, or Avastin, is a humanized monoclonal antibody binding to VEGF-A. It has been approved for the treatment of advanced non-squamous NSCLC. Phase III clinical trials showed that bevacizumab combined with carboplatin and paclitaxel significantly improved the therapeutic efficacy (29). Ramucirumab is a recombinant human IgG1 monoclonal antibody targeting VEGFR2. According to the results of the REVEL study, the FDA and European Medicines Agency (EMA) have approved the combination of Ramucirumab and docetaxel for the treatment of metastatic NSCLC and progressed disease after the treatment of platinum (30).

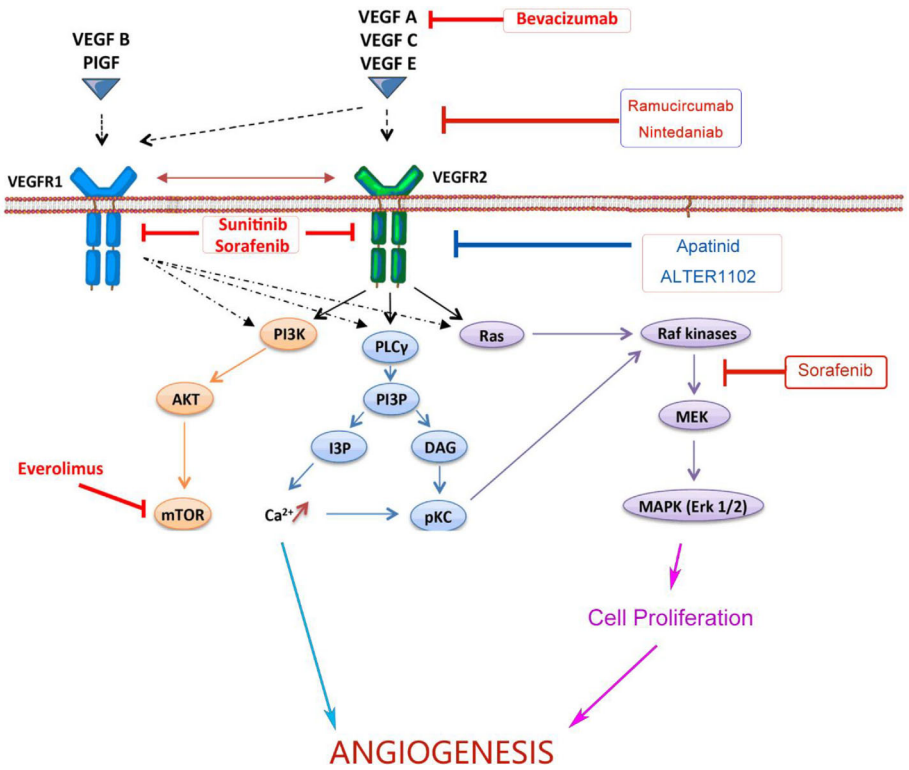

FIGURE 1 | Monoclonal antibodies and small molecules targeting VEGF/VEGFR signaling in tumor angiogenesis. Monoclonal antibodies and small molecule TKIs targeting the VEGFA/VEGFR-2/PLCγ/Raf/PI3K signaling pathway could inhibit tumor angiogenesis and improve the efficiency of anticancer treatments. VEGF, Vascular Endothelial Growth Factor; VEGFR, Vascular Endothelial Growth Factor Receptor; TKI, Tyrosine Kinase Inhibitor; PI3K, Phosphoitide 3-Kinase; AKT, serine/threonine-specific protein kinase; mTOR, mammalian target of rapamycin; PLCγ, Phospholipase C γ; PI3P, Phosphatidylinositol 3-Phosphate; IP3, Inositol Triphosphate; DAG, Diacyl Glycerol; pKC, Protein Kinase C; MEK, Mitogen-activated protein kinase; MAPK, Mitogen Activated Protein Kinase.

Nintedanib is a small molecular inhibitor targeting three critical receptors signaling in angiogenesis, VEGFR, fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR). The LUME-Lung 1 study showed that nintedanib in combination with pemetrexed significantly improved progress-free survival (PFS) of patients (31). It was approved by EMA as the second-line treatment for stage IV NSCLC. In addition, tyrosine kinase inhibitors (TKIs) including sorafenib, sunitinib and apatinib have also been clinically studied in advanced NSCLC, but no obvious overall survival (OS) benefit was observed. Anlotinib is another small molecular inhibitor targeting multiple receptor tyrosine kinases (RTKs), including VEGFR2 and VEGFR3. The results of the ALTER 0303 trial showed that anlotinib significantly prolonged the OS and PFS of patients with advanced NSCLC (32). It has been approved as the third-line treatment for advanced NSCLC.

Although a number of angiogenesis inhibitors have been tested in clinical trials, anti-angiogenesis alone showed limited therapeutic effect in cancer treatment (33). Most of the angiogenesis inhibitors were approved for the combination therapy with other drugs. Given that reduced vessels in tumor will result in decreased delivery of combinatory drugs as well, these results challenge the well-accepted mechanism of anti-angiogenesis in reducing vascular supply, and thus suppress tumor growth by starving tumor. This paradox is resolved by recent findings of vessel normalization, a process recovering the perfusion function and structure of vessels in tumor, which enhanced antitumor immune response by increasing immune cell infiltration and oxygen supply in tumor (33–36). Consistent with the mechanism of vessel normalization, low dose of anti-VEGFR2 antibody showed better effect on reprogramming the tumor microenvironment and displayed better therapeutic efficacy than the high-dose treatment (37). The vessel normalization theory provides novel perspectives in the combination of anti-angiogenesis with other drugs or therapies.

RATIONALE FOR COMBINATION OF ICI INHIBITORS WITH ANGIOGENESIS IN NSCLC

Angiogenesis Fosters An Immunosuppressive Tumor Microenvironment by Modifying The Recruitment of Immune Cells

TME is a dynamic ecosystem composed of tumor cells, immune cells, fibroblasts, stroma cells, blood vessels and various soluble

factors, which suppress antitumor immune response and promote resistance to immunotherapy (38). Excessive VEGF signaling drives aberrant angiogenesis in tumor. Compared to normal blood vessels in tissues, blood vessels in TME are leaky, tortuous, cystic dilation, interlaced and randomly connected. The tumor vascular endothelial cells have abnormal morphology, loose connections between pericytes and varied basement membrane thickness. These abnormalities of structure and function lead to the heterogeneity of tumor blood perfusion, and eventually form a microenvironment characterized by increased interstitial fluid pressure, hypoxia and acidosis (39). The hypoxic microenvironment induced by VEGF/VEGFR signaling suppresses the antitumor immune response through a variety of mechanisms (40, 41).

The TME is enriched with suppressive immune cells including regulatory T cells (Tregs), myeloid-derived suppressive cells (MDSCs), tumor associated macrophages (TAMs), and immature dendritic cells (imDC). Hypoxia facilitates the infiltration of these suppressive immune cells by inducing the expression of chemokines recruiting these immune cells. For example, C-C motif chemokine ligand 22 (CCL22) and C-C motif chemokine ligand 28 (CCL28) recruits Tregs into tumor (42); colony Stimulating factor 1 (CSF1), C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine ligand 12 (CXCL12) increases the recruitment of pro-inflammatory monocytes and TAMs, and convert TAMs from a pro-inflammatory M1-like type to a tumor-promoting M2-like type (43); Dendritic cells (DCs) are mainly recruited into tumor by C-C motif chemokine ligand 20 (CCL20), and granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukin-6 (IL-6), Interleukin-10 (IL-10) prevent maturation of recruited DCs (44). Moreover, the hypoxic environment inhibits the infiltration of effector T cells. VEGF can reduce the expression of adhesion molecules critical for T cell infiltration, such as integrin ligand vascular cell adhesion protein 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1), on immune cells and endothelial cells (ECs) (45). VEGF-A, IL-10 and prostaglandin E2 (PGE2) induce the expression of Fas ligand on endothelial cells, which causes cell death of endothelial cells and $CD8^+$ T cells through the Fas/FasL signaling pathway, and thus reduce T cell mobilization and infiltration (46). Consistently, blockade of the VEGF signaling reduced the recruitment of suppressive cells into tumor but increased the infiltration of effector T cells (37), indicating that anti-angiogenesis is a potential strategy to re-program the immunosuppressive TME, and thus improve the efficacy of immunotherapy.

Angiogenic Factors Directly Regulate Differentiation of Various Immune Cells

In addition to its effect on immune cell migration, the VEGF signaling directly regulates differentiation and proliferation of suppressive immune cells including Tregs, TAMs, MDSCs, and DCs (47, 48). VEGF (red stars) and angiopoietin-2 (ANG2) (green pentagons) are also produced by these immune cells, which foster both the paracrine and the autocrine VEGF (and/or ANG2) signaling in tumor (49). Immunosuppressive cytokines secreted by these suppressive immune cells, including IL-10, indoleamine 2,3-dioxygenase (IDO), and transforming growth factor beta (TGF-β) et al., further worsen the environment by inducing Tregs and inhibiting DC maturation, NK cell activation, T cell activation and proliferation (50). Therefore, angiogenesis inhibitors might normalize the aberrant vasculature in tumor, reduce the development of suppressive immune cells, enhance effector cell infiltration into tumor, and thus reprogram the immunosuppressive to immunosupportive (**Figure 2**).

VEGF Inhibits the Maturation and Differentiation of DCs

DCs are the professional antigen-presenting cells (APCs) which play a critical role in the antitumor immune cycle. Following the exposure to tumor antigens, DCs migrate to lymph nodes and become mature during the migration. They initiate adaptive antitumor immune response by activating T cells recognizing tumor antigens (51). Plenty of evidence has shown that VEGF could inhibit differentiation and maturation of DCs (52, 53). It was found that elevated VEGF levels in mice hindered the development of DCs (48). Studies have showed that VEGF-A inhibited the differentiation of monocytes to DC, and VEGF-A inhibition using bevacizumab or sorafenib restored this process (54).

Due to the lack of costimulatory molecules, immature DCs promote tolerance instead of activation of T cells. It was reported that the binding of VEGF to VEGFR-2 on the surface of DC restrains its maturation by inhibiting the nuclear factor κB (NF-κB) signaling pathway (55). VEGF inhibition increases antigen uptake and migration of tumor-associated DCs in mouse tumor models (56). The VEGFR inhibitor Axitinib promotes maturation of monocyte-derived human DCs, featured with elevated levels of activation markers, major histocompatibility complex (MHC) molecules and co-stimulatory genes such as CD80, CD86, and CD83 (57).

VEGF Increases the Number of Tregs

It is known that Tregs in tumor suppress T cell response against cancer (58). Studies have shown that the VEGF signaling contributes to the induction, maintenance and activation of Tregs in tumors. The expression of VEGF was found to be positively associated with the levels of Tregs in tumor, which indicate poor prognosis in many cancer types (59). Consistent with this finding, higher expression of VEGFR2 was found in Tregs compared to other $CD4^+$ T cells (59, 60), suggesting a preferential role of VEGF signaling in Tregs. Interestingly, neuropilin-1, an co-receptor increasing the binding affinity of VEGF for VEGFRs, is also highly expressed in Tregs (61), which mediates the activation of Tregs and thus enhances their suppressive function (62). VEGF can directly bind to Neuropilin 1 (Nrp-1) on Tregs and guide their migration into a tumor (63). Inhibition of VEGF signaling using sunitinib, bevacizumab or soluble VEGFR-1/-2 reduce Treg proportion in different mouse tumor models and in cancer patients (47, 64–66). Decreased proliferation of Tregs and reduced levels of peripheral Treg levels are also reported in some studies.

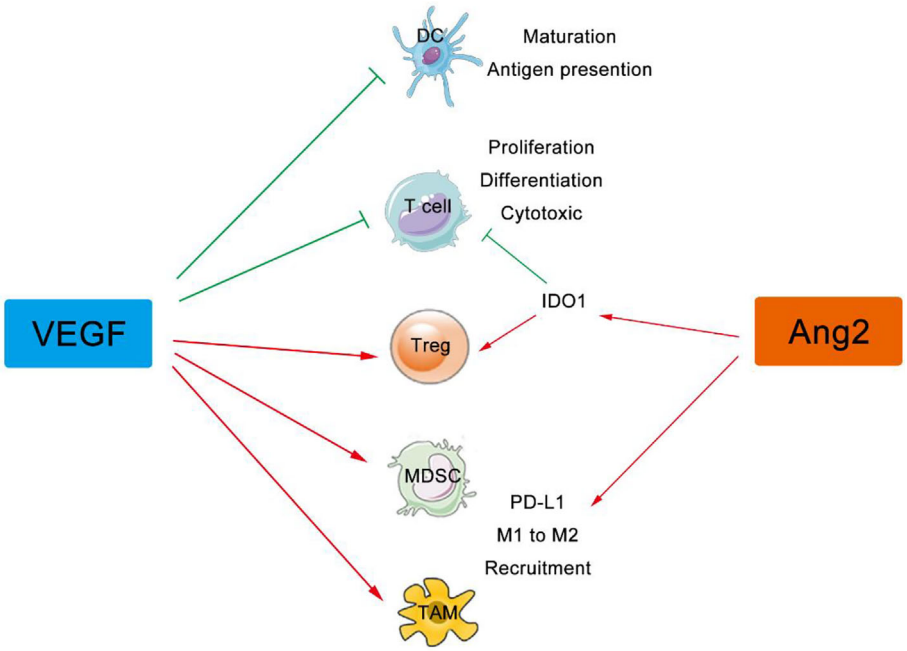

FIGURE 2 | VEGF and ANG2 regulate immune cells in tumor. The VEGF family can suppress the maturation, differentiation, and antigen presentation of APCs, DCs, NKs, and T cells, while both VEGF and Ang2 can improve the suppressive effect of Tregs, TAMs, and MDSCs. VEGF, Vascular Endothelial Growth Factor; ANG2, Angiogenin 2; APCs, Antigen Presenting Cells, DCs, Dendritic Cells; Treg, Regulatory T cells; NKs, Natural Killer Cells; TAMs, Tumor Associated Macrophages; MDSCs, Myeloid Derived Suppressor Cells.

Following the reduction of Tregs, enhanced antitumor immune response was detected in tumors.

VEGF Promotes the Expansion of MDSCs

MDSCs were initially defined as $CD11b^{+}Gr\text{-}1^{+}$ cells in tumors. There are two main major populations of MDSCs: monocytic MDSCs (M-MDSC) and polymorphonuclear MDSCs (PMN-MDSC). PMN-MDSCs are the dominant population of MDSCs in mouse tumor models, while M-MDSCs are mainly found in human tumors (67). MDSCs employ a number of mechanisms to suppress the antitumor immune response, for examples, consuming the nutrient of lymphocyte, reducing trafficking and viability of lymphocyte, generating oxidative stress, and inducing the differentiation of Tregs (67, 68).

The intratumoral level of MDSCs was found to be associated with the VEGF concentration in mouse tumor models. In addition, VEGF infusion significantly elevated levels of $Gr1^{+}$ cells in normal mice without tumor (48), suggesting that VEGF signaling is involved the differentiation of myeloid cells. It was reported that VEGF-A-induced excessive activation of Janus kinase 2/Signal transducer and activator of transcription 3 (Jak2/STAT3) signaling contributes to the abnormal myeloid cell differentiation in cancer (69). Inhibition of VEGF signaling by sunitinib decreased the levels of MDSC in the spleen, bone marrow, and tumor in mouse models, and showed combinatory effect with HPV vaccine for the treatment of tumors expressing human papillomavirus (HPV) antigens (70). Mechanistically, sunitinib downregulates STAT3 signaling and leads to apoptosis in MDSCs (71). In addition to the reduction in MDSC quantity, VEGF inhibition impairs their suppressive function. Axitinib treatment decreases the suppressive capacity of MDSCs isolated from spleens or tumors in mouse models. Moreover, axitinib promotes the differentiation of MDSC toward a phenotype with enhanced capacity of antigen presentation (72). Reduction of MDSCs was also observed in cancer patient treated with sunitinib, which led to stronger T cell immune response against cancer (73). A recent study also showed that bevacizumab-containing regimens had low levels of the granulocytic MDSCs than regimens without bevacizumab in patient tumor samples of NSCLC (74).

VEGF Induces the Differentiation of Macrophages From M1 to M2

TAMs promote angiogenesis by expressing a high level of VEGF. The lacked expression of costimulatory molecules on TAMs induces T cell tolerance and apoptosis. TAMs also promote immunosuppression in tumor by secreting cytokines that can suppress T cell recruitment and activation, such as IL-10, TGFβ, and prostaglandins (75). In addition to the recruitment of TAMs into tumor, VEGF signaling is also involved in the conversion of TAMs from the M1 to M2 phenotype. High levels of TAMs were observed in tumors with increased expression of stromal-cell-derived factor 1 alpha (SDF-1α), CXCL12, C-X-C motif chemokine receptor 4 (CXCR4) and VEGF in mouse tumor models (76, 77). Teresa E Peterson et al. have shown that dual inhibition of VEGFRs and Ang-2 reduced macrophage

recruitment and promoted the polarization of TAMs to a M1 antitumor phenotype (78). Deng et al. also found that VEGF blockade potentiated antitumor efficacy in glioblastoma by reducing TAM recruitment into tumor (79), The combination of VEGFR and CXCR4 inhibitors also showed therapeutic effect in glioblastoma multiforme (GBM) xenografts (80).

VEGF Inhibits the Development and Activation of T Cells

T cells play an essential role in the antitumor immune response by directly killing tumor cells. Boosting the T cell immune response against cancer has become the primary goal of most immunotherapies. Low expression of VEGF was detected in T cells from tumor (81), suggesting that T cells might also promote angiogenesis. Ohm et al. found that the infusion of VEGF-A to tumor-bearing mice led to severe thymic atrophy resulted from a dramatic reduction in $CD4^+/CD8^+$ thymocytes (82). The inhibition of thymocyte maturation is mediated by the VEGFR2. These findings indicate that the VEGF signaling could directly inhibit T cell development. In addition, studies have shown that VEGF-A produced in the tumor microenvironment promotes T cell exhaustion by inducing the expression of co-inhibitory molecules in $CD8^+$ T cell, and targeting VEGF-A/VEGFR signaling could reduce the expression of these suppressive genes (83).

VEGF-induced recruitment and expansion of suppressive immune cells in tumor inhibit the activation of tumor antigen-specific T cells. A lot of clinical and preclinical studies support that blockade of the VEGF/VEGFR signaling can enhance T cell response in tumor. Bevacizumab (Avastin) administration increased cytotoxic T cell levels in colorectal cancer and NSCLC patients (84, 85). Sunitinib treatment increase the levels of $CD4^+$ and $CD8^+$ T cell in mouse cancer models. Stronger cytotoxic activity and elevated expression of Th1 cytokine (Interferon-gamma, IFN-γ) were observed in these T cells from sunitinib-treated tumors (71). Similarly, Schmittnaegel et al. found that dual targeting of ANG2 and VEGFA increased the levels of effector $CD8^+$ T cells in tumors (86). Furthermore, IFN-γ secreted by activated T cells has strong anti-angiogenic activity, suggesting that immunotherapy can also be antiangiogenic. The IFN-γR signaling could directly modulate the function and phenotype of vascular endothelial cells, and thereby normalize tumor blood vessels and promote effector T cell infiltration (87).

Lenvatinib is a RTK that specifically inhibits the kinase activities of VEGF receptors 1-3. Studies have shown that Lenvatinib reduced TAMs and increased the levels of effector $CD8^+$ T cells. Combined with PD-1 blockade can further elevate the levels of activated $CD8^+$ T cells, and thereby enhance antitumor immunity *via* the IFN signaling pathway (88).

Synergism of Anti-Angiogenesis Inhibitors and ICB

Taken together, the VEGF signaling plays a pivotal role in the immunosuppressive TME which severely inhibits antitumor immune response. VEGF/VEGFR inhibition could reprogram the TME from immunosuppressive into immunostimulating by modulate the recruitment and function of immune suppressive cells and T cells. Therefore, anti-VEGF/VEGFR therapy not only has anti-angiogenic effects but also promotes immune response against cancer.

On the other hand, hypoxia-inducible factor 1-alpha (HIF-1α) up-regulates the expression of immune checkpoint molecules in tumor (83). VEGF-A directly increases the expression of PD-1 on activated $CD8^+$ T cells and Tregs through VEGFR2 (83). Besides, elevated levels of IFN-γ in tumor resulted from VEGF signaling inhibition could induce the expression of PD-L1 on tumor cells. These mechanisms provide a theoretical basis for the combined treatment of advanced NSCLC with ICB and anti-angiogenic agents.

IMMUNOTHERAPY AND ANTIANGIOGENIC AGENTS: PRECLINICAL STUDY

Plenty of preclinical evidence also indicates that combining immunotherapy with anti-angiogenic inhibitors can improve the therapeutic efficacy in advanced NSCLC. It was reported that endostatin could improve the therapeutic effect of adoptive transfer of cytokine-induced killer cells (CIKs) for the treatment of lung carcinomas (89). Another preclinical study also showed that the VEGF inhibitor bevacizumab improved the effect of CIKs therapy in treating NSCLC (90). These findings provide evidence for the combination of anti-angiogenesis therapy and immunotherapy to treat lung cancer. In addition, the effects of different doses of antiangiogenic inhibitors on the combination with immunotherapy are also studied. A small dose of apatinib was enough to increase T cells infiltration, reduce hypoxia, and decrease the recruitment of TAMs into tumor (37, 91). Consistently, the combination of low-dose apatinib and PD-L1 antibody can significantly inhibit tumor growth and increase the survival time in mouse models (91).

IMMUNOTHERAPY AND ANTIANGIOGENIC AGENTS: CLINICAL DATA

Given that both the potential molecular mechanism and preclinical evidence support the combination of immunotherapy with anti-angiogenesis therapy, a number of clinical trials are underway to evaluate the safety and efficacy of this new therapy in NSCLC (**Table 1**). Preliminary data indicate that immunotherapy combined with anti-vascular therapy is a promising approach for the treatment of NSCLC.

Nivolumab Combined With Bevacizumab

The combination between PD-1 blockade and bevacizumab was tested in the Checkmate012 phase I clinical trial (NCT01454102). Advanced NSCLC patients who failed in the first-line chemotherapy of platinum were divided into two groups, and treated with nivolumab or the combination of nivolumab with bevacizumab. The median PFS in the combination group was 37.1 weeks, while

TABLE 1 | Clinical trials of the combination of anti-angiogenic inhibitors with immune checkpoint blockade in NSCLC.

Clinical trial	Patients	Targeted Agent	Primary Endpoint	Phase	Status
NCT01454102 (CheckMate 012)	Stage IIIB/IV NSCLC, first or subsequent line of therapy	Bevacizumab + nivolumab	SAE	I	Active, not recruiting
NCT02574078 (CheckMate 370)	Stage IV NSCLC	Bevacizumab + Nivolumab	PFS, OS	I/II	Completed
NCT02681549	Untreated brain metastases from melanoma or NSCLC	Bevacizumab + Pembrolizumab	BMRR	II	Recruiting
NCT02039674 (KEYNOTE- 021)	In participants with unresectable or metastatic NSCLC	Pembrolizumab + paclitaxel + bevacizumab	DLTs	I/II	Active, not recruiting
NCT02366143 (IMpower 150)	Stage IV non-squamous NSCLC	Atezolizumab + bevacizumab carboplatin + paclitaxel	PFS, OS	III	Completed
NCT02856425 (PEMBIB)	Solid tumors including NSCLC of adenocarcinoma and squamous	Nintedanib + Pembrolizumab	MTD of nintedanib, Safety	Ib	Recruiting
NCT02443324	LA/Unresectable/Metastatic NSCLC 0–3 prior lines of therapy	Ramucirumab + pembrolizumab	DLTs	I	Active, not recruiting
NCT02572687	LA/unresectable/metastatic/thoracic Malignancies	Ramucirumab + MEDI4736	DLTs	I	Completed
NCT02174172	Advanced or metastatic NSCLC	Bevacizumab + Atezolizumab	Dose of Atezolizumab	Ib	Completed
NCT03377023	Advanced or metastatic NSCLC	Ramucirumab + durvalumab	MTD, ORR	I/II	Recruiting
NCT03713944	Stage IV Non-squamous NSCLC	Bevacizumab + Atezolizumab	PFS, ORR	II	Active, not recruiting
NCT03647956	EGFR-mutant Metastatic NSCLC	Bevacizumab + Atezolizumab	ORR	II	Unknown
NCT03527108	Recurrent, Advanced, Metastatic NSCLC	Ramucirumab + Nivolumab	DCR	II	Recruiting
NCT03689855 (RamAtezo-1)	Stage IV, NSCLC, after progression on immune checkpoint blockers (ICBs)	Ramucirumab + Atezolizumab	ORR	I/II	Active, not recruiting
NCT03786692	Stage IV NSCLC in never smokers or possess a driver mutation	Bevacizumab + Atezolizumab	PFS	II	Recruiting
NCT03836066	LA/metastasis/high-intermediate tumor mutation burden in First Line NSCLC	Bevacizumab + Atezolizumab	PFS, OS	II	Recruiting
NCT03616691	LA/metastatic NSCLC after Failure with atezolizumab monotherapy	Bevacizumab + Atezolizumab	DCR	II	Not yet recruiting
NCT03786692	Stage IV NSCLC in never smokers or possess a driver mutation	Bevacizumab + Atezolizumab	PFS	II	Recruiting
NCT03735121	Previously Treated LA/Metastatic NSCLC	Bevacizumab + rHuPH20	Drug serum concentration	Ib/III	Recruiting

SAE, Serious Adverse Events; PFS, Progression-free survival; OS, Overall survival; BMRR, brain metastasis response rate; DLT, Dose-limiting Toxicity; MTD, Maximum Tolerated Dose ORR, Objective Response Rate; DCR, Disease control rate; LA, Locally Advanced.

the nivolumab monotherapy group was 16 weeks in patients with squamous cancers and 21.4 weeks in patients with non-squamous cancers. Lower incidence of severe adverse events (AEs) (grade 3 and above) was observed in the combination. However, the objective response rates (ORR) are similar in these two groups. Follow-up studies are ongoing (12).

Pembrolizumab Combined With Ramucirumab

The combination between ramucirumab and pembrolizumab has been studying by a multicenter phase I study (NCT02443324) in different types of cancers. 27 patients were recruited in this study. The objective reactions in these NSCLC patients were 30%. The median treatment time is 6.8 months or longer, and the median response time is 1.45 months. The most common serious AEs related to treatment in NSCLC patients were fatigue and myocardial infarction (7%) (92). The team has also expanded a multi-center, open-label Phase 1a/b trial to study ramoxiimab plus pembrolizumab in the treatment of advanced newly-treated NSCLC (N=26) (11). The results showed that 22 (84.6%) patients had any grade of treatment-related AEs, and hypertension is the most common side-effect (n = 4, 15.4%). The ORR of the treatment group was 42.3%. The ORR in patients with high PD-L1 expression levels (tumor proportion score (TPS)≥50%) and low levels (TPS 1%-49%) were 56.3% and 22.2%, respectively. The median PFS was 9.3 months in the treated group, and the patients with PD-L1 TPS 1%-49% were 4.2 months. The patients with PD-L1 TPS≥50% did not reach the median PFS. The median OS was not reached in the treated population.

Atezolizumab Combined With Bevacizumab

The combination of bevacizumab with atezolizumab and chemotherapy was studied by IMpower150, which is a phase III randomized controlled clinical trial (NCT02366143). 1202 non-squamous NSCLC patients with stage IV or recurrent metastatic diseases who have not treated with chemotherapy were included. Patients were randomized 1:1:1 to receive atezolizumab combined

with carboplatin + paclitaxel (ACP) (n = 402), atezolizumab combined with carboplatin + paclitaxel + bevacizumab (ABCP) (n = 400), carboplatin + Paclitaxel + Bevacizumab (BCP) (n = 400), after 4-6 courses of treatment, receive atezolizumab or bevacizumab or both for maintenance treatment until the disease progresses or no clinical benefit. The results of the study show that immunotherapy on the basis of the combination of bevacizumab and chemotherapy can prolong patient survival. The median PFS of the ABCP was 8.3 months, and the BCP was 6.8 months (HR: 0.59, P<0.0001). The median OS was 19.2 months for the ABCP group, and 14.7 months for the BCP group (HR: 0.78, P=0.02). The incidence of treatment-related serious AEs was 25.4% for ABCP group and 19.3% for BCP group. However, 77.4% of ABCP patients had grade 1-2 AEs. This study shows that, regardless of the PD-L1 expression, VEGFR or anaplastic lymphoma kinase mutation status, the use of ABCP can significantly improve PFS and OS in patients with metastatic non-squamous NSCLC (93). According to this study, the FDA approved the combination therapy of ABCP as the first-line treatment for metastatic non-squamous NSCLC in December 2018. This combination is currently being tested in hepatocellular carcinoma (HCC) as well. At the 2019 (ESMO) annual meeting, it was reported that atilizumab combined with bevacizumab and bisorafenib had better OS and PFS in patients with unresectable hepatocellular carcinoma (94).

Apatinib Combined With SHR-1210

A single-arm phase II trial studying the combination of Apatinib with SHR-1210 was reported at the ASCO meeting in 2019. 96 patients were recruited in this study. Apatinib is a small TKI that primarily act on VEGFR-2, and SHR-1210 is another PD-1 antibody. These two drugs are developed in China. Patients failed at least one previous line of chemotherapy received intravenous infusion of SHR-1210 200 mg q2w combined with oral Apatinib 250 mg qd. The ORR of all evaluable patients was 30.8%. DCR was 82.4%. Median PFS was 5.9 months. The OS endpoint was not reached. Among the patients with bTMB 1.54 mutations/Mb, the ORR was 52.6%, and the DCR was 81.6%, suggesting that apatinib combined with SHR-1210 might have better therapeutic effect in patients with high tumor mutation burden (TMB) (95).

Overall, the combination of ICI and anti-angiogenic agents has shown encouraging results in treating advanced NSCLC. To achieve maximal therapeutic effect, a number of questions need to be addressed in future trails, including the effect of different anti-angiogenic inhibitors, the drug dose, the timing and schedule of the two type of drugs in the treatment etc.

CONCLUSION

In this paper, we overviewed the updated knowledge of ICB, anti-angiogenesis, and the combination of these two kinds of therapies. A lot of preclinical studies have revealed the potential mechanisms of abnormal angiogenesis in the regulation of antitumor immunity in mouse tumor models, and support the application of combining immunotherapy and anti-angiogenesis for cancer treatment. The combination of immunotherapy and anti-angiogenesis is expected to enhance the efficacy of immunotherapy by converting the immunosuppressive TME to immunosupportive. Results of the ongoing clinical trials also support that the combination of ICB and anti-angiogenesis is a promising approach for the treatment of NSCLC. Translational studies and innovative clinical trials are needed in the future to address important questions not resolved in current studies, including the identification of biomarkers precisely the response to the combination therapy, optimizing the drug dose, administration schedule and the timing of the treatment.

AUTHOR CONTRIBUTIONS

JS and PZ conceptualized the idea for the review. SR and XX performed the literature search, analyzed cited references and wrote the first draft of the manuscript. HY, JS, and PZ wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study is supported by grants from the National Key Research and Development Program of China (2016YFA 0500304), the National Nature Science Foundation in China (NSFC) (81802853, 81773052, 81572806, 82002400), the Natural Science Foundation of Zhejiang Provincial (Y19H160116, Q18H160119), the Postdoctoral Science Foundation in China (2018M633237), the Guangzhou Science Technology and Innovation Commission (201607020038), the Science and technology projects of Guangdong Province (2016A0 20215086), the Guangdong Innovative and Entrepreneurial Research Team Program (2016ZT06S638), and the leading talents of Guangdong province program.

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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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GLOSSARY

AE	Adverse event
ANG2	Angiopoietin-2
APC	Antigen-presenting cell
CCL2	C-C motif chemokine ligand 2
CCL20	C-C motif chemokine ligand 20
CCL22	C-C motif chemokine ligand 22
CCL28	C-C motif chemokine ligand 28
CIK	Cytokine-induced killer cell
CSF1	Colony stimulating factor 1
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C motif chemokine receptor 4
DC	Dendritic cell
EC	Endothelial cell
EMA	Exponential moving average
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
GBM	Glioblastoma multiforme
GM-CSF	Granulocyte-macrophage colony stimulating factor
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia-inducible factor 1-alpha
HPV	Human papillomavirus
ICAM1	Intercellular adhesion molecule 1
ICB	Immune checkpoint blockade
ICI	Immune checkpoint inhibitor
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-6	Interleukin-6
imDC	Immature dendritic cell
Jak2/STAT3	Janus kinase 2/Signal transducer and activator of transcription 3
MDSC	Myeloid-derived suppressive cell
MHC	Major histocompatibility complex
NF-κB	Nuclear factor κB
Nrp-1	Neuropilin 1
NSCLC	Non-small cell lung cancer
ORR	Objective response rate
OS	Overall survival
PD-1	Programmed cell death-1
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed death ligand 1
PFS	Progress-free survival
PGE2	Prostaglandin E2
PI3K	Phosphoinositide-3-kinase
PlGF	Placental growth factor
PLCγ	Phospholipase C gamma
RTK	Receptor tyrosine kinase
SDF-1α	Stromal-cell-derived factor 1 alpha
TAM	Tumor associated macrophage
TGF-β	Transforming growth factor beta
TKI	Tyrosine kinase inhibitor
TMB	Tumor burden
TME	Tumor microenvironment
TPS	Tumor proportion score
Tregs	Regulatory T cells
VCAM1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 June 2021
doi: 10.3389/fimmu.2021.663115

Immunoregulatory Monocyte Subset Promotes Metastasis Associated With Therapeutic Intervention for Primary Tumor

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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: 02 February 2021
Accepted: 12 May 2021
Published: 07 June 2021

Citation:
Shibuya T, Kamiyama A, Sawada H, Kikuchi K, Maruyama M, Sawado R, Ikeda N, Asano K, Kurotaki D, Tamura T, Yoneda A, Imada K, Satoh T, Akira S, Tanaka M and Yotsumoto S (2021) Immunoregulatory Monocyte Subset Promotes Metastasis Associated With Therapeutic Intervention for Primary Tumor. Front. Immunol. 12:663115. doi: 10.3389/fimmu.2021.663115

Systemic and local inflammation associated with therapeutic intervention of primary tumor occasionally promotes metastatic recurrence in mouse and human. However, it remains unclear what types of immune cells are involved in this process. Here, we found that the tissue-repair-promoting $Ym1^{+}Ly6C^{hi}$ monocyte subset expanded as a result of systemic and local inflammation induced by intravenous injection of lipopolysaccharide or resection of primary tumor and promoted lung metastasis originating from circulating tumor cells (CTCs). Deletion of this subset suppressed metastasis induced by the inflammation. Furthermore, transfer of $Ym1^{+}Ly6C^{hi}$ monocytes into naïve mice promoted lung metastasis in the mice. $Ym1^{+}Ly6C^{hi}$ monocytes highly expressed matrix metalloproteinase-9 (MMP-9) and CXCR4. MMP-9 inhibitor and CXCR4 antagonist decreased $Ym1^{+}Ly6C^{hi}$-monocyte-promoted lung metastasis. These findings indicate that $Ym1^{+}Ly6C^{hi}$ monocytes are therapeutic target cells for metastasis originating from CTCs associated with systemic and local inflammation. In addition, these findings provide a novel predictive cellular biomarker for metastatic recurrence after intervention for primary tumor.

Keywords: surgery, irradiation, inflammation, atypical monocyte, lung metastasis

INTRODUCTION

Systemic and local inflammation caused by cancer therapy is now recognized as an important risk factor for cancer recurrence. Surgical resection of primary tumor, chemotherapy, or radiation therapy can awake dormant cancer cells and induce metastatic outgrowth in distant organs through inflammation (1–7). In addition to these cancer treatments, it has also been reported that

inflammation caused by bacterial infection and cigarette smoke-exposure, promotes cancer dormancy escape and metastasis (8, 9). Such immune cells as neutrophils, macrophages, and monocytes are involved in cancer recurrence caused by inflammation. Recently, neutrophils have received increased attention with regard to their role in promoting cancer progression and metastasis associated with inflammation. For instance, neutrophils were reported to play critical roles in promoting lung metastases mediated by producing proinflammatory cytokines (10). Neutrophil extracellular traps (NETs) awake dormant cancer cells through interaction with cancer cells. NETs also trap circulating tumor cells (CTCs) and lead to increased formation of metastasis (9, 11, 12). In addition to neutrophils, monocytes and macrophages were also reported to be involved in cancer recurrence. The depletion of $CD11b^+$ macrophages reduces lung metastasis of breast cancer cells (13). Vascular endothelial growth factor A (VEGFA)-secreting macrophages promote the extravasation of cancer cells and lung metastasis (14). It was also reported that monocytes recruited to metastasis site by the CCL2-CCR2 axis differentiate into macrophages and promote extravasation and survival of cancer cells (14, 15). A recent report indicated that not only neutrophils, but also monocytes, awake dormant cancer cells (7). Considering these reports, the types of immune cells involved in cancer progression and metastasis presumably depend on the context of inflammation or experimental models. However, immune cells involved in actual cancer-related events in patients are not well understood.

Blood monocytes play critical roles in inflammation as a component of mononuclear phagocyte system. In the steady-state conditions, monocytes consist of two or three subpopulations in mouse or human, respectively (16, 17). Classical monocytes ($Ly6C^{hi}CCR2^+CX3CR1^-$ in mouse, $CD14^+CD16^-$ in human) are recruited into an inflamed site in a CCR2-dependent manner, and act as inflammation-promoting immune cells (14, 18, 19). In contrast, non-classical monocytes ($Ly6C^{low}CCR2^-CX3CR1^+$ in mouse, $CD14^{dim}CD16^+$ in human) are differentiated from $Ly6C^{hi}$ monocytes in an Nr4A-dependent manner, patrol the vasculature during homeostasis, and contribute to cancer immunosurveillance (20). Intermediate dim → + monocytes ($CD14^+CD16^+$) in human have been suggested to be responsible for the proliferation and stimulation of T cells (21). These monocyte subsets have been considered to coordinately engage in various immune responses in tissue injury or cancer. Recently, however, emergency hematopoiesis including monopoiesis during inflammation or other immune responses has been extensively studied, and several reports have identified bone marrow (BM)-derived atypical novel monocyte subsets that are rarely observed in the steady-state condition. In mouse, inflammation induced by microbial stimulation gives arise to neutrophil-like $Ly6C^{hi}$ monocytes derived from granulocyte-macrophage progenitors (GMPs), but not MDPs (22, 23). $Ceacam1^+Msr^+Ly6C^{low}$ monocytes called segregated-nucleus-containing atypical monocytes (SatM) emerge in lung of bleomycin-treated mouse and are involved in fibrosis (24). $Ly6C^{hi}MHCII^{hi}Sca\text{-}1^{hi}$ monocytes arise in BM of acute gastrointestinal infected mouse and are considered to regulate immune response *via* the production of prostaglandin E2 and IL-10 (25). These reports suggest the possibility that a novel inflammation-related subset of monocytes can modulate cancer progression and metastasis associated with inflammation. However, details of such a monocyte subpopulation remain unknown.

We previously reported that GMP-derived atypical $Ly6C^{hi}$ monocytes characterized by Ym1 expression ($Ly6C^{hi}Ym1^+$ monocytes) are produced in BM during the recovery phase of tissue injury. These monocytes share some characteristics with granulocytes and exhibit the immunoregulatory phenotype that contributes to tissue repair and regeneration (22). Here, we show that not neutrophils, but $Ym1^+Ly6C^{hi}$ monocytes contribute to promoting metastasis caused by inflammation associated with intervention for primary tumor. These findings demonstrate that the mechanisms of tissue repair are closely related to metastasis and provide a novel therapeutic target for the metastasis.

METHODS

Mice

C57BL/6J mice were obtained from CLEA Japan, Inc. CD204-DTR knock-in mice (26), Ym1-DTR knock-in mice, Ym1-Venus mice, and Lcn2 $^{-/-}$ mice were described previously (22, 27). All experiments using the mice described herein were approved by the Tokyo University of Pharmacy and Life Sciences Animal Use Committee (L18-22, L18-23, L19-20, L19-21, L20-17, and L20-18) and performed in accordance with applicable guidelines and regulations.

Reagents

For the induction of inflammation, lipopolysaccharides (LPS; *E. coli*, O111:B4) (Sigma), CpG-ODN (ODN1668; Hokkaido System Science), and Poly(I:C) (GE Healthcare Life Sciences) were used. For the depletion of monocytes and/or neutrophils, anti-Gr-1 (clone RB6-8C5, in-house purification) or anti-Ly6G (clone 1A8; BioXCell) was used. For the inhibition of MMP-9 activity and CXCR4, SB-3CT (Tokyo Chemical Industry) and AMD3100 (Sigma) were used respectively. Diphtheria toxin (DT) was purchased from Sigma. For the detection of IL-6 and TNF-alpha concentrations in serum, an ELISA MAX™ Standard Set was purchased from BioLegend. For analysis of cell surface marker expression, the following Abs were used: anti-CD11b-PE (clone M1/70), anti-CD62L-PE (clone MEL-14), anti-F4/80-PE (clone RM8), anti-C5aR-PE (clone 20/70), anti-MHC-II-PE (clone M5.114.15.2), anti-VCAM1-PE [clone 429 (MVCAM)], anti-Ly6G-PE (clone 1A8), anti-CXCR4-APC (L276F12), and anti-Treml4-PE (clone 16E5) were purchased from BioLegend. Anti-PD-L1-PE (clone MIH5) was purchased from Thermo Fisher Scientific. Anti-CD204-PE (clone REA148) was purchased from Miltenyi Biotec. Anti-CXCR2-APC (clone 242216) and anti-CCR2-APC (clone 475301R) were purchased from R&D Systems. Anti-CD131-PE (clone JORO50) was purchaced from BD Biosciences.

Cell Lines

The murine melanoma cell line, B16F10 (Riken Cell Bank, Ibaraki, Japan), was maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin-streptomycin at 37°C in a humidified incubator with 5% CO_2.

Preparation of Cells

BM monocytes were isolated by cell sorter or Monocyte Isolation Kit (#130-100-629 Miltenyi Biotech). For BM monocyte isolation using cell sorter, BM cells from WT- or Ym1-Venus mice were incubated with anti-CD16/32 (clone 93) and then with a cocktail of biotinylated-anti-Lin [CD4 (Clone GK1.5), CD8 (Clone 53-6.7), B220 (RA3-6B2), NK1.1 (Clone PK136), Ly6G (Clone 1A8) and Ter119 (Clone TER-119)] antibodies in MACS buffer (phosphate-buffered saline (PBS), pH 7.2; 2 mM EDTA; 0.5% bovine serum albumin), followed by incubation with anti-biotin microbeads (#130-090-485 Miltenyi Biotech). Lin^+ cells were depleted by magnetic sorting (autoMACS Pro Separator, Miltenyi Biotech). Lin^- cells were stained with anti-CD45.2-PE-Cy7 (BioLegend, clone 104), anti-Ly6G-APC (BioLegend, clone 1A8), anti-CD115-Brilliant Violet 421 (BioLegend, clone AFS98) and anti-Ly6C-PE (BioLegend, clone HK1.4) antibodies and then fractionated by a cell sorter (SH800, SONY, or AriaIII, BD Biosciences). For the analysis of the number of monocytes and tumor cells in lung, sorted monocytes and B16 cells were stained with PKH-26 (red fluorescence) and PKH-67 (green fluorescence) (Sigma), respectively, according to the manufacture's protocol. For the isolation of lung cells, lungs were fragmented and transferred to a conical tube containing digestion solution (0.2 U/mL Liberase TL (#5401020001, Roche), 1 μg/mL DNase I (#DN25, Sigma) in HBSS). Samples were incubated at 37°C under agitation for 25 min. After incubation, the cells were dispersed by pipetting and pelleted by centrifugation. The cells were then washed with MACS buffer. To deplete erythrocytes, the cells were treated with BD Pharm Lyse™ - Lysing Buffer (BD Biosciences) and then washed with MACS buffer. For the analysis of peripheral blood mononuclear cells, peripheral blood was collected in an EDTA-containing tube. Then, the red blood cells were lysed with BD Pharm Lyse™ - Lysing Buffer.

Experimental Metastasis Assay

B16 cells (1×10^5 cells) were injected intravenously into WT-, CD204-DTR-, or Ym1-DTR mice to generate lung metastases. The number of nodules reflecting lung metastasis of B16 was visually counted. To evaluate melanoma-related mRNA expression in lung, total RNA from snap-frozen-lung tissue was extracted with a FavorPrep Total RNA Extraction Column (Favorgen) according to the manufacturer's protocol. For qRT-PCR, cDNAs were synthesized using ReverTra Ace (TOYOBO). qRT-PCR was performed on cDNA with a THUNDERBIRD SYBR qPCR Mix (TOYOBO). Expression levels were normalized to 18s ribosomal RNA (rRNA). The following primer sequences were used for each gene: *Pmel* forward 5'-GCTTGTAGGTATCTTGCTGGTGTT-3', reverse 5'-CCTGCTTCTTAAGTCTATGCCTATG-3'; *Dct* forward 5'-GGCTACAATTACGCCGTTG-3', reverse 5'-CACTGAGAGAGTTGTGGACCAA-3'; and *18s rRNA* forward 5'-CGGACAGGATTGACAGATTG-3', reverse 5'-CAAATCGCTCCACCAACTAA-3'. For experimental metastasis assay with tumor resection, 1×10^6 B16 cells were implanted subcutaneously into the back of WT mice. Six or seven days after implantation, mice under anesthesia underwent tumor tissue resection through cutaneous incision. Twenty-four hours later, B16 cells (1×10^5 cells) were injected intravenously to generate lung metastases. Lung metastasis of B16 was estimated as above.

X-Ray Irradiation

B16 cells (1×10^6 cells) were implanted subcutaneously into the back of WT mice. Seven to eight days after implantation, mice under anesthesia were immobilized in a customized harness that allowed the implanted tumor to be exposed, whereas the remainder of the body was shielded by 3.5 cm of lead. Mice were irradiated in a Faxitron CP-160 irradiator (Faxitron X-ray Corporation).

Quantitative RT-PCR (qRT-PCR)

For the analysis of mRNA levels in $Ym1^+$- or $Ym1^-$ Mo, sorted $Ym1^+$- or $Ym1^-$ Mo RNA was extracted and converted into cDNA, and qRT-PCR was performed on the cDNA as above. Expression levels were normalized to 18s rRNA. The following primer sequences were used for each gene: *Chi3l3* forward 5'-AAAGACAAGAACACTGAGCTAAAAACTC-3', reverse 5'-GAATCTGATAACTGACTGAATGAATATC-3'; *MMP-9* forward 5'-CTTCCCCAAAGACCTGAAAAC-3', reverse 5'-CTGCTTCTCTCCCATCATCTG-3'; *Il1b* forward 5'-GGATGAGGACATGAGCACCT-3', reverse 5'-AGCTCATATGGGTCCGACAG-3'; *Vegfa* forward 5'-AAAAACGAAAGCGCAAGAAA-3', reverse 5'-TTTCTCCGCTCTGAACAAGG-3'; *Cox2* forward 5'-CCAGCACTTCACCCATCAGTTTTTCAAG-3', reverse 5'-CAGTTTATGTTGTCTGTCCAGAGTTTCA-3'; and *Lcn2* forward 5'-CCATCTATGAGCTACAAGAGAACAAT-3', reverse 5'-TCTGATCCAGTAGCGACAGC-3'.

RNA-Sequencing

Sorted cells were lysed and their total RNAs were extracted with RNeasy Mini kit (QIAGEN). Five hundred picograms of total RNA was subjected to DNA library preparation for RNA sequencing analysis using SMART-Seq v4 Ultra Low Input RNA Kit (TAKARA) and Nextera XT DNA Library Preparation Kit (Illumina). Sequencing was performed on a NextSeq 500 sequencer (Illumina) in the 75-bp single-end read mode. Data with the fragments per kilobase of exon per million reads (FPKM) were used for further analysis after mapping of the sequence reads. PCA analysis of RNA-sequencing was performed using AltAnalyze. The R package limma was used to identify differentially expressed genes. For PCA analysis, RNA-seq data in BM naïve monocytes, $Ym1^+Ly6C^{hi}$ monocytes, and $Ym1^-Ly6C^{hi}$ monocytes were retrieved from the Gene Expression Omnibus database (accession number GSE118032) (22).

Western Blotting

Lungs were thoroughly homogenized in a homogenizer (Bioprep-6, Allsheng, Hangzhou, China) at 3800 rpm for four cycles, and 0.2 s per cycle, in RIPA buffer (50 mM Tris HCl [pH 7.4], 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) with protease inhibitors (#11836145001, Roche). For 10 mg of tissue, 500 µL of RIPA buffer was used. After 30 min on ice, the samples were centrifuged at 10,000 × g for 20 min at 4°C, and protein concentration in the supernatant was determined using the bicinchoninic acid (BCA) protein assay (#23225, Thermo Fisher Scientific). Equal amounts of protein from each sample were loaded on SDS-polyacrylamide gel electrophoresed, separated, and transferred onto PVDF membranes. The immunoblots were incubated in blocking buffer [5% skim milk in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST)] for 60 min at room temperature and probed with anti-citrullinated histone H3 (#ab5103, Abcam) or anti-GAPDH mAb-HRP-DirecT (#M171-7, Medical & Biological Laboratories) overnight at 4°C. Then, the immunoblots were washed three times for 5 min in PBST, incubated with polyclonal goat anti-rabbit IgG-HRP (#P0448, Dako) for 30 min at room temperature in blocking buffer, and washed three times in PBST again. Immunodetection was performed using a SuperSignal™ West Pico PLUS Chemiluminescent Substrate (#34580, Thermo Fisher Scientific).

Immunohistochemistry

Lungs were harvested and embedded in OCT compound (SECTION-LAB, Japan). The cut surface was covered with an adhesive film (Cryofilm type IIC9, SECTION-LAB, Japan) and frozen sections (5 µm) were prepared with a macrotome (CM3050S Leica Microsystems, Germany). The resulting sections were post-fixed with 100% EtOH for 10 s and 4% PFA/PBS(-) for 10 s, rinsed with PBS(-) for 20 s, and incubated with TNB Blocking Buffer [0.1 M Trizma Base, pH7.5, 0.15 M NaCl, 0.5% (w/v) blocking reagent (PerkinElmer, FP1020)] for 1 h at room temperature. The sections were then incubated with anti-citrullinated histone H3 antibody (1/250), or MPO antibody (#AF3667, R&D Systems, 1/100) in TNB Blocking Buffer for 1 h at room temperature. After three washes with PBS (-), the sections were incubated with donkey anti-rabbit IgG-Cy3 (#406402, Biolegend, 1/1000), or donkey anti-goat IgG-Alexa 488 (#705-545-003, Jackson ImmunoResearch, 1/1000) in TNB Blocking Buffer for 1 h in the dark at room temperature. After two washes with PBS(-) and one wash with water, the sections were counterstained with DAPI, and the slides were covered with cover slips using mounting media (FluorSave Reagent, 345789, Merck Millipore).

Gelatin Zymography

A conditioned medium from monocytes (5×10^6 cells/mL, in a 24-well plates containing Advanced RPMI1640 medium (Thermo Fisher Scientific), grown on plastic for 24 h), was mixed 4:1 ratio with loading buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue). Then, the samples were loaded on Novex™ 10% Zymogram Plus (Gelatin) Protein Gels (#ZY00102BOX, Thermo Fisher Scientific). After electrophoresis, the gels were rinsed twice with water and incubated in washing buffer (50 mM Tris, pH 7.5, 0.016% NaN_3, 2.5% Triton X-100, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 30 min at room temperature. Then, the gels were rinsed with incubation buffer (50 mM Tris, pH 7.5, 0.016% NaN_3, 1% Triton X-100, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 10 min at 37°C and incubated in incubation buffer at 37°C for 16 h. The gels were stained with 0.5% Coomassie Blue R-250 (#031-17922, Wako; diluted with 40% ethanol and 10% acetic acid) and destained with 40% ethanol and 10% acetic acid.

Invasion Assay

$Ly6C^{hi}$ monocytes (2 or 5×10^6/mL) sorted from LPS-treated WT- or Ym1-Venus mice were incubated in serum-free medium (Advanced-RPMI1640, Thermo Fischer Scientific) at 37°C in 5% CO_2 for 24 h. The culture supernatant was centrifugated (10,000 ×g, 30 min at 4°C). The supernatants were collected for the invasion assay. Human melanoma cell line A375 (ATCC-CRL-1619, 1×10^6/mL) was suspended in serum-free medium (RPMI1640, Wako) and added into the upper chamber of a 24-well Transwell chamber that had been coated with Matrigel (Corning, BioCoat 354480) in the presence or absence of monocyte-culture supernatant. The lower chamber contained RPMI1640 containing 0.1% FBS as a chemoattractant. Assays were carried out at 37°C in 5% CO_2 for 24 h. At the end of the incubation, the non-invading cells on the upper surface of the filter were mechanically removed. The invading cells that migrated through the Matrigel and the 8-µm pore membrane, were fixed with 4% paraformaldehyde/PBS for 5 min, and stained with 0.1% crystal violet (WAKO) for 20 min. The proportion of invading cells was calculated using BZ-X710 software (Keyence).

Statistics

Data were analyzed either by analysis of variance (ANOVA) followed by multiple comparison, or by the t-test with Prism (GraphPad Software, CA). P values < 0.05 were considered significant.

RESULTS

$Ly6C^{hi}$ Monocytes, but Not Neutrophils, Promote Lung Metastasis Accelerated by Systemic Inflammation

Inflammation is one of the most important factors that promote cancer metastasis (2, 7–11, 28–30). The metastasis cascade involves multiple processes, including invasion of cancer cells into adjacent tissue, intravasation, survival in blood circulation, extravasation of circulating tumor cells (CTCs), and subsequent outgrowth at distant sites (31). Among these steps, the outgrowth of CTCs at distant sites was proven to be enhanced by systemic inflammation in an experimental metastasis model (8, 10, 11, 29, 30, 32). However, the precise mechanisms of inflammation-induced metastasis originating from CTCs remain unknown. To explore these mechanisms, we first compared some forms of systemic inflammation induced by different Toll-like receptor

(TLR) ligands from the perspective of promoting metastasis in an experimental metastasis model (**Figure 1A**). Mice were treated with different TLR ligands, followed by intravenous (i.v.) injection of B16 melanoma cells. Consistent with previous reports (8, 10, 30), the systemic injection of lipopolysaccharide (LPS) *via* tail vein promoted the formation of metastatic foci of B16 melanoma cells originating from CTCs in lung (**Figures 1B, C**). The mRNA expression levels of premelanosome protein (*Pmel)* and dopachrome tautomerase (*Dct)* genes, both of which are highly expressed in B16 melanoma cells (33), were significantly elevated in the lungs of LPS-treated mice (**Figure 1D**), indicating outgrowth of B16 melanoma cells in the lungs. On the other hand, CpG-ODN or Poly (I:C) had negligible effects on metastasis (**Figures 1B–D**), indicating that systemic-inflammation-induced enhancement of metastasis depends on the mode of inflammation.

It was reported that neutrophils and monocytes are involved in metastasis under inflammatory conditions (7, 9–11). In fact, both neutrophils and monocytes accumulated in lung in the early phase (Day1 to 2 after systemic injection of LPS) of inflammation (**Supplemental Figures 1A, B**). Therefore, we focused on the role of neutrophils and monocytes in the inflammation-induced promotion of metastasis. The depletion of neutrophils by anti-Ly6G monoclonal antibody (mAb) injection had no effects on lung metastasis (**Figures 1E, F**, and **Supplemental Figure 2**). On the other hand, anti-Gr-1 mAb, which depletes both neutrophils and $Ly6C^{hi}$ monocytes, but not $Ly6C^{low}$ monocytes, suppressed the metastatic formation (**Figure 1G** and **Supplemental Figure 2**), suggesting that $Ly6C^{hi}$ monocytes, but not neutrophils, contributed to promoting metastasis induced by systemic injection of LPS. We previously reported that BM and peripheral blood monocytes highly expressed CD204, a class A scavenger receptor (26), and that both $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes but not neutrophils were specifically deleted in peripheral blood by diphtheria toxin (DT) injection in CD204-DTR mice (26) (**Supplemental Figure 3**). In these mice, the number of metastatic foci was decreased by DT injection (**Figures 1H–J**), indicating that monocytes are responsible for the promotion of lung metastasis. The injection of anti-Gr-1 mAb did not increase inflammatory cytokine production induced by LPS (**Figure 1K**), indicating that the suppression of metastasis by anti-Gr-1 mAb is not attributed to the suppression of inflammatory cytokine production.

Albrengues et al. recently reported that neutrophil extracellular traps (NETs) play a critical role in the awakening of dormant cancer cells and the growth of metastatic lesions in lung, when mice were injected intranasally (i.n.) with LPS (9). Thus, we compared i.n. and i.v. routes of LPS administration in terms of NET formation in lung. As was previously reported, the i.n. injection of LPS induced the citrullination of histone H3, a specific marker for NET formation in lung. On the other hand, the injection of LPS *via* tail vein never induced NET formation in lung (**Figures 1P, Q**). These results strongly suggest that NET formation is not attributed to inflammation-induced promotion of metastasis in the case of systemic injection of LPS.

To further confirm the role of $Ly6C^{hi}$ monocytes in systemic-inflammation-induced metastasis originating from CTCs, we purified $Ly6C^{hi}$ monocytes from either naïve or LPS-injected mice and injected intravenously these monocytes into naïve mice. After that, we injected cancer cells (**Figure 1L**). As shown in **Figures 1M–O**, $Ly6C^{hi}$ monocytes from LPS-injected mice (LPS Mo) facilitated the formation of metastatic foci in lungs, whereas $Ly6C^{hi}$ monocytes from naïve mice (Naïve Mo) did not. We counted the number of transferred monocytes and B16 cells in lung soon after injection of these cells. However, there was no significant difference in the cell number of these cells in the lungs between Naïve Mo-transferred- and LPS Mo-transferred mice (**Supplemental Figure 4**), suggesting functional difference between Naïve Mo and LPS Mo in lung. Taken together, the systemic injection of LPS provides $Ly6C^{hi}$ monocytes with the ability to promote metastasis.

$Ym1^{+}Ly6C^{hi}$ Monocyte Subset Plays a Vital Role in Lung Metastasis

We next sought to reveal the properties of $Ly6C^{hi}$ monocytes in mice treated with LPS. We previously identified a subpopulation of $Ly6C^{hi}$ monocytes that are characterized by a high expression of Ym1 (22). $Ym1^{+}Ly6C^{hi}$ monocytes greatly expanded in BM during the recovery phase of systemic inflammation induced by LPS administration or tissue injury. These monocytes infiltrating into an injured site exhibited immunoregulatory and tissue-reparative phenotypes. These findings of the roles of $Ym1^{+}Ly6C^{hi}$ monocytes in tissue repair prompted us to speculate that $Ym1^{+}Ly6C^{hi}$ monocytes could play roles in systemic inflammation-induced metastasis. We first monitored the accumulation of $Ym1^{+}Ly6C^{hi}$ monocytes in lung after systemic inflammation by using Ym1-Venus mice. As expected, when Ym1-Venus mice were injected with LPS, a large number of $Ym1^{+}Ly6C^{hi}$ monocytes were accumulated in the lungs (**Figures 2A–C**). Intriguingly, a small number of $Ym1^{+}Ly6C^{hi}$ monocytes were found in the lungs of mice injected with either CpG-ODN or Poly(I:C) (**Figures 2A–C**), both of which had no effects on metastasis formation in lung (**Figures 1B–D**). An increase in the number of $Ym1^{+}Ly6C^{hi}$ monocytes was also observed in mice injected with both LPS and cancer cells (**Supplemental Figures 5A, B**), suggesting the role of $Ym1^{+}Ly6C^{hi}$ monocytes in lung metastasis.

We previously generated Ym1-DTR mice in which Ym1-expressing cells were deleted by DT injection (**Supplemental Figure 6**). As shown in **Figures 2D–F**, the transient deletion of Ym1-positive cells on Days 1 and 4 significantly suppressed lung metastasis induced by LPS injection. To further reveal the role of $Ym1^{+}Ly6C^{hi}$ monocytes in promoting metastasis originating from CTCs, we purified $Ym1^{+}Ly6C^{hi}$ or $Ym1^{-}Ly6C^{hi}$ monocytes from BM of LPS-treated Ym1-Venus mice and injected those cells into naïve mice. After that, we injected cancer cells (**Figures 2G, H**). The injection of $Ym1^{+}Ly6C^{hi}$ monocytes resulted in a large number of metastatic foci in lung compared with the injection of $Ym1^{-}Ly6C^{hi}$ monocytes (**Figures 2I, J**). These results clearly indicate that $Ym1^{+}Ly6C^{hi}$ monocytes have the ability to promote lung metastasis.

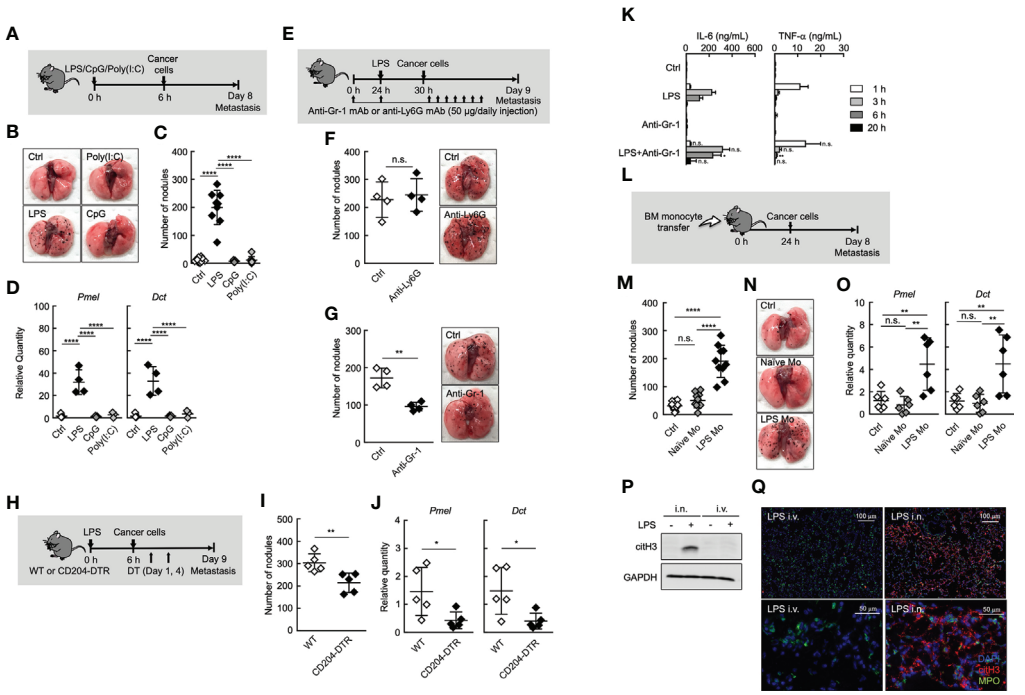

FIGURE 1 | Ly6C^{hi} monocytes promote lung metastasis in systemic inflammatory state. **(A–D)** Effects of TLR ligands on lung metastasis. **(A)** Experimental design for analyzing the effect of TLR ligands on metastatic progression. WT mice were injected with either PBS (Ctrl), 20 µg of LPS, 100 µg of CpG-ODN (CpG), or 100 µg of Poly(I:C) followed by i.v. injection of B16 cells (1 x 10^5 cells) 6 h later. The lungs were analyzed for metastasis on Day 8. **(B)** Representative images of lungs on Day 8. **(C)** Quantitative summary of the number of lung metastases on Day 8. **(D)** mRNA expression levels of B16 melanoma cell-specific genes were determined by qRT-PCR and are shown as fold change relative to control lungs. Average values are shown with SD. One-way ANOVA followed by Dunnett's test, n = 5-9 **(C, D)**. ****$P < 0.001$; n.s., not significant. Each symbol represents an individual animal. **(E–G)** Effects of immune cell deletion on lung metastasis. **(E)** Experimental design used to test the effect of anti-Gr-1 mAb and anti-Ly6G mAb on metastatic progression. WT mice were injected with 20 µg of LPS on Day 1 followed by i.v. injection of B16 cells (1 x 10^5 cells) 6 h later. For deletion of neutrophils alone (anti-Ly6G), or monocytes and neutrophils (anti-Gr-1), 50 µg/daily of indicated mAbs or PBS (Ctrl) were injected into these mice from Day 0 to Day 8. The lungs were analyzed for metastasis on Day 9. **(F, G)** Quantitative summary of the number of lung metastases (left), and representative images of the effect of the lungs metastasis (right). Average values are shown with SD. Unpaired two-tailed t-test, n = 4 **(F, G)**. **$P < 0.01$; n.s., not significant. Each symbol represents an individual animal. **(H–J)** Reduced number of lung metastases in CD204-DTR mice. **(H)** Experimental design used to test the contribution of CD204$^+$ cells to metastatic progression. WT and CD204-DTR mice were injected intravenously with 20 µg of LPS. Six hours later, B16 cells were injected intravenously, and this was followed by the i.p. injection of DT (500 ng/injection) on Days 1 and 4. The lungs were analyzed for metastasis on Day 9. **(I)** Quantitative summary of the number of lung metastases on Day 9. **(J)** mRNA expression levels of indicated genes in lungs are shown as fold change relative to lung of DT-treated WT mice. Average values are shown with SD. Unpaired two-tailed t-test, n = 5 (J and K). **$P < 0.01$; *$P < 0.05$. Each symbol represents an individual animal. **(K)** WT mice were injected intraperitoneally (i.p.) with anti-Gr-1 mAb (50 µg/injection) at -24 and 0 h and intravenously with LPS at 0 h. Sera were collected at 1, 3, 6, and 20 h after LPS injection. Serum cytokine concentrations were measured by ELISA. Average values are shown with SD. Unpaired two-tailed t-test at each time point, compared with LPS injection, n = 3-4. **$P < 0.01$; *$P < 0.05$; n.s., not significant. **(L–O)** Increased number of lung metastases in LPS Mo-transferred mice. **(L)** Experimental design used to test the effects of monocyte transfer on metastatic progression. WT mice were transferred intravenously with advanced RPMI1640 (Ctrl) or Ly6C^{hi} monocytes prepared either from naïve mice (Naïve Mo) or LPS-treated mice (LPS Mo) (5 x 10^5 cells). Twenty-four hours later, B16 cells were injected intravenously. The lungs were analyzed for metastasis on Day 8. **(M)** Quantitative summary of the number of lung metastases on Day 8. **(N)** Representative images of the lungs. **(O)** mRNA expression levels of indicated genes in lungs are shown as fold change relative to control lungs. Average values are shown with SD. One-way ANOVA followed by Dunnett's test, n = 6–10 **(M, O)**. ****$P < 0.001$; **$P < 0.01$; n.s., not significant. Each symbol represents an individual animal. **(P, Q)** Intranasal (i.n.) but not i.v. injection of LPS induces NET formation in lung. WT mice were injected i.v. or i.n. with LPS (20 or 10 µg, respectively). Twenty-four hours later, the lungs were analyzed. **(P)** Western blot analysis for citrullination of histone H3 (citH3) in lungs of LPS-treated mice. Western blot analysis of lung tissues was performed as described in Materials and Methods. **(Q)** Immunohistochemistry of lung section from LPS-treated WT mice. Images show representative immunostaining of myeloperoxidase (MPO: green), citH3 (red), and DAPI (blue) in the lung of mice treated with LPS. Original magnification, ×20 (upper panel) and × 100 (lower panel). The data shown are representative of two independent experiments.

Ym1$^+$Ly6C^{hi} Monocytes Express Metastasis-Related Genes

To elucidate the mechanisms underlying the promotion of metastasis by Ym1$^+$Ly6C^{hi} monocytes, we sought to characterize the Ym1-Venus$^+$Ly6C^{hi} monocyte subpopulation that accumulated in lung during systemic inflammation. Flow cytometry analysis revealed that the Ym1$^+$Ly6C^{hi} monocyte subpopulation expressed the same levels of several monocyte surface markers as the Ym1$^-$Ly6C^{hi} monocyte subpopulation (**Figure 3A**). Next, we globally compared the mRNA expression profiles of Ym1$^+$Ly6C^{hi} monocytes and Ym1$^-$Ly6C^{hi} monocytes from lungs of LPS-treated mice by RNA sequencing analysis.

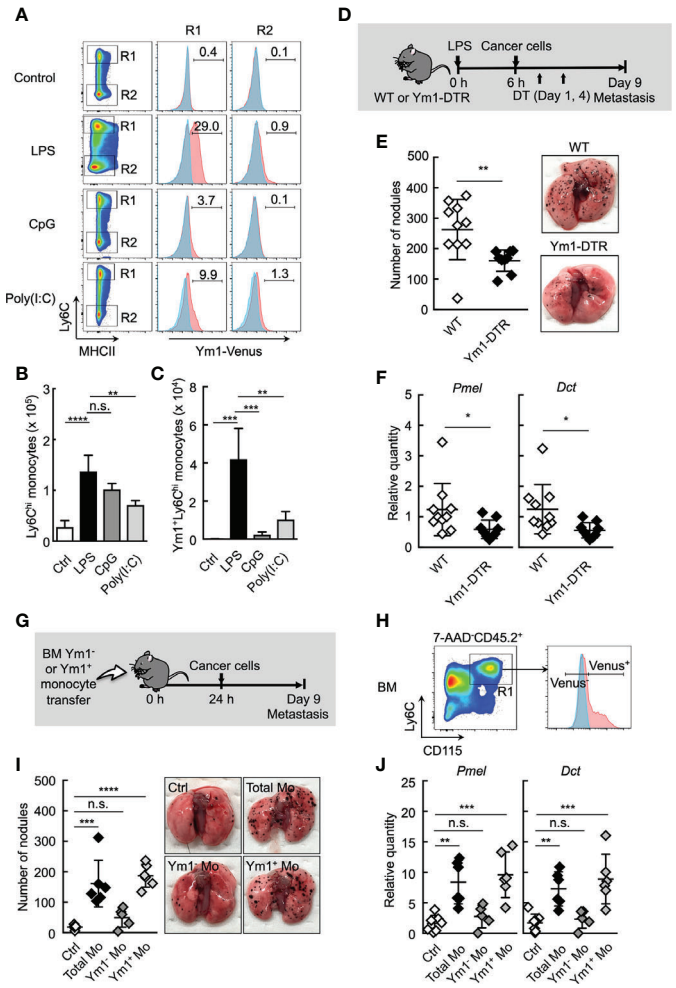

FIGURE 2 | Ym1$^+$Ly6C^{hi} monocyte subset plays a vital role in lung metastasis. **(A–C)** Flow cytometric analysis of the lung cells in WT- (shaded area in blue) or Ym1-Venus mice (in red). PBS (Ctrl), LPS, CpG, or Poly(I:C) was injected intravenously into WT- or Ym1-Venus mice. Forty-eight hours later, lung cells were stained for CD45.2, CD11c, CD11b, Ly6G, MHCII and Ly6C, and analyzed by flow cytometer as described in **Supplemental Figure 1A**. Numbers indicated percentage of Ym1$^+$ cells in CD45.2$^+$CD11c$^-$CD11b$^+$MHCII$^-$Ly6C^{hi}cells (R1; Ly6C^{hi} monocytes) or CD45.2$^+$CD11c$^-$CD11b$^+$MHCII$^-$Ly6C^{low}cells (R2; Ly6C^{low} monocytes) **(A)**. Absolute numbers of Ly6C^{hi} monocytes **(B)** and Ym1$^+$Ly6C^{hi} monocytes **(C)** in lungs. Average values are shown with SD. One-way ANOVA followed by Dunnett's test, n = 3-5. ****P < 0.001; ***P < 0.005; **P < 0.01; n.s., not significant. **(D–F)** Reduced number of lung metastases in the absence of Ym1$^+$Ly6C^{hi} monocytes. **(D)** Experimental design used to test the contribution of Ym1$^+$ cells to metastatic progression. WT- and Ym1-DTR mice were injected intravenously with LPS. Six hours later, B16 cells were injected intravenously, and this was followed by the i.p. injection of DT (500 ng/injection) on Days 1 and 4. The lungs were analyzed for metastasis on Day 9. **(E)** Quantitative summary of the number of metastases in lungs (left), and representative images of lung metastasis (right). **(F)** mRNA expression levels of indicated genes in lungs are shown as fold change relative to lung of DT-treated WT mice. Average values are shown with SD. Unpaired two-tailed t-test, n = 9-10. **P < 0.01; *P < 0.05. Each symbol represents an individual animal. **(G–J)** Increased number of lung metastases in Ym1$^+$Ly6C^{hi} monocyte-transferred mice. **(G)** Experimental design used to test the effects of transfer of Ym1$^+$ or Ym1$^-$ Ly6C^{hi} monocytes (Ym1$^+$ Mo or Ym1$^-$ Mo, respectively) on metastatic progression. Ym1$^+$Ly6C^{hi} and Ym1$^-$Ly6C^{hi} monocytes were sorted from BM of Ym1-Venus mice 48 h after LPS (20 μg) treatment. WT mice were transferred intravenously with advanced RPMI1640 medium (Ctrl), Ym1$^+$ Mo or Ym1$^-$ Mo (5 x 10^5 cells). Twenty-four hours later, B16 cells (1 x 10^5 cells) were injected intravenously. The lungs were analyzed for metastasis on Day 9. **(H)** Identification of Ym1$^+$ Mo or Ym1$^-$ Mo in BM for cell sorting. Samples were pregated on live CD45.2$^+$ cells. **(I)** Quantitative summary of the number of lung metastases (left), and representative images of the lungs metastasis (right). **(J)** mRNA expression levels of indicated genes in lungs are shown as fold change relative to control lungs. Average values are shown with SD. One-way ANOVA followed by Dunnett's test, n = 5-7 (I and J). ****P < 0.001; ***P < 0.005; **P < 0.01; n.s., not significant. Each symbol represents an individual animal.

PCA analysis demonstrated that the two monocyte subsets exhibited obvious differences in gene expression after infiltrating into lung (**Figure 3B**). In addition, the gene expression of lung monocyte subsets clearly differed from previously reported that of BM monocyte subsets (22) (**Figure 3B**). While *Chi3l3* (Ym1-coding gene), known as a marker of M2 macrophages (34), is highly expressed in Ym1$^+$Ly6C^{hi} monocytes, Ym1$^+$Ly6C^{hi} monocytes did not show higher expression of any other M2 genes (**Supplemental Figure 7**). Interestingly, the expression of several metastasis-related

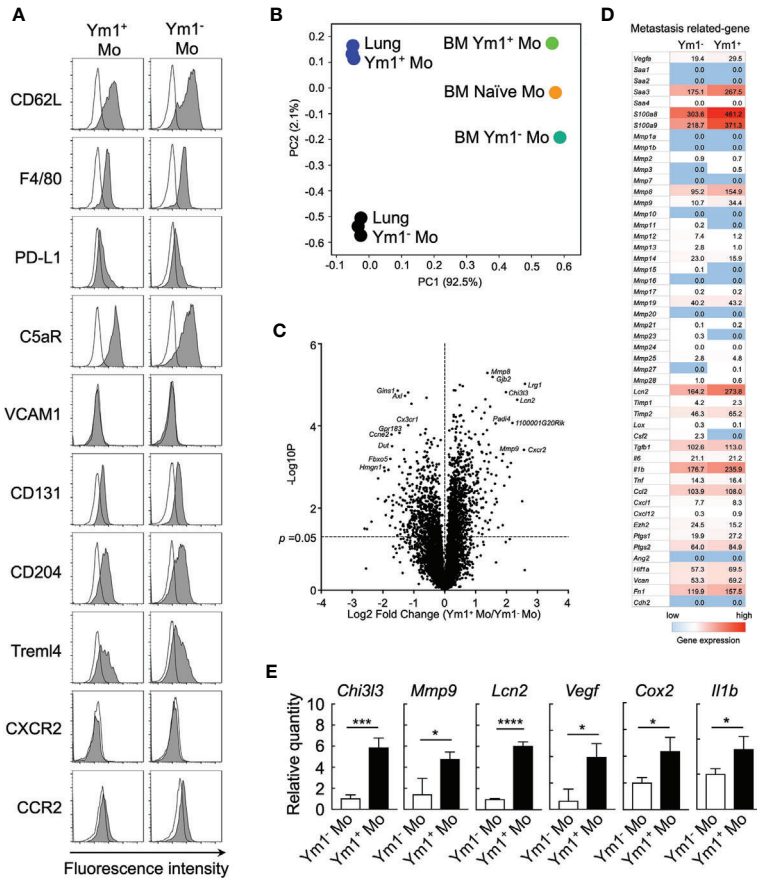

FIGURE 3 | Ym1-Venus^{+}Ly6C^{hi} monocyte express metastasis-related genes. **(A)** Ym1-Venus mice were injected intravenously with LPS (20 μg). Forty-eight hours later, the expression of surface markers of Ym1^{+} Mo and Ym1^{-} Mo in lung was analyzed by flow cytometry. Black lines indicate isotype control. **(B, D)** Gene expression profiles of Ym1^{+} Mo or Ym1^{-} Mo in lung and BM were globally compared by RNA-sequencing analysis. PCA **(B)**, volcano plots **(C)**, and heatmap of indicated genes **(D)**. **(E)** mRNA expression levels in Ym1^{+} Mo and Ym1^{-} Mo of lung. Ym1-Venus mice were injected intravenously with LPS (20 μg) followed by i.v. injection of B16 cells (1 x 10^{5} cells) 6 h later. Ym1^{+} Mo and Ym1^{-} Mo were sorted from BM of Ym1-Venus mice 48 h after LPS treatment. the expression of mRNA levels was analyzed. Average values are shown with SD. Unpaired two-tailed t-test, n = 3. $^{****}P < 0.001$; $^{***}P < 0.005$, $^{*}P < 0.05$.

genes (35) such as *Mmps*, *Vegf, Cox2*, and *Il1b* was enhanced in Ym1^{+}Ly6C^{hi} monocytes, while expression levels of inflammatory cytokines except *Il1b* were not different between two subsets (**Figures 3C, D** and, **Supplemental Figure 7**). In addition to these genes, *Lcn2*, which is reported to enhance matrix metalloproteinase-9 (MMP-9) activity by stabilizing MMP-9 (36), was also expressed in higher levels in Ym1^{+}Ly6C^{hi} monocytes. The high expression of those genes in Ym1^{+}Ly6C^{hi} monocytes was also confirmed by PCR analysis (**Figure 3E**).

MMP-9 Is Essential for Ly6C^{hi} Monocyte-Promoting Lung Metastasis

MMP-9 plays an important role in the invasion and metastasis of cancer cells (37–40). Thus, we next sought to examine the roles of MMP-9 in metastasis promotion by Ym1^{+}Ly6C^{hi} monocytes. We first examined the protein levels of MMP-9 in the culture supernatant of purified Ly6C^{hi} monocytes using gelatin zymography. The higher protein levels of proMMP-9, latent form of MMP-9 was observed in the culture supernatant of LPS Mo compared with Naïve Mo (**Figure 4A**). We also confirmed that Ym1^{+}Ly6C^{hi} monocytes in LPS Mo showed higher protein levels of proMMP-9 than Ym1^{-}Ly6C^{hi} monocytes in LPS Mo (**Figure 4A**). We next demonstrated whether the culture supernatant of LPS Mo promoted cancer cell invasion *in vitro* using the Matrigel invasion assay. The culture supernatant of LPS Mo induced a significant increase in cancer cell invasion compared with that of Naïve Mo (**Figure 4B**). We next demonstrated the *in vivo* contribution of Ym1^{+}Ly6C^{hi} monocyte derived-MMP-9 to metastasis. The sequential injection of MMP-9 inhibitor suppressed LPS-promoted lung metastasis (**Supplemental Figure 8**). However, previous report suggested that MMP-9

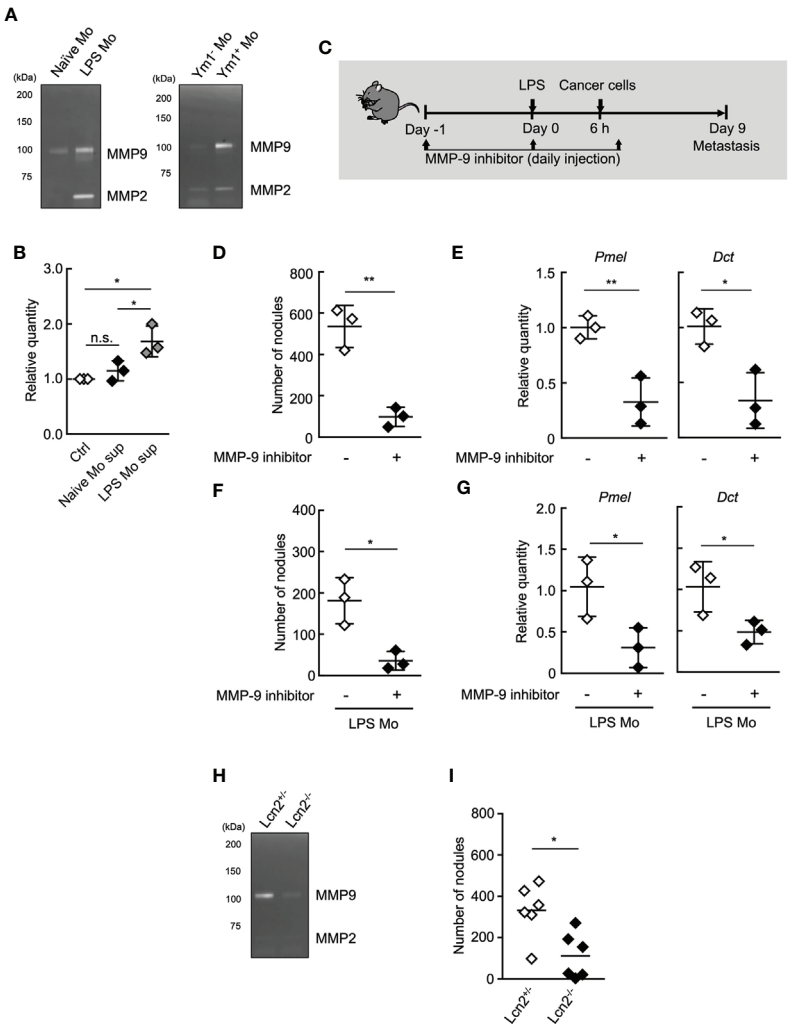

FIGURE 4 | MMP-9 is essential for Ly6C^{hi} monocyte-promoting lung metastasis. **(A)** Gelatin zymography of culture supernatant of monocytes. Ly6C^{hi} monocytes isolated from naïve- or LPS-treated WT mice (left), or Ym1^{+} or Ym1^{-}Ly6C^{hi} monocytes isolated from LPS-treated Ym1-Venus mice (right) were cultured for 24 h. The culture supernatants were assayed for gelatinase activity. Experiments were repeated twice with similar results. **(B)** Invasion assay of cancer cells in the presence of culture supernatant of Ly6C^{hi} monocytes isolated from naïve or LPS-treated WT mice. Average values are shown with SD. One-way ANOVA followed by Tukey's test, n = 3. *P < 0.05; n.s., not significant. Each symbol represents an individual animal-derived culture supernatant. **(C–E)** Effects of MMP-9 inhibitor on LPS-promoted metastatic progression. **(C)** Experimental design used to test the effects of MMP-9 inhibitor injected at the early points of metastasis. WT mice were injected with 20 μg of LPS on Day 0 followed by i.v. injection of B16 cells (1 x 10^5 cells) 6 h later. These mice were injected with 10% DMSO/PBS [inhibitor (-)] or MMP-9 inhibitor (SB-3CT, 250 μg) on Day -1, 0, 1. The lungs were analyzed for metastasis on Day 9. **(D)** Quantitative summary of the number of lung metastases. **(E)** mRNA expression levels of indicated genes in lungs are shown as fold change relative to control lungs. Average values are shown with SD. Unpaired two-tailed t-test, n = 3. **P < 0.01; *P < 0.05. Each symbol represents an individual animal. **(F, G)** Effects of MMP-9 inhibitor on metastasis promoted by LPS Mo transfer. WT mice were transferred intravenously with Ly6C^{hi} monocytes prepared from LPS-treated mice (LPS Mo) (5 x 10^5 cells) on Day -1. Twenty-four hours later, B16 cells were injected intravenously. These mice were injected with 10% DMSO/PBS [inhibitor (-)] or MMP-9 inhibitor (SB-3CT, 250 μg) on Day -1, 0, 1. The lungs were analyzed for metastasis on Day 9. **(F)** Quantitative summary of the number of lung metastases on Day 9. **(G)** mRNA expression levels of indicated genes in lungs are shown as fold change relative to control lungs. Average values are shown with SD. Unpaired two-tailed t-test, n = 3. *P < 0.05; n.s., not significant. Each symbol represents an individual animal. **(H)** Gelatin zymography of culture supernatant of Lcn2$^{-/-}$ monocytes. Ly6C^{hi} monocytes isolated from LPS-treated Lcn2$^{+/-}$ or Lcn2$^{-/-}$ mice were cultured for 24 h. The culture supernatants were assayed for gelatinase activity. Experiments were repeated twice with similar results. **(I)** Reduced number of metastatic foci in lung by the injection of Lcn2$^{-/-}$ LPS Mo. LPS Mo were prepared from BM of Lcn2$^{+/-}$ or Lcn2$^{-/-}$ mice 48 h after LPS (20 μg) injection, and these monocytes were transferred intravenously into WT mice. Twenty-four hours later, B16 cells (1 x 10^5 cells) were injected intravenously. The lungs were analyzed for metastasis on Day 9. Average values are shown. Unpaired two-tailed t-test, n = 6. *P < 0.05. Each symbol represents an individual animal.

induces not only the awakening of dormant cancer cells in lung through extracellular matrix remodeling (ECM) but also outgrowth of tumor growth in the late phase of metastasis (9). Thus, to inhibit *in vivo* MMP-9 enzymatic activity only at early time points, WT mice were injected with MMP-9 inhibitor on Days -1, 0, and 1 only (**Figure 4C**). Treatment of WT mice with the inhibitor at these time points also suppressed the number of metastatic foci (**Figures 4D, E**). The metastasis-promoting effects

of injecting LPS Mo were also canceled by the early injection of the inhibitor (**Figures 4F, G**). We further tried to demonstrate that $Ym1^{+}Ly6C^{hi}$ monocyte-derived MMP-9 is responsible for the progression of metastasis by using lipocalin 2 (Lcn2) -deficient mice (27). As described above, Lcn2 is reported to be responsible for stability of MMP-9 (36). In fact, the protein levels of MMP-9 in $Lcn2^{-/-}$ LPS Mo were lower than those in $Lcn2^{+/-}$ LPS Mo (**Figure 4H**). The injection of $Lcn2^{-/-}$ LPS Mo resulted in the reduced number of metastatic foci in lung compared with the case of $Lcn2^{+/-}$ LPS Mo injection (**Figure 4I**). Taken together, these results indicate that $Ym1^{+}Ly6C^{hi}$ monocyte derived-MMP-9 has a strong impact on the promotion of lung metastasis.

$Ym1^{+}Ly6C^{hi}$ Monocytes Contribute to the Promotion of Lung Metastasis Induced by Tumor Resection

It was reported that the resection of primary tumor triggers a high frequency of tumor-dormancy escape and metastatic relapse in cancer (41–43). In mouse, inflammation associated with surgery triggered the outgrowth of distinct tumors or promoted metastasis (2, 7). Thus, we first sought to examine whether $Ym1^{+}Ly6C^{hi}$ monocytes are involved in the formation of metastatic foci after resection of tumors. B16 cells were inoculated subcutaneously. Subcutaneous tumors were removed by resection followed by i.v. injection of B16 cells 24 h after the resection. As shown in **Figures 5A, B**, the

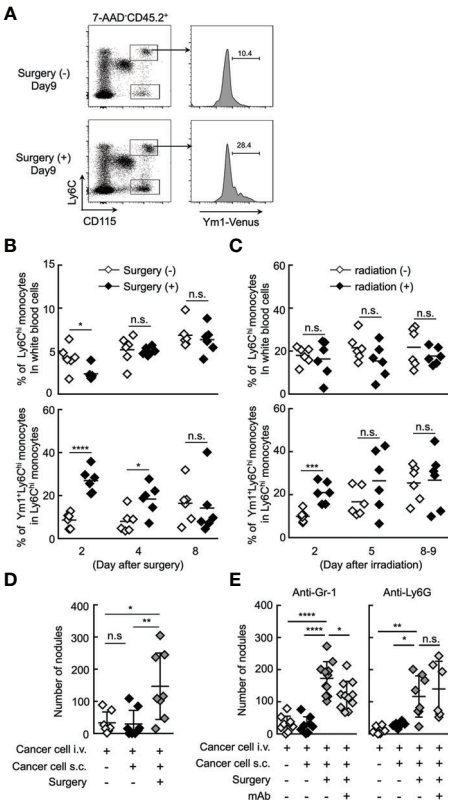

FIGURE 5 | $Ym1^{+}Ly6C^{hi}$ monocytes contribute to the promotion of lung metastasis induced by tumor resection. **(A, B)** Increased frequency of $Ym1^{+}Ly6C^{hi}$ monocytes after tumor resection. B16 cells (1 x 10^6 cells) were subcutaneously (s.c.) inoculated in the flank of Ym1-Venus mice. Seven days later, the primary tumor was removed by surgery. **(A)** The frequency of monocytes in peripheral blood were analyzed at the indicated time points. Representative flow cytometric profiles of the percentage of $Ym1^{+}$ cells in $Ly6C^{hi}$ monocytes on day 9. **(B)** Percentage of $Ly6C^{hi}$ monocytes in white blood cells (upper) and $Ym1^{+}$ cells in $Ly6C^{hi}$ monocytes (lower) in peripheral blood. Unpaired two-tailed t-test, n = 5-6. $^{****}P < 0.001$; $^{*}P < 0.05$; n.s., not significant. **(C)** Increased frequency of $Ym1^{+}Ly6C^{hi}$ monocytes after radiation exposure to primary tumor. B16 cells (1 x 10^6 cells) were subcutaneously (s.c.) inoculated in the flank of Ym1-Venus mice. Seven to eight days later, the animals were randomized into one of two treatment groups: no irradiation or 30 Gy irradiation. The frequency of monocytes in peripheral blood were analyzed at the indicated time points. Percentage of $Ly6C^{hi}$ monocytes in white blood cells (upper) and $Ym1^{+}$ cells in $Ly6C^{hi}$ monocytes (lower) in peripheral blood. Unpaired two-tailed t-test, n = 6. $^{***}P < 0.005$; n.s., not significant. Each symbol represents an individual animal. **(D)** Increased number of lung metastases after tumor resection. B16 cells (1 x 10^6 cells) were s.c. inoculated in the flank of WT mice. Seven days later, the primary tumor was removed by surgery followed by i.v. injection of B16 cells (1 x 10^5 cells) 24 h later. The lungs were analyzed for metastasis on Day 16. Quantitative summary of the number of lung metastases. Average values are shown with SD. One-way ANOVA followed by Dunnett's test, n = 7-8. $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant. Each symbol represents an individual animal. **(E)** Effects of immune cell deletion on lung metastasis after tumor resection. B16 cells (1 x 10^6 cells) were s.c. inoculated in the flank of WT mice. Seven days later, the primary tumor was removed by surgery followed by i.v. injection of B16 cells (1 x 10^5 cells) 24 h later. For deletion of $Ly6C^{hi}$ monocytes and neutrophils (anti-Gr-1), or neutrophils alone (anti-Ly6G), 50 μg/daily of indicated mAbs or PBS were injected into these mice from Day 6 to Day 15. The lungs were analyzed for metastasis on Day 16. Quantitative summary of the number of lung metastases. Average values are shown with SD. One-way ANOVA followed by Dunnett's test, n = 6–11. $^{****}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., not significant. Each symbol represents an individual animal.

proportion of $Ym1^{+}Ly6C^{hi}$ monocytes, but not total $Ly6C^{hi}$ monocytes, was drastically increased within 2 days after tumor resection. In addition to resection of primary tumor, it has already been reported that radiation exposure to primary tumor promotes cancer metastasis in mouse (44, 45). As expected, an increase in $Ym1^{+}Ly6C^{hi}$ monocytes was also observed in tumor-bearing mice treated with irradiation therapy (**Figure 5C**). Furthermore, we found that resection of the tumors promoted metastasis originating from CTCs (**Figure 5D**). We then tried to identify the immune cells responsible for the promotion of metastasis induced by resection. As shown in **Figure 5E**, anti-Ly6G mAb had no effects on metastasis. On the other hand, anti-Gr-1 mAb suppressed the metastatic formation. Taken together, we concluded that $Ym1^{+}Ly6C^{hi}$ monocytes contributed to the promotion of metastasis induced by tumor resection or radiation exposure to tumor.

Inhibition of CXCR4 Signaling Reduces Lung Metastasis by $Ym1^{+}Ly6C^{hi}$ Monocytes

It is presumably critical for $Ym1^{+}Ly6C^{hi}$ monocytes to accumulate *in situ* for the promotion of lung metastasis. Chong et al. reported that the lung accumulation of $Ly6C^{hi}$ monocytes is dependent on the CXCR4-CXCL12 signaling axis in the LPS-induced inflammation state (46). In fact, CXCR4 expression was observed on $Ly6C^{hi}$ monocytes, but not B16 cells (**Figure 6A**). AMD3100, a CXCR4 antagonist, inhibited the accumulation of $Ly6C^{hi}$ monocytes, but not neutrophils (**Figure 6B**) in lung associated with systemic inflammation. We then examined whether AMD3100 suppressed lung metastasis promoted by the surgical resection of primary tumor resection. The promotion of lung metastasis was inhibited by the treatment with AMD3100 (**Figures 6C, D**). These findings suggest that CXCR4 is a novel therapeutic target for controlling lung metastasis associated with surgical intervention for cancer by inhibiting the accumulation of $Ym^{+}Ly6C^{hi}$ monocytes in potential metastatic organs.

DISCUSSION

In this study, we demonstrated that $Ym1^{+}Ly6C^{hi}$ monocytes, but not neutrophils, promote inflammation-induced lung metastasis associated with intervention for primary tumor. Inhibition of the accumulation of $Ym1^{+}Ly6C^{hi}$ monocytes in lung or inhibition of MMP9 reduced lung metastasis, suggesting that $Ym1^{+}Ly6C^{hi}$ monocytes are a therapeutic target for the metastasis. Recent reports highlighted the critical roles of NETs in metastasis associated with inflammation in mouse. For instance, intranasal injection of LPS triggers marked neutrophil recruitment, detects numerous NET formation, and promotes lung metastasis (9). The induction of peritonitis results in NET formation in liver and facilitates liver metastasis (11). The inhibition of NET formation with DNase and neutrophil elastase inhibitor suppresses lung and liver metastases (11). In these experimental models, inflammation is elicited by local infection or tissue injury. However, such local inflammation at a distant site from the primary tumor dose not seems to occur in tumor-bearing patients. In this study, we demonstrated that a novel monocyte subset, but not neutrophils, play a critical role in the progression of metastasis associated with inflammation caused by primary tumor resection or irradiation. Although neutrophils were recruited into lung in this type of inflammation, NET formation hardly occurred at this site. These results indicate that the context of inflammation determines the types of immune cells primarily responsible for promoting metastasis, and suggest that therapeutic target cells for metastasis prevention need to be carefully selected according to the actual situation of cancer patients.

Recent reports have shown that a functionally distinct monocyte subset is differentiated in BM in response to certain inflammatory stimuli (16, 23, 25). This monocyte subset is differentiated from granulocyte-monocyte progenitors (GMPs) and shares some characteristics with granulocytes. In line with this concept, we showed in our previous report that immunoregulatory $Ym1^{+}Ly6C^{hi}$ monocytes were generated from GMPs in BM during the recovery phase of tissue injury and contributed to inflammatory response associated with tissue repair (22). It is well known that tissue repair and wound healing after tissue injury consist of multiple processes including regeneration of parenchymal cells, ECM remodeling and angiogenesis (47). These processes also contribute to tumor progression and metastasis (32). For example, the high expression of wound-response gene increased the risk of metastasis in human (48). Increased MMPs, which are critical molecules for ECM remodeling in injured tissue, are correlated with low overall survival rate in cancer patients (49). In fact, lung metastasis of B16F10 was decreased in MMP9-deficient mouse (40). Regarding the regeneration of blood vessels in wound tissue, the hypoxic condition is detected by hypoxia inducible factor alpha (HIF1a), which induces the production of vascular endothelial growth factor (VEGF) to promote angiogenesis (50). The same mechanisms also apply in cancer tissue, and neutralizing antibody against VEGF or VEGF receptor was reported to inhibit lung metastasis (51, 52). In this respect, the tissue repair process shares common features with cancer progression and metastasis. This study, together with our previous report, reveals a link between tissue repair and cancer progression from the perspective of cell population. In the future, the relationship between these monocytes and primary tumor progression should be investigated.

Patients who undergo resection of primary tumors face the risk of metastatic recurrence that peaks sharply 12 to 18 months after surgery (41–43). Although the cause of early metastatic relapse has been debated, a recent report has indicated that systemic inflammation induced by resection triggers the outgrowth of distant dormant tumors in mouse, implicating that $Ly6C^{hi}$ monocytes are essential effector cells for the induction of the outgrowth (7). However, it is unclear what monocyte subset is involved in tumor progression induced by resection. In the present study, we showed that immunoregulatory $Ym1^{+}Ly6C^{hi}$ monocytes promoted lung metastasis of CTCs in an MMP-9- and CXCR4-dependent manner. MMP-9 secreted by $Ym1^{+}Ly6C^{hi}$ monocytes may degrade extracellular matrix and promote infiltration of cancer cells into metastatic tissues.

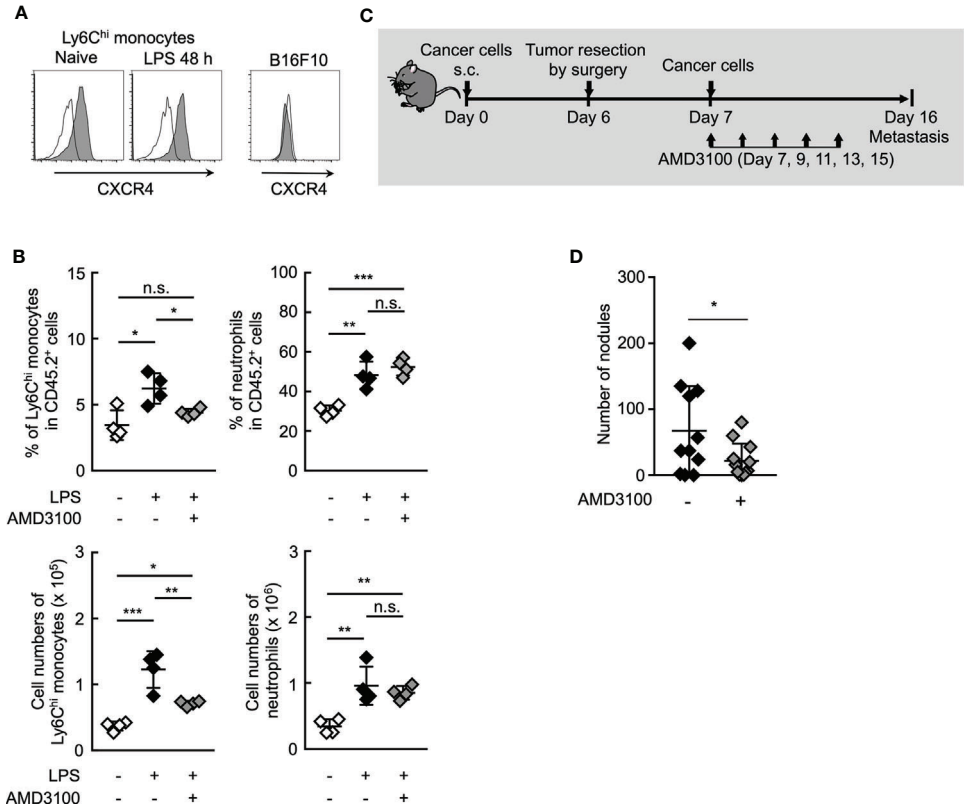

FIGURE 6 | Inhibition of CXCR4 signaling reduce lung metastasis by Ym1^{+}Ly6C^{hi} monocytes. **(A)** Expression of surface CXCR4 on peripheral blood Ly6C^{hi} monocytes isolated from naïve or LPS-treated mice, or B16 cells. Black lines indicate isotype control. **(B)** Inhibition of lung accumulation of Ly6C^{hi} monocytes by treatment with CXCR4 antagonist. WT mice injected intravenously with LPS (20 μg) and treated i.p. with AMD3100 (5 mg/kg, -1 h before and 24 h after LPS injection) or PBS [inhibitor (-)]. Average values are shown with SD. One-way ANOVA followed by Dunnett's test, n = 4. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$; n.s., not significant. Each symbol represents an individual animal. **(C, D)** Effects of CXCR4 antagonist on lung metastasis after tumor resection. **(C)** Experimental design used to test the effect of AMD3100 treatment on tumor-resection-induced metastatic progression. B16 cells (1 x 10^6 cells) were s.c. inoculated in the flank of WT mice. Six days later, the primary tumor was removed by surgery followed by i.v. injection of B16 cells (1 x 10^5 cells) 24 h later. AMD3100 (5 mg/kg) or PBS [inhibitor (-)] was injected into these mice on Day 7, 9, 11, 13, and 15. The lungs were analyzed for metastasis on Day 16. **(D)** Quantitative summary of the number of lung metastases. Average values are shown with SD. Unpaired two-tailed t-test, n = 10–11. *$P < 0.05$. Each symbol represents an individual animal.

These results suggest that the risk of metastatic recurrence after resection can be reduced by developing a therapeutic method targeting immunoregulatory Ym1^{+}Ly6C^{hi} monocytes.

The prediction and prevention of metastasis is a vital clinical task in cancer treatment. A previous report suggested that the high expression of wound response signature in tumors is a predictor of poor patient survival and increased risk of metastasis in human (48). In this study, we observed the rapid increase of Ym1^{+}Ly6C^{hi} monocyte numbers in peripheral blood in the early stage of lung metastasis. Given that the emergence of Ym1^{+}Ly6C^{hi} monocytes is implicated in the initial step of wound healing or tissue repair, monitoring of these monocytes in peripheral blood after surgical intervention for primary tumor may be a useful predictive cellular biomarker for metastasis. For this purpose, the human counterpart of mouse Ym1^{+}Ly6C^{hi} monocytes should be identified. A recent study that employed single-cell RNA sequencing uncovered a four-monocyte population in healthy human peripheral blood (53). Furthermore, in cancer patients, monocytic myeloid-derived suppressor cells have emerged as the major negative regulators of immune responses (54–56). In any case, estimation of the emergence of novel and atypical monocyte subsets associated with inflammation is important for the development of therapeutic strategies for metastasis.

DATA AVAILABILITY STATEMENT

The RNA-seq data has been deposited to the GEO - accession number is GSE174199.

ETHICS STATEMENT

The animal study was reviewed and approved by Tokyo University of Pharmacy and Life Sciences Animal Use Committee.

AUTHOR CONTRIBUTIONS

TS: Data curation, Formal analysis, Validation, Investigation, Methodology. AK: Validation, Investigation. HS: Validation, Investigation. KK: Data curation, Funding acquisition. MM: Validation, Investigation. RS: Validation, Investigation. NI: Conceptualization. KA: Funding acquisition, Writing-review and editing. DK: Data curation, Investigation. TT: Data curation, Investigation. AY: Resources. KI: Resources. TSa: Resources. SA: Resources. MT: Conceptualization, Supervision, Funding acquisition, Methodology, Writing-original draft, Project administration, Writing-review and editing. SY: Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Methodology, Writing-original draft, Writing-review and editing. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We would like to thank Dr. Fumiko Itoh, and Dr. Naoki Yoshida for helpful comments. This work was supported in part by a Grants-in-Aid for Scientific Research (B) (17H04068, and 20H03473 to MT, 17H04052 to KA), Scientific Research (C) (19K08894 to SY), and JSPS Research Fellows (19J10017 to KK) from Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research on Innovative Areas (Homeostatic regulation by various types of cell death) (26110006) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan, the MEXT Joint Usage/Research Center Program at the Advanced Medical Research Center, Yokohama City University (to TT), AMED-CREST, AMED under Grant Number JP20gm1210002 (to MT), and AMED under Grant Number 20cm0106374h0001 (to MT). We thank H. Yokoi for secretarial assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.663115/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 June 2021
doi: 10.3389/fimmu.2021.633361

The Adverse Impact of Tumor Microenvironment on NK-Cell

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NK cells are considered an important component of innate immunity, which is the first line of defensing against tumors and viral infections in the absence of prior sensitization. NK cells express an array of germline-encoded receptors, which allow them to eliminate abnormal cells and were previously considered a homogenous population of innate lymphocytes, with limited phenotypic and functional diversity. Although their characteristics are related to their developmental origins, other factors, such as tumors and viral infections, can influence their phenotype. Here, we provide an overview of NK cells in the context of the tumor microenvironment, with a primary focus on their phenotypes, functions, and roles in tumor micro-environment. A comprehensive understanding of NK cells in the tumor microenvironment will provide a theoretical basis for the development of NK cell immunotherapy.

Keywords: NK cells, tumor microenvironment, cytotoxicity, migration, metabolism

OPEN ACCESS

Edited by:
Anahid Jewett, University of California, Los Angeles, United States

Reviewed by:
Gabriella Pietra, University of Genoa, Italy
Jian Zhang, Shandong University, China

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

Received: *25 November 2020*
Accepted: *19 May 2021*
Published: *11 June 2021*

Citation:
Hu Z, Xu X and Wei H (2021) The Adverse Impact of Tumor Microenvironment on NK-Cell. Front. Immunol. 12:633361. doi: 10.3389/fimmu.2021.633361

INTRODUCTION

Natural killer (NK) cells, which originate in the bone marrow, were first identified in 1975 (1, 2). NK cells are generated from common lymphoid progenitor cells, which develop common innate lymphoid cell progenitors. The common innate lymphoid cell progenitors subsequently give rise to the NK-restricted NK cell progenitors. NK cells are defined as $CD3^{+}CD56^{-}$ lymphocytes, and are distinguished into $CD56^{bright}$ and $CD56^{dim}$ subsets. $CD56^{bright}$ NK cells usually express CD122, NKp46, and NKp80, while $CD56^{dim}$ NK cells express more markers, including CD16, CD57, and PEN5. NK cells can be found in peripheral, lymph node, spleen, liver, lung, and bone marrow (3–6). More than 90% of peripheral blood, lung, bone marrow, and spleen NK cells belong to the $CD56^{dim}CD16^{+}$ subset, which exhibit marked cytotoxic function on interaction with target cells. In contrast, most NK cells in lymph nodes belong to the $CD56^{bright}CD16^{-}$ subset, and have predominantly immune regulatory characteristics (7). When NK cells meet with stressed cells, they produce lytic granules, containing factors such as perforin, granzymes, and granulysin, which can induce cell death. Further, NK cells can induce apoptosis of target cells by binding to their FAS or TRAILR receptors. NK cells produce an array of cytokines [interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-10], growth factors (granulocyte-macrophage colony-stimulating factor), and chemokines (CCL3, CCL4, CCL5, XCL1), and can shape immune responses through their interactions with dendritic cells (DCs), macrophages, and T cells (8–10).

The NK cell cytotoxic attack is immediate, and does not require prior antigen-priming or MHC-restriction, and NK cell status depends on the balance of activating and inhibitory signals among the various receptors interacting with their ligands (11). Activating receptors include the cytotoxicity

receptors (NCRs; NKp46, NKp30, and NKp44), C-type lectin receptors (CD94/NKG2C, NKG2D, NKG2E/H, and NKG2F), and killer cell immunoglobulin-like receptors (KIRs) (KIR-2DS and KIR-3DS). Barrow and colleagues reported that natural killer cell p44-related protein (NKp44) can recognize platelet-derived growth factor-DD, which is produced by proliferating tumor cells and can activate NK cells (12). Inhibitory receptors include C-type lectin receptors (CD94/NKG2A/B) and KIRs (KIR-2DL and KIR-3DL). MHC class I (MHC-I) molecules are present on most cells, and NK cell inhibitory receptors (KIRs and CD94/NKG2A/B) can bind to them to prevent NK cell-mediated killing (13–17); however, when stressed cells downregulated MHC-I expression, NK cells are activated through "missing-self recognition" by losing their inhibitory signals. When cancer cells show elevated level of NK cell receptor, like NKG2D in response to stress, "self-induced" activation mechanism occur and leading the engagement of NK cells. Despite the expression of the inhibitory receptor, the activation of the "induced self" override the inhibitory signals present on cancer cells. These two mechanisms are not contradictory, and may co-regulated the overall response of NK cells to pathogens (18).

Carrega et al. showed that, in patients with non-small cell lung cancer, tumor-infiltrating NK cells express several activation markers, including NKp44, CD69, and HLA-DR, yet showed profoundly impaired cytotoxic potential (19). Further, an inverse correlation was demonstrated between circulating or tumor-infiltrating NK cell levels and the presence of metastases in patients with various types of solid tumours (20–22). In this review, we summarize recent developments and gaps in knowledge relating to tumor-infiltrated NK cells.

TUMOR-INFILTRATED NK CELLS EXHIBIT AN ALTERED PHENOTYPE

NK cells have been observed in many types of tumors, including primary tumors, metastases, and tumor-infiltrated lymph nodes (23–25). In non-small cell lung cancer, $CD56^{bright}$ and $CD16^{-}$ NK cells were observed in the tumor stroma. Although these NK cells displayed some activation markers, such as NKp44, CD69, and HLA-DR, their cytolytic potential was lower than that of NK cells in the peripheral blood and normal lung tissue (19). NK cells are rarely detected in colorectal carcinoma tissue; however, adjacent normal mucosa contained normal levels of NK cells (26). Besides, high levels of $CD57^{+}$ NK cell infiltration is associated with good prognosis, while $NKp46^{+}$ infiltrate has no prognostic value (27). Few NK cells are detected in endometrial tumors and tumor-resident $CD103^{+}$ NK cells express more co-inhibitory molecules, such as TIGIT and TIM3, depending on the severity of the disease (28). Zhang et al. demonstrated that TIGIT is associated with NK cell exhaustion in tumor-bearing mice and patients with colon cancer, and blockade of TIGIT prevents NK cell exhaustion and promotes NK cell-dependent tumor immunity in several tumor-bearing mouse models (29). The tumor associated circulating NK from the patients of prostate cancer increased the expression of markers of exhaustion (PD-1, TIM-3) and were impaired in their degranulation capabilities (30). Izawa et al. found that the ratio of tumor infiltrating $CD56^{dim}$ NK cells gradually decreased, according to disease progression, due to the relatively higher sensitivity of $CD56^{dim}$ NK cells to apoptosis in response to H_2O_2 in the tumor microenvironment. Further, exposure of NK cells to H_2O_2 results in impaired antibody-dependent cellular cytotoxicity (31). Moreover, Carrega et al. reported that the percentage of NK cells was lower in neoplastic tissue than in equivalent normal tissue. These researchers also found that, in lung and breast cancer, levels of $CD56^{bright}$ $perforin^{low}$ NK cells were significantly higher than those in matched normal tissue (32). In addition, several studies have indicated that there is a correlation between high NK cell infiltration and better prognosis in renal cell carcinoma (33–36).

Natural Cytotoxicity Receptors (NCRs) include of NKp44, NKp46 and NKp30 and they can play an important role in most functions exerted by NK cells. NKp44 is a transmembrane glycoprotein and it has three mRNA splice variants which display different signaling capability. NKp44-1has the ITIM in their cytoplasmic tail but NKp44-2 and 3 are not. NKp44 ligands which expressed by tumor cells comprises cellular and cell-released forms. Mixed-lineage leukemia protein-5 (MLL5), termed 21spe-MLL5, Cell surface-associated heparan sulfate (HS) proteoglycans (HSPGs) and Proliferating Cell Nuclear Antigen (PCNA) are expressed on the surface of tumor cells, while Platelet-Derived Growth Factor (PDGF)-DD and Nidogen-1 (NID1) glycoprotein are secreted by tumor cells as soluble molecules to interact with NKp44. Among these NKp44 ligands, 21spe-MLL5, HSPG and (PDGF)-DD interact with NKp44 result in activation of NK cells. However, PCNA and NID1 inhibit the cytolytic function of NK cells. Tumor-infiltrated NK cells expressed higher level of KLRC1(NKG2A) gene and KLRD1 (CD94). Also, KLRB1 gene (CD161) was expressed on tumor-infiltrated NK cells, which could bind to the CLE2D ligand on tumor cells to inhibit NK cell-mediated cytotoxicity (37–40). Small cell lung cancer primary tumors expressed very low level of NKG2DL mRNA and small cell lung cancer lines express little to no surface NKG2DL at the protein level, which caused the evading NK surveillance (41). Although the activating receptor NKG2D induces NK cell-mediated killing of metastasizing tumor cells by recognition of the stress-induced ligands MICA, MICB, and ULBP1-6. However, platelets enable escape from this immune surveillance mechanism by obstructing the interactions between NK cells and tumor cells or by cleaving the stress-induced ligands (42).

Anahid Jewett et al. recently indicated that NK cells could select and kill cancer stem cells/undifferentiated tumors. Cancer stem-like cells had a specific genetic signature and sustained tumor growth due to their self-renewal capacity. NK cells triggered differentiation of CSCs/undifferentiated tumors primarily *via* secreted and membrane bound forms of IFN-γ. Thus, NK cells played an important and unique role in targeting stem-like tumors or poorly differentiated tumors (43, 44). CD94, NKG2A, NKp46 and CD69 were considered as the phenotype as memory-like NK cells and the memory-like NK cells could be induced by IL-12, IL-15 and IL-18. Memory-like NK cells able to lyse autologous tumor cells can

also be generated from patients with solid malignancies. The anti-tumor activity of allogenic and autologous memory-like NK cells is significantly greater than that displayed by NK cells stimulated overnight with IL-2. Also, memory-like NK cells displaying high levels of anti-tumor activity and low levels of reactivity against non-malignant cells, which could be transferred to future clinical trials of adoptive NK cell therapy (45).

Huergo Zapico et al. found that when NK cells were co-cultured with melanoma cells, melanoma cells up-regulated the expression of stem cell marker CD271 and CD166. In addition, melanoma cells showed cadherin switching, increased fibronectin expression and cytoskeletal recombination, indicating F-actin stress fiber production. Melamo cells could induce down regulation of NKp30, NKG2D and DNAM-1 on NK cells. However, the melanoma cell lines had little effect on the expression of Tim-3. Compared with regions far from NK cells, the expression of E-cadherin was lower in regions close to NK cells, and the expression of N-cadherin was higher in the region close to NK cells. These data clearly suggest that, at least in some cases, NK cells can influence the EMT at the tumor site (46).

Scavenger receptor MARCO, which is expressed on a specific subpopulation of TAMs in the tumor. When using anti-MARCO treatment to mouse model, the killing ability of NK cells were enhanced and the amount of IL-15 is the serum was also enhanced. The author indicated that IL-15 production induced by anti-MARCO locally in the tumor and possibly the draining lymph node will support the proliferation, migration, and cytotoxic capacity of NK cells (47). Sialic acids, extracellular matrix/collagen or aminophospholipids was expressed on the surface of tumor respectively, which could be recognized by sialic acids, extracellular matrix/collagen or aminophospholipids expressed on the surface of NK cells. Thus, NK cell function was inhibited (48).

THE CYTOTOXICITY OF TUMOR-INFILTRATED NK CELLS IS IMPAIRED

The tumor microenvironment is a complex milieu, full of inhibitory cells and factors. Tumor-associated macrophages (TAMs) accumulate at the tumor site, as well as circulating monocytes, recruited by the tumor-derived chemotactic factor, CCL2. Initially, these monocytes polarize into M1 cells, which exhibit cytotoxicity against tumor cells. M1 cells secrete cytokines, including IFN-γ and IL-12, which activate NK cells; however, with carcinoma progression and metastasis, TAMs polarize into cells and secrete large amounts of IL-10 and TGF-β, which suppress NK cell cytotoxicity. In contrast, M2 cells stimulate regulatory T cells (Tregs) and Th2 cells, which generate an immunosuppressive environment for NK cells (49). Other immunosuppressive cells within tumors are myeloid derived suppressive cells (MDSC), which include granulocytes, macrophages, and dendritic cells that are blocked at various stages of maturation MDSCs produce immunosuppressive factors such as IL-10, TGF-β, and IL-4. TGF-β inhibits the expression of two NK cell receptors, NKp30 and NKG2D, which are critical for tumor cell recognition and killing, as well as for functional interaction between NK cells and DC. IL-4 strongly reduces the ability of NK cells to kill sensitive targets and produce cytokine. Indoleamine 2,3-dioxygenase (IDO) promotes the production of the immunosuppressive tryptophan catabolite, L-kynurenine, which interferes with the IL-2-induced upregulation of NKp46 and NKG2D expression, thereby reducing the ability of NK cells to recognize and kill tumor cells (50–52). The expression of CD16 is down-regulated in most solid tumors-infiltrated NK cells, which may be related to the reduced proportion of CD56dim NK cells (32). CD57 is a marker of NK cell terminal differentiation, and CD57+ NK cells have high cytotoxic potential (53, 54). $CD56^{dim}KIR^{+}CD57^{+}$ NK cells were observed in the peripheral blood of patients with acute myeloid leukemia (55). In addition, tumor-infiltrating NK cells in non-small-cell lung cancer and melanoma metastatic lymph nodes exhibit downregulation of the activation markers, CD69, NKp44, and HLA-DR (19, 56), while TIGIT, TIM-3, LAG-3, and PD-1 were upregulated in tumor-infiltrated NK cells, indicating that they tended toward exhaustion (57). In the tumor microenvironment $CD49a^{-}CD49b^{+}Eomes^{+}$, NK cells can convert into $CD49a^{+}CD49b^{+}Eomes^{+}$ and $CD49a^{+}CD49b^{-}Eomes^{int}$ NK cells (type 1 innate lymphoid cells) in response to cytokine-TGFβ signaling; however, intermediate group 1 innate lymphoid cells and group 1 innate lymphoid cells could not control local tumor growth and metastasis, and TGF-β signaling in $NKp46^{+}$ cells suppress NK cell-mediated tumor immunosurveillance (57) (**Figure 1**).

NK CELL METABOLISM IS DYSREGULATED BY THE TUMOR MICROENVIRONMENT

Cong et al. investigated the role of NK cells in the tumor microenvironment by using credibly induced KrasG12D(KRAS) knockout into mouse lung cancer models. Further, they applied anti-NK1.1 monoclonal antibody, PK136to depletion of number of NK cells, or Kras mice with an Nfil3-/- mouse knockout model, and showed that depletion of NK cells significantly accelerated tumor development during tumor initiation, while depletion of NK cells during promotion and development had no effect on tumor development. The author hypothesized that NK cells could effectively prevent the occurrence of tumor, but could not control the occurrence and development of lung cancer. In addition, they found that the quantity of NK cells, T cells, B cells, and MDSCs in the lungs declined progressively, while the number the macrophages were increased, particularly the quantity of M2 cells, which function as immune suppressive cells. Further, tumor-infiltrated NK cells showed significantly attenuated cytotoxicity, and the expression levels of granzyme B, perforin, CD107a, IFN-γ, and TNF-α were gradually reduced in lung NK cells during lung cancer development. In contrast, the expression of molecules associated with activation and cytotoxicity, including NKp46, CD69, CD44, CD226, CD16/32, FasL, TRAIL, and CD122, and the inhibitory molecules, CTLA4, CD96, CD94, PD-1, PD-L1, Tim3, CD276, LAG3, and CD244, were unchanged in lung NK cells during lung cancer development.

As established, glucose metabolism is essential for the function of human and mouse NK cells; hence, dysregulation of glucose metabolism can lead to NK cell dysfunction. FBP1 is important in

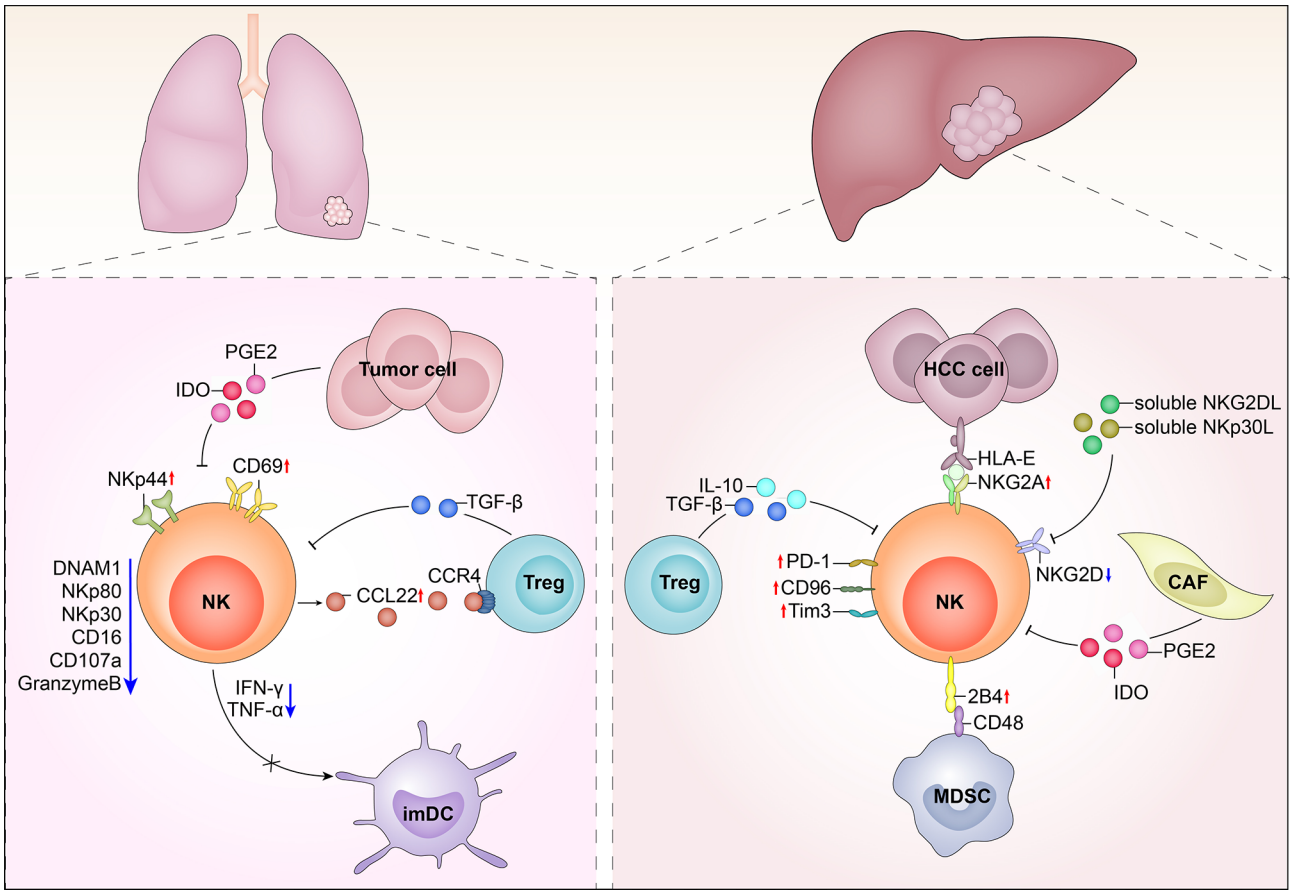

FIGURE 1 | Tumor infiltrates NK cells in hepatocellular carcinoma (HCC) and lung cancer. Left image: In the tumor microenvironment of lung cancer, NK cells up-regulated the expressions of CD69 and NKp44 and down-regulated the expression of NKp30, NKp80, DNAM-1 and CD16. Series of soluble molecules were secreted by tumor cells, like IDO, PGE2, and TGF-β. Tumor cells also could express membrane molecules that can shed receptors on the surface of NK cells. In addition, intratumoral NK cells showed impaired IFN-γ secretion, which may lead to inefficiency in DC maturation. Right image: In the tumor microenvironment of HCC, NK cells up-regulated KIR, NKG2A, PD1, TIM2, and CD96 inhibitory receptors while down-regulated NKG2D. Treg cells produced IL-10 and TGF-β. In HCC, TGFβ, prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO), produced by tumor-associated fibroblasts inhibit the cytotoxic activity and cytokine secretion of NK cells.

glucose metabolism and inhibits glycolysis in human tumor cells and hematopoietic progenitor cells. Levels of FBP1 showed a 69-fold increase in tumor-infiltrated NK cells, in which glycolysis was inhibited; however, when FBP1 was inhibited, NK cell glycolysis function was restored. Thus, FBP1 weakens the cytotoxicity of tumor-infiltrated NK cells by inhibiting glycolysis; however, FBP1 can also impair the viability of tumor-infiltrated NK cells directly and independently of glycolysis. Lactic acid accumulated in the tumor microenvironment is a potent inhibitor of NK cell effector function and viability. Intracellular acidification and decreased ATP synthesis caused by lactic acid may be related to impaired IFN-γ production by NK cells (58).

Zheng et al. observed that the tumor-infiltrated NK cells mainly had small, fragmented, distinct mitochondria in the cytoplasm, whereas liver and peripheral NK cells primarily had large, tubular, and densely packed mitochondria. There was a positive correlation between mitochondrial length and granzyme B levels. Furthermore, tumor-infiltrated NK cells had a significantly lower mitochondrial mass than paired tumor-adjacent normal liver NK cells. Moreover, the cells also had increased mitochondrial ROS levels and tumor infiltrated NK cells had upregulated expression of numerous mitochondrial fission-related genes, including *INF2*, *MIEF2*, *FIS1*, and *GDAP1*; high expression of fission genes drives mitochondrial fragmentation. Hypoxia is a key feature of the tumor microenvironment and tumor infiltrated NK cells are enriched for hypoxia signatures, including expression of HK2, SLC7A5, SLC2A3, and KDM3A. In addition, NK cells cultured under hypoxia show reduced expression of granzyme B, IFN-γ, and CD107a after activation, suggesting impaired functionality. Drp1 is the main regulator of mitochondrial fission, and drives division at specific points along mitochondria. Compared with paired tumor-adjacent normal liver NK cells, tumor-infiltrated NK cells upregulate Drp1 pro-fission activity through phosphorylation of its Ser616 residue. Restoration of mitochondrial morphology by knocking down Drp1, and in response to mdivi-1 treatment, enabled tumor-infiltrated NK cells to kill tumor cells. Therefore, mitochondrial fragmentation is correlated with decreased NK cell antitumor capacity (59) (**Figure 2**).

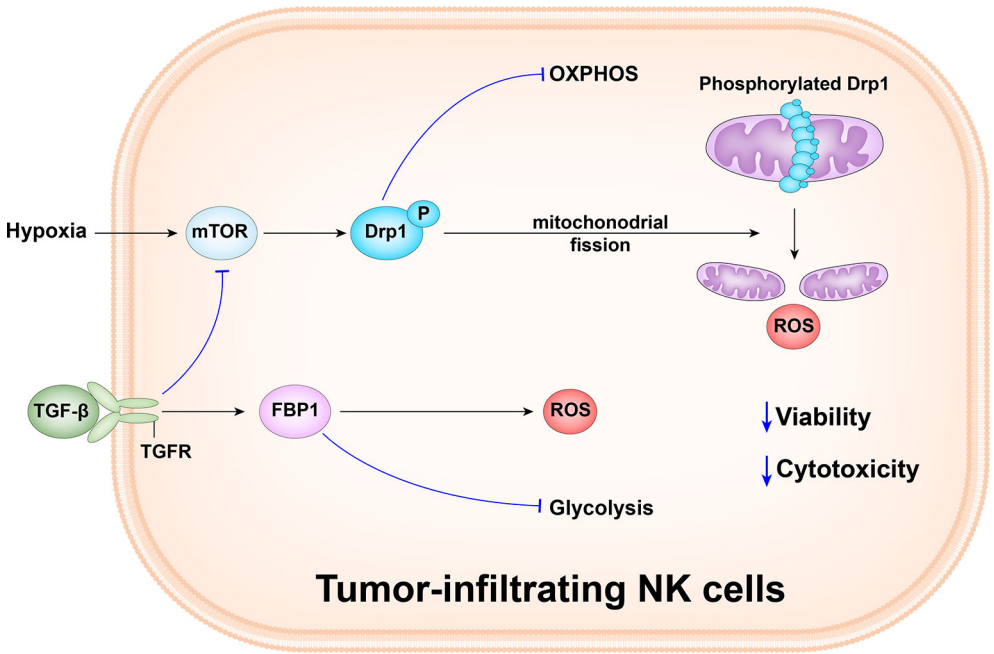

FIGURE 2 | Tumor-infiltrating NK cells in human liver cancer have small, fragmented mitochondria in their cytoplasm, while tumor and peripheral NK cells in the liver have normal large, tubular mitochondria. Mitochondrial fragmentation causes tumor to evade NK cell-mediated surveillance. The hypoxic tumor microenvironment promotes the continuous activation of the mechanism target of rapamycin-GTPase motor associated protein 1(mTOR-DRP1) in NK cells, leading to excessive mitochondrial fragmentation and enhance the viability and anti-tumor ability of NK cells. Besides, the abnormally expressed gluconeogenic enzyme, FBP1, in response to TGFβ, can inhibit NK cell glycolysis and promotes the production of reactive oxygen species (ROS), and causes dysfunction by inhibiting glycolysis and reducing activity.

Obesity induces lipid accumulation driven by peroxisome proliferation-activated receptor (PPAR) in NK cells, resulting in complete "paralysis" of cell metabolism and transport, which reduces the anti-tumor response of NK cells and fails to reduce tumor growth *in vivo* experiment with obesity (55). Frank Cichocki et al. found that in adaptive NK cells, AT-rich interaction domain 5B (ARID5b), a short isoform of chromatin modified transcriptional regulator, was selectively induced by DNA hypopethylation. Knockdown and overexpression studies have shown that ARID5b plays a direct role in promoting mitochondrial membrane potential, gene expression encoding electron-transport chain components, oxidative metabolism, survival, and IFN-γ production (56). In addition, mTOR is sensitive to nutrient utilization and can be inhibited by TGF-β, thereby inhibiting NK cell metabolism and function. Therefore, it can be speculated that TGF-β production is also higher in nutrient-deficient TMEs, and mTOR may be inhibited, thereby limiting the effector function of NK cells (57). Studies by Antonie Marcaus et al. showed that after high concentration of IL-15 was exposed to NK cells, metabolic checkpoint kinase mTOR was activated and promoted bioenergy metabolism. This process is essential for maintaining the proliferation of NK cells during development and for achieving anti-tumor cell lysis (60).

Róisín M. Loftus et al. found that NK cells isolated from human solid tumors were deficient in their pro-inflammatory functions, including production of IFN-γ and tumor cytotoxicity. Tumor cells are known to have a high demand for glutamine in addition to glucose, so it is likely that low levels of glutamine are also present in the tumor microenvironment. Glutamine limited tumor microenvironment can inhibit the expression of cMyc in NK cells, resulting in reduced NK cell metabolism and inhibition of anti-tumor NK cell function (61, 62).

EFFECTS OF THE TUMOR MICROENVIRONMENT ON NK CELL MIGRATION

High NK-cell infiltration is often believed to be an indicator of better prognosis (28), and $CD56^{bright}$ cells may be preferentially recruited tumor sites (19, 63); however, $CD56^{bright}$ cells have been associated with poor cytolytic function. CCR5 is a receptor for MIP-1b, which is an adhesive signal that leads to the arrest of leukocytes within tissue. Several studies have indicated that only $CD56^{bright}$ $CD16^{-}$ peripheral blood NK cells express CCR5, which may explain their accumulation in tumor tissues. NK cells are not normally associated with secondary lymphoid organs. Indeed, $CD16^{+}$ NK cells both lack CCR7 and fail to respond to CCR7 ligands; however, $CD16^{-}$ NK cells express high levels of CCR7, and respond very well to CCR7 ligands. Further, $CD56^{bright}$ NK cells express CD62L, CCR7, CCR5, and CXCR3, which are responsible for their preferential migration into secondary lymphoid organs. Gillard-Bocquet et al. found that CXCR5 and CXCR6 were overexpressed, while the expression of CX3CR1 and

S1PR1 was downregulated, relative to non-tumor NK cells (64, 65). A recent study on melanoma revealed that the chemoattractant, chemerin, greatly favors the infiltration of conventional NK cells, T cells, and DCs, but not MDSCs, into tumors, thereby modifying the tumor microenvironment from a tolerogenic to a tumor-suppressive state (26, 66, 67). Matteo Gallazzi et al. found that when co-cultured the healthy donor-derived pNK cells with three different prostate cancer cell lines, together with increased production of pro-inflammatory chemokines/chemokine receptors CXCR4, CXCL8, CXCL12, reduced production of TNF-α, IFN-γ and Granzyme-B (30).

De Andrade et al. found that NK cell frequencies were lower in tumor compared with matching blood samples. They also found that XCL1 and XCL2 were highly expressed among TI-NK cells than blood NK cells, which played the critical role in recruiting DCs to tumor. Besides, TI-NK cells express high level of CCL3, CCL4, CCL4L2 and CCL5, which could bind to CCR5 and other chemokine receptors to recruit T cells and other immune cells. These tumor NK cell populations may thus create distinct microenvironments. NK cells not only kill tumor cells but also recruit key immune cell populations required for protective tumor immunity (46, 68).

NK CELLS AFFECT OTHER IMMUNE CELL EFFECTOR FUNCTIONS

Mailloux and colleagues observed that Tregs accumulated within Lewis lung cancer (LLC)-bearing lungs. Further, these Tregs had upregulated CCR4, which can bind chemokines to attract Tregs into LLC-bearing lungs. They also found that LLC-bearing lung tissue secreted elevated levels of CCL22, which can also attract many Tregs to the tumor microenvironment. Surprisingly, the CCL22 was secreted by NK cells, with the phenotype, $NK1.1^{+}CD11b^{dim}CD49b^{+}CD122^{+}CD27{+}CD19^{+}CD3^{-}$. Moreover, NK cells and Tregs co-localized in the tumor microenvironment, indicating that Tregs were recruited by NK cells. Thus, if NK cells can stimulate up-regulated CCL22 secretion in the tumor microenvironment, then they may have the unexpected side effect of indirectly contributing to tumor-induced immune suppression, through Treg recruitment (69–71); however, Roy et al. found that NK cells lysed Tregs which expanded in response to an intracellular pathogen, indicating a potential new role for NK cells in maintaining the delicate balance between the regulatory and effector functions of the immune response (72–75).

Russick et al. indicated that some subsets of tumor-infiltrated NK cells express inhibitory markers, including KLRC1 and CTLA4, and that these NK cells may weaken the function of $CD8^{+}$ T cells. When NK cells and DCs are co-cultured, DC maturation is reduced; however, this can be partially reversed by the addition of CTLA4 (19, 76, 77). Neo et al. found that CD73-positive NK cells overexpress multiple alternative immune checkpoint receptors, including LAG3, VISTA, PD1, and PD-L1, and defined this subset of NK cells as regulatory NK cells (78–81). Regulatory NK cells produce IL-10, and/or express the immune checkpoint molecule, CD73, and inhibit autologous $CD4^{+}$ T cell proliferation (29, 82–84).

Erin E. Peterson et al. reported that NK cells and cDC1s engage in intercellular cross-talk integral to initiating and coordinating adaptive immunity to cancer. The NK cell-cDC axis was associated with increased overall survival and anti-PD1 immunotherapy response in patients with metastasis melanoma (85).

CONCLUSIONS

In summary, NK cells are powerful effectors of innate immunity that constitute a first line of defensing against cancer; however, the tumor-microenvironment is highly complex, containing numerous immune-inhibited cells and factors. NK cells can infiltrate primary solid tumors, metastases, and tumor-infiltrated lymph nodes. Tumor-infiltrated NK cells exhibit an altered phenotype, with downregulation of NKp30, NKp80, DNAM-1, and CD16. In addition, expression and secretion of CD107 are impaired. Tumor cells produce soluble molecules, such as IDO, PEG2, TGF-β, and a series of membrane molecules, including PD1, PD-L1, LAG3, TIGIT, and CTLA4. Simultaneously, NK cell metabolism is markedly altered within the tumor-microenvironment, as many molecules, such as FBP1, can directly impair tumor-infiltrated NK cell viability, independent of glycolysis. Further, NK cells can be inhibited by TGF-β produced by Tregs. Tumor-infiltrated NK cells display impaired IFN-γ secretion, which can lead to inefficient DC maturation. NK cells secrete CCL22 to recruit Tregs *via* CCR4 within the tumor and it may intensify the level of immune-inhibition.

Use of cytokines like IL-2, IL-15, IL-12, IL-21 and IL-18 is considered a promising approach to induction of more efficient NK cell activation at tumor sites, while IL-15 and IL-21 can enhance NK cell cytotoxicity (86). Moreover, IL-18-primed NK cells can cooperate with DCs to recruit effector T cells to tumor sites. Immune checkpoint inhibitors, such as lirilumab, which targets KIRs, or monalizumab, which targets NKG2A and the tyrosine kinases inhibitors Imatinib and Sorafenib which aims to enhance the effector function of NK cells by promoting DC-mediated NK-cell activation may improve anticancer responses (87). A better understanding of the roles of tumor-infiltrated NK cells will provide more options for cancer immunotherapy and represents an attractive target to focus on to improve NK cell-based immunotherapies. Also, the comprehensive view of NK cells in the tumor microenvironment will give us inspiration to envisage a future scenario on the research of NK cells and make more favorable clinical outcomes.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. ZH drafted the manuscript and XX drafted the figures. HW edited/reviewed the article. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the key project of the National Natural Science Foundation of China (#81872318 and #81602491).

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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: 15 June 2021
doi: 10.3389/fimmu.2021.673248

Targeted Next-Generation Sequencing Combined With Circulating-Free DNA Deciphers Spatial Heterogeneity of Resected Multifocal Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) has a high risk of recurrence after surgical resection, particularly among patients with multifocal HCC. Genomic heterogeneity contributes to the early recurrence. Few studies focus on targeted next-generation sequencing (tNGS) to depict mutational footprints of heterogeneous multifocal HCC.

Methods: We conducted tNGS with an ultra-deep depth on 31 spatially distinct regions from 11 resected multifocal HCC samples. Matched preoperative peripheral circulating-free DNA (cfDNA) were simultaneously collected. Genomic alterations were identified and compared to depict the heterogeneity of multifocal HCC.

Results: Widespread intertumoral heterogeneity of driver mutations was observed in different subfoci of multifocal HCC. The identified somatic mutations were defined as truncal drivers or branchy drivers according to the phylogenetic reconstruction. *TP53* and *TERT* were the most commonly altered truncal drivers in multifocal HCC, while the most frequently mutated branchy driver was *TSC2*. HCC patients with a higher level of intertumoral heterogeneity, defined by the ratio of truncal drivers less than 50%, had a shorter RFS after surgical resection (HR=0.17, p=0.028). Genome profiling of cfDNA could effectively capture tumor-derived driver mutations, suggesting cfDNA was a non-invasive strategy to gain insights of genomic alterations in patients with resected multifocal HCC.

Conclusions: Truncal mutations and the level of genomic heterogeneity could be identified by tNGS panel in patients with resected multifocal HCC. cfDNA could serve

OPEN ACCESS

Edited by:
Jianmei Wu Leavenworth, University of Alabama at Birmingham, United States

Reviewed by:
Ruibin Xi, Peking University, China
Qiang Gao, Fudan University, China
Lili Feng, Beth Israel Deaconess Medical Center and Harvard Medical School, 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: *27 February 2021*
Accepted: *27 May 2021*
Published: *15 June 2021*

Citation:
Lin J, Zhao S, Wang D, Song Y, Che Y, Yang X, Mao J, Xie F, Long J, Bai Y, Yang X, Zhang L, Bian J, Lu X, Sang X, Pan J, Wang K and Zhao H (2021) Targeted Next-Generation Sequencing Combined With Circulating-Free DNA Deciphers Spatial Heterogeneity of Resected Multifocal Hepatocellular Carcinoma. Front. Immunol. 12:673248. doi: 10.3389/fimmu.2021.673248

as a non-invasive and real-time auxiliary method to decipher the intertumoral heterogeneity and identify oncodrivers of multifocal HCC.

Keywords: hepatocellular carcinoma, heterogeneity, circulating-free DNA, somatic mutation, immunotherapy

BACKGROUND

Hepatocellular carcinoma (HCC) ranks the first leading pathological types of primary liver cancer and the third leading cause of cancer-associated death worldwide (1). Intrahepatic tumor dissemination is the most common route of metastasis for advanced HCC, resulting in little chance to undertake radical resection for these patients. Over 50% of the HCC patients were reported to have multifocal lesions at their initial diagnosis (2), and prognoses varied among patients with resected multifocal HCC.

Previous studies have been well demonstrated that HCC is featured as a highly heterogenous malignance (3) through comprehensive multi-omics analyses including whole-genome sequencing (WGS) and transcriptome sequencing (4). Different tumoral lesions in HCC exhibited *de novo* carcinogenesis or tended to share a common primary tumor clone. Clinically, patients with multifocal HCCs tend to have early recurrences with a grim prognosis despite receiving aggressive therapeutic interventions (5). Therefore, it is imperative to decipher the evolutionary relationship among multiple tumors of multifocal HCC based on molecular profiling, so that precise personalized therapy against multifocal HCC may be established (6, 7). Existing researches commonly utilized WGS or whole-exome sequencing (WES) to differentiate intratumor heterogeneity through clonal evolution analysis for genomic alterations. However, due to the high cost, it is currently difficult to be widely applied in clinical practice. In recent years, targeted next-generation sequencing (tNGS)-based panel, which captured critical cancer-related genes and structure variations, has been specifically designed and implemented in routine clinical practice (8). Nonetheless, this strategy has not been fully investigated in multifocal HCC. It is encouraging to apply tNGS panel to identify spatial heterogeneity and clonal relationship of multifocal HCC.

Herein, we applied an ultra-deep tNGS-based assay of Cancer Sequencing YS (CSYS) panel (9) to detect genomic alterations in 31 surgically resected tumor tissues and the paired preoperative circulating-free tumor DNA (cfDNA) samples from 11 multifocal HCC patients. We explored the mutational similarity and spatial heterogeneity on the basis of alterations of 466 cancer-related genes captured by CSYS panel. Finally, we further tracked clonal relationship from cfDNA and deciphered the clonal relationship among various tumor foci in these multifocal HCC patients.

MATERIALS AND METHODS

Patients and Sample Collection

31 HCC tumor samples and 11 preoperative blood samples were obtained from 11 patients with resectable multifocal HCC who underwent primary and curative (tumor-free margin) surgical resections in our center (Peking Union Medical College Hospital, PUMCH). All tumor tissues were assessed by professional pathologists to confirm the diagnosis of HCC. The study's protocol was approved by the Ethics Committee of PUMCH. All patients signed informed consent forms, and their clinical follow-up data were available.

DNA Extraction and Sequencing

Only samples with estimated tumor purity >20% based on histopathological assessment were further subjected to genomic profiling. DNA was extracted from the fresh-frozen tumors and circulating leucocytes using a DNA Extraction Kit (Qiagen) according to the manufacturer's protocols. Preoperative peripheral blood lymphocytes and plasma were separated through centrifugation at 1,600 g for 10 min. Supernatant plasma was then transferred to a new 2 mL centrifuge tube and centrifuged at 16,000 g for 10 min. MagMAX^{TM} Cell-Free DNA isolation kit (Life Technologies, California, USA) was used to extract cfDNA in the plasma according to its manufacturer's instructions. Tiangen whole blood DNA kit (Tiangen, Beijing, China) was used to extract DNA from peripheral blood lymphocytes according to the manufacturer's instructions. DNA concentration was measured using Qubit dsDNA HS Assay kit or Qubit dsDNA BR Assay kit (Life Technologies, California, USA).

The tNGS panel (CSYS) for hybrid selection and the target-specific enrichment chip were designed and manufactured (OrigiMed, Shanghai) by custom pipeline. CSYS panel captured all coding exons of 466 key cancer-related genes and selected introns of 36 genes commonly rearranged in solid tumors (**Supplementary Table S1**). In addition, the probe density was increased to ensure high efficiency of capture in the conservatively low read depth region. With the input DNA at least 100ng for each library, CSYS panel was sequenced with a pre-set mean coverage of 900X for tumor DNA samples and 300X for matched blood samples on an Illumina NextSeq-500 Platform (Illumina Incorporated, San Diego, CA).

Somatic Variants Calling and Tumor Mutation Burden (TMB)

Data quality was inspected and controlled by examining sequencing coverage and uniformity, and a suite of customized bioinformatics pipelines was applied as previous reports (10) for somatic variants calling, including single nucleotide variations (SNVs), short and long insertions/deletions (indels), copy number variations (CNVs) and gene rearrangements. We used MuTect (11) (version 1.7) to identify SNVs and used Pindel (12) (version 0.2.5) to identified indels. The lengths of short indels were required <50 bp, while those >50 bp were considered as

long indels. For each alteration, we performed a manual review process to ensure no false positives or mistakes based on our in-house database. The annotations for these alterations were based on SnpEff 3.0 (13). CNVs were identified by Control-FREEC (14) (version 9.7) with the following parameters: step = 10,000 and Window = 50,000.

The processing for raw reads from cfDNA sequencing was followed as previously described (15). Briefly, cutadapt (version 1.18) (16) was used to filter out high-quality reads, and BWA (17) was used to map these reads into human genome by the reference from UCSC hg19 sequences. BaseRecalibrator tool from GATK (version 3.8) was applied to recalibrate base quality, and Picard was employed to remove PCR duplicates. Mutect2 was used to detect variants from cfDNA, and CNVs information was computed by CNVKit (18). All somatic variants were annotated by ANNOVAR (version 2017.07.17) with RefSeq (19).

TMB was estimated following the methods of Chalmers et al. (20). Briefly, SNVs and indels occurred in somatic and coding regions were counted. In order to reduce sampling noise, synonymous mutations were counted, while non-coding alterations and known germline alterations in dbSNP were excluded. To calculate the TMB per megabase (Mut/Mb), the total number of mutations counted was divided by the size of the coding region of the targeted territory.

Determination of Potential Driver Mutation Genes

The definition of potential HCC-driver mutation genes referred to a published study. Briefly, potential driver mutation genes included significantly mutated in TCGA-LIHC (21) program of HCC's genome ($q<0.1$), and mutations presented in TARGET database (v3.0, https://software.broadinstitute.org/cancer/cga/target).

Clonality Analysis

To gain insights into the genetic phylogeny of multifocal HCC, tumor phylogeneties were reconstructed for each multifocal HCC case using LICHeE (Lineage Inference for Cancer Heterogeneity and Evolution) algorithm (22). LICHeE is a computational method to decipher cancer cell lineages using somatic mutations from tumor samples. The parameters of *LICHeE* were set as follows: minVAFPresent of 0.01, maxVAFAbsent of 0.01, and maximum number of trees of 1, and the others with default values. The phylogenetic tree was constructed according to the output tree of *LICHeE*, and length of trunks and branches were proportional to the corresponded mutations.

Statistical Analysis

Assessments of differences in the means or medians of continuous variables were performed using SPSS software version 23 (IBM Corporation, Chicago, IL). The Mann-Whitney U test was employed to assess differences in the distributions of continuous variables between two groups. Fisher's exact test was applied to examine the dependency of two binary variables. Spearman correlation tests were applied to analyze the relationship between two variables. A two-tailed p value <0.05 was considered significant. The "survival" R package was used for the survival analysis using the Kaplan-Meier estimator, and p values were calculated with the log-rank test. Estimations for hazard ratios (HRs) were applied with Kaplan-Meier estimator. The statistical analyses were performed using R software (R-3.5.1).

RESULTS

Spatial Intertumoral Heterogeneity in Cancer Genome of Multifocal HCC

Surgically resected fresh-frozen tissues from 31 tumoral focal of 11 patients with pathologically-confirmed multifocal HCC (**Table 1**) were obtained to examine the genomic profiles. Preoperative peripheral blood leukocyte DNA was used as germline control for each patient. 9 of 11 patients had hepatitis B virus (HBV) infection, one patient had a history of hepatitis C virus (HCV) infection, and 3 patients were AFP-negative HCC at their initial diagnosis.

CSYS was performed in these tumor and blood samples, with an average of 1304× sequencing depth. Among the coding exons of 466 genes and various genomic regions previously shown to be involved in HCC including *TERT* promoter, oncogenic fusions and hepatitis B or C virus genomic integrations, we identified a

TABLE 1 | Summary of baseline clinicopathological characteristics of 31 tumoral focal from 11 HCC patients.

Patient ID	Tumor ID	Age, yrs	Sex	Pathological differentiation	Vascular tumor thrombus	Hepatitis history	Hepatic cirrhosis	Preoperative AFP
HCC01	T1/T2	59	M	Moderate	None	None	None	571
HCC02	T1/T2	56	F	Moderate- poor	None	HBV	Yes	15114
HCC03	T1/T2/T3	64	M	Moderate	None	HBV	Yes	53.3
HCC04	T1/T2/T3	58	F	Poor	None	HBV	Yes	7.6
HCC05	T1/T2/T3	75	M	Well	None	HBV	Yes	184.8
HCC06	T1/T2/T3	61	M	Moderate	None	HCV	None	4.1
HCC07	T1/T2/T3	36	M	Moderate- poor	Portal vein	HBV	None	525.7
HCC08	T1/T2/T3	51	M	Moderate	Microvascular	HBV	Yes	1420
HCC09	T1/T2/T3	40	M	Poor	Portal vein	HBV	Yes	10.2
HCC010	T1/T2/T3	40	M	Moderate	Microvascular	HBV	Yes	663.6
HCC011	T1/T2/T3	55	M	Well- moderate	None	HBV	Yes	112.1

M, Male; F, Female; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein (ng/ml, range of normal values: 0-20).

total of 132 somatic mutations across 98 genes (**Supplementary Table S2**), including 101 single-nucleotide variants (SNVs) and 31 short insertions and deletions (indels). High concordance among putative driver genes was observed among different foci in the individual patient (**Figure 1A**). As the most commonly altered driver genes, mutations in *TP53* and *TERT* were both identified in 7 of 11 (64%) HCC patients, and different tumor lesions shared *TP53* or *TERT* promoter alterations except for three patients (HCC05, HCC07 and HCC08's T3). Besides, somatic copy number alterations (SCNAs) in these cancer-related genes were shared by different tumor foci in the same patient, especially in HCC07 and HCC09. Overall, we observed a high proportion of shared events in SNVs, indel, or SCNAs (**Figure 1B**), whereas three patients including HCC02, HCC03 and HCC09 had moderate numbers of unique somatic alterations. Moreover, we also estimated tumor mutation burden (TMB) for each tumor sample, and the average value of TMB was 5.4 (IQR: 3.8 – 7.7, SD: 2.9) Mut/Mb. Among this group of patients, the change of TMB is consistent with the mutation changes. For instance, the difference of mutation type between P02 and P09 is obvious, and the corresponding TMB change is also relatively significant (**Figure 1B**). These data implied that the genomic heterogeneity of multifocal HCC affected the assessment of TMB, which is a challenge for existing TMB-guided immune checkpoint blockade.

Hierarchical clustering of all HCC samples based on the genomic alterations revealed that almost all multifocal HCC patients in the present study could be categorized into intrahepatic metastasis spreading tumors, with the exception of T3 from HCC08 patient, which was considered as a multicenter originated tumor lesion (**Figure 1C**). These outcomes indicate that multifocal HCC shared large proportion of onco-driver mutations among different tumor lesions.

Phylogenetic Reconstructions Identified Truncal and Branchy Drivers

We dug into the substantial tumor heterogeneity and branched evolution in all 11 multifocal HCC patients to construct phylogenetic trees for these tumors. All HCC tumors showed a branched evolutionary pattern among the detected cancer-related genes (**Figure 2A**), which was consistent with previous studies proposed that genomic evolution of multifocal HCC was not a linear model (22). It should be emphasized that some focal tumors presented as an inconspicuous branching relationship under the narrow spectrum of cancer-related genes enrolled in CSYS, including HCC03, HCC05 and HCC08. In these patients, the inconspicuous branching relationship is mainly characterized by the short trunk of the evolutionary tree, indicating that there are fewer common mutations among the different lesions. Two patients (HCC04 and HCC06) showed a convergent tumor from other foci, because the T1 of these two patients was supposed to share the mutational features from both the other two superior tumors.

Next, putative driver mutations associated with HCC were mapped onto the phylogenetic trees to address whether specific driver genes were predominantly altered on trunks or branches.

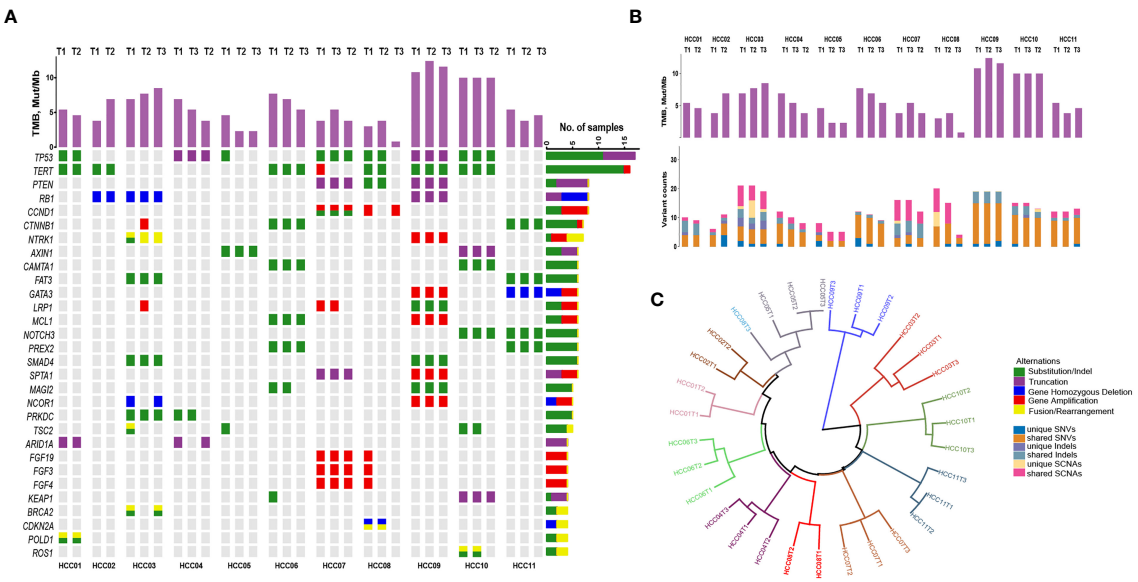

FIGURE 1 | The mutational landscape and spatial heterogeneity of genetic divergence in different tumoral focal from 11 patients with resected multifocal HCC. **(A)** Concordance among somatic alterations (SNVs, indels and SCNAs) in detected cancer-related genes through tNGS panel among different primary intrahepatic tumor lesions. Stacked bar plots illustrate the tumor mutation burden (TMB) value of each tumor. **(B)** The absolute counts of somatic mutations (SNVs, indels and SCNAs) that are shared or subfoci-unique in all multifocal HCC samples. **(C)** Hierarchical clustering of all HCC samples based on the mutational landscape. T3 of HCC08 is separated away from other foci in patient.

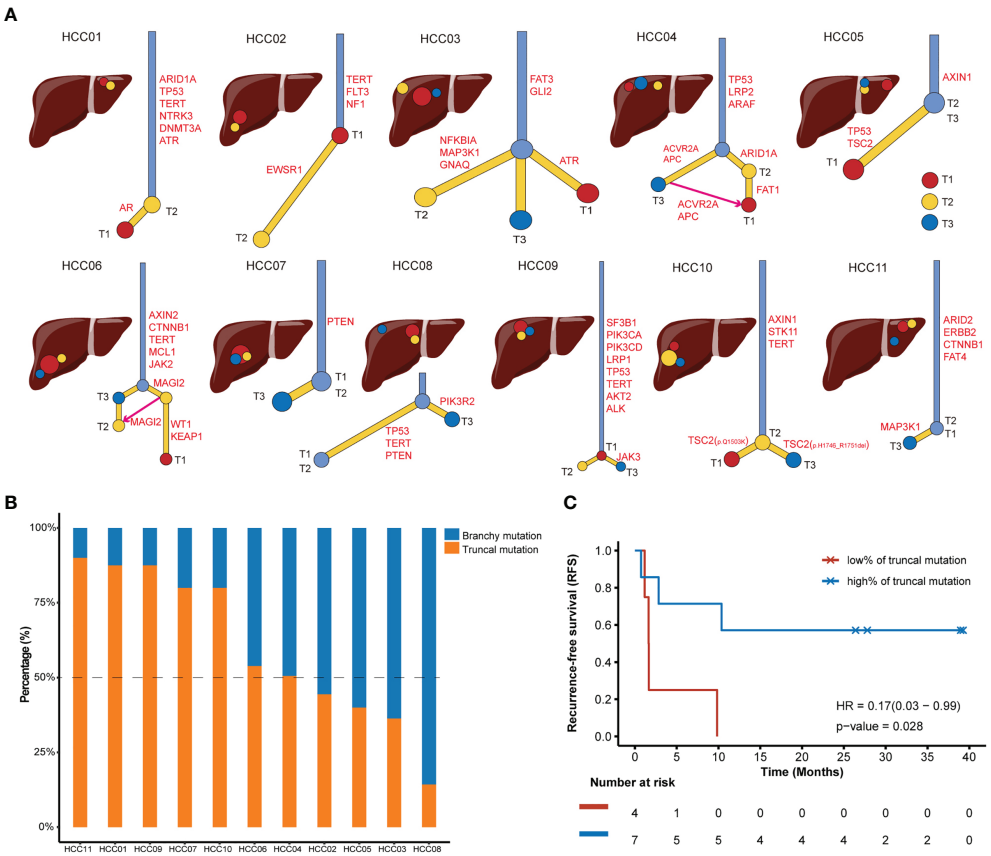

FIGURE 2 | Phylogenetic trees of multifocal HCC cases and recurrence outcomes after radical surgical resections. **(A)** Phylogenetic trees are constructed using somatic mutations. Intrahepatic tumoral focal are arrayed in the liver around the anatomical diagram. T1 is the red circle, T2 is the yellow circle and T3 is the blue circle. The putative HCC driver mutations are also annotated in the trunks or branches of phylogenetic trees. **(B)** The tumor mutation burden (TMB) value and percentage of truncal mutations and branchy mutations in each patient with multifocal HCC. Blue bar and orange bar represent the proportion of branchy mutation and truncal mutation, respectively. **(C)** Kaplan-Meier curve showing poor recurrence-free survival (RFS) in patients with a low ratio (<50%) of truncal mutation (Log-rank test).

The identified somatic mutations could be classified into HCC drivers if it belonged to the frequently mutated genes of HCC that were proposed by *TCGA-LIHC* program (21) or presented in TARGET database (v3). To further investigate the evolutional phenotype of each driver mutation in individuals, we defined truncal drivers as mutations shared by all foci, which presented in trunks of the phylogenetic trees; and branchy drivers as mutations not shared by all foci or held by only one lesion. Therefore, both truncal drivers and branchy drivers were highlighted in each phylogenetic tree (**Figure 2A**). Among these 11 multifocal HCC patients, the most frequent truncal drivers were *TP53* mutants (4/11, 36.4%) and *TERT* promoter mutations (4/11, 36.4%), suggesting these two alterations occurs early in carcinogenesis of HCC. Moreover, *TSC2* mutations (3/11, 27.3%) was the most frequently mutated branchy driver, while sporadically mutated branchy drivers, such as *CCND1*, *CDKN2A*, *LRP1*, *MAP3K1* and *PTEN*, were also observed in multifocal HCC, which were supposed to drive the complicated heterogeneity and multiple phenotypes in HCC. Intriguingly, T1 and T3 of HCC10 showed distinct branches for these two tumor regions possessed different mutational loci in *TSC2* (**Figure 2A**), even though they shared a common ancestor in mutations including *AXIN1*, *STK11* and *TERT*.

To further quantify and appreciate the heterogeneity among different foci, we further determined the percentage of truncal drivers in all identified HCC drivers for each patient (**Figure 2B**). We supposed that the lower the proportion of truncal drivers were, the greater the genomic heterogeneities existed among different tumor lesions. The proportion of truncal drivers varied from patient to patient. Intriguingly, we observed a significantly poorer recurrence-free survival (RFS) after radical resection in patients with a low rate (<50%) of truncal drivers (HR=0.17, p=0.028, **Figure 2C**), suggesting highly heterogeneous multifocal HCC patients were speculated to have an underprivileged prognosis after receiving surgical resections. This outcome was consistent with clinical observations that patients with lower intratumor heterogeneity had better survival prognosis than those with higher level of heterogeneity (23), demonstrating

that it's imperative to timely infer the heterogeneous levels for resected multifocal HCC patients, and personalized postoperative adjuvant therapy should be considered for those with high risk of tumor recurrence determined by multi-regions genomic sequencing.

cfDNA Tracked Mutations in Primary Multi-Focal HCC

Tumor truncal or branchy drivers may inform prognosis and recurrence risk after the surgical resection, which is worthwhile to be monitored for patients with resectable multifocal HCC. cfDNA has been proved as a non-invasive liquid biopsy for HCC's somatic alterations (24). Herein, we simultaneously performed cfDNA sequencing at prior-surgery status on all 11 patients with available HCC foci tissues. On the day before the surgery, cfDNA detected a total of 46 SNVs and 5 indels, through an ultra-deep sequencing under an average depth of 5397×.

We focused on tumor-derived driver mutations captured by cfDNA in each patient. 7 of 11 (64%) multifocal HCC patients had cfDNA captured tumor-derived driver mutations, while branchy drivers were detected in only one patient (HCC08). This could be perfectly explained by previous analyses of genomic of multifocal tumors, whose results indicated that it was MO-HCC without truncal drivers (**Figure 3A**). For 6 patients with cfDNA-detectable truncal drivers, ubiquitous variants were commonly observed than unique variants that only existed in one or some of the tumors (**Figure 3B**). Importantly, cfDNA-captured mutational loci were highly consistent with these alterations occurred in tumor tissues (**Figure 3C**), suggesting that cfDNA could possibly retrieve intertumoral genomic heterogeneities and might be utilized to capture truncal drivers in tumor specimens of multifocal HCC.

Finally, we explored whether cfDNA could be used for discovering or tracking potential driver variants by dynamic monitors during antitumor treatment of HCC patients. Herein, we dynamically tracked tumor mutations in 3 cfDNA samples at different time-points from one advanced HCC patient who achieved the objective response after receiving combinational treatment of lenvatinib plus pembrolizumab (**Figure 3D**). The patient's tumor sample is a large lesion that includes multiple regions with different densities under CT. At baseline of the initial cfDNA, this patient received one dose of transhepatic arterial chemotherapy and embolization (TACE) and then was treated with sorafenib for two months. His disease progressed

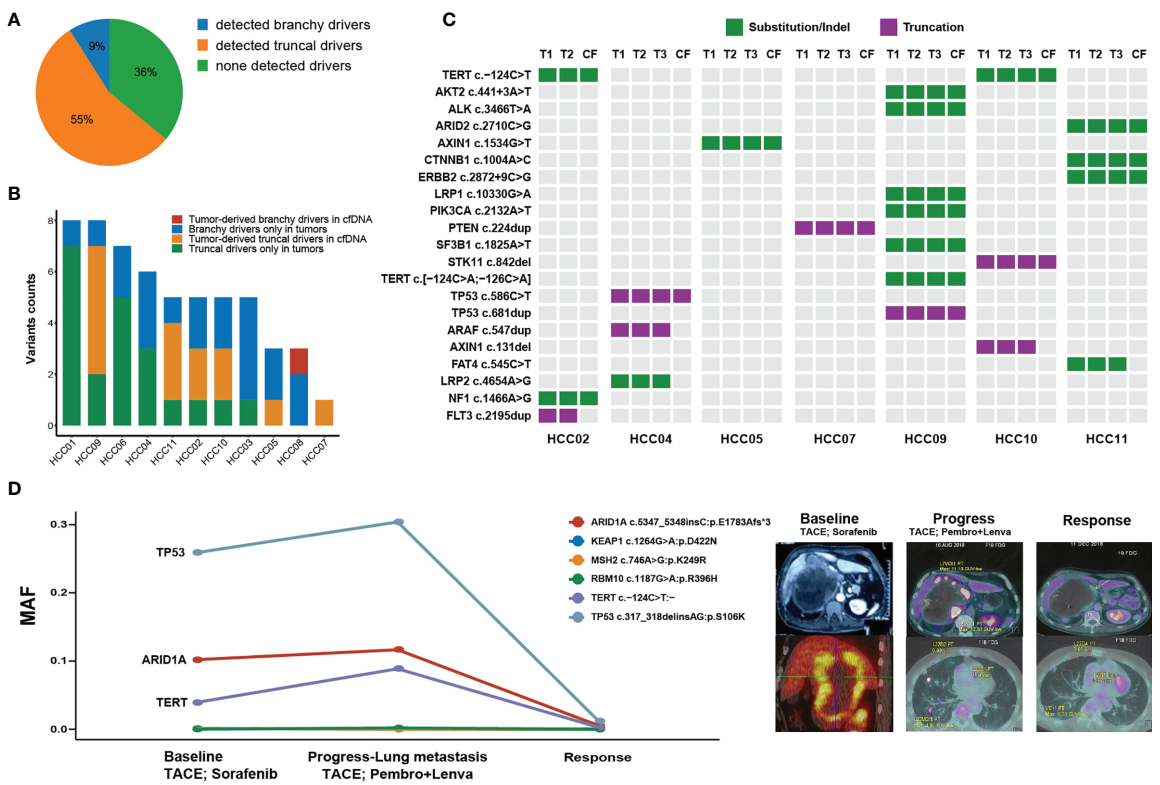

FIGURE 3 | Overview of altered putative HCC driver mutations uncovered through circulating-free DNA (cfDNA). **(A)** The proportion of cfDNA-capturing tumor-derived driver mutations in 11 multifocal HCC patients. **(B)** The absolute counts of somatic HCC driver mutations that are simultaneously or independently captured by tumor tissues and cfDNA. **(C)** The distribution of HCC driver mutations that were mutant in at least one foci of each patient which partly presented in circulating-free (CF) samples. **(D)** Longitudinal tracking of tumor progression and therapeutic response by cfDNA to pembrolizumab plus lenvatinib in one patient with metastatic HCC. The line chart shows the mutational allele frequencies (MAFs) of major driver mutations in cfDNA which occurred at the time of baseline, disease progression with emerging lung metastasis, while almost absented at the time of objective response.

with newly emerging metastasis in lungs. The second cfDNA analysis found mutation allele frequencies (MAFs) of three ubiquitous somatic variations (*TP53*, *ARID1A* and *TERT*) elevated in the circulating blood when compared with the initial cfDNA. Then, he received the second TACE treatment and was simultaneously treated by pembrolizumab (200 mg/3 weeks) combined with lenvatinib (8 mg/day) for the next two months. Along with the responsive status of obviously shrunk tumors, the third cfDNA analysis found extremely low MAFs of all previously detected ubiquitous somatic variations. Therefore, cfDNA preliminarily provided a promising tool to dynamically track genomic alterations or even truncal driving mutations during immunotherapy for HCC patients, and thereby, could inform disease progression or therapeutic responses.

DISCUSSION

This study investigated intertumoral genomic heterogeneities based on ultra-deep tNGS captured 466 cancer-related genes in patients with resected multifocal HCC. We deciphered an evolutionary trajectory in multifocal HCC through this methodology. We found varying levels of intertumoral genomic heterogeneity existed in different foci of multifocal HCC, even though only putative cancer-driving genes were considered. Of importance, these results indicate that diverse drivers in multifocal HCC are the underlying contributors that lead to the heterogeneity of clinical prognosis and therapeutic efficacy.

Our study proposed a clinical application model and analysis method to distinguish truncal or branchy drivers among different subfoci of multifocal HCC. Frequently mutated somatic driver mutations, including *TP53* and *TERT*, play a core role in carcinogenesis and tumor progression in HCC, which are still indestructible drug targets for HCC treatment. Besides, *TSC1* or *TSC2* alterations seems to be heterogeneity makers to hatch out subclones of primary HCC lesions. HCC patients carrying *TSC1/2* mutations were demonstrated to predispose a deregulated mTOR activity, and thereby inhibition of mTOR signaling has been widely investigated in clinical trials (25, 26). However, mTOR inhibitor like everolimus showed an unsatisfactory efficacy in advanced HCC patients (27), possibly due to its brachy role in HCC's progression. As such, the data strongly supported that comprehensive insights of genetic landscape for multifocal HCC could reveal the most crucial target to facilitate the design of combinational targeted therapies (28, 29).

To explore the varying survival prognosis of resected multifocal HCC, we demonstrated the proportion of truncal drivers could be used as an assessable assay for intertumoral genomic heterogeneity through tNGS, and low ratio of truncal drivers (<50%) informed a significantly elevated recurrence risk after surgical resections for multifocal HCC patients. Accumulating evidences have revealed that intertumoral heterogeneity in multifocal HCC would foster tumor evolution, metastasis and chemotherapeutic resistance, suggesting that molecular heterogeneity is a key challenge in HCC treatment (30, 31). Considering the increased cost-effectiveness of tNGS, due to it is a lower cost, shorter cycle time and higher operability than either WES or WGS, this strategy provides a solution for the clinical application and personalized postoperative managements for patients with resected multifocal HCC.

In addition, preoperative cfDNA-based detection could sensitively capture most of the tumor-derived driver mutations, suggesting ultra-depth cfDNA could serve as a non-invasive and real-time auxiliary method to decipher the intertumoral heterogeneity and identify oncodrivers of multifocal HCC. It should be noted that in view of the limited capture of ctDNA, not all cancer species can effectively identify tumor heterogeneity. Whether circulating tumor DNA (ctDNA) profile could represent these ITH in different tumor type still needs to be confirmed by further research (32). Besides, we used cfDNA to dynamically monitor the therapeutic effect in a case with unresectable and metastatic HCC, implying that it is promising to track patients' therapeutic responses through cfDNA detection. Although cfDNA demonstrated lower mutation detection efficiency, less genetically informative and robust repeatability than tumor tissues biopsy, cfDNA could provide a more comprehensive mutational footprints by revealing intertumoral heterogeneity in multifocal HCC (24). Impressively, cfDNA levels fluctuated consistently with pathophysiological conditions (33), bringing an emerging path to meet the demand of alpha-fetoprotein negative HCC patients.

CONCLUSION

In conclusion, multifocal HCC shows a significant intertumoral genomic heterogeneity among tumor-associated genes and driver mutations. Through performing ultra-deep tNGS on global foci, both truncal and branchy drivers can be economically and effectively identified, which potentially provide a basis for decision-making for personalized therapy at postoperative or recurrent stage.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Peking union medical college hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JZL, SHZ and DXW collected the data and wrote the manuscript. YS, YC, XY, JZM, FCX, JYL, XBY helped to collect literature and participated in discussions. XBY, LZ, JB, XL, XTS, KW, JP and HTZ designed and verified the study. KW, JP and HTZ finally

examined the study. All authors contributed to the article and approved the submitted version.

FUNDING

HZ is supported by the International Science and Technology Cooperation Projects (2016YFE0107100 and 2015DFA30650), the CAMS Innovation Fund for Medical Science (CIFMS) (2017-I2M-4-003), the Beijing Natural Science Foundation (L172055), the National Ten-thousand Talent Program, the Beijing Science and Technology Cooperation Special Award Subsidy Project and the CAMS Initiative for Innovative Medicine (CAMS-2018-I2M-3-001).

ACKNOWLEDGMENTS

We thank the patients who volunteered to participate in this study and the staff members at the study site who cared for these patients.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.673248/full#supplementary-material

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Conflict of Interest: SZ, YC and KW were employed by OrigiMed, Shanghai, China.

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

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REVIEW
published: 23 June 2021
doi: 10.3389/fimmu.2021.692360

Shaping Immune Responses in the Tumor Microenvironment of Ovarian Cancer

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

Received: 08 April 2021
Accepted: 02 June 2021
Published: 23 June 2021

Citation:
Luo X, Xu J, Yu J and Yi P (2021) Shaping Immune Responses in the Tumor Microenvironment of Ovarian Cancer. Front. Immunol. 12:692360. doi: 10.3389/fimmu.2021.692360

Reciprocal signaling between immune cells and ovarian cancer cells in the tumor microenvironment can alter immune responses and regulate disease progression. These signaling events are regulated by multiple factors, including genetic and epigenetic alterations in both the ovarian cancer cells and immune cells, as well as cytokine pathways. Multiple immune cell types are recruited to the ovarian cancer tumor microenvironment, and new insights about the complexity of their interactions have emerged in recent years. The growing understanding of immune cell function in the ovarian cancer tumor microenvironment has important implications for biomarker discovery and therapeutic development. This review aims to describe the factors that shape the phenotypes of immune cells in the tumor microenvironment of ovarian cancer and how these changes impact disease progression and therapy.

Keywords: ovarian cancer, tumor microenvironment, immune, genetic, epigenetic, cytokine

INTRODUCTION

Ovarian cancer (OvCa) is the fifth most common cause of cancer death in women and has high mortality, with a 5-year overall survival rate of < 50% (1). Due to a lack of typical symptoms and effective early diagnostic measures, most patients are diagnosed at advanced stages (III and IV), when treatment options are limited (2, 3). Despite complete remission after debulking surgery combined with first-line chemotherapy, recurrence occurs in 70–80% of patients within 2–5 years, and chemotherapeutic resistance will eventually develop in all recurrent OvCa patients, leading to death (4, 5). The mechanism underlying recurrence and metastasis in OvCa is not clear, and may be related to changes in the immune system (6). The immune system consists of various cells and mediators, which protect against foreign pathogens and eliminate damaged cells to maintain tissue homeostasis (7). During tumor progression, immune cells often exhibit phenotypic and functional instability and transdifferentiate into different cell types or states, which can promote or inhibit tumor growth and metastasis (8, 9). Moreover, the infiltration of various immune cells into the tumor microenvironment (TME) is associated with clinical outcomes of OvCa (10). Therefore, understanding the cancer-associated changes in immune cells of the TME may clarify the mechanisms of OvCa pathogenesis and reveal novel biomarkers and therapeutic targets for OvCa (11).

The immune cell types in the OvCa TME and their functions have been extensively studied (12). However, the changes that occur in immune cells of the OvCa TME during cancer progression and how these insights might guide therapy are less clear. Here, we review how immune responses in the OvCa TME are shaped by the interactions between tumor cells and immune cells, which provides potential therapeutic targets and highlights the need for innovative therapeutic approaches.

INFILTRATING IMMUNE CELLS IN THE OVARIAN CANCER TUMOR MICROENVIRONMENT

The tumor microenvironment (TME) refers to the niche, either primary or metastatic, where tumor cells interact with the host stroma including immune cells, endothelial cells, fibroblasts and metabolites. The important contribution of the TME to ovary cancer could manifest by the co-evolution of cancer and stromal cells which formed pre-metastatic niches and facilitated the peritoneal metastasis, such as neutrophil influxed into the omentum and extruded neutrophil extracellular traps (NETs), rendering the premetastatic omental niche conducive for implantation, was a prerequisite step for peritoneal metastasis in orthotopic ovarian cancer models (13); endothelial cells had activated Notch1 receptors (N1ICD) expression, facilitated peritoneal metastasis and associated with shorter survival in ovarian cancer-bearing mice, since sustained N1ICD activity induced EC senescence, expression of chemokines and the adhesion molecule VCAM1, promotes neutrophil recruitment and tumor intravasation (14).

The tumor immune microenvironment (TIME) is the immune contexture acting as a crucial orchestrator for cancer development, progression and metastasis, mainly composed with the infiltrated immune cells, their chemokines and cytokines (12). The relationship of TIME function and the clinical correlation were analyzed in ovarian carcinoma based on The Cancer Genome Atlas (TCGA) cohort, and four TIME molecular subtypes of the global immune-related genes were obtained, the high immune scoring subtype with the upregulated tumour-infiltrating immune cells had a high BRCA1 mutation, high expression of immune checkpoints, and optimal survival prognosis (15, 16). Cândido et al. evaluated the immune response patterns through analysis of type 1 (Th1), type 1 (Th2), and type 17(Th17) cytokines in patients with epithelial ovarian cancer (EOC), and found higher levels of TNF-α/IL-4/IL-6/IL-10 in EOC patients compared to the control, IL-10 and TNF-α concentrations were higher in stage III/IV and associated with higher CA125, higher Th1 immune response was observed when the cytoreduction was considered optimal, while higher concentrations of Th2 cytokines were associated with unsatisfactory cytoreductive surgery and undifferentiated tumors (17).

The infiltrated immune cells can either limit or promote cancer development depending on the composition of immune cells and their phenotypic states. Notably, some infiltrated immune cells serve as tumor-associated immune cells, such as immature/tolerogenic dendritic cells (DCs), M2 macrophages, regulatory T (Treg) cells and, myeloid-derived suppressor cells (MDSCs). These cells maintain immune tolerance and suppress anti-tumor immunity, leading to OvCa therapeutic resistance (9). In contrast, mature DCs, M1 macrophages, natural killer (NK) cells, αβ T cells and γδ T cells can directly inhibit tumor growth or increase the susceptibility to checkpoint inhibitor therapies for OvCa (18, 19). Importantly, infiltration of $CD4^+$ and $CD8^+$ T cells into the tumor has been associated with improved overall and progression-free survival in OvCa patients (20). In **Figure 1**, we summarized the functions of immune cells in the OvCa TME.

The infiltrated immune cells functioned as a profound network regulating each other in the TIME. Several immunosuppressive cell types have been found migrating into OvCa tissues to promote immune escape by suppressing NK cells

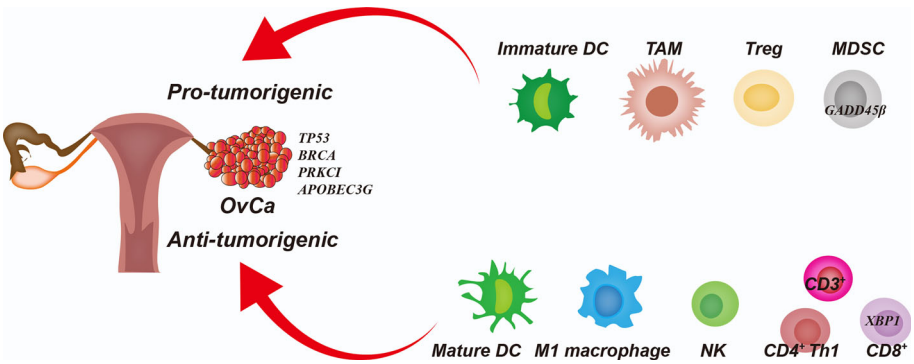

FIGURE 1 | Interplay Among Cancer Cells and Immune Cells in the Ovarian Cancer Tumor Microenvironment. The immature dendritic cells (DCs), tumor-associated macrophages (TAMs), regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) promote immunoresistance and therapeutic resistance in the ovarian cancer (OvCa) cells. Mature DCs, M1 macrophages, natural killer (NK) cells, and cytotoxic T lymphocytes (CTLs) inhibit tumor growth and increase the therapeutic susceptibility of OvCa cells.

and cytotoxic T cells (21). For instance, M2 macrophages act as tumor-associated macrophages (TAMs) to subvert adaptive immunity and inflammatory circuits to promote tumor growth and progression (22). TAMs are the most abundant immune cells in advanced stage OvCa and foster tumor growth, invasion, angiogenesis, metastasis, and drug resistance (23). TAMs secrete IL10, IL6, TGF-β, CCL18, and CCL22, which attract regulatory T cells and promote differentiation of T cells towards the Th2 phenotype. IL10 and TGF-β also inhibit the cytotoxic activity of NK cells and cytotoxic T lymphocytes. Furthermore, CCL18 promotes T-cell anergy and unresponsiveness (24). In addition, Th17 cells and Tregs, which are subsets of $CD4^+$ cells, maintain immunological self-tolerance and dampen anti-tumor activity in the TME, which is pro-tumorigenic in OvCa (22). A higher prevalence of Treg cells has been detected in tumors and malignant ascites of OvCa patients. The Treg cells directly inhibit other subsets of T cells by secreting the inhibitory cytokines IL-35, IL-10, and TGF-β or through binding checkpoint inhibitor receptors such as programmed cell death protein 1 (PD-1, also called PDCD1), cytotoxic T lymphocyte antigen 4 (CTLA4), and lymphocyte activation gene 3 (LAG3) (5). MDSCs are myeloid cells that suppress T cell responses and include myeloid progenitors and immature myeloid cells (25). MDSCs have been shown to accumulate in the circulation of cancer patients, and MDSC numbers generally correlate with an inferior prognosis (26). Advanced OvCa is associated with a myeloid bias that increases the frequencies of circulating granulocyte-monocyte progenitors (27). Tumor-derived factors, such as G-CSF (also known as CSF3), GM-CSF (also known as CSF2), and IL-6 drive this myeloid bias and increase the circulating and tumor-infiltrating MDSC population, which accelerates tumor progression by suppressing T cell responses and releasing metabolic factors (28). Furthermore, DCs are crucial for promoting and maintaining the anti-tumor immune response, which can coordinate the adaptive and acquired immune response to activate T cells (29).

GENETIC ALTERATIONS IN OvCa CELLS AND IMMUNE CELLS IN THE TME

OvCa, especially high-grade serous OvCa (HGSOC), has been found to predominantly harbor mutations in *TP53*, loss of heterozygosity for *TP53*, mutations in *BRCA1/BRCA2*, loss of *PTEN*, and copy number abnormalities for other genes involved in homologous recombination (HR) DNA repair, resulting in high genomic instability (30, 31). OvCa cells with genomic instability also show has altered infiltration of immune cells in the TME (32). Non-homologous end-joining (NHEJ) occurs more frequently but may cause less severe mutations than HR and therefore is less studded in OvCa (30, 33).

TP53 mutation is the most common event associated with poor clinical prognosis in HGSOC (34). The *TP53* status of the cancer cell has a profound impact on the immune response (35). TP53 controls the expression of multiple immunosuppression-associated proteins such as PD-L1 (also known as CD274), VISTA (also known as VSIR), NKG2D (also known as KLRK1), and FOXP3; loss or mutation of *TP53* in cancers changes cytokine secretion, resulted in reshaping the immune microenvironment to promote immune escape of cancer (36, 37). In OV-90 OvCa cell line, *TP53* loss promotes the recruitment of MDSCs and homing of the monocytes to the ascites through tumorigenic production of CCL2 (38). *TP53* deficiency in OvCa cells also increases the peripheral and intratumoral Treg populations, which are involved in suppressing effector T cells (39). Moreover, the interaction between TAMs and mutant *TP53* HGSOC promotes angiogenesis and epithelial-mesenchymal transition by increasing release of GATA3 exosome from TAMs, which is involved in the regulation of M2 macrophage polarization in the HGSOC TME (40). Taken together, these findings lead us to conclude that *TP53* mutation in OvCa cells acts as a critical player for the immunosuppressive effects of MDSCs, Tregs, and TAMs.

BRCA1/BRCA2-mutant tumors are often deficient in repairing double-stranded DNA breaks using HR, and these tumors exhibit increased therapeutic sensitivity to platinum-containing therapy and inhibitors of poly-(ADP-ribose)-polymerase (PARP) (41, 42). Somatic or germline *BRCA* mutations are present in approximately 25% of HGSOCs, which can give rise to a 10-fold increased risk of developing HGSOC (43). One study showed that HGSOCs with *BRCA1* disruption had more infiltration of $CD8^+$ T cells in the TME than HR-proficient HGSOCs (44). This finding could be explained by the ability of BRCA1 to regulate cellular responses to inflammation, oxidative stress, and hypoxia, such as the direct role of BRCA1 in TNF-α and IL-1β signaling through NFκB, and interferon signaling through STAT1 (45). Moreover, survival analysis showed that *BRCA1/BRCA2*-mutant HGSOCs with high numbers of lymphocytes in the TME have a favorable prognosis (46). These findings document the relationship among *BRCA1/BRCA2*-mutation status, immunogenicity, and patient survival, suggesting that *BRCA1/BRCA2*-mutant HGSOCs may be more sensitive to immunotherapy than HR-proficient HGSOCs.

PRKCI, a gene encoding a serine-threonine kinase belonging to the atypical protein kinase C (aPKC) family, is located in the 3q26 locus, which is amplified in about 70% of HGSOC cases (44). Sharmistha et al. showed that *PRKCI* is amplified and overexpressed in OvCa and acts as an OvCa-specific oncogene. Furthermore, *PRKCI* overexpression in OvCa cells promoted nuclear localization of YAP1, leading to up-regulation of TNF expression, which then contributed to an immunosuppressive TME with an abundance of MDSCs and poor infiltration of cytotoxic T cells and NK cells (44). Thus, the PRKCI-YAP1 regulation of tumor immunity could provide an important window of diagnostic and therapeutic implications for OvCa (47).

In addition to somatic or germline mutations in OvCa cells, genomic amplifications are also found in the immune cells of the TME, which can regulate their phenotypes (48). APOBEC3G, one of the APOBEC family of antiviral DNA cytosine deaminases, is expressed broadly in human tissues (49).

Leonard et al. showed that the expression levels of APOBEC3G are surprisingly high in cytotoxic (CD8A) and helper T ($CD4^+$) lymphocytes in HGSOC and correlate positively with improved HGSOC patient outcomes (50). Engineering T cells with boosted APOBEC3G could be interesting to as a cellular immunotherapy against HGSOC. Unlike APOBEC3G, which confers immunosensitivity, elevated *GADD45B* expression confers poor clinical outcomes in most human cancers. *GADD45B* is an important myeloid-intrinsic factor for proinflammatory macrophage activation and the immunosuppressive activity of the TME, which restricts $CD8^+$ T-cell trafficking into tumors (51). To explore the function of *GADD45B* in OvCa, Daniela et al. performed flow cytometry analysis of an OvCa allograft mouse model and found that conditional knockout of *GADD45B* in myeloid cells restores proinflammatory TAM activation and intratumoral $CD8^+$ T-lymphocyte infiltration, resulting in reduced tumor growth (51). Moreover, a study revealed that upregulation of *XBP1* in $CD4^+$ and $CD8^+$ T cells isolated from OvCa specimens was associated with decreased infiltration of T cells into tumors and with reduced *IFNG* mRNA expression. *XBP1*-deficient T cells in the metastatic OvCa milieu exhibited global transcriptional reprogramming and improved effector capacity (52). Accordingly, mice that bear OvCa and lack *XBP1* selectively in T cells demonstrate superior anti-tumor immunity, delayed malignant progression, and increased overall survival; interestingly, the role of *XBP1* in NK cells may be opposite (53). Targeting *XBP1* may help to restore the metabolic fitness and anti-tumor capacity of T cells in cancer hosts (52). Therefore, all three genes as new candidate biomarkers for effective T-cell responses and provide potential enhancers of cellular immunotherapy for OvCa.

These data show that genetic alterations, which cause phenotypic changes both within the OvCa cells and in the immune cells of the TME, can impact immune cell infiltration and cancer prognosis. These genetic alterations are summarized in **Table 1**.

EPIGENETIC EFFECTS OF NONCODING RNAS IN THE OvCa TME

There is increasing evidence that epigenetic regulation by noncoding RNAs (ncRNAs) plays an important role in OvCa by reprogramming the phenotypes of immune cells in the TME (55). ncRNAs have especially been linked to immunosuppressive activities such as TAM polarization, MDSC recruitment, Treg development, and functional defects in NK cells and cytotoxic T cells in the OvCa TME (24).

The term ncRNAs includes a range of epigenetic regulatory RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (56). ncRNAs mediate many fundamental cellular processes, such as development, differentiation, proliferation, transcription, post-transcriptional modifications, apoptosis, and cell metabolism (57). Recently, it was discovered that the expression of most ncRNAs is perturbed in cancer, and these up- or down-regulated ncRNAs are significantly correlated with numbers and types of immune cell infiltration in TME (58). Xu and colleagues identified miR-424(322) as a negative regulator of several mRNAs encoding immune regulatory proteins, including the T cell inhibitory ligands PD-L1and CD80, in chemoresistant OvCa cells (59). High levels of miR-424(322) in tumors are correlated with improved progression-free survival and, in a syngeneic OvCa mouse model, overexpression of miR-424(322) in the OvCa cells increased the number of cytotoxic $CD8^+$ T cells and decreased the number of MDSCs and Tregs in the TME, reduced tumor growth, and enhanced the efficacy of chemotherapy (59). Moreover, Xie et al. found that miR-20a is overexpressed in human OvCa tissues and enhances long-term cellular proliferation and invasion capabilities by suppressing NK cell cytotoxicity through directly binding 3'-untranslated region (3'UTR) of MICA/B mRNA and downregulating its expression on the membrane of OvCa cells. MICA/B are ligands of the natural killer group 2 member D (NKG2D) receptor found on NK cells, $\gamma\delta^+$ T cells and $CD8^+$ T cells (60). The reduction of membrane-bound MICA/B proteins allows OvCa cells to evade immune-mediated killing (60). Furthermore, a study by An and Yang investigated the role of miRNAs in immune cells and indicated that miR-21 in macrophages could modulate M0 polarization into M2 by increasing the expression of M2 macrophage markers CD206 and IL-10, and decreasing the expression of M1 macrophage markers iNOS and TNF-α. Then, co-cultured M2 macrophages with miR-21 overexpression and OvCa cells found that M2 macrophages promote the chemoresistance of OvCa by activating PI3K/AKT signaling of tumor cells (61). Another miRNA with an inhibitory effect on polarization of M2 macrophages is miR-217. Transfection of OvCa cells with miR-217 suppresses expression of the secreted factor IL6, which attenuates M2 macrophage polarization through JAK/STAT3 signaling (62). In addition, it has been reported that lncRNAs are correlated with reprogramming of immune cells in OvCa. In a study by Shang et al., the authors found that the lncRNA HOTTIP

TABLE 1 | Genes regulate immune system in OvCa.

Cell type	Gene alterations	Pathogenetic role	Ref
OvCa	TP53 deficiency	Increases MDSCs, Tregs and TAM populations	(39, 40, 54)
	BRCA mutation	Increases infiltration of $CD4^+$ and $CD8^+$ T cells	(45, 46)
	PRKCI amplification	Enhances MDSCs and reduces $CD8^+$ T cells and NK cells infiltration	(44)
	APOBEC3G high level	Increases T cell infiltration	(50)
MDSCs	GADD45β deletion	Restores proinflammatory TAM activation and $CD8^+$ T cells infiltration	(51)
T cells	XBP1 deficiency	Restores the metabolic fitness and antitumour capacity of T cells	(52)

OvCa, ovarian cancer; MDSCs, myeloid-derived suppressor cells; DCs, dendritic cells; TAM, tumor-associated macrophage; Treg, regulatory T; NK, natural killer.

was highly expressed in OvCa tissues, and overexpressing HOTTIP in OvCa cells promoted the expression of IL6 by binding to JUN. IL6 secretion then conferred PD-L1 expression on neutrophils, reduced $CD3^+$ T cell proliferation, and reduced response to tumor immunotherapy (63). In another study, Colvin et al. revealed that high MIR155HG expression in cancer-associated fibroblasts (CAFs) in OvCa patients was associated with higher infiltrates of immune cell subsets, including $CD8^+$ T cells, $CD4^+$ memory activated T cells, follicular helper T cells, $\gamma\delta^+$ T cells, M1 macrophages, and eosinophils, and with longer survival (64). A functional RNA co-expression enrichment analysis revealed that the Gene Ontology terms for RNAs co-expressed with MIR155HG could be grouped into categories associated with T cell activation, antigen processing and presentation, leukocyte migration, and activation of an immune response. A similar analysis revealed that the RNAs co-expressed with MIR155HG included Kyoto encyclopedia of genes and genomes (KEGG) pathways related to immune diseases and the immune system, suggesting a role for MIR155HG in regulating the immune microenvironment (64). However, the specific mechanisms and cells involved remain unknown.

One important aspect to consider in the regulatory role of miRNAs in the TME is that miRNAs can be transported beyond their cells of origin. Indeed, miRNAs can be transported inside extracellular vesicles (EVs) and delivered to recipient cells, regulating their biological functions (65). This miRNA-mediated cell-to-cell communication represents active crosstalk involving multiple cellular components of the TME, which include cancer cells, mesenchymal stromal cells, CAFs, endothelial cells, and immune cells. Interactions between OvCa cells and TAMs in promoting cancer progression have been reported to be mediated by miRNAs packaged in exosomes (66). One study reported that the exosomal miR-1246 derived from OvCa cells is abundantly expressed in OvCa exosomes and is taken up by M2 macrophages, which confers chemoresistance in OvCa cells through targeting Cav-1 mRNA of M2 macrophages and regulating p-gp interaction (67). Moreover, epithelial ovarian cancers (EOC) released exosomal miR-222-3p downregulates SOCS3 expression and activates STAT3 signaling pathways in macrophages, which induces polarization of the M2 phenotype and enhances the growth and metastasis of EOC cells (68). Similarly, the high expression of miR-940 in exosomes derived from EOC stimulated M2 phenotype polarization and promoted EOC proliferation and migration at the hypoxia environment (69). In addition, under the hypoxic condition, EOC cell-derived exosomes deliver miR-21-3p, miR-125b-5p and miR-181d-5p to macrophages and induce the polarization of M2 macrophages by regulating the SOCS4/5/STAT3 pathway at M0 macrophages, which promoted EOC cell proliferation and migration (70). Zhou et al. identified miR-29a-3p and miR-21-5p enriched in the exosomes derived from TAMs and led to imbalance of Treg/Th17 ratio to facilitate EOC progression and metastasis. Meanwhile, co-culture experiments involving TAMs and T cells or over-expressed the miR-29a-3p and miR-21-5p in $CD4^+$ T cells also significantly increased the Treg/Th17 ratio in EOC. The mechanism suggests the supernatant release of two miRNA exosomes from TAMs in OvCa could target STAT3 of $CD4^+$ T cells (22). Also, Czystowska et al. reported that small exosomes found in the ascites and plasma of OvCa patients contains ARG1 (arginase-1). ARG1-containing exosomes suppress proliferation of $CD4^+$ and $CD8^+$ T-cells *in vitro* and *in vivo* in OvCa mouse models by distributing ARG1 from tumor cells to antigen-presenting cells in secondary lymphoid organs. High expression of ARG1-containing exosomes contributes to tumor growth and tumor escape from the host immune system, and increased ARG1 activity in plasma is associated with worse prognosis in OvCa patients (71). Tumor-derived exosomes have also been reported to enhance immune suppression by promoting the differentiation of inhibitory immune cells, including TAMs and Treg cells.

The regulatory mechanisms linking OvCa and immune cell function *via* ncRNAs are detailed in **Figure 2** and **Table 2**. These findings underline the importance of continued research to identify ncRNA-modulated immune changes in the OvCa TME, as they may reveal novel insights, diagnostic strategies, and potential therapeutic targets for OvCa.

REGULATION OF IMMUNE CELLS IN THE OvCa TME *VIA* CYTOKINES

Although genetic and epigenetic factors regulate the immune cell phenotypes in the OvCa TME, the final effect on cell function depends on the expression of secreted factors (72). OvCa cells continuously secrete cytokines that regulate tumorigenicity in both autocrine and paracrine fashions. Cytokines mediate cell-to-cell interactions and regulate cell growth, differentiation, maturation, and immune response, participating in inflammatory reactions, wound healing, and tumor progression (73). Increasing evidence shows that immune cells reprogram their environments by interacting with cytokines, such as interleukins, chemokines, and growth factors (74).

Chronic inflammation is implicated in tumorigenesis and tumor progression. Cytokines mediate chronic inflammation and are involved in cancer progression by regulating the immune system (75). Increased levels of IL6 have been observed in many cancers, especially OvCa. In the OvCa TME, cancer cells secrete IL6, which inhibits the maturation of DCs and induces immunosuppressive alternatively activated TAMs, which compromise the activation of tumor-infiltrating T cells (76). On the other hand, IL6-producing MDSCs suppresses Th1 differentiation of $CD4^+$ T cells, which decreases their ability to help $CD8^+$ T cells and DCs, resulting in impaired adaptive immune responses against the development of OvCa (77). Moreover, a study by Isobe et al. found that M2-polarized TAMs were the primary IL6-secreting cells in peritoneal fluid from metastatic OvCa (77). IL6 induces JAK/STAT3 signaling by binding to the IL6 receptor (IL6R) to enhance OvCa cell growth and chemotherapy resistance (77). Also, multiple interleukins, including IL4, IL6, IL10, and IL13, are released from OvCa cells and other cells of the TME and strongly polarize TAMs into M2-like phenotypes in OvCa (24). In contrast, studies have found that

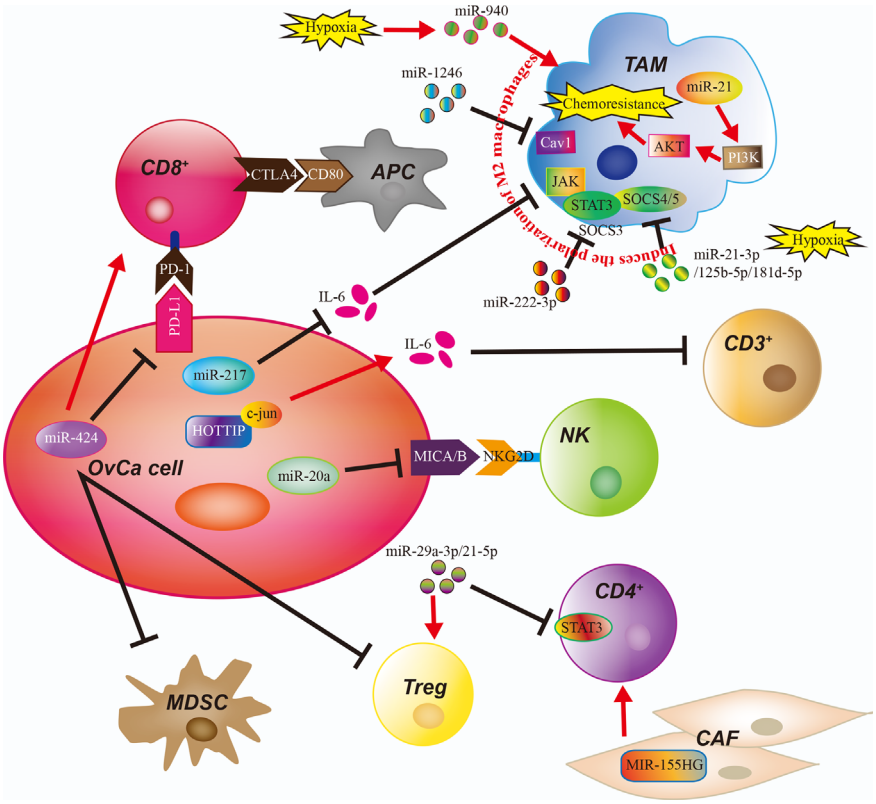

FIGURE 2 | The Role of Noncoding RNAs in the Ovarian Cancer Tumor Microenvironment. The immune cells of the ovarian cancer (OvCa) tumor microenvironment are regulated by noncoding RNAs and exosomes containing micro-RNAs (miRs). APC, antigen-presenting cell; CAF, cancer-associated fibroblast; $CD3^+$, CD3-expressing T cell, $CD4^+$, CD4-expressing T cell; $CD8^+$, CD8-expressing T cell; MDSC, myeloid-derived suppressor cell; NK, natural killer cell; TAM, tumor-associated macrophage.

TABLE 2 | miRNAs and lncRNAs regulate immune cells in OvCa TME.

Names	Function	Mechanism	Ref
miR-424(322)	Promotes proliferation $CD8^+$ T cells and inhibition of MDSC and Treg cells	Regulates PD-L1/PD-1 and CD80/CTLA-4	(59)
miR-20a	Suppresses NK cell cytotoxicity	Binds MICA/B 3'-UTR	(60)
miR-21	Repolarizes M2 macrophages into M1	Activates PI3K/AKT signaling	(61)
miR-217	Suppresses M2 macrophage polarization	Inhibits IL-6/IL-6R/JAK2/STAT3 signaling	(62)
HOTTIP	Inhibits $CD3^+$ T cell proliferation	Binds c-jun to promote the expression of IL-6	(63)
CAFs MIR155HG	Promotes higher infiltrates of immune cell subsets	No mention	(64)
miR-1246 exosome	Induces the polarization of M2 macrophages	Inhibits expression of Cav1	(67)
miR-222-3p exosome	Induces the polarization of M2 macrophages	Regulates SOCS3/STAT3 pathway	(68)
miR-940 exosome	Induces the polarization of M2 macrophages	Hypoxia induces the high expression of miR–940	(69)
miR-21-3p/125b-5p/-181d-5p exosome	Induces the polarization of M2 macrophages	Regulate SOCS4/5/STAT3 pathway	(70)
miR-29a-3p/21-5p exosome	Unbalance of Treg/Th17 cells	Suppresses expression of STAT3	(22)

OvCa, ovarian cancer; TME, tumor microenvironment; CAFs, cancer-associated fibroblasts; MDSCs, myeloid-derived suppressor cells; Treg, regulatory T; NK, natural killer.

NK cells preactivated briefly with IL2, IL15, and IL18 induce proliferation of NK cells to enhance IFNG production and NK-cell-mediated killing of OvCa *in vitro and in vivo* (78, 79). Significantly, IL12 secreted by genetically modified chimeric antigen receptor (CAR) T cells have also been shown to modulate the OvCa TME through multiple mechanisms, including reactivation of anergic tumor-infiltrating lymphocytes, inhibition of Treg-mediated suppression of effector T cells, and induction of Th1 $CD4^+$ T cells to the tumor site (80). Furthermore, Ullah et al. demonstrated that IL1B-producing tumor cells mediate immune suppressive effects such as increased Tregs and diminution of NK and memory T cells by upregulating HLA-G expression through the NFKB pathway in OvCa (81). Overall, interleukins are responsible for the dysfunction of innate and adaptive immunity against OvCa, and an interleukin-targeting approach has achieved good results in animal experiments, indicating that interleukins might be therapeutically effective when combined with current immunotherapies (82).

Chemokines are the largest subfamily of cytokines and can be divided into CC chemokines, CXC chemokines, C chemokines, and CX3C chemokines, based on the location of the first two cysteine (C) residues. They play a critical role in tumor growth and metastasis as key mediators of the inflammatory response (83). A complex chemokine-signaling network has been proposed to influence the development and progression of OvCa by regulating the trafficking of infiltrating immune cells (83). Macrophage-derived chemokine CCL22 in the TME and malignant ascites facilitate Treg infiltration to the OvCa, which inhibits anti-tumor immunity (48). Katrina et al. showed that high expression of STAT1 and STAT1 target genes (CXCL9, CXCL10, and CXCL11) are strongly correlated with improved chemotherapy response in OvCa (84). The Th1 immune response recruiting NK cells and effector $CD8^+$ T cells was enhanced by CXCL9, CXCL10, and CXCL11 derived from tumor cells, which can limit the diffusion and migration of OvCa cells (84). The chemokine landscape of OvCa is heterogeneous with high expression of lymphocyte recruiting chemokines (CCL2, CCL4, and CCL5) in tumors with intraepithelial T cells, whereas CXCL10, CXCL12, and CXCL16 are expressed quasi-universally, including tumors lacking intraepithelia T cells (85). Zsiros et al. found that dendritic cell (DC)-vaccine primed T cells expressed the cognate receptors for the above chemokines that were strongly correlation with the presence of tumor-infiltrating $CD8^+$ T cells in OvCa. Importantly, *Ex vivo* CD3/CD28 costimulation and expansion of vaccine-primed T cells upregulated CXCR3 and CXCR4, and enhanced their migration toward universally expressed chemokines in OvCa (85). Thus, vaccine primed and CD3/CD28 costimulated T cells can prepare for adoptive therapy to expand the available pool of tumor-reactive T cells in OvCa TME. Moreover, the intraepithelial tumor-infiltrating lymphocytes recruited by tumor chemokine CCL5 release IFN-γ to activate TAMs and DCs to secrete CXCL9, which in turn establishes a positive loop effectively amplifying T cell recruitment in EOC. CCL5 and CXCL9 co-expression reveals immunoreactive tumors with longer survival and response to checkpoint blockade, including OvCa (86). However, another study found that CCL5 expression in OvCa cancer stem cells recruited Tregs to promote immunoresistance and tumor metastasis *via* intercellular CCL5-CCR5 interactions, and co-culture with ovarian cancer cell lines induced the expression of MMP9 in Tregs, which promoted the invasion and metastasis of OvCa cells (87). Moreover, Taki et al. found that SNAIL (also known as SNAI1) expression in OvCa cells induces OvCa progression *via* upregulation of CXCR2 ligands (CXCL1 and CXCL2) and recruitment of MDSCs. *Snail* knockdown in mouse OvCa cells reduces the expression of the CXCL1/CXCL2 chemokines, which attract MDSCs to the tumor *via* CXCR2. Blocking CXCR2 inhibits MDSC infiltration and delays progression of Snail-high mouse tumors (88). Interestingly, Idorn et al. found that lentiviral transduction of tumor ascites lymphocytes (TALs) with chemokine receptor CXCR2 significantly increased migration of TALs towards rhIL8 and autologous ascites, which provides the proof of concept that engineering TALs with a chemokine receptor is feasible and can improve homing of transduced TALs towards the OvCa microenvironment (89). In brief, many chemokines are associated with OvCa by mediating immune responses that may favor or inhibit tumor progression.

STATs belong to a family of cytoplasmic transcription factors that communicate signals from the cell membrane to the nucleus (90). Upon the binding of cytokines or growth factors to cognate receptors on the cell surface, STATs are tyrosine phosphorylated, particularly by the JAK, ABL or SRC kinase families (91). The STAT family includes seven structurally and functionally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. They have essential roles in fundamental processes, including sustaining proliferation, evading apoptosis, inducing angiogenesis, promoting invasion, and suppressing antitumor immunity (92). Each STAT protein appears to have distinct physiologic functions in the immune response of OvCa. STAT3 and STAT5 are known to bind to the promoter and increase the transcription of FOXP3 in $CD4^+$ T cells; this expression is essential for the conversion of naive $CD4^+$ T cells into Tregs in the OvCa TME (93). Thus, activation of STAT3 in $CD4^+$ T cells generates an inflammatory environment around the OvCa, which promotes tumor growth by stimulating angiogenesis and suppressing anti-tumor response (90). In addition, ascites from OvCa patients polarized macrophages toward the M2 phenotype through STAT3 activation in OvCa cells (90). A study reported that when tumor supernatants from the epithelial OvCa cell lines OVCAR3, CAOV3, and SKOV3 were co-cultured with $CD8^+$ T cells, STAT5 phosphorylation was reduced, which diminished $CD8^+$ T cell proliferation (94). Moreover, STAT1 activation recruit $CD8^+$ T cells at the site of induction by inducing the production of the chemokines CXCL9, CXCL10 and CXCL11 that bind to the common chemokine receptor CXCR3 in OvCa. High level of STAT1 in OvCa cells was significantly correlated with levels of CD8A transcripts from intratumoral $CD8^+$ T cells and increased prognostic in patients with HGSOC (84). However, recent research found that OvCa patients with high intratumoral STAT1 activation exhibited poor prognosis compared with patients with low STAT1 activation *via* immunohistochemical analysis, indicating STAT1 may have a dual role in tumor development (95). Cytokines can transmit

signals to STATs, and STATs can regulate the expression of cytokines by binding promoters, thus forming a circular pathway to promote OvCa immunosuppression and metastasis.

Therefore, cytokine signaling components in the OvCa TME include interleukins, chemokines, and STATs. They play crucial roles in immune cell recruitment in the TME to influence OvCa clinical outcomes (96). Immune cells and OvCa cells interact through cytokines to generate a comprehensive network at the tumor site, which is responsible for the overall progression of the tumor (**Figure 3**). The roles of cytokines in OvCa are summarized in **Table 3**.

PRECLINICAL AND CLINICAL APPLICATION: TARGETING IMMUNE RESPONSES FOR THE TREATMENT OF OvCa

Due to nearly 75% of OvCa patients are diagnosed at a late stage with widespread intra-abdominal metastasis, cytoreductive surgery and primary chemotherapy with platinum agent and taxane have not been very effective (97). The majority (over 70%) of patients will relapse, with 5-year survival rates of approximately 30% and the proportion of patients who remain cancer-free at 10 years is less than 15% (98). Based on the detailed evidence with existing studies, certain disease mechanisms can be chosen as treatment targets. Currently, several targeted drugs have been approved by the Food and Drug Administration (FDA) and some of them are being tested in randomized controlled trials including mutant gene repairers, immune checkpoint inhibitors, Poly (ADP-ribose) polymerase (PARP) inhibitors and angiogenesis inhibitors (99). Despite these effects were promising, these targeted drugs were difficultly adopted as first-line therapy, because that remains poor response and increased risk of drug toxicity and death (100). For example, the response rate of anti-PD1 and anti-CTLA-4 treatments in OvCa clinical trial is 10-20%, because the majority of patients have high PD-L1 expression or lack T cells with appropriate anti-tumor reactivity (101). PARP inhibitors is only limited to populations with *BRCA* mutation associated OvCa with the FDA approval and the efficacy is somewhat limited (102). Therefore, novel clinical biomarkers and new therapeutic strategies should be developed.

In OvCa, the tumor mutational burden (TMB) is a positive relationship with the presence of neoantigens on cancer (103). Vaccine-induced tumor-associated antigen-specific immune response that could eliminate OvCa at its earliest stages is an

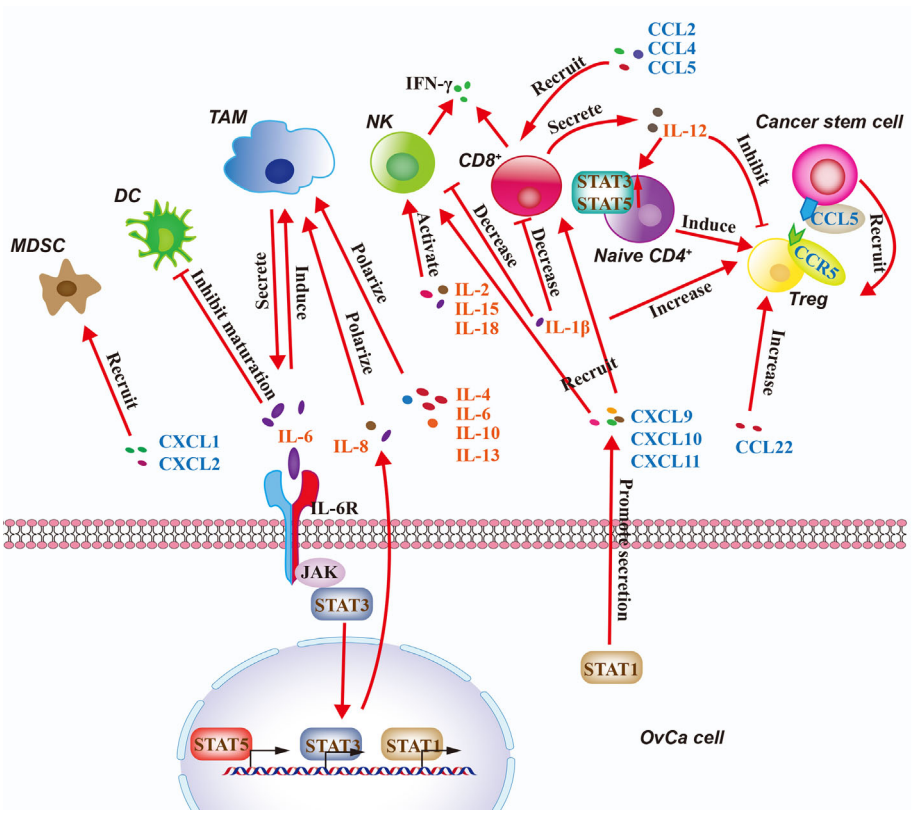

FIGURE 3 | Regulation of the Immune Microenvironment in OvCa by Cytokine Signaling. Cytokine signaling pathways in the ovarian cancer (OvCa) tumor microenvironment include interleukins, chemokines, with intracellular regulation *via* STAT1/3/5. Immune cells and OvCa cells interact through cytokines and STATs to generate a comprehensive network at the tumor site. CSC, cancer stem cell; DC, dendritic cell; $CD4^+$, CD4-expressing T cell; $CD8^+$, CD8-expressing T cell; MDSC, myeloid-derived suppressor cell; NK, natural killer cell; TAM, tumor-associated macrophage; Treg, regulatory T cell.

TABLE 3 | Cytokines and STATs involved in regulating immune cells of OvCa.

Immune cells	Interleukin	Chemokine	STATs
DCs	IL-6	NA	STAT3
Macrophages	IL-4/-6/-10/-13	CCL5	STAT3
MDSCs	NA	CXCL1/2	NA
NK cells	IL-2/-15/-18 and IL-1β	CXCL9/10/11 and CCL2/4/5	NA
$CD4^{+\ Foxp3-}$ T cells	IL-6/-12	CXCL9/10/11	STAT3
$CD8^{+}$ T cells	IL-6	CXCL9/10/11	STAT1/3/5
Tregs	IL-12	CCL5/22, CXCL2	STAT3/5
Ref	(77–82)	(85–90)	(81, 91, 94–96)

DC, dendritic cell; OvCa, ovarian cancer; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; NK, natural killer; STAT, signal transducer and activator of transcription.

attractive notion. The vaccine group with notable amplified T cell response and prolonged survival compared to a mock vaccine, but the heterogeneous character of OvCa makes it difficult to select an appropriate antigen to candidate as vaccine (104). Moreover, epigenetic therapies for OvCa can reinvigorate the antitumor immunity in tumor cell lines and mouse models (105). In particular, DNMT and HDAC inhibitors can reverse immune evasion and sensitize to subsequent immune checkpoint blockade by inducing an interferon response *via* upregulation of surface tumor antigens and key immunomodulatory proteins (105). Stone et al. demonstrated that the activation of type I interferon signaling in response to DNMT inhibitor 5-azacytidine (AZA) was a key requirement for efficient stimulation of $CD45^{+}$ immune cells, $CD8^{+}$ cytotoxic T cells and NK cells, restriction of macrophages and MDSCs in the OvCa (106). In support, Sara et al. demonstrated the enhanced expression of cancer-testis antigens and class I major histocompatibility complex (MHC)-encoded molecules in OvCa cells that were treated with DNMT inhibitors and subsequently increased infiltration of $CD8^{+}$ cytotoxic T cells, NK cells, and NKT cells and decreased infiltration of MDSCs and PD-1hi CD4 T cells in OvCa microenvironment (107). Additionally, reports have shown that HDAC inhibitors suberoylanilide hydroxamic acid (SAHA) can also inhibit OvCa growth and enhance the host immune response against cancer cells *via* the suppression of Tregs and FoxP3 expression, upregulation of NK cell-activating ligands, MHC molecules (class I and II), enhancement of NK cell and $CD8^{+}$ T cell cytotoxicity and production of proinflammatory cytokines (108). However, clinical trials with single-agent epigenetic therapy demonstrated disappointing effects in OvCa and showed severe toxicity profile of these drugs including fatigue, vomiting, and neutropenia (105). In addition, cytokine therapy is easily translated with small molecule drugs that has advantages in clinical treatment (109). Indeed, pre-clinical trials revealed that anti-IL-6 monoclonal antibody exerted anti-tumor efficacy for OvCa patients (110). However, therapies targeting cytokines also show limitations in treating OvCa. In phase I/II trial, anti-cytokine drugs had not improved response and clinical benefits in advanced OvCa patients (111). These drug therapies are all clearly listed in **Table 4**, which also shows the importance of the targeted mechanism.

Mono-immunotherapy has not achieved satisfactory clinical results in the most HGSOC patients, but a positive effect has been observed after combined therapy (125). Recent studies have demonstrated that poly (ADP-ribose) polymerase inhibitors (PARPis) exhibit anti-tumor immunity that occurs in a stimulator of interferon genes (STING)-dependent manner and is augmented by immune checkpoint blockade (126). In OvCa, combined PARPi and anti-PD-1/PD-L1 therapy has yielded encouraging preliminary results in two early-phase clinical trials (127). Moreover, combining PD-1 blockade with a single dose of the cancer vaccines GVAX or FVAX resulted in enhanced clonal expansion of antigen-specific $CD8^{+}$ T cells and tumor control in OvCa (8). Similarly, PD-1 blockade and IL-10 neutralization were inefficient as monotherapies, but the combination of these two led to improved survival and delayed tumor growth in OvCa. This survival benefit was accompanied by augmented anti-tumor T and B cell responses and decreased infiltration of immunosuppressive MDSCs (128). Furthermore, studies showed that using DNMT or HDAC inhibitors in combination with anti-PD-1 or anti-CTLA-4 therapy enhances the antitumor immune response, reduces tumor burden and improves treatment outcomes in OvCa mouse models compared to each drug alone (105). In addition, recent research found that the microelement manganese ($Mn2^{+}$) promoted DC and macrophage maturation and tumor-specific antigen presentation, augmented $CD8^{+}$ T cell and NK cell activation and increased the number of memory $CD8^{+}$ T cells in a STING-dependent way. Patients with platinum and/or anti-PD-1 antibody-resistant metastatic OvCa achieved partial response following the administration of $Mn2^{+}$ (129). The balance between immune-stimulating and immunosuppressive factors in the TME has revealed a complex regulatory mechanism in OvCa. Thus, it has been broadly considered that combination cancer immunotherapy vs. monotherapy is the future direction of OvCa treatment, such as PARPis combined with immunotherapy, angiogenesis inhibitors combined with PARPis or immunotherapy (129).

The limitations of the drug therapies reviewed above in the treatment of OvCa prepare the groundwork for the use of novel immune cell therapies to treat this disease, either innate or adaptive immune cell therapies. Adoptive cellular therapy (ACT) that ex vivo–induced antigen-specific immune cells are infused back to patients to overcome immunosuppression (130). The chimeric antigen receptor T (CAR-T) cell therapy is a potential strategy in adoptive antitumor treatment (131). Four CAR-T cell therapies have been approved by the FDA for lymphoblastic leukemia, but neither approach applies to OvCa (132). Recently, FDA approves Abecma (idecabtagene vicleucel) as the first B-cell maturation antigen (BCMA)-CAR T cell immunotherapy for the treatment of relapsed or refractory multiple myeloma, which led to objective

TABLE 4 | Major selected drugs and therapy regimens in clinical studies for ovarian Cancer.

Therapeutic regimen	Drug name	Function	Clinical trial identifier	Ref
Targeted therapy	Avelumab	Blocks PD-L1	NCT01772004	(112)
	Nivolumab	Blocks PD-1	UMIN000005714	(113)
	Ipilimumab	Blocks CTLA-4	NCT01611558	(114)
	APR-246	Binds TP53 *via* cysteine 277	NCT03268382	(115)
	Olaparib	Prevents the cell from repairing single-stranded DNA breaks	NCT0247764	(116)
	Bevacizumab	Inhibits VEGF	NCT01305213	(117)
	Aflibercept	Inhibits VEGF and PlGF	NCT00327444	(118)
	Apatinib	Inhibits VEGFR2	NCT02867956	(119)
	catumaxomab	Inhibits the EpCAM	NCT00326885	(120)
Vaccine	MUC1-vaccine	Targets MUC1	NCT01068509	(10)
	NY-ESO-1 vaccine	Targets NY-ESO-1	NCT00616941	(121)
Epigenetic therapy	DNMTi (AZA)	Removes methylation from ERVs	NCT01897571	(122)
	HDACi (SAHA)	Upregulates the expression of ERVs	NCT02915523	(105)
Cytokine therapy	Siltuximab	Inhibits IL-6	NCT00841191	(111)
	Tocilizumab	Inhibits IL-6 receptor	NCT01637532	(123)
	Carlumab	Inhibits CCL2	NCT00992186	(124)

VEGF, vascular endothelial growth factor; PlGF, placental growth facto; EpCAM, epithelial cell adhesion molecule; DNMTi, DNA methyltransferase inhibitor; HDACi, histone deacetylase inhibitor; AZA, 5-azacytidine; SAHA, suberoylanilide hydroxamic acid; ERVs, endogenous retroviruses.

responses in 72% of heavily treated patients (133). For OvCa patients, CAR-T cells targeting the CA-125 tumor antigen are being developed and have shown promise against human xenograft models and plans to evaluate their safety in in-human phase I clinical trials have been reported (134, 135). Moreover, CAR-T cell therapy for OvCa with other common target antigens include mesothelin (MSLN), HER2 and FRα, which proliferate steadily *in vivo* and accumulate specifically in tumor tissues to enhance the antitumor effect (135). Fang et al. generated CAR-T cells with *piggyBac* (PB) transposon vector encoding scFV for MSLN and full-length antibody for PD-1 (αPD-1-mesoCAR-T cells) that been used in patients with refractory OvCa combined with an anti-angiogenic drug, apatinib. The patient achieved partial response with inhibition of liver metastatic nodules and survived for 17 months and had mild side effects with only grade 1 hypertension and fatigue (136). CAR-T cells offer the promise of prolonged remission after a single infusion, but challenges include the need to wait for the patient's own cells to be engineered ex vivo, the risk of cytokine storms and graft-*versus*-host disease, and high production costs (137, 138). On the other hand, NK cells do not require human leukocyte antigen (HLA) matching to a specific patient, it is feasible and safe to transfer cells across allogeneic barriers (139). Thus, NK cell lines or ex vivo-expanded NK cells from third-party donors could be used as "off-the-shelf" cellular therapies, with the potential for lower costs and shorter wait times (140, 141). Recently, CAR-NK92 cells targeting CD24 were shown to kill CD24-expressing OvCa cell lines *in vitro* by producing high levels of IFN-γ (142). With more *in vivo* experiments and clinical studies ongoing, NK cell therapies may achieve revolutionary advances in the treatment of OvCa (143–147). However, the source of the NK cells, as well as the persistence, expansion, homing, and trafficking of the NK cells after being transferred into the patient, are great challenges (148). In addition, CAR-macrophage (CAR-M) has been demonstrated antigen-specific phagocytosis and pro-inflammatory M1 polarization in vitro, which was able to cross-present antigen and activate T cells (149). Interestingly, there are now many ongoing clinical trials evaluating the effects of combinatorial immune checkpoint blockade (targeting either PD1 or PDL1) with CD19-targeted CAR-T cells, the early results suggest that combinatorial treatment is safe and has a low toxicity profile and prolonging T cell function and limiting exhaustion (150). Innovative approaches to increase trafficking and limit suppression by anti-inflammatory cytokines and cells in the TME are also in development (151). Overexpression of IL-7 and CCL19 in CAR-T cells increased infiltration of pro-inflammatory dendritic cells and T cells into solid tumor tissues and enhances tumor regression in mouse models (151). In human OvCa cells, the HDAC inhibitor valproate (VPA) was reported to upregulate various NKG2DLs in human OvCa cells and enhance their susceptibility to CAR T cell-mediated attack (152). Adoptive transfer of NY-ESO-1–specific $CD8^+$ TCR gene-engineered T cells, in combination with the demethylating agents decitabine and SGI-110, elicited synergistic inhibition of tumor growth, curing a fraction of OvCa mice (153). Thus, the combination of adoptive cell therapy and drug therapy has shown promising results as a novel treatment strategy for OvCa patients. A limitation of genetically reprogrammed immune cell therapeutics is the use of viral vectors that have expensive and long production times for clinical use (151). Researchers are developing a new non-viral method for delivering DNA sequences to primary immune cells and exploring the proper cocktail of cytokines for growth conditions of immune cells (151). Finally, we describe the mono-therapy and combination therapy in OvCa patient (**Figure 4**).

CONCLUSIONS AND PERSPECTIVES

The immune system plays an important role in the occurrence and development of OvCa, and immune dysregulation can lead to immune escape and resistance (154). Studies of immune cells in the OvCa TME have focused on T cells, DCs, MDSCs, macrophages, NK, and γδ T cells, as well as B cells (9). The B cells, mature DCs along with NK cells and T cells, are recognized as the main effector cells of immunity, which suppress tumor progression by secreting immunoglobulins or perforin/

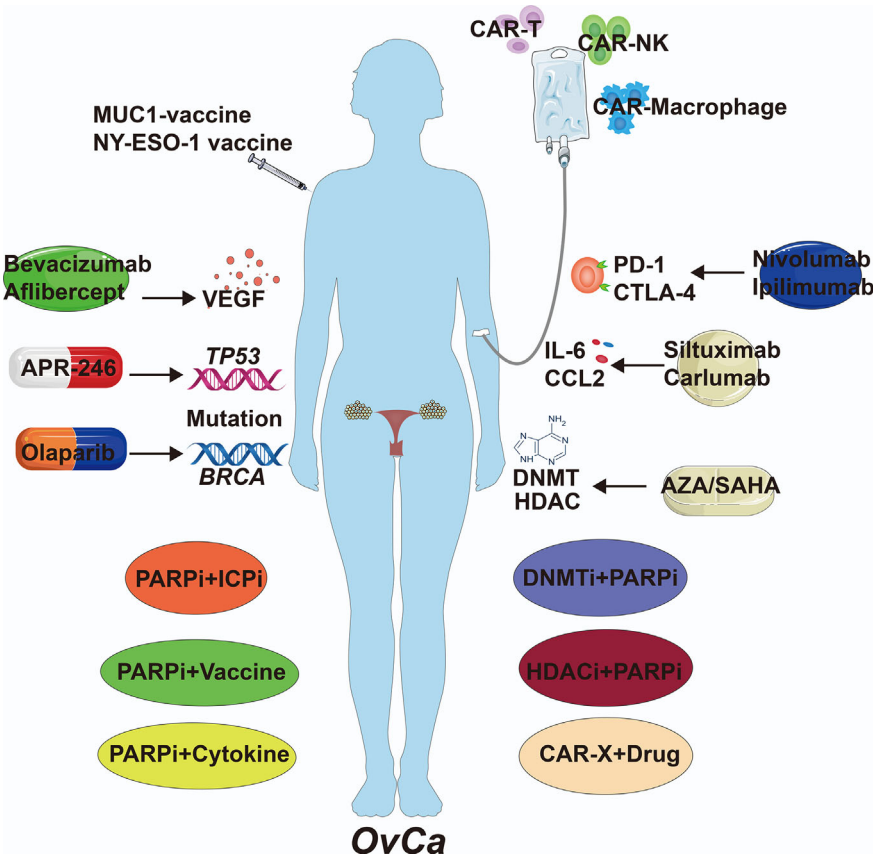

FIGURE 4 | The Clinical Therapies of OvCa. Immunological therapies of OvCa include drug, cell and combination therapy. VEGF, vascular endothelial growth factor; DNMT, DNA methyltransferase; HDAC, histone deacetylase; AZA, 5-azacytidine; SAHA, suberoylanilide hydroxamic acid; CAR, chimeric antigen receptor.

granzyme, thereby promoting immune response, and killing cancer cells directly. However, some immune cells play immunosuppressive roles in the OvCa TME, such as immature DCs, Tregs, MDSCs, and M2 macrophages, which serve as immunosuppressive factors to inhibit the cytotoxic functions of NK and $CD4^+$/$CD8^+$ T cells (155). In this review, we mainly describe various factors that affect the phenotype of immune cells in OvCa, including transcriptional and post-transcriptional factors, as well as cytokine signals. The main genes that affect the phenotype of immune cells are those that are frequently mutated or amplified in OvCa. In addition to mutations in the tumor cells, mutations also accumulate in the immune cells themselves, especially myeloid cells. Furthermore, ncRNAs, including miRNAs and lncRNAs, regulate the activity of immune cells in OvCa by binding target genes (156). Many recent studies have shown that OvCa cells and TAMs can release miRNA exosomes, thereby regulating immune cell phenotypes. Finally, cytokine signaling components, including interleukins, chemokines, and STATs, often mediate the interaction between immune cells and tumor cells in the OvCa TME to regulate immune system reorganization. The immune cells can be regulated by many factors in the development of OvCa, and elucidating how these factors shape immunity in the TME should provide insight to develop novel therapeutics to treat OvCa. Aimed at the genomic instability in HGSOC, therapeutic drugs have been developed by targeting mutation of *TP53* and *BRCA* (105). Then, in our review, we found that *PRKCI, APOBEC3G, GADD45B* and *XBP1* also could be potential target for OvCa therapy, and their remarkable regulation of immune *in vitro* or *in vivo* has been confirmed. Moreover, ncRNAs are important to carcinogenesis of OvCa and regulation of immune system, but the therapeutic strategies focused on ncRNA are few studies. The prognosis of HGSOC is generally poor and mono-therapy often exerts low response rates and serious side effects. To broaden the clinical benefit and safety and minimize the therapeutic costs, cellular engineering therapies with NK cells and combination of different immunotherapies and/or chemotherapies are considered to be the future direction of OvCa treatment. However, the present clinical benefit is only available for a fraction of OvCa patients.

Understanding the precise cellular and molecular mechanisms is a critical task to further improve the current immunotherapies or develop new therapeutic avenues. Recent applications of single-cell RNA sequencing (scRNA-seq) in the TME have provided important insights into the biology of tumor-infiltrating immune cells, including their heterogeneity, dynamics, and potential roles

in both disease progression and response to immunotherapies (157). ScRNA-seq has been used in a variety of tumor research, including OvCa (1). However, most of the single-cell studies focused on OvCa cells and malignant ascites, and just one study revealed the tumor immune phenotypes of OvCa (158–160). It is believed that there will be single cell research on immune cells of ovarian cancer in the near future, which will further reveal the causes of phenotypic changes of immune cells, and provide novel gene targets to pursue as well as promising gene-based biomarkers to stratify patients for clinical actions.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

The authors regret that it was not possible to include many interesting studies in the field due to limited space.

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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

MINI REVIEW
published: 28 July 2021
doi: 10.3389/fimmu.2021.717421

Lineage Reprogramming of Effector Regulatory T Cells in Cancer

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Regulatory T-cells (Tregs) are important for maintaining self-tolerance and tissue homeostasis. The functional plasticity of Tregs is a key feature of this lineage, as it allows them to adapt to different microenvironments, adopt transcriptional programs reflective of their environments and tailor their suppressive capacity in a context-dependent fashion. Tregs, particularly effector Tregs (eTregs), are abundant in many types of tumors. However, the functional and transcriptional plasticity of eTregs in tumors remain largely to be explored. Although depletion or inhibition of systemic Tregs can enhance anti-tumor responses, autoimmune sequelae have diminished the enthusiasm for such approaches. A more effective approach should specifically target intratumoral Tregs or subvert local Treg-mediated suppression. This mini-review will discuss the reported mechanisms by which the stability and suppressive function of tumoral Tregs are modulated, with the focus on eTregs and a subset of eTregs, follicular regulatory T (T_{FR}) cells, and how to harness this knowledge for the future development of new effective cancer immunotherapies that selectively target the tumor local response while sparing the systemic side effects.

Keywords: anti-tumor immunity, effector regulatory T cells, follicular regulatory T cells, Foxp3, Treg lineage stability, humoral antibody response

OPEN ACCESS

Edited by:
Khashayarsha Khazaie, Mayo Clinic College of Medicine and Science, United States

Reviewed by:
Salman M. Toor, Hamad bin Khalifa University, Qatar
Arya Biragyn, National Institute on Aging (NIH), 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: *30 May 2021*
Accepted: *14 July 2021*
Published: *28 July 2021*

Citation:
Dixon ML, Leavenworth JD and Leavenworth JW (2021) Lineage Reprogramming of Effector Regulatory T Cells in Cancer. Front. Immunol. 12:717421. doi: 10.3389/fimmu.2021.717421

INTRODUCTION

An effective immune system must be capable of maintaining self-tolerance while generating robust responses to foreign antigens. Tregs are important components participating in such immune regulation (1, 2). In both human and mice, Tregs are characterized by their high expression of both the IL-2 receptor α-chain (CD25) and the transcription factor Foxp3, which are essential for their development, suppressive activity and stability (3–8). $Foxp3^+$ Tregs comprise both central Treg (cTreg) and eTreg subsets (9, 10). Accumulation of Tregs, particularly eTregs, within the tumor represents a major obstacle to the development of effective anti-tumor immunity (11–13). The

frequency of Tregs among tumor-infiltrating lymphocytes (TIL) is often associated with poor prognosis of patients with many types of cancer (14), although Tregs can also be beneficial during early stages of inflammation-related cancers, such as colorectal cancer, and correlate with better prognosis (15–18). Substantial reviews have discussed the homeostatic regulation of Tregs and their suppressive function, including the most recent one centering on tumoral Tregs (19). This review will cover Treg stability with a focus on eTregs and T_{FR} cells, and how their stability affects cancer progression and how it can be targeted for therapy.

Treg AND eTreg BIOLOGY

Tregs mediate suppression through various mechanisms including obstructing CD80/CD86 co-stimulation *via* the surface receptor CTLA-4, limiting IL-2 availability for effector T-cells (Teff) and secreting inhibitory molecules IL-10, IL-35 or TGF-β (20). However, Tregs are phenotypically and functionally diverse. Based on the developmental origin, Tregs are defined as either thymic or peripheral Tregs. Thymic Tregs (tTregs) begin as CD4 single positive thymocytes with TCRs displaying high affinity for self-antigens. Peripheral Tregs (pTregs) develop from naïve $CD4^+$ T-cells in the periphery that experience antigen and receive specific environmental stimuli, such as TGF-β and IL-2 (21, 22). Although the definitive markers distinguishing tTregs from pTregs remain obscure, all Tregs in the periphery reside in multiple lymphoid and non-lymphoid tissues to maintain tolerance or suppress ongoing inflammatory responses. In the circulation and lymphoid organs, the majority of Tregs that express the homing receptors CD62L and CCR7, but low level of CD44, are cTregs and are largely IL-2-dependent (9). In contrast, a large population of Tregs in the non-lymphoid tissues that have a $CD44^{hi}CD62L^{lo}CCR7^{lo}$ surface phenotype resembling activated or effector conventional T-cells are eTregs (9, 23). In the presence of TCR, CD28 and IL-2 signaling, cTregs differentiate into eTregs accompanying the upregulation of IRF4 and Blimp1 (23, 24). eTregs can further undergo stimulus-specific differentiation that is regulated by signals and transcription factors typically associated with the differentiation of conventional T-helper (T_H) cells. This polarization allows Tregs to regulate specific immune responses mediated by their analogous effector $CD4^+$ T-cells in addition to their generic suppressive capacity (23). In addition to the high level of CD44, eTregs express effector markers, including ICOS and GITR (10, 24). Analogous subsets also exist for human Tregs, including resting $FOXP3^{lo}CD45RA^+$ and effector $FOXP3^{hi}CD45RA^-$ suppressive subsets, while $FOXP3^{lo}CD45RA^-$ cells are non-suppressive cytokine-secreting subsets (25). Importantly, CD15s has been identified as a biomarker for most suppressive human $FOXP3^{hi}$ eTregs (26). Although eTregs are predominantly found in non-lymphoid tissues, B-cell follicles in the lymphoid or lymphoid-like organs contain a subset of eTreg, known as T_{FR} cells, which are responsible for regulating the follicular helper T (T_{FH})–B-cell interaction in the germinal center (GC), and thus the production of high-affinity antibody (27–30).

T_{FR} CELL BIOLOGY

T_{FR} cells share many features with T_{FH} cells, but they express Foxp3 and belong to eTregs. Like T_{FH} cells, T_{FR} cells express high levels of PD-1 and CXCR5, which allows them to traffic to B-cell follicles following the chemokine CXCL13 gradients (27–30). Both T_{FR} and T_{FH} cells require ICOS and CD28 signaling for their development and maintenance and are dependent of antigen presenting cells and B-cells in the GC (27–31). T_{FH} and T_{FR} cells express high levels of Bcl6, however, unlike T_{FH} cells, T_{FR} cells also co-express Blimp1, which antagonizes Bcl6. While Bcl6 is critical for the development of T_{FR} cells as depletion of Bcl6 results in an almost complete loss of T_{FR} cells, Blimp1 is important for the regulation of T_{FR} suppressive function (31–36). Additionally, PD-1 and IL-2 signals are critical for T_{FR} cells. Mice deficient in PD-1 or its ligand PD-L1 have increased T_{FR} cell abundance with enhanced suppressive activity (37), while high IL-2 concentrations at the peak of influenza infection prevent T_{FR} cell development (38). However, the maintenance of developed T_{FR} cell stability appears to require the IL-2 signaling that is regulated by Blimp1 (34).

While T_{FR} cells are capable of regulating a variety of immune responses similar to conventional Tregs, they are uniquely known for their ability to regulate GC response and antibody production (27–30). Despite the low frequency, the importance of T_{FR} cells has been re-emphasized in a recent study in which a mouse model with a selective depletion of T_{FR} cells displays a profound alteration of immune responses, including increased self-reactive antibody (39). Several mechanisms for T_{FR}-mediated suppression have been reported, including the one mediated by CTLA-4. Genetic deletion or blockade of CTLA-4 impairs T_{FR} cell development and function, leading to spontaneous T_{FH} differentiation and GC expansion (40, 41). T_{FR} cells are also shown to inhibit specific effector molecules, central metabolic and anabolic pathways in both T_{FH} and GC B-cells, but retain their transcriptional signature (42). This type of suppression appears durable and persists in their absence, and can be overcome by IL-21 signals (42). However, it remains unclear if T_{FR} cells directly target T_{FH} and/or B-cells during GC responses, and whether T_{FR} cells can regulate memory B-cells or plasma cells directly.

Treg/T_{FR} STABILITY

Tregs must maintain their anergic phenotype and suppressive activity during ongoing inflammatory responses (43–45). This functional stability reflects a lack of effector activity by Tregs (i.e., expression of pro-inflammatory cytokines) and may or may not require maintenance of Foxp3 expression (44–46). Loss of Foxp3 (even a slight reduction) often results in the generation of ex-Tregs (47), while conversion into effector T-cells with unaltered Foxp3 expression is referred as Treg "fragility" (48). Several factors appear to be important for Treg stability/fragility, including CD25/STAT5 signals (43), PTEN/Akt/Foxo1/3a pathway (49–51), CARMA1–BCL10–MALT1 (CBM)

signalosome complex (52), autophagy (53), Ezh2 (54, 55), Helios (56), Eos (57) and Nrp1 (48, 58). While the former 6 pathways regulate Foxp3, ablation of the latter 2 factors does not affect Foxp3 expression. Many of these pathways implicated in the context of tumor will be discussed in *Treg/T_{FR} Stability in the TME*. Here we focus on the CD25/STAT5/Foxp3-dependent regulation of Treg stability and function.

Foxp3-Dependent Treg Stability

Foxp3 is crucial for maintaining Treg identity. Loss of Foxp3 results in Treg instability, dysfunction, and potential life-threatening autoimmune diseases (59–62). At steady state, Foxp3 expression and tTregs are incredibly stable (63). However, Tregs often become unstable under inflammatory conditions. Treatment of Tregs *in vitro* with proinflammatory cytokines like IL-4 and IL-6 results in the downregulation of Foxp3 and the upregulation of effector cytokines such as IFNγ (43, 64). Adoptive transfer of $Foxp3^+$ Tregs into lymphodepleted mice also results in the loss of Foxp3 expression by a substantial population of Tregs, which appears to be limited to the $CD25^{lo}Foxp3^+$ subset as the majority of $CD25^{hi}Foxp3^+$ cells retain Foxp3 expression (65–67). While a portion of the $Foxp3^-$ population, ex-Tregs, acquires Teff function, others are capable of reacquiring Foxp3 expression upon activation (66), suggesting the heterogeneity of Tregs and their ability to accommodate their function by adapting to environmental stimuli. These ex-Tregs are consistently reported to be autoreactive and pathogenic, causing autoimmune diseases upon adoptive transfer (35, 67–69).

Mechanisms for Foxp3-Dependent Treg Stability

Mechanisms to reinforce Foxp3 expression and Treg stability have been extensively studied. TCR stimulation, along with the recruitment of transcription factors, such as NFAT, Foxo1 and Foxo3, to the *Foxp3* promoter, is the primary step in triggering *Foxp3* gene transcription (70–73). Additionally, the conserved non-coding sequence (CNS) elements at the *Foxp3* locus are important for Treg fate determination and lineage stability (74–76). The pioneer element CNS3 facilitates Foxp3 induction and increases the generation of both tTregs and pTregs. While tTregs do not rely on CNS1 for Foxp3 induction, CNS1 is indispensable for pTreg generation as it contains a TGF-β-NFAT response element and is dependent of TGF-β signaling to induce histone acetylation in the *Foxp3* enhancer region (76–78). CNS2, which contains the Treg specific demethylation region (TSDR), is crucial for the maintenance of Foxp3 expression in dividing Tregs (43, 76). CNS2, the CpG-rich region, is fully methylated in conventional T-cells, but largely demethylated in tTregs and partially methylated in pTregs. Upon TSDR demethylation, Foxp3, along with STAT5, NFAT and Cbfβ-Runx1, binds to CNS2, stabilizing Foxp3 expression through positive feedback mechanisms (62, 79–83). The availability of IL-2 and activation status of CD25/STAT5 signals that are modulated by several factors, including Helios and Blimp1 (34, 56), are essential for CNS2 to sustain Foxp3 expression, preventing Treg differentiation into Teff by counteracting proinflammatory cytokine signaling (43), which explains why $CD25^{hi}Foxp3^+$ cells are more stable than $CD25^{lo}Foxp3^+$ cells.

Blimp1-Mediated Regulation of Treg/T_{FR} Stability

eTregs are marked by the expression of Blimp1 (10), however, its role in eTregs have been largely restricted to its regulation of IL-10 expression until recent findings from our group and others showing that it is important for Treg lineage stability and suppressive activity (34, 35). Consistent with the finding that expression of Blimp1 in the thymus is very low and Blimp1 unlikely regulates early T-cell development (84), mice with a Treg-specific deletion of Blimp1 do not show overt autoimmune phenotype (34, 35). However, Tregs from these mice are unstable with reduced Foxp3 expression and produce inflammatory cytokines after immunization, and these mice develop severe experimental autoimmune encephalitis (EAE) (34, 35, 68). At the peak of EAE, the presence of IL-6 activates the DNA methylating enzyme Dnmt3a, resulting in CNS2 methylation. Blimp1 is able to inhibit Dnmt3a upregulation and CNS2 methylation, thereby preventing the acquisition of a Teff phenotype (35). Additionally, Blimp1 can repress IL-23R-STAT3 signaling while retaining the CD25-STAT5 pathway in eTregs to sustain Foxp3 expression (34). Blimp1 is also critical for both T_{FR} lineage stability and their proper entry into the GC (34). Blimp1-deficient T_{FR} cells display an impaired suppressive phenotype *in vivo* with reduced Foxp3 and CTLA-4 expression, while increasing proinflammatory cytokines like IL-17A and IFNγ. These unstable T_{FR} cells prematurely migrate into the GC and differentiate into T_{FH}-like cells, resulting in T_{FH} and GC B-cell expansion along with increased antibody and autoantibody production. Furthermore, adoptive transfer of Blimp1-deficient T_{FR} cells can promote pathogenesis associated with dysregulated GC responses (34, 68). Taken together, these studies have revealed Blimp1 as a new and central regulator of eTreg and T_{FR} lineage stability and suppressive capacity.

Treg/T_{FR} STABILITY IN THE TME

Tregs are often recruited to the tumor microenvironment (TME) *via* various chemokines, such as CCL20, where they become highly activated and suppressive (11–13, 19, 85–87). Many pathways have been implicated in the regulation of TIL Treg stability.

Pathways to Regulate Foxp3-Dependent TIL Treg Stability

A significant portion of TIL Tregs express PTEN and Foxo3a. The PTEN/Akt/Foxo3a pathway is important for the suppression of responses to apoptotic cells, including apoptotic tumor cells (49). Disruption of the PTEN/Akt/Foxo3a pathway through inhibition of PTEN results in Treg instability and the transitioning of suppressive $Foxp3^+$ Tregs to proinflammatory ex-Tregs, leading to a more immunogenic microenvironment

and substantial tumor regression (49–51). Disruption of the CBM signalosome complex also results in the acquisition of an anti-tumor effector phenotype by TIL Tregs, i.e., production of IFNγ, and reduced tumor growth. Increased IFNγ activates macrophages and upregulates PD-L1 by tumor cells. Accordingly, PD-1 blockade therapy along with CARMA-1 or MALT1 disruption eradicates tumors that do not respond to anti-PD-1 monotherapy, suggesting that induction of Treg instability confers the sensitivity to checkpoint inhibitor (52). Similarly, disruption of Ezh2 activity or depletion of Helios in Tregs leads to Foxp3 instability with an increased expression of effector cytokines like IFNγ and TNFα, enhanced anti-tumor immunity, and decreased tumor growth and progression (54, 55, 88). Importantly, colorectal cancers with abundant infiltration of $FOXP3^{lo}$ non-suppressive T-cells display better prognosis than those infiltrated mainly with $FOXP3^{hi}$ Tregs (18).

Pathways to Regulate Foxp3-Independent TIL Treg Stability

Tregs can become unstable with an intact Foxp3 expression. The transcription factor Eos functions as a Foxp3 co-repressor to inhibit downstream target genes and to maintain Treg suppressive phenotype (89). In response to proinflammatory cytokines like IL-6, Eos but not Foxp3 is downregulated, leading to Treg reprogramming and the acquisition of a T_H phenotype with the upregulation of CD40L, IL-2, and IL-17A (57, 90). Co-transfer of "Eos-labile" Tregs results in more robust anti-tumor responses and better tumor control compared to transfer of Eos-stable Tregs. Moreover, reprogrammed Tregs upregulate CD40L and are able to facilitate DC cross-presentation to activate $CD8^+$ T-cell anti-tumor response after vaccination with an tumor antigen (91).The Nrp1-Sema4a pathway is another mechanism for reinforcing TIL Treg function and limiting anti-tumor immune responses, while it is dispensable for the suppression of autoimmunity and the maintenance of immune homeostasis by Tregs. Ligation of Nrp1 on Tregs by Sema4a increases Treg survival and potentiates stable suppression with the increased production of IL-10 and IL-35, due to diminished Akt activation *via* the recruitment of PTEN (58, 92). Interestingly, loss of Nrp1 in Tregs results in high expression of IFNγ that drives the instability of surrounding wild-type Tregs. Consequently, mice with Nrp1-deficient Tregs display enhanced anti-tumor immunity and tumor clearance, prolonged survival and increased responsiveness to anti-PD-1 therapy without autoimmune abnormalities (48).

Metabolic Pathways to Regulate TIL Treg Stability

Unlike Teff, Tregs favor oxidative phosphorylation but keep glycolysis under strict control, which plays an important role in shaping Treg identity and function (93, 94). The TME creates a low-glucose and high lactate environment that often promotes Treg suppressive function (95–99). Tregs may couple the survival mechanism, like autophagy to metabolic homeostasis by limiting glycolysis and reducing PI3K/Akt/Myc activation to ensure their integrity in the hostile TME (53). A most recent study has further elucidated that high-glucose conditions impair the function and stability of Tregs (100). However interestingly, Tregs have evolved to benefit from the symbiosis with tumors by utilizing the glycolytic by-product lactic acid to proliferate and prevent the destabilization effects of high glucose. This alternative pathway appears to be exclusively important for the stability and suppressive identity of tumoral but not peripheral Tregs. Similarly, limiting lipid uptake or metabolism by genetic or pharmacologic inhibition of FABP5 disrupts mitochondrial respiration, but also enhances Treg suppression by increasing IL-10 expression, suggesting another layer of complexity for the regulation of TIL Tregs (101).

New Pathways to Regulate TIL Treg and T_{FR} Stability

Our recent study has revealed the importance of Blimp1 in the regulation of eTreg/T_{FR} stability and suppressive function under immune and autoimmune conditions (34, 68). However, the specific impact of $Blimp1^+$ eTregs on, and mechanisms of action within, tumors are not yet explored. Since a majority of TIL Tregs express Blimp1 in some tumor models (102), and Blimp1 is suggested to be used for outcome prediction of cancer patients (103), loss of Blimp1 in eTregs may reprogram these cells into Teff, and potentially lead to increased anti-tumor immunity and decreased tumor progression, although this awaits further investigation. Importantly, these effects are likely restricted to TIL Tregs, since Blimp1 is expressed at low levels by Tregs at steady state (24). Despite a few reports showing that T_{FR} cells are significantly increased in cancer patients compared to healthy controls (104, 105), their mechanisms of action in the tumor are unclear. The increased TIL T_{FH} and B-cells, as likely observed in mice with the Treg-specific deletion of Blimp1, and tertiary lymphoid structure formation are associated with favorable outcomes in certain types of cancer and better responses to immunotherapy (106–112). Thus, it is important to define the contribution of T_{FR} cells to tumor progression and the impact of Blimp1 on T_{FR} function in the tumor.

THERAPEUTIC APPROACHES TARGETING Treg STABILITY

Current cancer immunotherapy, particularly checkpoint inhibitor and CAR T-cell transfer, have shown great promise in some types of cancer. However, the success rates remain suboptimal (113–115), and some of these approaches are complicated with systemic immune-related adverse effects (116–118). Since Tregs, particularly eTregs, are one of major suppressive immune components in many cancers, most of these approaches are complicated with negative outcomes from Tregs in addition to positive effects on anti-tumor effector cells. For example, IL-2 can potently activate both T-cells and nature killer cells, and is potentially applicable for tumor control. However, IL-2 has the propensity to amplify Tregs, representing a major barrier for IL-2-based cancer therapy. The next generation of IL-2 that specifically targets tumor and preferentially boosts $CD8^+$ T-cell response without inducing Treg responses appears to be

promising (119). Similarly, high PD-1 expression is deleterious to Treg and T_{FR} suppression; anti-PD-1 may promote $CD8^+$ T-cell anti-tumor response while inducing potent Treg/T_{FR}-mediated suppression (37, 120). Therefore, the PD-1 expression balance between Teff and Tregs can predict the clinical efficacy of PD-1 blockade therapy, and needs to be considered when anti-PD-1 or anti-PD-L1-based therapy is applied (121). Interestingly, another checkpoint inhibitor, CTLA-4 blockade, has been recently shown to drive Treg instability in glycolysis-low tumors (122), a new mechanism beyond the conventional role of anti-CTLA-4 therapy in inducing Treg depletion.

Depletion of Tregs has been demonstrated to enhance anti-tumor responses, however, this ablation also results in lethal autoimmunity (60–62, 123). Studies from us and others suggest that a more effective approach would entail the specific reprogramming of TIL Tregs and reshaping the TME by employing the features of Treg instability, while not altering the stability of Tregs in the periphery (44, 45) (**Figure 1**). Disruption of the CBM signalosome complex or targeting Helios or Nrp1 or ligation of GITR in Tregs is shown to be effective for tumor control without peripheral autoimmune effects reported (48, 52, 88, 124). Based on the profound effect of Blimp1 depletion on the stability and suppressive ability of eTreg and T_{FR} cells, our findings suggest that targeting $Blimp1^+$ eTreg may generate similar anti-tumor effects while limiting systemic toxicity. In addition to inducing eTreg destabilization (34), targeting $Blimp1^+$ eTregs may also induce potent anti-tumor humoral responses, thus achieving multifaceted anti-tumor effects.

CONCLUSION/PERSPECTIVE

It is important to recognize that Treg stability can be manipulated to induce changes of immune responses, achieving the therapeutic benefit. Notably, loss of TIL eTreg stability in various tumors leads to remodeling of the TME from a suppressive state to an effective anti-tumor state and decreased tumor progression. Current and future challenges include the ability to selectively induce these changes in specific subsets of Tregs and in the TME but not systemically. As the field of cancer immunology progresses, understanding factors that regulate Tregs specifically in the tumor, yet have limited impact on Tregs in the periphery, is highly desirable and important for treating nearly every cancer patient, particularly any patient treated with immunotherapy, as it will direct the development of effective, targeted immunotherapies with reduced adverse

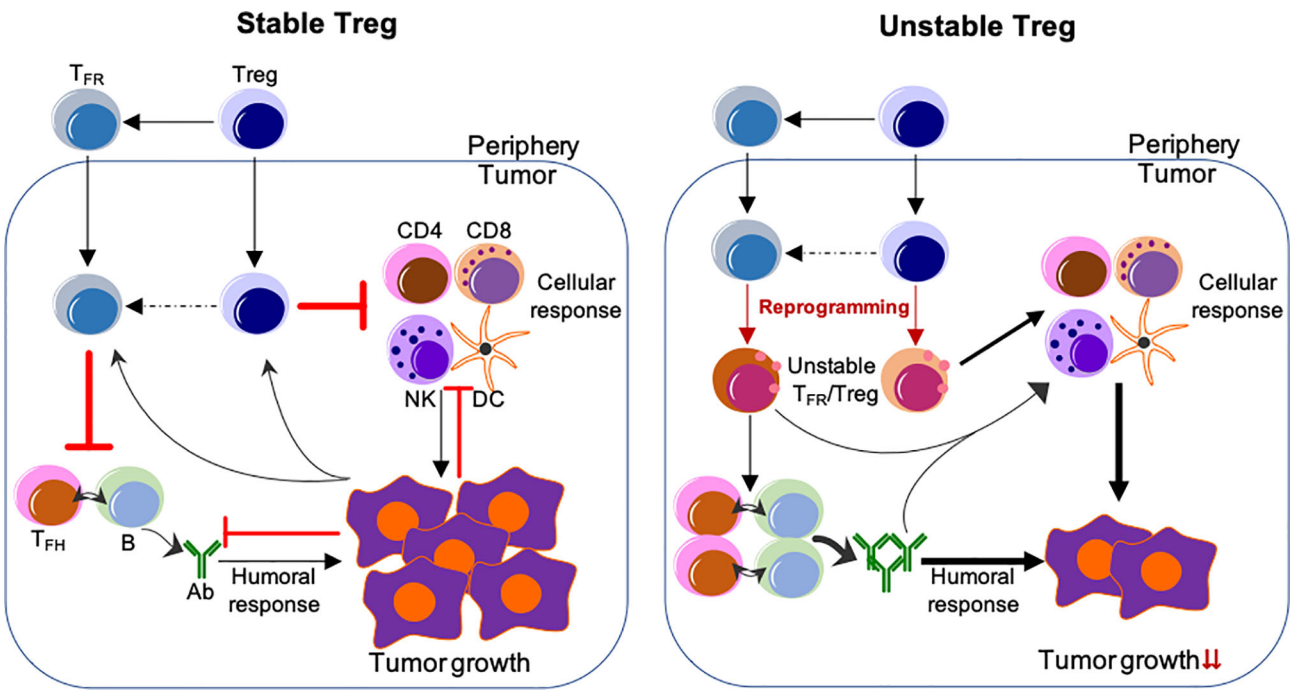

FIGURE 1 | Reprogramming of TIL Tregs to control tumor by targeting their stability. *Left*, Stable Treg. Treg and T_{FR} cells mainly suppress the cellular and humoral anti-tumor immune responses, respectively. Conversely, tumor cells impose suppression on both cellular and humoral immune responses, but foster the immune suppression by Treg and T_{FR} cells. *Right*, Unstable Treg. Factors or approaches destabilize or reprogram Treg and T_{FR} cells into effector-like cells, which display impaired suppressive activity, but instead cooperate with both cellular and humoral anti-tumor components to control tumor growth and progression. The peripheral events are not depicted, but strategies used to selectively reprogram TIL Tregs, but not Tregs in the periphery, are expected to be most effective without systemic adverse effects. The unclear events are indicated by dashed lines. Not depicted: Peripheral T_{FH} and B-cells and their migration into the tumor; expansion of Treg/T_{FR} cells and anti-tumor effector cells; other cells regulating anti-tumor responses (e.g., myeloid-derived suppressor cells and macrophages, etc.).

events. This represents a new direction for how to manipulate Treg activity for cancer treatment.

AUTHOR CONTRIBUTIONS

MLD, JDL, and JWL drafted the manuscript and revised it critically. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This work was supported by the University of Alabama at Birmingham faculty start-up funds to JWL. MLD is supported by NIH pre-doctoral training program (T32 AI007051). JWL is also supported by DoD W81XWH-18-1-0315 and NIH grant R01AI148711. Due to the limited space, the authors regret that this minireview article cannot include all interesting studies in the field.

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GLOSSARY

Bcl6	B-cell lymphoma 6 protein
BCL10	B-cell lymphoma/leukemia 10
Blimp1	B lymphocyte-induced maturation protein 1
CAR	chimeric antigen receptor
CARMA1	caspase recruitment domain-containing membrane-associated guanylate kinase protein-1
Cbfβ	core-binding factor subunit beta
CBM	CARMA1–BCL10–MALT1
CD40L	CD40 ligand
CNS	conserved non-coding sequence
CCR7	CC receptor 7
CTLA-4	cytotoxic T lymphocyte antigen 4
cTreg	central Treg
CXCR5	C-X-C chemokine rector 5
CXCL13	C-X-C chemokine ligand 13
Dnmt3a	DNA (cytosine-5)-methyltransferase 3a
EAE	experimental autoimmune encephalitis
eTreg	effector Treg
Ezh2	enhancer of zeste homolog 2
FABP5	fatty acid binding protein 5
Foxo3	forkhead box O3
Foxp3	forkhead box protein P3
GC	germinal center
GITR	glucocorticoid-Induced tumor necrosis factor receptor
ICOS	inducible T cell costimulatory
IFN	interferon
IL	interleukin
IL23R	IL-23 receptor
IRF4	interferon regulatory factor 4
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1
mTOR	mechanistic target of rapamycin
NFAT	nuclear factor of activated T-cells
Nrp1	neuropilin-1
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog
pTreg	peripheral Treg
Runx1	runt-related transcription factor 1
Sema4a	semaphorin 4a
STAT	signal transducer and activator of transcription
TCR	T-cell antigen receptor
Teff	effector T-cells
T_{FH}	follicular helper T
T_{FR}	follicular regulatory T
TGF-β	transforming growth factor β
T_H	T helper
TIL	tumor-infiltrating lymphocytes
TME	tumor microenvironment
TNF	tumor necrosis factor
Treg	regulatory T-cells
TSDR	Treg specific demethylation region
tTreg	thymic Treg

frontiers in Immunology

REVIEW
published: 20 August 2021
doi: 10.3389/fimmu.2021.694833

Emerging Complexity in CD4$^+$T Lineage Programming and Its Implications in Colorectal Cancer

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The intestinal immune system has the difficult task of protecting a large environmentally exposed single layer of epithelium from pathogens without allowing inappropriate inflammatory responses. Unmitigated inflammation drives multiple pathologies, including the development of colorectal cancer. CD4$^+$T cells mediate both the suppression and promotion of intestinal inflammation. They comprise an array of phenotypically and functionally distinct subsets tailored to a specific inflammatory context. This diversity of form and function is relevant to a broad array of pathologic and physiologic processes. The heterogeneity underlying both effector and regulatory T helper cell responses to colorectal cancer, and its impact on disease progression, is reviewed herein. Importantly, T cell responses are dynamic; they exhibit both quantitative and qualitative changes as the inflammatory context shifts. Recent evidence outlines the role of CD4$^+$T cells in colorectal cancer responses and suggests possible mechanisms driving qualitative alterations in anti-cancer immune responses. The heterogeneity of T cells in colorectal cancer, as well as the manner and mechanism by which they change, offer an abundance of opportunities for more specific, and likely effective, interventional strategies.

Keywords: CD4$^+$T cell, effector T cell, regulatory T cell (Treg), T follicular helper cell (Tfh), T follicular regulatory cell (Tfr), lineage programming, plasticity, colorectal carcinoma

OPEN ACCESS

Edited by:
Maria Manuela Rosado, University Roma "Sapienza", Italy

Reviewed by:
Nevil Singh, University of Maryland, Baltimore, United States
Jesse Goyette, University of New South Wales, Australia

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Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *13 April 2021*
Accepted: *04 August 2021*
Published: *20 August 2021*

Citation:
DiToro D and Basu R (2021) Emerging Complexity in CD4$^+$T Lineage Programming and Its Implications in Colorectal Cancer. Front. Immunol. 12:694833. doi: 10.3389/fimmu.2021.694833

INTRODUCTION

Despite being exposed to billions of microbes and their products, the basal tone of a healthy gut immune system is overtly tolerogenic. A strong tolerogenic capacity is beneficial to the host. Inappropriate activation of gut immunity underlies multiple inflammatory diseases. Chronic inflammation carries additional risk: it is a key factor in the development and progression of colorectal carcinoma (CRC) (1). This suppression cannot be absolute, however. Overcoming it is critical for mounting responses to pathogens, and for developing effective anti-cancer immune responses. The capacity to switch between tolerogenic and inflammatory states is one of the most critical aspects of gut immunity. This delicate balance is orchestrated by counteracting classes of CD4$^+$T cells.

Naïve CD4$^+$T cells are pluripotent precursors that differentiate into phenotypically and functionally distinct subsets uniquely tailored to operate in a specific inflammatory context. The

differentiation of naïve, antigen-inexperienced CD4+T cells is a multi-step process and represents the integration of qualitative and quantitative variations in diverse signaling events guiding their development (2). Rational exploitation of CD4+T cell differentiation and function represents a potentially powerful avenue for therapeutic intervention. A nuanced understanding of the molecular determinants guiding these processes is a prerequisite for designing effective and safe therapies. Recent evidence has challenged long held notions regarding the conceptual and functional organization of T cell subsets, and our understanding of the roles these cells play in health and disease. These advances have illuminated an increasingly complex web of overlapping transcriptional networks. Emerging patterns hint at an underlying simplicity that may instruct potential therapeutic strategies.

CD4+T CELL HETEROGENEITY – A HISTORICAL PERSPECTIVE

Heterogeneity among CD4+T cells was first revealed by Mossman and Coffman in 1986, with the identification of Th1 and Th2 cells (3). This groundbreaking work lead to a period of intensive investigation and rapid discovery. The signaling and transcriptional events guiding these cell fates were identified, leading to the concept of 'master regulator' transcription factors (4–6). Additional effector subsets, including Th17 and Th22 cells, and the molecular determinants guiding their development, were discovered (7–11). The manner in which these distinct effector populations modulate cellular processes at the site of inflammation was carefully scrutinized.

The possibility that CD4+T cells also suppress inflammation was first proposed in 1970 by Gershon and Kondo (12, 13). The field became mired in controversy, however, and was effectively abandoned. The identification of distinct functional subsets by Mossman and Coffman led to a re-examination of this putative role. In 1995, Shimon Sakaguchi conclusively demonstrated the existence of regulatory T cells (Tregs) (14).

The role of T cells in driving antibody responses was also re-examined. T cells were known to be required for germinal center formation and class switched affinity matured antibody responses since the 1960's, but the nature of this interaction and the specific cells participating in it remained unknown (15). Following establishment of the Th1/Th2 paradigm by Mossman and Coffman, it was proposed that, while Th1 cells regulate peripheral cellular events, Th2 cells functioned to provide help to B cells. This inference was based on their production of interleukin 4 (IL-4), which was shown to promote B cell proliferation in 1982 (15). However, deletion of Th2 genes, including *IL4*, failed to reduce germinal center and total IgG levels. Identification of Treg cells by Sakaguchi effectively overturned the nascent Th1/Th2 paradigm, and suggested germinal centers could depend on an as yet undiscovered subset. By the late 2000's it was understood that help to B cells was provided by a distinct functional subset of CD4+T cells, termed T follicular helpers (Tfh) (16). Recently, a suppressive counterpart to Tfh, known as T follicular regulatory cells (Tfr), were identified (17).

This heterogeneity of form and function is established *via* competing developmental signals driving lineage defining transcriptional events. The role of these cells, and the molecular determinants guiding their differentiation, are discussed below and summarized in **Figure 1**.

EFFECTOR CD4+T CELL SUBSETS

Th1

Th1 cells develop in response to intracellular pathogens (Type I responses). They promote the destruction of infected cells by inducing apoptosis and enhancing cytotoxic and phagocytic activity. Th1 cells also promote destruction of cancer cells, and drive much of the tissue damage seen during inflammation. Differentiation of Th1 cells is initiated by interleukin-12 (IL-12), a heterodimer consisting of a p35 and p40 subunit (**Figure 1A**) (18). Ligation with the IL-12 receptor, IL12R, drives STAT4-mediated expression of the transcription factor TBET (5, 19–21). Re-exposure to antigen and IL-12 at the site of inflammation induces maturation, allowing production of cytokines including interferon-γ (IFN-γ). Autocrine IFN-γsignaling further contributes to maturation of Th1 cells *via* STAT1-mediated stabilization of *TBET* (22).

Th2

Type II responses to extracellular multicellular pathogens like helminths drive production of interleukin-4 (IL-4), which promotes STAT6-mediated transcription of *GATA3* and acquisition of a Th2 fate identity (**Figure 1B**) (6, 23). Peripheral maturation of Th2 cells permits secretion of a variety of cytokines, including IL-4 and interleukins 5 and 13 (IL-5, IL-13), which promote degranulation of eosinophils and mast cells. Dysregulated Th2 development this leads to hypersensitivity diseases, including asthma and allergy (24).

Th17

Th17 cells promote responses to extracellular single cell pathogens (Type III responses). They recruit neutrophils and macrophages to the site of inflammation and stimulate phagocytosis of the invading microbes (25). Differentiation of Th17 cells is guided by the transcription factor RORγt, which is expressed in response to the cytokines TGF-β and interleukin 6 (IL-6) (**Figure 1D**) (7–11). Priming of Th17 cells by IL-6 up-regulates the IL-23 receptor (IL23R). Peripheral maturation of Th17 cells is driven by interleukin-23 (IL-23), a heterodimer composed of the IL-12p40 subunit complexed with a p19 subunit (26). IL-23 and IL1-β can activate STAT4 in Th17 cells, leading to induction of TBET and IFN-γ. Co-production of IFN-$\gamma$is pathogenic in many autoimmune and immune mediated diseases, though it is protective in anti-tumor responses (discussed in greater detail below).

Th22

Th22 cells are critical regulators of epithelial barrier integrity and remodeling (27–30). Th22 cells secrete the cytokines interleukin-

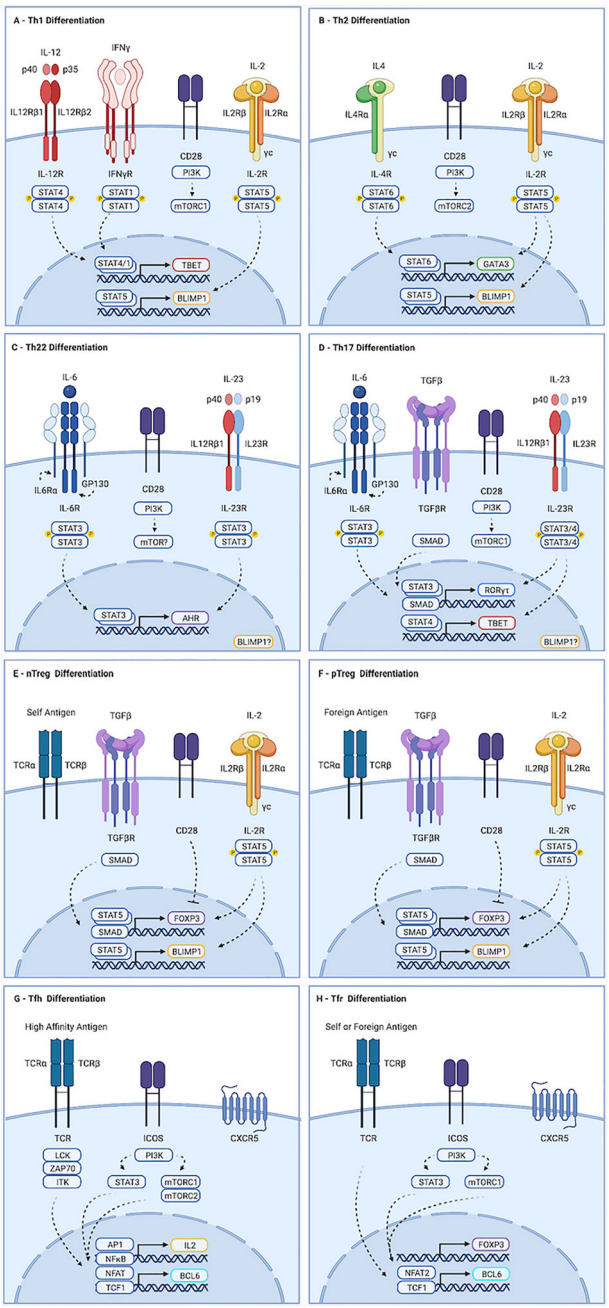

FIGURE 1 | Molecular determinants guiding CD4+ T cell differentiation. **(A)** Th1 development is initiated by IL-12 mediated STAT4 dimerization, driving expression of *TBX21*. Activation of mTORC1, primarily by CD28, is also required. Maturation occurs in response to IL-12, and to STAT1 activation by autocrine IFN-γ. **(B)** Th2 differentiation is driven by IL-4, which promotes STAT6-dependent transcription of GATA3, and by mTORC2. **(C)** Th22 cells form in response to IL-6 driven STAT3 activation, leading to production of *AHR*. The contributions of mTORC1 and mTORC2 to this process remain unclear. **(D)** IL-6 in the presence of TGF-b-mediated SMAD activation and strong activation of mTORC1 drives transcription of *ROR-γt*, which primes cells to acquire a Th17 fate. Maturation occurs downstream of IL-23 mediated STAT3 activation. IL-23 and IL-1b can also promote STAT4-mediated induction of *TBX21* in Th17 cells, leading to production of IFN-γ and GM-CSF. **(E, F)** nTreg cells develop in the thymus following exposure to self-antigen. pTreg cells develop in the periphery in response to foreign antigen. Both require TGF-β and IL-2 to activate SMAD and STAT5 signaling, respectively, which drive transcription of *FOXP3*. While strong activation of AKT and mTOR favors effector cell development, weak induction favors regulatory cells. **(G)** Strong TCR stimulation and ICOS ligation by dendritic cells promotes Tfh differentiation. ICOS activates AKT, but also drives STAT3-mediated production of *TCF1*, which promotes expression of *BCL6*. Maturation requires continued TCR and ICOS stimulation by B cells. Recently activated cells fated to become Tfh produce IL-2. Signaling is largely paracrine, and drives STAT5 mediated induction of *BLIMP1*, a mutual antagonist of BCL6, in non-Tfh. **(H)** Events guiding Tfr differentiation overlap substantially with those of Tfh. Tfr are thought to be derived from FOXP3-positive precursors. As with Tfh, ICOS-mediated STAT3-dependent induction of *TCF1* promotes *BCL6* expression. However, Tfr appear to depend exclusively on mTORC1, whereas Tfh require both mTORC1 and mTORC2. Similarly, induction of CXCR5 in Tfr appears to require NFAT2, which is dispensable for Tfh development. Created with BioRender.com.

22 (IL-22) and tumor necrosis factor alpha (TNF-α), but do not produce IL-17A or IFN-γ. Development of Th17 cells requires STAT3 activation by IL-6 in the absence of TGF-β (**Figure 1C**). IL-23 enhances production of IL-22 from Th22 cells. Though no single lineage specifying transcription factor has been identified, aryl hydrocarbon receptor (AHR) is required for their optimal development. Th22 cells also express TBET and RORγt, albeit at levels below those seen in Th1 and Th17 cells, and deletion of these transcription factors reduces Th22 numbers.

Tfh

Tfh cells orchestrate germinal center B cell responses. They are required for most class-switched affinity matured antibody responses (16). Strong antigenic stimulation and ICOS ligation by dendritic cells (DCs) drives expression of the transcription factor BCL6, the surface receptor PD-1, and the chemokine receptor CXCR5 (**Figure 1G**) (31–34). Primed cells, sometimes referred to as pre-Tfh, migrate to B cell follicles along a CXCL13 gradient. Maturation of Tfh cells occurs in response to sustained TCR and ICOS stimulation by B cells (31, 32, 35). Tfh develop in response to all major classes of pathogens. They are also seen in autoimmune diseases, and play physiologically relevant roles in response to some cancers (16). Abortive development of Tfh is seen even in response to organisms like Listeria monocytogenes that do not require or support germinal center reactions, suggesting early commitment to Tfh fate may be a universal feature of T cell activation (36).

REGULATORY CD4+T CELL SUBSETS

nTregs & pTregs

CD4+T cells are also essential for maintaining tolerance to self-antigens, commensal microbes and dietary antigens (37). Tolerance to self-antigens is mediated by natural regulatory T cells (nTreg), which develop in the thymus in response to moderately-high affinity antigen (38–40). Treg cells specific to foreign antigens develop in the periphery (pTregs) (41–43). While strong induction of the PI3K-AKT-mTOR pathway by co-stimulation and cytokine-mediated activation of STAT3, STAT4, or STAT6 promote pro-inflammatory outcomes, Treg fate determination is favored by TGF-β-mediated SMAD activity, STAT5 activation downstream of interleukin-2 (IL-2), and weak PI3K-AKT-mTOR stimulation (**Figures 1E, F**) (44, 45). Development of Tregs requires the transcription factor FOXP3. Suppression of inflammation by Treg cells is mediated by contact-dependent mechanisms, including CTLA and PD-1 ligation, and secretion of the cytokine interleukin-10 (IL-10). Importantly, their influence often manifests in unpredictable ways: In many contexts, Treg cells are required for optimal inflammatory responses (46).

Tfr

T follicular regulatory (Tfr) cells constrain germinal center (GC) processes (17, 47, 48). They develop in a wide range of inflammatory contexts, including infection, autoimmunity, and cancer. Tfr cells prevent production of auto-reactive antibodies and taper GC reactions during resolution of inflammation. As with Treg cells, the constraint provided by Tfr can also be required for optimal inflammatory responses (49, 50). Tfr are predominantly derived from nTreg cells, but can also develop from naïve precursors (47, 51, 52). The preponderance of naïve *versus* nTreg derived cells varies by tissue, with gut associated lymphoid tissues containing higher numbers of Tfr specific to foreign antigens and derived from naïve cells (53). Both BCL6 and FOXP3 are required for Tfr development, in parallel with their pro-inflammatory Tfh and suppressive Treg counterparts (**Figure 1H**) (47, 54). The molecular determinants guiding Tfr fate acquisition overlap substantially with that of Tfr, and include ICOS-mediated STAT3-dependent induction of TCF1, which promotes transcription of *BCL6* (55, 56). However, whereas NFAT2 is dispensable in Tfh, it is required by Tfr. Furthermore, while mTORC1 and mTORC2 contribute to Tfh development, Tfr appear to depend exclusively on mTORC1 (57, 58).

OVERLAPPING TRANSCRIPTIONAL NETWORKS

The historic progression of discoveries in the field of lymphocyte biology led to a model whereby one master regulator transcription factor is necessary and sufficient for one cell type. Master regulator transcription factors are commonly understood to be both necessary and sufficient for the acquisition of a cell fate. While this framework proved useful in identifying important transcriptional networks, further investigation revealed these factors are not sufficient for complete lineage programming and, in some cases, not absolutely required. For example, RORγt is insufficient for complete Th17 programming, Bcl6 is not sufficient for Tfh programming and ectopic Foxp3 expression confers only partial Treg identity (59, 60). Cooperation with additional transcription factors is necessary (61, 62).

Nor are these factors unique to specific populations. Indeed, there is substantial overlap in genetic programming between lymphocyte subsets. The Tfh compartment provides a useful illustration of this phenomenon. Tfh exhibit similar heterogeneity to that seen in non-Tfh effectors (63). During type I responses, Tfh cells express low levels of TBET and IFN-γ (31, 64). They express GATA3 and IL-4 during type 2 responses, and can produce IL-13 and IL-15 (65, 66). Tfh have also been shown to express RORγt and IL-17A (67–69). Production of these cytokines by Tfh guides isotype switching in B cells (70).

These transcriptional networks also regulate the function of regulatory cells. Tfr cells transiently express TBET during Type I responses. TBET, GATA3, and RORγt are expressed in a subset of FOXP3+ Treg cells termed effector regulatory T (eTreg) cells (48, 71). eTreg cells are enriched in peripheral tissues and are the primary mediators of suppressive functions. Expression is dependent on the local inflammatory context, correlates with the effector response, and is required to elicit optimal suppressive

capacity. Conversely, some eTreg cells demonstrate compromised suppressor function and promote anti-tumor immunity, including in colorectal carcinoma (CRC) (72). This phenomenon, discussed in greater detail below, also appears dependent on expression of canonical effector transcription factors.

Their influence extends beyond CD4+T cells. TBET is often expressed in B cells, and is required for optimal antibody production during Type I responses (73, 74). Both innate lymphoid cells and invariant natural killer T cells express TBET, GATA3, or RORγt depending on the inflammatory environment (75, 76). Thus, rather than functioning as bona fide master regulators, it appears these proteins may overlay context-specific programming onto multiple lymphocyte lineages.

As traditional lines blur, others come into focus. BCL6 and BLIMP1, encoded by the gene *PRDM1*, are mutually antagonistic transcription factors. Tfh express BCL6, and effector cells produce BLIMP1 (77, 78). This bifurcation begins soon after activation. A limited and discrete subset of activated cells produce the cytokine IL-2 (79). These cells are marked by early expression of BCL6 and supply the Tfh compartment (**Figure 1G**) (80). IL-2 signaling at early time points is largely paracrine, inducing BLIMP1 in IL-2-negative cells *via* STAT5. BLIMP1 inhibits BCL6 and IL-2, reinforcing a non-Tfh fate, and collaborates with TBET and GATA3 to promote Th1 and Th2 development and function (**Figures 1A, B**) (81–84).

The role of IL-2, STAT5 and BLIMP1 in Th17 and Th22 cells is less clear. In mice, activation of STAT5 downstream of IL-2 inhibits Th17 development (45). In humans, however, IL-2 is crucial for optimal Th17 responses (85). *In vitro* primed murine Th17 cells express little to no BLIMP1 (86). Early studies crossing CD4-Cre or proximal Lck-Cre mice to *PRDM1* floxed mice, leading to deletion of *PRDM1* in the thymus, revealed colonic inflammation mediated by increased Th17 numbers, suggesting BLIMP1 opposes Th17 function (87). However, thymic deletion generates multiple developmental defects. Peripheral deletion of BLIMP1 using distal Lck-Cre mice leads to a reduction in Th17 numbers and amelioration of Th17-mediated inflammation (88). In this study, IL-23 was shown to mediate induction of BLIMP1 *via* STAT3, suggesting BLIMP1 may play a role in Th17 maturation (**Figure 1D**). Unfortunately, the role of BLIMP1 in Th22 cells remains largely unexamined. Th22 cells notwithstanding, this evidence suggests BCL6 and BLIMP1 mark pro-inflammatory cells that primarily support humoral *versus* cellular responses across multiple inflammatory contexts.

Both Tfh and non-Tfh effector cells exist in mutual opposition with a FOXP3+ suppressive counterpart. Intriguingly, BLIMP1 is required for optimal production of IL-10 and suppression of peripheral inflammation by eTreg cells (71, 89, 90). Expression occurs downstream of TCR-mediated activation of IRF4, and STAT5 phosphorylation by IL-2 (**Figures 1E, F**) (87). In contrast, BCL6 is indispensable for Tfr. Thus BLIMP1 appears essential to most, and possibly all, peripheral subsets, while BCL6 is required by central, follicular T cells. It is therefore tempting to suggest the complexity of CD4+T cell differentiation may be collapsed into outcomes along two functional dimensions. One dimension describes a cooperative relationship between cells in distinct locations, the other an antagonistic relationship between cells occupying the same niche (**Figure 2**).

There is reason to suspect this model may hold some validity. The conceptual organization is reflected in the underlying transcriptional programming, and is highly generalizable to different inflammatory settings. Indeed, these four subsets may be a necessary result of the both function and architecture of the adaptive immune system. The ubiquity of host-pathogen interactions and commensal microbial communities coupled with the destructive nature of immune responses necessitate a system capable of both driving and suppressing inflammation. The low copy number and exceptional diversity of receptor clonotypes necessitate localization in specialized tissues that permit deep sampling of the repertoire. The need to modulate events at the site of inflammation requires cell types that egress from these tissues, while complicated highly compartmentalized processes like germinal center reactions require cells dedicated to central events. Given this, Tfh, Tfr, Treg and effector cells may represent fundamental functional states, while overlapping transcriptional networks modify these core states to suit specific inflammatory settings, thereby increasing the diversity of potential outcomes.

PLASTICITY OF EFFECTOR & REGULATORY CD4+T CELL SUBSETS

The transcriptional programs that guide these fate outcomes are not mutually exclusive, nor are they necessarily static. Lymphocyte phenotypes change at the population level as inflammatory responses mature. This is seen in multiple contexts, including the late emergence of distinct cytokine producing effector subsets, or the development of memory cells. These changes can be accomplished *via* two non-exclusive mechanisms; selective amplification of underlying heterogeneity, and the conversion of cells from one phenotype to another.

Data suggests the dynamic heterogeneity of effector responses may in part be due to lineage plasticity (**Figure 2**). Naïve cells primed *in vitro* under conditions promoting Th1, Th2 or Th17 differentiation can acquire different phenotypes upon re-stimulation (91). Th17 cells appear to be particularly adept at acquiring the functions and phenotypes of other lineages (92–94). De novo co-expression of IFN-γ by Th17 cells occurs *in vivo* and represents a key source of IFN-γ in multiple pathologies. *In-vitro* generated Th17 cells can convert into IL-17A–negative IFN-γ producers in response to STAT4 activation downstream of IL-23 following adoptive transfer (95–97). At least one study utilizing IL-17A fate reporter mice suggests trans-differentiation into Th1 cells may also occur *in vivo* (97). TGF-β, a potent repressor of Th22 cells, can also induce AHR and IL-22 in Th17 cells (98). Co-expression of GATA3 and Th2 cytokines in Th17 cells is also documented (99).

Expression of TBET, GATA3, and RORγt, and their associated cytokines, by Treg and Tfh cells is variably described as plasticity in the literature. This terminology is somewhat controversial. Co-expression of canonical effector

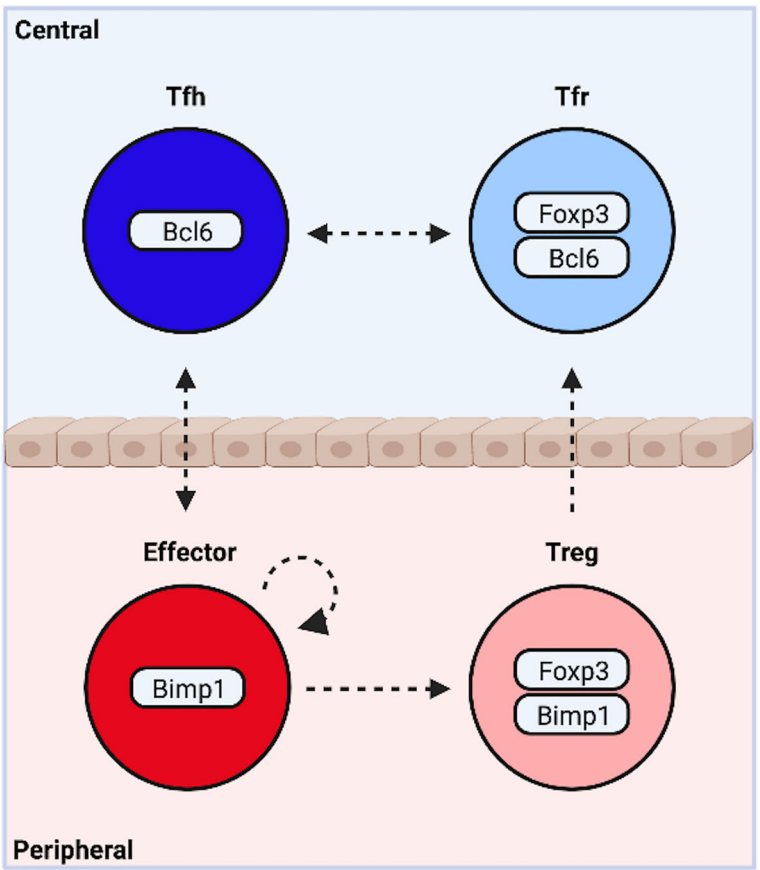

FIGURE 2 | Functional Bifurcations Among CD4+ T Cells. Following activation, naïve cells are programmed to modulate central or peripheral processes. Similarly, activated cells either drive or suppress inflammation. These functional bifurcations are coincident and sufficiently independent to allow for the simultaneous generation of all four potential outcomes. Pro-inflammatory T follicular helper (Tfh) cells organize germinal center responses, while traditional non-Tfh effector subsets promote cellular responses at the site of inflammation. Both exist in mutual opposition with a suppressive counterpart. T follicular regulatory cells (Tfr) modify central events, while peripherally induced regulatory T cells (pTregs) suppress peripheral inflammation. The dynamic heterogeneity of CD4+ T cell responses may be due in part to plasticity between subsets (indicated by arrows). Created with BioRender.com.

transcriptional modules is required for optimal function and may simply represent normal developmental maturation. De novo transition from one effector module to another *in vivo* has not been shown. However, it seems reasonable to consider pro-inflammatory eTreg cells in CRC an example of plasticity. While these cells do not fully extinguish FOXP3, they alter their core transcriptional networks and adopt a fundamentally different functional state. Certainly this represents meaningful functional plasticity, if not bona fide lineage conversion. Nevertheless, the role of plasticity in driving the heterogeneity seen within Treg and Tfh populations remains murky. Studies addressing the duration and stability of these states *in vivo* are needed.

More substantial evidence indicates plasticity between effector, Treg, Tfh and Tfr lineages may also occur (**Figure 2**). nTreg cells supply the majority of the Tfr compartment. Some studies suggest Tfr may convert into Tfh *in vivo*, and Tfh can be converted into Tfr *in vitro* (53, 100–102). Fate mapping indicates former IL-17A-producing cells can transition into pTreg cells downstream of TGF-β-mediated induction of AHR (103). Lineage reporter mice also suggest Treg cells can lose FOXP3 and develop into pro-inflammatory ex-Tregs displaying Th1 or Th17 effector phenotypes (104, 105). Conversion of effector cells to Tfh appears negligible in many contexts. However, former IL-17A producing cells can exhibit a Tfh-like phenotype and guide IgA production in Peyer's Patches (106). Similarly, while deletion of IL-2 producing Tfh precursors does not affect Th1 and Th2 numbers, it can lead to a reduction in Th17 cells (80). These findings suggest Tfh and Th17 development may be uniquely related. Peripheral Tfh-like cells may also indicate overlap between Tfh and effector lineages (107, 108). These cells exhibit qualities consistent with both effector and Tfh lineages, organize ectopic lymphoid tissues, and are capable of providing help to B cells. However, it remains unclear if they represent Tfh that migrated to the periphery, effectors that acquired a Tfh-like phenotype, or the *de novo* generation of an intermediate phenotype. Together these data suggest limited plasticity between Tfh, Tfr, Treg and effector cells is possible. Notably, interconversion between Tfh and Treg cells, and effector and Tfr

cells, has not been observed, suggesting plasticity may be restricted along individual functional dimensions.

The cellular sources and molecular mechanisms underlying this apparent lineage plasticity remain uncertain. Many studies indicate mature Treg, Tfh and effector cell phenotypes are remarkably stable (109–113). In contrast, substantial evidence supports the existence of a window early in T cell differentiation in which activated cells maintain a state of pluripotency. Limiting dilution adoptive transfer experiments indicate single naïve $CD4^+$T cells can give rise to both Tfh and effector cells (33). Recently activated cells exhibit epigenetic instability that is extinguished upon initiation of cell cycle progression and developmental maturation (114, 115). Furthermore, some cells transiently co-express multiple lineage programming transcription factors shortly after activation (116, 117). Indeed, this phenomenon complicates interpretation of lineage reporter experiments and may underlie results initially interpreted as supporting conversion of Treg cells to effectors (109, 110). Co-expression is likely mediated by convergent signaling events. Th17 development, in particular, exhibits substantial overlap with other lineages. TGF-β is required for Th17 and regulatory T cell development. STAT3 is required by Th17, Th22, Tfh and Tfr cells. STAT4 promotes IFN-γ production in both Th17 and Th1 cells. Thus plasticity between functional states may plausibly result from incomplete development following cell priming, and partial overlap between competing developmental pathways.

Caution, however, is warranted in interpreting data regarding cellular plasticity. Many studies utilize *in vitro* generated cells and adoptive transfer techniques. But *in vitro* polarized cells are not equivalent to mature *in vivo* effectors, and adoptive transfer into inflamed hosts may not reflect normal physiologic processes. Even *in vivo* experiments utilizing lineage reporter mice suffer from limitations. The fidelity with which a reporter gene indicates a given cell fate can be compromised, For example, while the vast majority of IL17A producers are Th17 cells, some Tfh produce IL17A, confounding efforts to address the relationship between these cells. In addition, transient expression can permanently activate a reporter construct without stable adoption of a cell fate. However, even with these limitations in mind, the abundance and diversity of data supporting plasticity strongly suggest it is both real and relevant to many physiologic and pathophysiologic contexts, including CRC.

COLORECTAL CANCER

Colorectal carcinoma (CRC) is the third most frequently diagnosed cancer in both men and women in the United States, with >140,000 cases diagnosed each year (CDC). It is also the third leading cause of cancer deaths, depriving >50,000 patients of their lives each year. CRC represents 98% of colonic cancers, and the WHO recognizes 6 distinct tumor subtypes. Most tumors develop as a result of sequential mutations driving progression along multiple potential pathways (118). Chronic inflammation is a well-recognized driver of tumorigenesis (1). Microbial dysbiosis is common in colorectal carcinoma, and may also contribute to tumorigenesis (119, 120). In the colon, Th1, Th17, Th22, pTreg and nTreg cell subsets exist in a state of dynamic equilibrium at epithelial barrier sites. Tfh additionally modulate colonic inflammation *via* the organization of ectopic lymphoid structures. Dysregulation of these cell populations can lead to chronic inflammation and dysbiosis. Immunotherapy therefore holds tremendous promise in treating CRC **(Figure 3)** (121).

ROLE OF EFFECTOR $CD4^+$T CELL SUBSETS IN COLORECTAL CANCER

Increased tumor infiltration by Th1 cells correlates with better prognosis (122, 123). This protection is likely mediated by the anti-proliferative, pro-apoptotic and anti-angiogenic actions of IFN-γ, as well as through enhanced recruitment of cytotoxic CD8 T cells (124). Th17 and Th22 cells, in contrast, are elevated in advanced disease and correlate with poor prognosis (125). Limited production of IL-22 can protect against genotoxic stress, but prolonged exposure drives uncontrolled proliferation of colonic epithelium, and promotes cancer stemness and chemo-resistance (126–129). IL-17A directly stimulates tumor growth and progression (130–132). IL-17A also stimulates angiogenesis *via* production of VEGF (133). Tumorigenic Th17 cells accumulate in response to IL-23, which is produced following microbial colonization of tumors due to barrier defects (134). Evidence indicates effector lineage plasticity may contribute to the pathogenesis of CRC. Th1-like IFN-γ+ Th17 cells exhibit potent anti-tumor properties (135, 136). In contrast, induction of IL-22 in Th17 cells downstream of TGF-β and AHR ligand promotes tumorigenesis (98).

While some microbial species promote tumorigenic Th17 cells, others predict enhanced responses to chemo- and immune-therapy (137–140). Colonization by protective organisms is associated with increased numbers of Tfh and the development of ectopic lymphoid structures (141). Accumulation of Tfh is associated with prolonged survival in humans (142). In mouse models, both Tfh and B cells are required for the protective effects conferred by these microbial species. Intriguingly, Tfr cells also accumulate at tumor sites, and may regulate Tfh functions (143).

ROLE OF REGULATORY $CD4^+$T CELL SUBSETS IN COLORECTAL CANCER

Treg cells exhibit conflicting roles CRC. Preclinical and clinical studies indicate Treg cells suppress effector T cell-mediated immune responses to cancer (144, 145). Treg infiltration in CRC has been associated with tumor progression, lymphatic invasion and metastasis (146–148). However, eTregs, which are abundant in the intestine, can also promote anti-tumor immunity to, and induce regression of, intestinal cancers (149, 150). Indeed, tumor infiltrating Treg cells are associated with improved prognosis in many studies (72, 151–153).

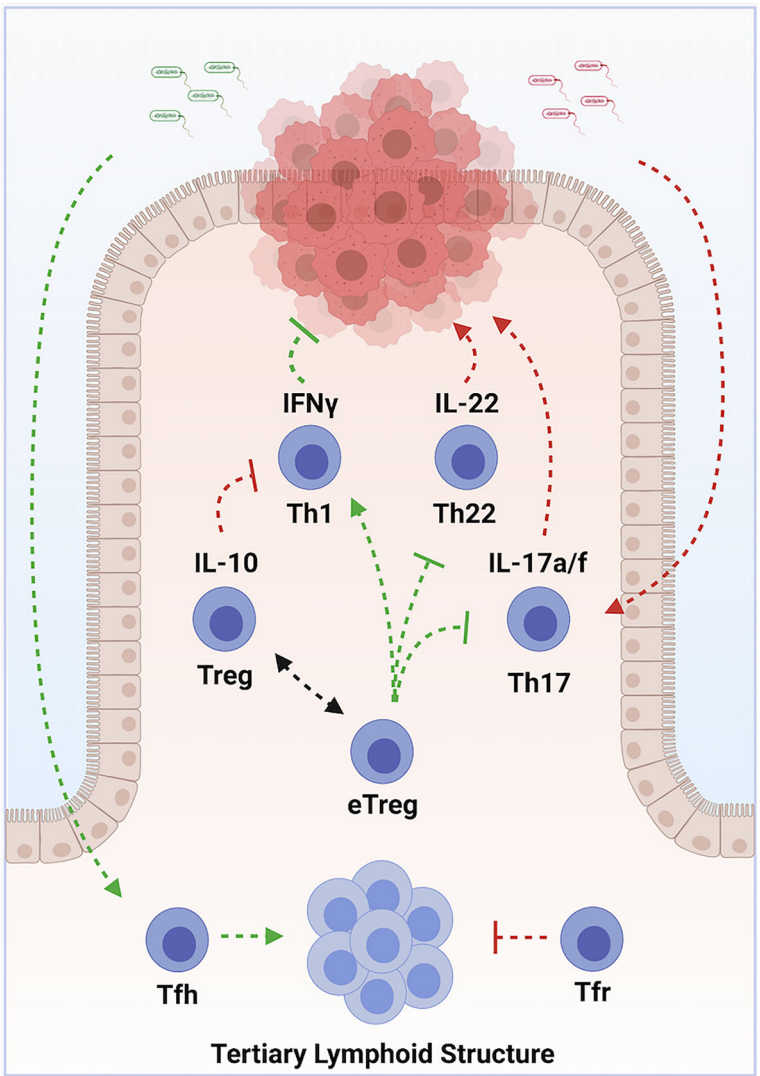

FIGURE 3 | Multilayered roles of various subsets of CD4+ T Cells in Colorectal Carcinoma. Chronic inflammation, driven by Th17 cells in response to commensal organisms, promotes tumor development. Sustained exposure to IL-22, produced by Th22 cells, contributes to tumorigenesis. Th1 cells promote tumor cell destruction via production of IFN-γ. Treg cells oppose tumor development by suppressing chronic inflammation, but contribute to progression by opposing optimal tumor responses. Some types of pro-inflammatory eTreg cells, in contrast, promote tumor immune responses. Tumor colonization by protective commensal species drives accumulation of Tfh, which organize tertiary lymphoid structures. These structures enhance tumor immune responses and predict responses to chemo- and immune-therapeutics. Arrows indicate positive modulation; perpendicular lines indicate inhibitory relationships. Green indicates an overall anti-tumor effect, while red indicates an overall pro-tumorigenic effect. Created with BioRender.com.

These discordant results may be due to heterogeneity within the Treg compartment. During inflammatory responses, Treg cells can be divided into 3 main compartments; Suppressive CD45RA+ FOXP3-high naïve-like cells, suppressive CD45RA– FOXP3-high eTreg cells, and pro-inflammatory CD45RA– FOXP3-low eTreg cells. ROR-γτ+ IL-17A+ FOXP3-high eTreg cells exhibit potent T cell suppression, but fail to restrain innate inflammation. They increase with tumor stage in human CRC, and promote tumor development in colitis-associated mouse models (154, 155). In contrast, FOXP3-low eTreg cells exhibit reduced T cell suppressive capacity and promote anti-tumor immunity (156, 157). Indeed, tumors harboring FOXP3-low eTreg cells that secrete IL-17A and/or IFN-γ are associated with significantly better prognosis (72). Tumors containing these cells exhibit increased expression of IL-12, has been speculated promote acquisition of this pro-inflammatory state. Cell lineage and target antigen may also influence this functional divide: While TCR sequences of Th17-like eTreg cells overlap with pTreg cells, Th1-like eTregs appear to be thymically derived (158).

TARGETING SUBSETS OF CD4+T CELLS IN CRC: THERAPEUTIC IMPLICATION

Treatment of CRC is guided by tumor stage and grade, but commonly involves surgical resection (159). Peri-operative chemotherapy is the standard of care for Stage III and IV tumors, and may be considered for stage II tumors. Established nearly two decades ago, Oxaliplatin, 5-fluorouracil and leucovorin (FOLFOX) still remains the first line regimen, although inhibition of VEGF or Ras signaling may offer statistically significant but limited improvement of outcomes in some cases. However, overall survival of localized, regional and metastatic CRC is only 91%, 72% and 13%, respectively (159). Therefore, additional therapeutic options are needed for therapeutic intervention.

Given the importance of T cells in modulating its pathophysiology, therapeutic approaches targeting lymphocyte function represent a promising addition to CRC treatment regimens. Defective mismatch repair (dMMR) leads to an abundance of tumor neoantigens. dMMR tumors are heavily infiltrated by Th1 cells and confer improved prognosis (122). Furthermore, dMMR tumors commonly exhibit elevated expression of PD-1 and PD-L1. Increased neoantigen burden and PD-1/PD-L1 mediated immune evasion suggest these tumors may be susceptible to checkpoint inhibition. Indeed, early trials examining the efficacy of PD-1 inhibition in dMMR tumors generated promising results (160). However, dMMR tumors are more commonly identified in earlier stages, and represent only 3-6% of advanced cases. Interventions targeting lymphocyte functions independent of checkpoint blockade are likely required for therapeutic efficacy in the majority of tumors.

Three general approaches to targeting CD4+T cells could be considered for CRC therapy: **A.** Direct inhibition CD4+T cell-derived tumor promoting factors. **B.** Interventions manipulating heterogeneity within CD4+T cell functional categories (Th1, Th17, Th22, Treg, eTreg, Tfh, Tfr etc.). **C.** Manipulation of the colonic microbiota. Importantly, successful implementation of each approach is currently impeded by an incomplete understanding of the relevant biology. Limited insight confers a limited capacity to intervene.

Direct Inhibition of CD4+T Cell-Derived Tumor Promoting Factors

Direct inhibition of effector cytokines known to drive tumor progression may improve outcomes. The suppressive cytokine IL-10 is a potential target to elicit a robust anti-tumor immunity. Serum IL-10 is positively correlated with tumor stage and negatively correlated with prognosis in CRC patients (161, 162). IL-10 is increased in the CRC microenvironment, and IL-10RA levels correlate with KI67 staining (163). IL-10 blocking antibodies drive accumulation of tumor-infiltrating lymphocytes (TILs), release of granzyme B, and tumor cell necrosis in an *in vitro* human CRC culture system (164). Systemic blockade of IL-10 or IL-10RA, however, carries substantial risk. Targeted approaches may be required. Intra-tumor injection of lentivral vectors encoding IL-10 shRNA reduces IL-10 expression and potentiates bone marrow derived dendritic cell vaccine efficacy in a mouse model of CRC (165). IL-10 shRNA alone was not effective, and IL-10 production by T cells was unaffected. Caution, however, is warranted. Mouse models indicate IL-10 can actually augment cancer responses. Indeed, exogenous IL-10 is being investigated as a therapeutic option in multiple cancer types, including CRC (166, 167). Identification of the specific cellular sources of IL-10 that inhibit tumor immunity and targeted suppression of IL-10 production in those cells, or inhibition of IL-10RA signaling in tumor cells, may offer improved safety and efficacy. Regardless, the seemingly contradictory findings surrounding IL10 make it abundantly clear that our understanding of the underlying biology is profoundly limited. It is difficult to predict outcome of actions without an accurate model of what is being acted upon.

Given the roles of Th17 and Th22 cells in promoting tumor development, IL-17A, IL-17F, and IL-22 are also promising targets in CRC. Deletion of Il17a or Il17f reduces tumor development in an APC-driven mouse model of CRC (130, 168). Blockade of the IL-17/IL-17RA axis may also improve the efficacy of anti-VEGF therapies. Anti-IL22 antibodies inhibit CRC cell proliferation *in vitro* (169). Gene therapy designed to drive expression of IL-22BP, a secreted binding protein that inhibits IL-22 signaling, reduces tumor burden in mice (170). Again, caution is warranted as some studies indicate disruption of Th17 and Th22 cell function can promote tumor development and progression (171). The cause of these disparate outcomes is not fully understood, but may relate to the specific mechanism of CRC pathogenesis and the role of T cells in promoting appropriate *versus* chronic, dysregulated inflammatory responses. Further elucidation of the role of these cells in CRC is required.

Interventions Manipulating Heterogeneity Within CD4+T Cell Functional Categories

T-bet, GATA3 and RORγt are key regulators of lymphocyte behavior. Interventions designed to modulate these factors could influence functional heterogeneity within multiple lineages simultaneously. They are potentially powerful therapeutic targets. TBET and RORγt are particularly important in CRC. Expression of T-bet in both effector and regulatory lineages correlates with enhanced tumor response and improved outcome. RORγt exhibits more nuanced effects. Effector and regulatory cells that express RORγt promote tumor progression. Co-expression with T-bet, however, confers potent anti-tumor activity. Interventions should be designed to promote activation of the T-bet transcriptional program and minimize the proportion of RORγt single-positive cells. Complete abrogation of RORγt, however, could prove counterproductive. A balance may have to be found.

The mechanisms by which to exert this pressure must also be determined. TGF-β is one potential source of influence. It promotes Treg differentiation, type III (RORγt-mediated) inflammation, and inhibits TBET. Empiric evidence indicates potential utility. Elevated TGF-β is a marker of poor prognosis in CRC (172). Upregulation of Smad7, a negative mediator of

TGF-β signaling, drives accumulation of TBET+ Th17 cells and improves tumor responses in a mouse model of CRC (136). Furthermore, antibody-mediated inhibition of TGF-β signaling in a mouse model of CRC promotes a rapid and long lasting Th1 response far more potent than checkpoint inhibition and capable of preventing metastasis (173). In mice with pre-existing metastases, TGF-β blockade renders tumors susceptible to checkpoint inhibition. Disruption of TGF-β signaling is an excellent candidate for therapeutic intervention in CRC.

The IL-6/STAT3 pathway is another promising target. IL-6 favors RORγt and is aberrantly activated in many tumor microenvironments. Myeloid-derived soluble IL-6 receptor can blunt Th1 and CD8 responses (174, 175). Concurrent inhibition of IL-6 and PD-1 leads to elevated Th1 levels and enhances response to checkpoint blockade in multiple mouse models (176, 177). Blockade of IL-6 signaling may yield similar effects in CRC. Pharmacologic inhibition of SIRT1, required for dimerization of STAT3 downstream of IL-6, reduces Th17 numbers in CRC patients and tumor development in mice (178). Care must be taken, however, to examine potential effects on dual Tbet+ RORγt+ cells when blocking this pathway.

IL-23, which also signals through STAT3, promotes tumorigenic Th17 cell differentiation in CRC. Blockade of IL-23 may therefore blunt pathogenic Th17 differentiation and, as with STAT3 inhibition, redirect developing cells to a Th1-like phenotype. But IL-23 is a member of the IL-12 family of cytokines and can promote IFN-γ production in Th17 cells *via* STAT4. Interference with this pathway also has the potential for unintended consequences.

Direct administration of IL-12 can promote type I (TBET-mediated) responses. When administered to mice harboring a toxigenic strain of *B. fragilis*, IL-12 monotherapy leads to increased tumor CTL numbers, though no change in tumor burden was seen. Co-administration of IL-10 also reduces tumor Th17 numbers, and dramatically improves tumor burden (167). This cooperative effect is promising, and suggests additional interactions could be similarly exploited. But its mechanism is incompletely understood, and it is difficult to anticipate which additional combinations will prove beneficial.

Selective amplification of Tfh may represent an alternative potential therapeutic avenue. Given its role in Tfh development, ICOS stimulation may promote accumulation of Tfh-like cells and development of ectopic lymphoid structures in CRC. ICOS levels correlate with survival in CRC, while its expression is reduced in distant metastases (179). ICOS ligation may additionally modulate the effector response. Intratumor ICOS+ T cells exhibit elevated TBET and IFN-γ expression, and ICOS-based chimeric antigen receptor T cells generate anti-tumor bipolar TBET+ RORγt+ effectors cells (179, 180).

Exploitation of Treg biology represents one of the most promising mechanisms for combatting CRC. Tumors can be classified into two groups based on the relative abundance of FOXP3-high and FOXP3-low eTregs. Infiltration by FOXP3-low eTregs confers significantly better prognosis (72). Conversion of FOXP3-high eTreg cells to pro-inflammatory FOXP3-low eTregs would release the pressure pro-inflammatory cells and potentiate interventions design to promote them. Unfortunately, very little is known about the signaling and transcriptional events that guide this transition. Both IL-12 and TGF-β are elevated in CRC tissue infiltrated by FOXP3-low eTregs, suggesting these factors could promote acquisition of a pro-inflammatory phenotype. Augmentation of IL-12 signaling may therefore benefit Treg responses as well, but enhanced TGF-β signaling may have undesirable effects on the balance of Th17 and Th1 cells, and could potentially increase total Treg numbers. Similarly, BLIMP1 has been shown to prevent production of inflammatory cytokines in RORγt+ Treg cells. But inhibition of BLIMP1 would be expected to have deleterious effects on the effector response. As with other proposed interventions, targeted approaches localizing effects to specific cell populations might be required. Bi-specific antibodies, for example, could be used to block signaling events in specific subsets of T cells, including Tregs. Even so, these interventions are highly speculative. Our understanding of eTreg cell states is limited. The molecular determinants guiding their development must be elucidated before viable interventions can be developed.

Manipulation of the Colonic Microbiota

Tumors preferentially develop in the distal colon and rectum, which harbors the highest concentration of microbial species (181). Early studies using germ free animals confirmed a role for microbial organisms in the development of CRC (182). 16S rRNA sequencing has identified differences in fecal and tumor mucosal microbiota between CRC patients and healthy controls (183). This dysbiosis is transferable, as fecal transplantation from tumor-bearing mice to conventionalized germ-free mice results in increased colon inflammation and tumorigenesis (184). Fecal transplants from CRC patients into germ-free mice also results in increased tumor burden (185). Interestingly, microbial patterns and signatures vary substantially between colon cancer tissue and adjacent non-malignant colon tissues (186). Thus, localized dysbiosis of intestinal microbiota can trigger inflammation leading to an increased permeability of the epithelial barrier and enhanced bacterial translocation, which in turn, promotes chronic inflammation by provoking a persistent immune response. This generates reactive oxygen and nitrogen species that lead to oxidative stress, DNA damage, and abnormal cellular proliferation, eventually culminating in the development of CRC.

While disparities between studies preclude the identification of a CRC-specific microbiome, substantial evidence supports causal roles for some species, including *Fusobacterium nucleatum* and *Bacteroides fragilis*. *Fusobacterium* is enriched in human CRC mucosa, predicts poor response to chemotherapy and prognosis and promotes tumor development in mice (183). Colonization persists even in distal metastases (187). Toxigenic *Bacteroides fragilis* is also enriched in CRC lesions, and promotes tumor development in mice. Interestingly, while toxigenic strains of *B. fragilis* promote tumor development, non-toxigenic strains confer protection by promoting infiltration of Tfh and development of ectopic lymphoid structures (137, 141).

Interventions should be designed to alter microbial populations to promote a beneficial immune response. Due to

the localized nature of dysbiosis, direct sampling of colonic mucosa may be required to identify relevant organisms. Species level identification may not be sufficient given the strain dependent effects of *B. fragilis*. In addition, commensal organisms form a complicated, inter-dependent network. Manipulations affecting single species could prove insufficient to alter function. More sophisticated approaches should be considered. The potential therapeutic utility is apparent but, as before, our ability to exploit this potential is hampered by an abridged appreciation of biology.

CONCLUSION & PERSPECTIVE

The gastrointestinal (GI) tract is a large surface lined by a single layer of epithelium exposed to trillions of microbes and innocuous substances from the diet. It harbors the largest collection of immune cells in the body. The gut immune system maintains a state of dynamic equilibrium, monitoring luminal contents to sustain tolerance to dietary and commensal antigens while retaining the ability to rapidly respond to invading pathogens. $CD4^+$ T cells are essential for both arms of this delicate balancing act. In recent years, increasing awareness of the diversity of $CD4^+$ T cell form and function, and the relationships between these cells, has exposed limitations to the established paradigm. Many fundamental questions will have to be addressed before a new model can be developed. The increasing complexity of lineage diversity and functional heterogeneity have made these questions harder to answer. But they must be answered. $CD4^+$ T cells are a tremendously powerful tool. It will be very difficult to wield this tool for clinical benefit without understanding how it works.

A deeper understanding of the intersection between $CD4^+$ T cells and CRC is also needed. What underlies the seemingly contradictory roles played by some cells? Both nTregs and pTregs are beneficial in controlling the inflammation that serves as the nidus for CRC, but are harmful after inflammation leads to cancer. And yet some Tregs shed their suppressive role, become eTregs, and participate in anti-cancer immune responses, much as effector cells do. Similarly, Th17 and Th22 cells promote pathogen clearance and epithelial barrier function, respectively. Effective clearance and barrier integrity minimize exposure of epithelial cells to noxious inflammatory stimuli. But the sustained activity of these cells promotes tumor development. In contrast, Th17 cells that also express TBET are an important component of anti-cancer responses. Similarly, the concerted influence of follicular T cells and the colonic microbiota can both promote and oppose CRC. The development of these populations, and their influence on inflammatory responses to CRC, must be resolved in greater detail so that they can be exploited to improve disease outcomes.

Regardless of the target, interventions must be designed with pleiotropic, combinatorial effects in mind. Independent effects on both effector and regulatory cell populations must be examined carefully. Potential effects on follicular T cells should also be considered, as should interactions with innate, epithelial and tumor cells. Given potentially counterproductive effects on disparate cell types, targeted interventions may afford enhanced efficacy.

In summary, the manipulation of $CD4^+$T cells represent a potentially powerful tool in CRC. Current attempts are limited by an incomplete understanding of the underlying biology. A more nuanced understanding of lineage diversity and plasticity in inflammatory responses during CRC is needed. The contributions of specific cell populations must be better delineated to understand the best way to implement therapeutic approaches. The relationships between these cells, and the molecular determinants guiding their development, must be understood. Much remains to be done. But we are close enough to see the reward far outweighs the cost.

AUTHOR CONTRIBUTIONS

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

FUNDING

This study has been supported by a Career Development Award grant to RB (Corresponding author) from Crohn's and Colitis Foundation of America (Identifier# 347717) and by start-up funds from the University of Alabama School of Medicine (to RB).

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ORIGINAL RESEARCH
published: 06 September 2021
doi: 10.3389/fimmu.2021.714244

PAC1 Receptor Mediates Electroacupuncture-Induced Neuro and Immune Protection During Cisplatin Chemotherapy

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Platinum-based chemotherapy is an effective treatment used in multiple tumor treatments, but produces severe side effects including neurotoxicity, anemia, and immunosuppression, which limits its anti-tumor efficacy and increases the risk of infections. Electroacupuncture (EA) is often used to ameliorate these side effects, but its mechanism is unknown. Here, we report that EA on ST36 and SP6 prevents cisplatin-induced neurotoxicity and immunosuppression. EA induces neuroprotection, prevents pain-related neurotoxicity, preserves bone marrow (BM) hematopoiesis, and peripheral levels of leukocytes. EA activates sympathetic BM terminals to release pituitary adenylate cyclase activating polypeptide (PACAP). PACAP-receptor PAC1-antagonists abrogate the effects of EA, whereas PAC1-agonists mimic EA, prevent neurotoxicity, immunosuppression, and preserve BM hematopoiesis during cisplatin chemotherapy. Our results indicate that PAC1-agonists may provide therapeutic advantages during chemotherapy to treat patients with advanced neurotoxicity or neuropathies limiting EA efficacy.

Keywords: neuromodulation, chemotherapy, immunosuppression, hematopoiesis, neurotoxic, electroacupuncture

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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 Immunology

Received: *24 May 2021*
Accepted: *12 August 2021*
Published: *06 September 2021*

Citation:
Li S, Huang J, Guo Y, Wang J, Lu S, Wang B, Gong Y, Qin S, Zhao S, Wang S, Liu Y, Fang Y, Guo Y, Xu Z and Ulloa L (2021) PAC1 Receptor Mediates Electroacupuncture-Induced Neuro and Immune Protection During Cisplatin Chemotherapy. Front. Immunol. 12:714244. doi: 10.3389/fimmu.2021.714244

INTRODUCTION

Platinum-based chemotherapy, such as cisplatin, carboplatin, and oxaliplatin are widely used in multiple tumors (1–3), but they produce severe side effects including neurotoxicity (4), anemia (5), immunosuppression (6), nephrotoxicity (7), and gastrointestinal toxicity (8). Cisplatin-induced neurotoxicity has been associated with pain neuropathies and deficient neuromodulation contributing to multiple disorders. Cisplatin-induced immunosuppression limits anti-tumor immune responses, treatment efficacy, and increases the risk of infections (9, 10). Thus, chemotherapy is often combined with complementary treatments to prevent immunosuppression,

such as EA or treatment with stimulating factors such as colony stimulating factor (CSF) to promote myeloid cell differentiation in the bone marrow (BM) (11). However, CSF is not effective in restoring the proliferation of hematopoietic stem/progenitor cells (HSPCs), induces multiple complications such as bone pain (12), and increases the risk of tumor growth and metastasis by inducing myeloid-derived suppressor cells (13, 14). Thus, there is an unmet clinical need to find safe and effective adjuvant treatments for chemotherapy-induced neurotoxicity and immunosuppression.

Acupuncture is a common complementary and integrative therapy as proved by profuse clinical studies and used by millions of people worldwide (15). The World Health Organization recommends acupuncture to prevent toxicity and leukopenia during radio- and chemotherapy (16). Acupuncture is safe and its effects have been confirmed in multiple clinical trials with different types of tumors including breast (17) and lung cancer (18). Systematic analysis of 31 clinical trials showed that acupuncture alleviated chemotherapy-induced myelosuppression (leukopenia, hemoglobin, and platelet reduction) and preserved immune responses including IL-2 production and lymphocyte counts in lung cancer patients during chemotherapy (19). A pilot, randomized, sham-controlled clinical trial also showed that acupuncture reduced chemotherapy-induced leukopenia in patients with ovarian cancer (20). However, the use of acupuncture is still debated because its inefficacy in some patients. Despite its clinical implications, the mechanism of acupuncture to treat chemotherapy-induced leukopenia is still unknown, and thus its efficacy in many patients but not in others with similar symptoms.

Although the mechanism of acupuncture is unknown, multiple studies reported the critical role of the sympathetic nervous system to modulate BM hematopoiesis. Hematopoietic stem (HSCs) and HSPCs reside in specific BM niches with a complex cellular and molecular environment including mesenchymal stem cells (21), osteoblasts (22), endothelial cells (23), and sympathetic projections (24, 25). Among these, the sympathetic projections are the most critical factors orchestrating BM cell proliferation, differentiation, and egress (24–26). Sympathetic terminals produce multiple factors orchestrating different cell types depending on the physiologic needs. Neurogenic factors produced by these terminals induce different factors such as catecholamines (dopamine and epinephrine) can activate HSCs proliferation and differentiation. The sympathetic system also modulates BM hematopoiesis indirectly by evoking multiple cells to produce stimulating factors, such as granulocyte-colony stimulating factor, which enhances hematopoietic cell proliferation and migration (24). Conversely, sympathetic signals can also inhibit CXCL12 production in mesenchymal stem cells and osteoblasts to induce BM egress of HSCs (27–29). Thus, sympathetic innervations induce complex signals to orchestrate the proliferation, differentiation, and egress of multiple cell types at different levels depending on the physiological needs (27–29). This complexity has made it difficult to design alternative treatments for patients with limited response to acupuncture.

Pituitary adenylate cyclase activating polypeptide (PACAP) is a multifunctional neuropeptide of the glucagon-secretin-vasoactive intestinal peptide (VIP) family, with 67% similarity to VIP (30). There are two isoforms of PACAP: PACAP27 and PACAP38, with the latter being dominant in mammalian tissues in most physiological and pathological conditions (31–33). However, several studies found PACAP levels of different tissue samples are altered under pathological conditions, with lower PACAP immunoreactivity in different human samples of primary small cell lung cancer, colon, and kidney cancers as compared to healthy tissues, while higher PACAP27 immunoreactivity was found in prostatic cancers as compared to benign prostatic hyperplasia (32, 33). PACAP binds to three G-protein coupled receptors, a higher affinity PACAP-specific receptor (PAC1), and two VIP/PACAP receptors (VPAC1 and VPAC2) with similar affinity for VIP and PACAP (34). PACAP has been found to be involved in neuroprotection, prevents apoptosis (35, 36), promotes cell proliferation (37), neurogenesis and axonal regeneration in the central and peripheral nervous systems (38, 39), and modulates immune and inflammatory responses (40, 41). We previously reported that PACAP is secreted by sympathetic nerve endings projected into the BM, and can modulate HSPCs proliferation *via* PAC1 signaling (42).

Multiple studies have shown that cisplatin chemotherapy causes neurotoxicity and multiple neuropathies (43, 44). We reasoned that this neurotoxicity can prevent sympathetic neuromodulation of BM hematopoiesis and thereby induce immunosuppression and leukopenia. In line with our hypothesis, BM hematopoiesis is prevented by neurotoxic agents such as 4-methylcatechol or glial-derived neurotrophic factor and chemotherapy-induced BM nerve injury impairs hematopoietic regeneration (45). Thus, we reasoned that electroacupuncture (EA) may activate BM sympathetic fibers, and protect them from chemotherapy-induced neurotoxicity to preserve hematopoiesis during chemotherapy. Here, we analyze whether EA induces sympathetic neuroprotection and preserves BM hematopoiesis in normal and cancer mice with Lewis lung carcinoma (LLC) cells. We also identify the neurogenic factor that mediates the protective effects of EA during chemotherapy.

Abbreviations: ANOVA, Analysis of variance; BM, bone marrow; Bdnf, brain derivedneurotrophic factor; CLPs, common lymphoid progenitors; CSF, colonystimulating factor; CMPs, common myeloid progenitors; DEG, common differentially expressed genes; EA, electroacupuncture; FBS, fetal bovine serum; FC, fold change; GMPs, granulocyte-macrophage progenitors; HSCs, hematopoietic stemcells; H&E, Hematoxylin & Eosin; HSPCs, hematopoietic stem/progenitor cells; HMGB1, high-mobility group Box 1; i.p, intraperitoneal; KEGG, Kyoto Encyclopedia of Genes and Genomes; LLC, lewis lung carcinoma; LT-HSCs, longterm HSCs; MPPs, multipotent progenitors; MEPs, megakaryocytic/erythroid progenitors; Ngf, nerve growth factor; PACAP, pituitary adenylate cyclase activating polypeptide; PBS, phosphate-buffered saline; PI, propidium iodide; PPI, protein-protein interaction; RT-qPCR, reverse transcription-quantitative and polymerase chain reaction; RIN, RNA integrity number; SEM, standard error of mean; ST-HSCs, short term HSCs; Th, tyrosine hydroxylase; TGF-a, transforming growth factor-a; COL1A1, type I collagen a1 chain; VIP, vasoactive intestinal peptide.

MATERIALS AND METHODS

Animals

All experimental procedures were performed in accordance with the Tianjin University of Traditional Chinese Medicine guidelines for the care and use of laboratory animals, and approved by the Animal Care and Use Committee of Tianjin

University of Traditional Chinese Medicine (Permit Number: TCM-LAEC2019057). Male Balb/c (8 weeks old, *n*=180) and C57/BL6 (6 weeks old, *n*=50) mice weighting 18-24 g were purchased from the experimental animal center of Beijing Wei Tong Li Hua Experimental Animal Technology Co., Ltd. (Beijing, China). License number: SCXK (Beijing) 2016-0006. All mice were maintained under a 12/12-hour light/dark cycle at 24-26°C in cages at a controlled humidity of 40-50%, and allowed free access to food and water. All mice were anesthetized with 4% isoflurane with oxygen as the carrier (Shenzhen RWD Life Technology Co., Ltd. China) before sacrificing for sample collection.

Materials and Reagents

Cisplatin (Jiangsu haosen pharmaceutical group Co., Ltd., China) was administered at 3-5 mg/kg in 0.9% sodium chloride solution intraperitoneal (i.p.), twice per week for two weeks. Control mice were treated with an equal amount of saline solution. The role of PACAP was analyzed by using PACAP6-38, a PAC1 antagonist at different concentrations (Low dose:10 μg/kg, High dose:100 μg/kg, i.p., Selleck Chemicals, Houston, USA), and PACAP1-38, PAC1 agonist (Low:10 μg/kg, High:50 μg/kg, i.p., Selleck Chemicals, Houston, USA) (46).

Establishment of LLC-Bearing Mice Model

LLC cells were cultured with Dulbecco's modified Eagle's medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 0.1 mg/ml streptomycin, and were maintained in a humidified chamber at 37°C in a 5% CO_2 atmosphere. One week after the C57/BL6 mice are acclimated and injected 1×10^5 LLC cells in 0.1 ml phosphate-buffered saline (PBS) buffer subcutaneously into the right groin (47). Tumor dimensions were measured by digital calipers at days 7, 10, 14, 17, and 21, and the tumor volume (mm^3) was calculated as (length × $width^2$)/2 (48).

Electroacupuncture Treatment

EA treatment was initiated on the same day that the mice received cisplatin. Mice were restrained using the soft cloth fixation method, the skin around the bilateral acupoint ST36 (49, 50) (*Zusanli* acupoint, located 2.0 mm lateral to the anterior tubercle of the tibia in the anterior tibial muscle and 4.0 mm distal to the knee joint lower point) and SP6 (51) (*Sanyinjiao* acupoint, located 2.0 mm proximal to the upper border of the medial malleolus, between the posterior border of the tibia and the anterior border of the Achilles tendon) were disinfected with alcohol swabs. The acupuncture needles (diameter=0.25 mm, length=13 mm, Huatuo Brand, Suzhou Medical Appliance Factory, Jiangsu, China) were inserted in bilateral ST36 and SP6 acupoints, with 3.0 and 2.0 mm depth, respectively. Then, the needles were connected to the SDZ-V EA device (Huatuo Brand, Suzhou Medical Appliance Factory, Jiangsu, China) with the dilatational wave at 5/25 Hz and 0.76 mA stimulation for 15 min. Experimental mice received EA three times per week for two weeks, and control mice received the same treatment without EA stimulation.

Blood Examination

Peripheral blood was collected in polypropylene tubes with ethylenediaminetetraacetic acid (Beijing Nobleryder technology co. Ltd. China) from the orbital sinus of mice anesthetized with isoflurane. Hematological parameters including leukocyte and lymphocyte counts were measured by an automated hematology analyzer (MEK-7222K, Nihon Kohden, Japan).

Flow Cytometry Assay

Hemocyte Panel

200 μL of blood was collected from each sample and incubated with cell membrane markers including LY-6G-PE, LY-6C-APC, CD3-PE-Cy7 and CD19-FITC (Biolegend, San Diego, California, USA) for 20 min at room temperature protected from light. Then, lysing buffer (BD Bioscience, Franklin Lakes, New Jersey, USA) was used to remove red blood cells. Samples were washed before resuspension in 0.5 ml PBS containing 2% FBS. The acquisition was conducted on an Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA), and the concentration of target population (events/μL) were analyzed.

HSPCs Subpopulation Panel

Mice tibias were harvested, the epiphyses of the bones were cut and immersed in 15 ml conical tubes with 1.0 ml PBS. Total BM cells were collected by centrifugation at 3,000×rpm for 10 min, and red blood cells were removed with lysis buffer. For HSPC subsets detection, 10^6 cells were stained with FITC-conjugated anti-Lin, PE-Cy7-conjugated anti-Sca-1, APC-conjugated anti-CD34, Brilliant Violet 421™-conjugated anti-CD16/32, PE-conjugated anti-CD127 (IL-7R) or Brilliant Violet 510™-conjugated anti-CD127, APC-Cy7-conjugated anti-CD117 (c-Kit), PerCP-Cy5.5-conjugated anti-CD90.1 (Thy1.1), PE-Cy5-conjugated anti-CD135 (Flk2) or PE-conjugated anti-CD135 (Biolegend, San Diego, California, USA) for 20 min at room temperature protected from light. Samples were then washed again before resuspension in 0.5 ml PBS containing 2% FBS. Acquisition was conducted on an Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA). All the data were analyzed as following: Positive cells events (%) = (the events in target gate/the total cell) × 100.

Cell Cycle Panel

Cell cycle was determined by nuclear staining with propidium iodide (PI) of BM cells. Briefly, suspensions of single cells were fixed in 75% ethanol at -20°C overnight. Samples of cells were incubated with 0.5 ml PI (TxCyclePI/RNAse, BD Bioscience, Franklin Lakes, New Jersey, USA) at 4°C for 15 min. Acquisition was conducted the same as the above panel, and analyzed by ModFit 3.1 software (Verity Software House, Topsham, ME).

Hematoxylin & Eosin (H&E) Staining

Tibial bone was collected and fixed as described (42). The OCT-embedded bone samples were sliced to a thickness of 5.0 μm with a Lecia frozen slicer (1950) and were stained with H&E. The histological sections were observed and photographed under a

light microscope (NIKON Eclipse Ci-L, Japan), the section within each group (n=4, each sample has two tissue slices) of randomly selected perspective in three pictures. Then BM hematopoietic cellularity was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) and calculated as follows, BM hematopoietic cellularity (%) = [1 - (white area pixels/total area pixels)] × 100% (52). BM cell density was measured using StrataQuest v7.0.176 software (TissueGnostics, Vienna, Austria). Total cells were identified based on hematoxylin staining. The number and density of cells were counted by the software after excluded cell debris, and BM cell density=total cells counts/total areas (mm^2).

Gene Chip (GCT) and Data Analysis

BM sample extraction (n=6) was performed as described above in flow cytometry assay. RNAs were extracted purified with a standard Affymetrix protocol according to Shanghai Biotechnology Corporation (Shanghai, China), and equal amount of RNA from each sample was pooled (n=1) in the same group and tested on a microarray. The raw chip data are accessible from the BioProject ID PRJNA 687726 in the public database of the NCBI BioProject (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA687726). Briefly, total RNA was isolated and RNA integrity number (RIN) value to inspect RNA integration was checked (53). Only RNA with RIN value greater than 7.0 and a 28S/18S ratio greater than 0.7 were used for microarray analyses. The gene chip results were scanned by Gene Chip Scanner 3000 (Cat#00-00213, Affymetrix, Santa Clara, CA, US) and analyzed by Command Console Software 4.0 (Affymetrix, Santa Clara, CA, US), the qualified data were normalized at the gene and exon levels, respectively by the Expression Console software (Affymetrix, Santa Clara, CA, US) (53), and the normalized signal value was the signal value calculated by Log_2. Then the differentially expressed genes (DEGs) were screened by threshold method, and the genes with a fold change (FC) > 2 were considered as DEGs as shown in **Figure 3A** of scatter plot. Also, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways obtained in the drawing by DEGs through website in http://enrich.shbio.com/. The KEGG obtained were sorted in descending order of size according to the value of the enriching factor and considering the top 30 pathways. The protein-protein interaction (PPI) network is based on the above analysis results of DEGs of different groups (Cis *vs* Veh, EA *vs* Cis) were further analyzed by STRING.

Reverse Transcription-Quantitative and Polymerase Chain Reaction (RT-qPCR)

RNA samples from each group returned by the company were verified by RT-qPCR. The RNA concentration was measured by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US) and total RNA was used for reverse transcription with the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol.

The cDNA was amplified by SYBR™ Select Master Mix (Applied Biosystem, Thermo Fisher Scientific, Inc), and the RT-qPCR procedure according to the manufacturer's protocol. Applied ABI Quant Studio 3 - Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform RT-qPCR under the following conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec, and finally the melt curve stage (95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec). The associated primers were synthesized by Suzhou GENEWIZ Biological Technology Co. Ltd, which were listed in **Additional File1: Table S1**. Relative gene expression was calculated using the double-standard curve method.

Immunofluorescence Staining

The bone fixation method was consistent with HE staining. The bone slice thickness of 8.0 μm was rinsed with 0.05% PBST and Proteinase K (BOSTER, WuhFan, China) incubation antigen-repaired for 15 min at room temperature. The following experimental method of immunofluorescence staining was referred to our previous protocol (42). Briefly, the sections were incubated with the primary antibodies rabbit anti-Th (1:50, BOSTER, Wuhan, China) overnight at 4°C. After a 0.05% PBST rinsed, the sections were incubated with Alexa Fluor 594-labeled anti-rabbit IgG (1:400, Abcam, Cambridge, UK) as secondary antibodies for 60 min at room temperature. The sections were observed and photographed under a fluorescence microscope (NIKON Eclipse Ci-L, Japan). Th^+ immunofluorescence staining analyzed the mean number of nerve fibers in five fields randomly was quantified and plotted as per mm^2 (45).

ELISA

BM samples were crushed while frozen and then suspended in cell lysis buffer (Solarbio life sciences, Beijing, China) with protease inhibitor cocktail (1%; Solarbio life sciences, Beijing, China), standing for 30 min at 4°C. Next, samples were centrifuged at 12,000 g for 10 min at 4°C for protein extraction and the clear supernatant extracts were stored at -80°C. PACAP levels including PACAP27 and PACAP38 were measured by using a sandwich enzyme immunoassay special for mouse (Product No. SEB347Mu, Cloud Clone Corp, Wuhan, China) according to the manufacturers' instructions.

Heated Pad Assay

Latency time response of mice to thermal nociception was analyzed with hot-plate tests performed at day 0, 3, 7, 10, 14 (54). The hot-plate temperature was set at 55 ± 0.2°C. Mice were individually placed on the top of the heated surface and the time of the first episode of nociception (jumping or paw licking) was measured, and the cut-off time was 30 s. The heated surface was cleaned up completely by ethanol in two tests and the temperature was allowed to stabilize.

Statistical Analysis

Results are presented as the mean ± standard error of mean (SEM). When the data were normally distributed, the results were analyzed by analysis of variance (ANOVA) for independent samples compared differences between two groups. Comparison of weight, latency, tumor volumes were assessed two-way repeated-measures ANOVA, other indicators were assessed

one-way ANOVA. LSD test was used if the data meet test of homogeneity of variances, if not, Dunnett's T3 test was used. For non-normal distributions, a nonparametric test with Kruskal Wallis was performed with SPSS 23.0. $P < 0.05$ was considered statistically significant. GraphPad Prism software (GraphPad, San Diego, CA, USA) was used for mapping.

RESULTS

Prevention of Cisplatin-Induced Leukopenia and Normal Hematopoiesis Preservation by Electroacupuncture

First, we analyzed whether EA prevents cisplatin-induced leukopenia by performing hematologic analyses of peripheral blood from control and cisplatin-treated mice with or without EA (**Figure 1A**). Cisplatin treatment induced leukopenia and EA prevented leukopenia and preserved the normal count of peripheral leukocytes. Next, we analyzed specific subpopulations of leukocytes as they are mainly composed of neutrophils, lymphocytes, and monocytes. Cisplatin decreased peripheral blood counts of all leukocytes but it was more detrimental to neutrophils and monocytes. We further confirmed our results with flow cytometry analyses of neutrophils (LY6G$^+$), monocytes (LY6C$^+$), and noted a similar effect on the subpopulations of lymphocytes T (CD3$^+$) and B (CD19$^+$) cells. EA was again effective at inhibiting cisplatin side effects and preserves normal peripheral counts of all these leukocytes and more protective on neutrophils and monocytes (**Figures 1B, C**). Cisplatin also induced about 25% mice body weight loss within 10 days, and EA preserved normal body weight over 14 days (**Figure 1D**). These results show that cisplatin induces leukopenia affecting all leukocytes although it was more detrimental to myeloid cells including neutrophils and monocytes, whereas EA preserved normal blood leukocyte counts.

Next, we analyzed the effects of cisplatin and EA in BM hematopoiesis. Histological hematoxylin & eosin (H&E) staining show normal BM morphology with proliferating hematopoietic cells in control mice. Cisplatin induced a sparse and scattered cell distribution, whereas EA preserved normal BM morphology (**Figure 2A**). We confirmed these results with semi-quantitative analyses BM hematopoietic cellularity showing that cisplatin decreased BM cells percentages, whereas EA improved it (**Figure 2B**). As shown in **Figures 2C, D**, we also performed the BM cell density at high configuration, and the results showed that cisplatin reduced BM cell counts, and EA treatment have increased tendency. Then, we analyzed the effects of cisplatin and EA in BM hematopoiesis by analyzing specific hematopoietic cell subpopulations (**Figure 2E**). Hematopoiesis starts with hematopoietic stem/*progenitor* cells (HSPCs; Lin$^-$Sca-1$^+$CD117$^+$) undergoing a sequential differentiation into self-renewal *long-term* (LT-HSCs; Lin$^-$/Sca-1$^+$/CD117$^+$/CD90.1$^+$/CD135$^-$), and *short-term* hematopoietic stem cells

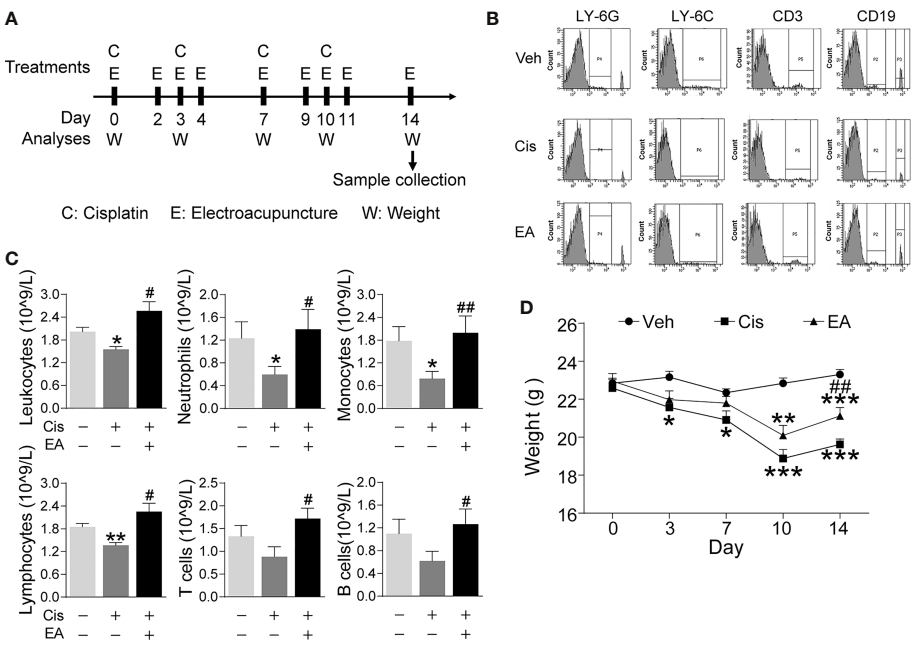

FIGURE 1 | Electroacupuncture prevented cisplatin-induced leukopenia. **(A)** Experimental flowchart depicting the time of the treatments of Cisplatin (C), electroacupuncture (E), and the analyses of body weight (W) and sample collection. **(B)** Representative peripheral blood flow cytometry analyses of neutrophils (LY6G$^+$), monocytes (LY6C$^+$), T (CD3$^+$), and B (CD19$^+$) lymphocytes and **(C)** Blood counts of specific subpopulation of leukocytes of mice with control (Veh), cisplatin alone (Cis; 3 mg/kg), or with electroacupuncture (EA) treatment (leukocytes, lymphocytes: n=6 per group; neutrophils, monocytes, T and B lymphocytes: Veh, n=6; Cis, n=6; EA, n=7). **(D)** Mice body weight curves treatment at day 0, 3, 7, 10, 14 (n=6 per group), P values were calculated using two-way repeated-measures ANOVA. Data are mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Veh; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Cis.

(ST-HSCs; $Lin^-/Sca\text{-}1^+/CD117^+/CD90.1^+/CD135^+$), which differentiate into non-self-renewing *multipotent* progenitors (MPPs; $Lin^-/Sca\text{-}1^+/CD117^+/CD90.1^-/CD135^+$). These progenitors can then differentiated into either common *lymphoid* (CLPs; $Lin^-/Sca\text{-}1^+/CD117^+/CD127^+$ for lymphocytes and NK cells) or common *myeloid* progenitors (CMPs; $Lin^-/Sca\text{-}1^-/CD117^+/CD127^-/CD34^+/CD16/32^-$), which ensuing differentiate into either *megakaryocytic/erythroid* (MEPs; $Lin^-/Sca\text{-}1^-/CD117^+/CD127^-/CD34^-/CD16/32^-$) or *granulocyte-macrophage* progenitors (GMPs; $Lin^-/Sca\text{-}1^-/CD117^+/CD127^-/CD34^+/CD16/32^+$ for neutrophils, monocytes, basophils, and eosinophils) (55, 56). Flow cytometry analyses showed that cisplatin was more detrimental in reducing HSPCs, MPPs, and myeloid ontogenesis (CMPs, GMPs, and MEPs), but not self-renewing stem cells (LT-HSCs, ST-HSCs) or lymphoid ontogenesis (CLPs). EA preserved the normal counts of all hematopoietic cells (MPPs, CMPs), and the proportion of HSPCs and MEPs have increased tendency, but not GMPs (**Figures 2F, G**). These results show that cisplatin inhibited BM hematopoiesis and specifically myeloid ontogenesis, whereas EA preserved BM hematopoiesis.

We next studied hematopoietic cell proliferation and cycle profile in BM by propidium iodide nuclear staining. Cisplatin inhibited hematopoietic cell proliferation by decreasing the transition from S to G_2/M phase, whereas EA preserved normal cell proliferation (**Figures 2H, I**). At the molecular level, we analyzed the expression of cell cycle genes by quantitative RT-qPCR. Cisplatin specifically reduced the expression of *Ki67* and *Ccna2* without significantly affecting *Ccnd1* and *Ccne1*, whereas EA preserved normal expression of these genes (**Figures 2J**). These results show that cisplatin inhibits the progression of S into the G_2/M phase by inhibiting DNA replication and the expression of critical factors such as *Ki67* (associated with ribosomal RNA synthesis) and *Ccna2* (cyclin A2). Again, EA preserved normal cell proliferation of BM hematopoietic cells.

Activated Bone Marrow Pathways in Cisplatin-Treated Mice by Electroacupuncture

We further analyzed the molecular mechanisms of cisplatin and EA by gene chip analyses (**Figure 3A**). Cisplatin modified the expression of 1,414 BM genes as compared to normal tissue, and EA modified 1,684 genes as compared to cisplatin (**Figure 3B**). Differential gene KEGG pathway analyses revealed that cisplatin main effects ($P < 0.01$; enrichment > 3) were activating pathways related to extracellular matrix receptor interaction, B cell and toll-like receptors signaling, the p53, PPAR signaling, osteoblast differentiation and NF-kB pathways (**Table 1**). KEGG analyses also showed the potential of EA to mainly activate pathways related to ribosome biogenesis ($P < 0.01$; enrichment > 28) (**Table 1**). KEGG analyses revealed 163 common differentially expressed genes (DEG) in both cisplatin and EA groups. The factors modulated by both cisplatin and EA further emphasizes the role of three major pathways ($P < 0.01$) related to ribosome biogenesis (*Rpl14*, *Gm6344*, *Rpl29*, *Rpl32*; enrichment > 12), PPAR signaling (*Fabp4*, *Scd1*; enrichment > 9), and collagen extracellular matrix receptor interaction (*Col1a1*, *Col1a2*, enrichment > *8*) (**Table 1**). These results were consistent with the protein-protein interaction (PPI) analyses that revealed the potential of cisplatin to induce 71 genes mostly related to ribosome biogenesis (*Rps13*, *Rpl14*, *Rpl32*, *Rpl34*, > 15 counts) and collagen extracellular matrix (*Col1a1*, *Col1a2*, 3 counts/each) (**Figure 3C** and **Table 2**). EA was again protective against cisplatin and preserving the expression of 48 genes mostly related to ribosome biogenesis (*Rpl14*, *Rps11*, *Rps13*, *Rpl32*, *Rpl34*, > 10 counts) (**Table 2**). The levels of common DEG detected by gene chip in both cisplatin and EA further emphasized the role of ribosome biogenesis, collagen extracellular matrix receptor interaction, and PPAR signaling (**Additional File 2: Table S2**). These results were then confirmed by RT-qPCR. Cisplatin significantly induced *Col1a1, Col1a2*, expression as shown in KEGG analyses, whereas EA preserved *Col1a1* normal expression consistent with the gene chip analyses (**Figure 3D**). These results suggest that cisplatin induces type I collagen α1 chain (*Col1a1*) and disrupts BM extracellular matrix, whereas EA preserves normal collagen BM expression and extracellular matrix composition for normal hematopoiesis.

Sympathetic Nerve Released PACAP Mediating Electroacupuncture Alleviation of Cisplatin-Induced Leukopenia

We next reasoned that cisplatin-induced neurotoxicity may affect hematopoiesis, and EA may preserve BM sympathetic neuromodulation. Thus, we analyzed the sympathetic fibers in BM sections by staining tyrosine hydroxylase (Th), the enzyme that converts tyrosine to dopamine essential for catecholamine biosynthesis in sympathetic innervations. These results showed the significant neurotoxicity induced by cisplatin, and the potential of EA to preserve BM sympathetic innervations (**Figures 4A, B**). Then, we performed RT-qPCR analyses to determine the neurogenic factors mediating EA-induced neuroprotection. Protein expression was confirmed by ELISA analyses. Cisplatin inhibited the production of critical neurogenic factors but especially nerve growth factor (*Ngf*), brain-derived neurotrophic factor (*Bdnf*), and PACAP. EA preserved normal production of all these factors, but was more effective in inducing PACAP expression (**Figure 4C**). Thus, we reasoned that PACAP may contribute to EA-induced neuroprotection during chemotherapy, and we analyzed whether PACAP inhibition prevents EA-induced neuroprotection using functional analyses of nociception. Previous studies reported that cisplatin neurotoxicity induces peripheral nerve injury affecting nociception (54). Thus, we analyzed whether EA preserves sensory nerve activity using thermal pain tests, and whether this effect is mediated by PACAP. Cisplatin increased mice latency time in the hot-plate tests showing neurotoxicity preventing thermal pain, whereas EA preserved thermal nociception (**Figure 4D**). Next, we analyzed whether PACAP is required for EA-induced neuroprotection by inhibiting the specific receptor for PACAP, PAC1. PACAP6-38, a competitive PAC1 inhibitor, abrogated the potential of EA to preserve nociception in thermal tests in a concentration-dependent manner (**Figure 4D**). Then, we analyzed whether the effects of PAC1 on neuroprotection correlated with hematopoiesis. Similar to neuroprotection, EA prevented cisplatin-

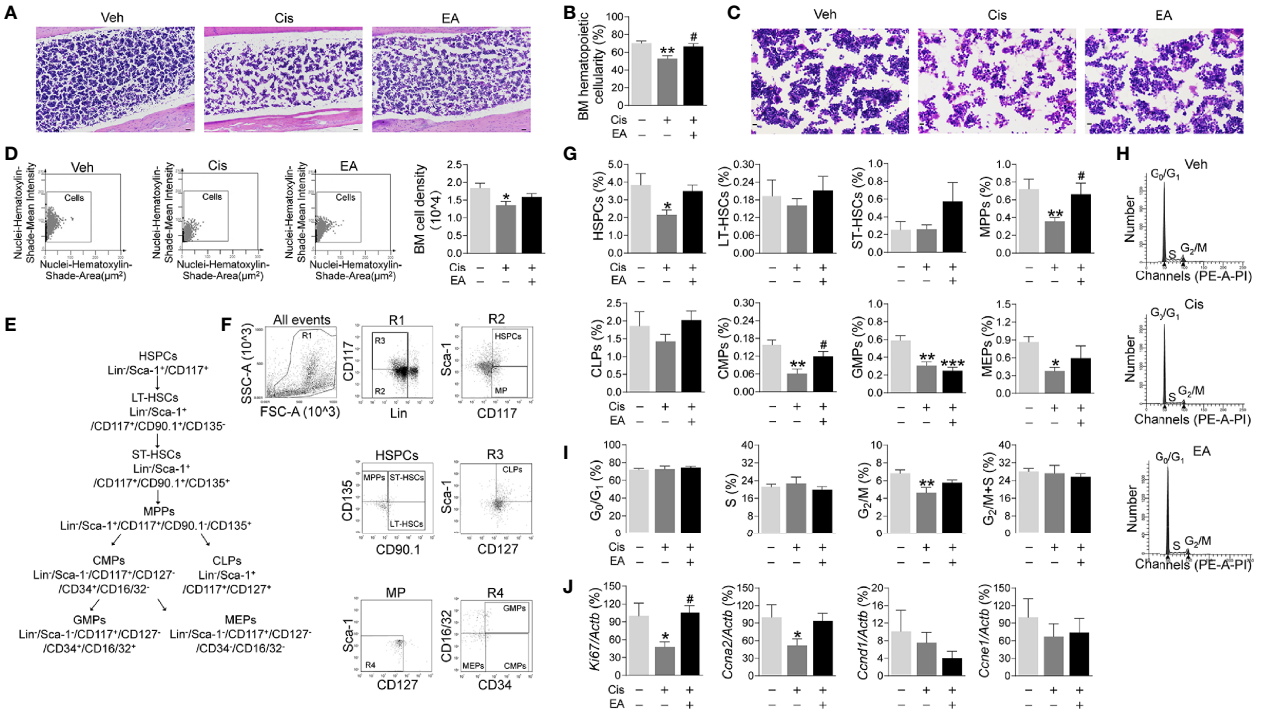

FIGURE 2 | Electroacupuncture preserved hematopoiesis in mice with cisplatin chemotherapy. **(A)** Representative H&E staining of tibia BM from mice with control (Veh), cisplatin alone (Cis; 3 mg/kg), or with electroacupuncture (EA) treatment (scale bar=20.0 μm) and **(B)** Histogram representation of BM hematopoietic cellularity of H&E staining analyzed by Image-Pro Plus 6.0 software (n=4 per group). **(C)** Representative H&E staining of tibia BM from mice with Veh, cisplatin alone or with EA treatment at high configuration (scale bar=10.0 μm). **(D)** Representative HistoFAXS Tissue Analysis of BM cell nuclei hematoxylin-shade-mean intensity, and quantitative analysis of BM cell density (n=4 per group). **(E)** Flowchart of hematopoiesis and hematopoietic cells markers. **(F)** Representative flow cytometry analyses and **(G)** quantification of hematopoietic BM cell subpopulations (Positive cells events (%) = (the events in target gate/the total cell) × 100) (n=6 per group). **(H)** Representative PI nuclear staining flow cytometry analyses in BM cell cycle (G_0/G_1, S, G_2/M phases) and **(I)** Quantification of PI nuclear staining of BM cells in G_0/G_1, S, G_2/M phases by ModFit 3.1 software (n=6 per group). **(J)** Expression of cell cycle related genes in BM cells (*Ki67*: Veh, n=7; Cis, n=5; EA, n=7. *Ccna2*: n=7 per group. *Ccnd1*: Veh, n=5; Cis, n=4; EA, n=6. *Ccne1*: Veh, n=6; Cis, n=4; EA, n=5). Data are mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Veh; $^{\#}P < 0.05$ *vs* Cis.

induced leukopenia, but not in mice pretreated with high doses of PAC1 inhibitor (**Figure 4E**). Likewise, PAC1 inhibitor also prevented the potential of EA to preserve BM hematopoiesis and counts of HSPCs and myeloid progenitors (MPPs) during cisplatin chemotherapy (**Figure 4F**). Control treatments with PACAP6-38 itself affected neither BM hematopoiesis nor HSPCs/MPPs counts. Furthermore, PAC1 inhibitor also prevented the potential of EA to preserve hematopoietic cell proliferation (**Figure 4G**). Together, these results show that inhibition of PACAP receptor PAC1 prevents the protective effects of EA during cisplatin chemotherapy, suggesting that the protective effects of EA are mediated by PACAP production.

Next, we reasoned that PAC1-agonists may mimic the protective effects of EA during cisplatin chemotherapy. PAC1-agonist, PACAP1-38, mimics EA-induced neuroprotection and preserves thermal nociception in cisplatin-treated mice in a concentration-dependent manner (**Figure 5A**). The high dose of PACAP1-38 preserves BM hematopoiesis and normal peripheral counts of leukocytes, including neutrophils and lymphocytes (**Figure 5B**). The high dose of PAC1-agonist also mimics the potential of EA to preserve hematopoiesis including HSPCs and myeloid progenitors (MPPs) but not GMPs (**Figure 5C**). The high and low dose of PAC1-agonist also preserved BM hematopoietic cell proliferation through the G_2/M phase (**Figure 5D**). Thus, treatment with high dose of PAC1-agonist, PACAP1-38, mimicked the potential of EA to preserve thermal nociception, peripheral counts of leukocytes, BM myeloid ontogenesis, and hematopoietic cell proliferation in mice with cisplatin chemotherapy.

Preservation of BM Hematopoiesis in Lung Carcinoma Mice by Electroacupuncture

We next analyzed the effects of EA in cancer mice with LLC cells. Mice were injected LLC cells, cisplatin chemotherapy with or without EA was started one week later, and tumor growth and hematopoiesis were analyzed at different time points (**Figure 6A**). Tumor volume dramatically increases after 14 days, and cisplatin treatment (5 mg/Kg; i.p.) significantly reduces tumor growth by over 60% by day 21 (**Figure 6B**). EA did not prevent the potential of cisplatin to inhibit tumor growth, actually EA showed a tendency to further decrease tumor growth to some extent as compared to cisplatin treatment alone. Cisplatin also induces peripheral leukopenia inhibiting all leukocyte subpopulations including neutrophils, monocytes, and lymphocytes, and it was more detrimental on T ($CD3^+$) than B ($CD19^+$) lymphocytes in cancer

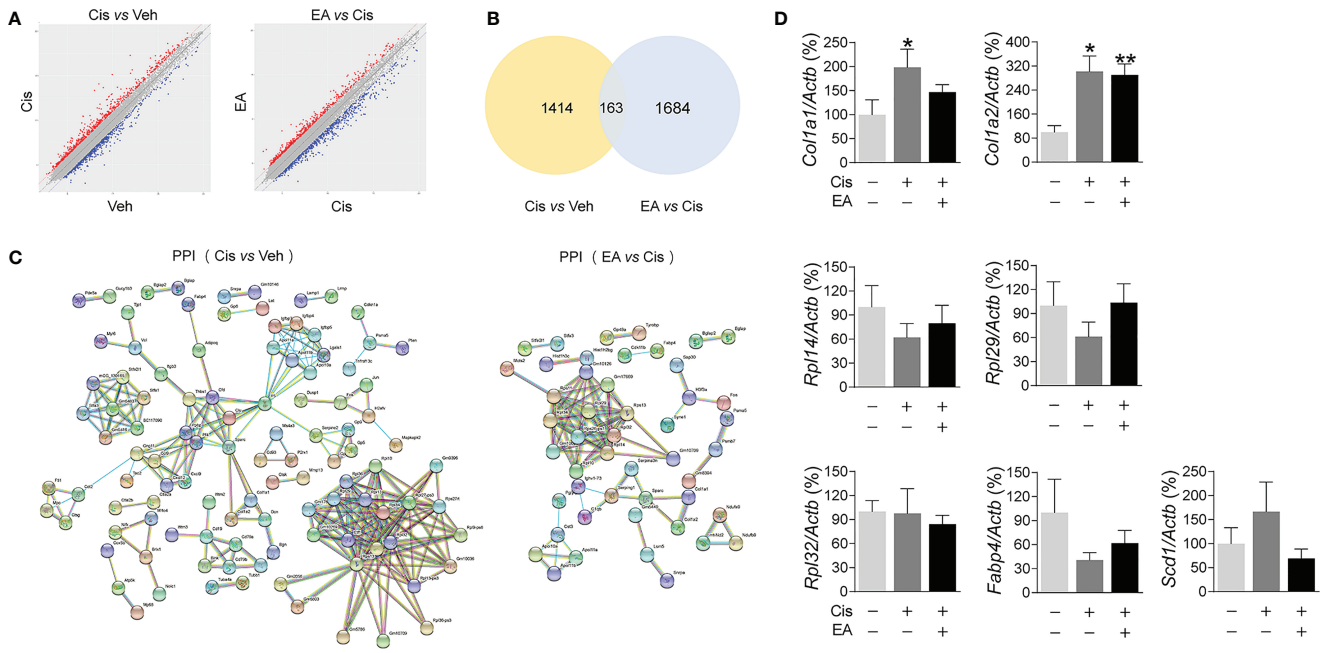

FIGURE 3 | Analyses of expression and enrichment of electroacupuncture in the bone marrow of mice with cisplatin. **(A)** Scatter plots of differentially expressed genes (DEG)s in Cis *vs* Veh and EA *vs* Cis group Each probe is represented by a point with red and blue points showing up- and down-regulated genes defined above Log2 FC > 2. **(B)** Venn diagram and **(C)** PPI network analyses of DEGs results. **(D)** RT-qPCR analyses of factors related to extracellular matrix (*Col1a1*, *Col1a2*), ribosome (*Rpl14*, *Rpl29*, *Rpl32)*, and PPAR signaling (*Fabp4*, *Scd1)* (*Col1a1*, *Col1a2*: *n*=7 per group. *Rpl14*, *Rpl29*, *Scd1*: *n*=6 per group. *Rpl32*, *Fabp4*: Veh, *n*=6; Cis, *n*=5; EA, *n*=6). Data are mean ± SEM, *$P < 0.05$, **$P < 0.01$ *vs* Veh.

TABLE 1 | KEGG enrichment of co-expressed DEGs.

Description	*P* value	Counts	Genes	Enrich factor
KEGG enrichment of co-expressed DEGs in cisplatin *vs* control group				
Extracellular matrix receptor interaction	<0.01	9	*Thbs1 Gp5 Reln Gp6 Col1a2 Col1a1 Gp9 Gp1ba Itgb3*	8.11
B cell receptor signaling pathway	<0.01	7	*Jun Cd79a Fos Blnk Cd79b Cd19 Cd72*	7.27
Hematopoietic cell lineage	<0.01	7	*Gp5 Gp9 Cd19 Gp1ba Il1a Itgb3 Il7r*	5.51
Toll-like receptor signaling pathway	<0.01	5	*Jun Cxcl9 Fos Ctsk Ifna4*	3.78
p53 signaling pathway	<0.01	4	*Thbs1 Ccng1 Cdkn1a Pten*	4.21
NF-kappa B signaling pathway	<0.01	5	*Cxcl12 Tnfrsf13c Blnk Lat Vcam1*	3.59
PPAR signaling pathway	<0.05	4	*Scd1 Fabp4 Lpl Adipoq*	3.52
Osteoclast differentiation	<0.05	6	*Jun Fos Ctsk Blnk Il1a Itgb3*	3.50
Th17 cell differentiation	<0.05	4	*Jun Fos Irf4 Lat*	2.93
Serotonergic synapse	0.07	4	*Gng11 Dusp1 Kcnj5 Alox12*	2.27
Apoptosis	0.08	4	*Jun Fos Ctsk Tuba4a*	2.20
Cellular senescence	0.09	5	*Mapkapk2 Slc25a5 Cdkn1a Il1a Pten*	2.01
KEGG enrichment of co-expressed DEGs in EA *vs* cisplatin group				
Ribosome biogenesis in eukaryotes	<0.01	33	*N-r5s100 Gm25212 N-r5s123 N-r5s134 Rn5s N-r5s128 N-r5s124 N-r5s136 N-r5s121 Gm23284 N-r5s117 Gm22109 N-r5s108 N-r5s122 N-r5s105 Gm22291 Rmrp N-r5s143 N-r5s139 N-r5s111 N-r5s103 N-r5s138 Gm25018 N-r5s146 N-r5s113 N-r5s142 N-r5s149 Gm26391 N-s5s110 N-r5s144 N-r5s133 N-r5s104 N-r5s141*	28.44
Biosynthesis of unsaturated fatty acids	0.09	1	*Scd1*	3.12
Extracellular matrix receptor interaction	0.11	2	*Col1a1 Col1a2*	2.41
PPAR signaling pathway	0.11	2	*Fabp4 Scd1*	2.35
Retrograde endocannabinoid signaling	0.14	3	*Ndufb9 Nd2 Ndufa9*	2.00
KEGG enrichment of co-expressed DEGs in cisplatin *vs* control group and EA *vs* cisplatin group				
Ribosome	<0.01	4	*Rpl14 Gm6344 Rpl29 Rpl32*	12.20
PPAR signaling pathway	<0.01	2	*Scd1 Gabp4*	9.19
Extracellular matrix receptor interaction	<0.01	2	*Col1a1 Col1a2*	8.65

Enrich factor = (the number of DEGs in a term/the total number of DEGs) / (the total gene number in a term of database/the total number of genes in the database).

TABLE 2 | The node counts between proteins with PPI.

Nodes	Counts	Nodes	Counts	Nodes	Counts
The node counts between proteins with PPI in cisplatin *vs* control group					
Rps13	21	*Cfd*	7	*Cd79a*	4
Rpl14	19	*Gng11*	7	*Cd79b*	4
Rpl32	19	*Igfbp4*	7	*Dcn*	4
Rpl34	16	*Igfbp5*	7	*Serpine2*	4
F5	14	*Igfbp7*	7	*Blnk*	3
Rpl13	13	*Lgals1*	7	*Brix1*	3
Rpl27-ps3	13	*Thbs1*	7	*Col1a1*	3
Rps27rt	13	*Bc117090*	6	*Col1a2*	3
Etf1	12	*Ccl9*	6	*Ctsg*	3
Rpl36	12	*Clu*	6	*Fos*	3
Rpl29	11	*Gm5416*	6	*Ftl1*	3
Pf4	10	*Gm5483*	6	*Gm10709*	3
Ppbp	10	*mCG_130165*	6	*Gm5786*	3
Rpl10	10	*Rpl9-ps6*	6	*Gp1ba*	3
Gm10269	9	*Stfa1*	6	*Gp5*	3
Gm17669	9	*Stfa3*	6	*Gp9*	3
Sparc	9	*Cd19*	5	*H2afv*	3
Gm10036	8	*Cxcl12*	5	*Mpo*	3
Rpl13-ps3	8	*Cxcl9*	5	*Psma5*	3
Apol10a	7	*Gm9396*	5	*Rpl36-ps3*	3
Apol11a	7	*Stfa2l1*	5	*Vcl*	3
Apol11b	7	*Cct2*	4		
The node counts between proteins with PPI in EA *vs* cisplatin group					
Rpl14	11	*Gm17669*	6	*Col1a1*	2
Rps11	11	*Cst3*	4	*Col1a2*	2
Rps13	11	*Serping1*	4	*Ighv1-73*	2
Rpl32	11	*Sparc*	4	*Lsm5*	2
Rpl34	10	*Apol10a*	3	*mt-Nd2*	2
Rps26-ps1	9	*Apol11a*	3	*Ndufa9*	2
Rpl29	8	*Apol11b*	3	*Psmb7*	2
Gm10020	7	*Gm10709*	3	*Serpina3n*	2
Gm10126	7	*H3f3a*	3		
Rpl10	7	*C1qb*	2		

mice. Furthermore, EA diminished leukopenia and neutropenia but not monocytopenia and lymphopenia in cancer mice (**Figure 6C**). Cisplatin inhibited hematopoiesis at different levels and significantly reduced the counts of multipotent (MPPs) and GMPs in cancer mice. EA preserved normal levels of both MPPs and GMPs in cancer mice. Furthermore, EA increased the levels of HSPCs, myeloid (CMPs), and megakaryocytic/erythroid progenitors (MEPs) in cancer mice (**Figure 6D**). At the cellular level, cisplatin significantly decreased BM cell counts in S phase, whereas EA preserved normal cell proliferation through the cell cycle in cancer mice (**Figure 6E**). These results show that EA diminished cisplatin-induced leukopenia and preserves BM hematopoiesis in cancer mice with Lewis lung carcinoma cells.

DISCUSSION

Despite the profuse clinical evidence showing the potential of EA to relieve leukopenia during chemotherapy, its mechanism is unknown, and thus why it is effective in some patients but not in others with similar symptoms and how the treatment can be improved. EA activates mechanisms that have physiologic limitations, and they are ineffective in patients with multiple comorbidities (57, 58). One typical example is that EA on ST36 improves organ function and survival in experimental sepsis by inducing dopamine production in the adrenal glands (49, 59). However, many septic patients have adrenal insufficiency, and thus they render insufficient dopamine production for EA to induce significant effects (49, 60, 61). Chemotherapy is another major clinical challenge that causes neurotoxicity, anemia, and immunosuppression that limit anti-tumor efficacy. Here, we show that EA on ST36 and SP6 prevents neurotoxicity, preserves BM hematopoiesis, and myeloid ontogenesis during cisplatin chemotherapy. EA induces neuro and immune protection by inducing neurogenic production of PACAP, which preserves BM hematopoiesis *via* PAC1 receptor. Thus, PAC1-agonists mimic EA potential to preserve BM hematopoiesis during chemotherapy and may provide therapeutic advantages to treat cancer patients with advanced neurotoxicity and neuropathies limiting EA efficacy.

Cisplatin is an effective chemotherapy treatment toxic to proliferating cells such as cancer cells. However, cisplatin is not specific for cancer cells and it also inhibits BM hematopoietic cells inducing anemia and immunosuppression that prevent anti-tumor immune responses (58, 62–64). Low concentrations of cisplatin (3 mg/kg) in normal mice decreased blood counts of all leukocytes but specially neutrophils and monocytes. Higher concentrations of cisplatin (5 mg/kg) are required to induce similar effects in cancer mice probably because it is absorbed by the cancer cells. In cancer mice, cisplatin also inhibited all leukocytes subpopulations and it was more detrimental to T than B lymphocytes. These results further reveal the potential of cisplatin to induce immunosuppression and limit anti-tumor immune responses.

Cisplatin causes leukopenia by inhibiting hematopoiesis. Cisplatin inhibited hematopoietic stem/progenitor, multipotent progenitors, and myeloid ontogenesis (CMPs, GMPs, and MEPs), but not self-renewing stem cells (LT-HSCs, ST-HSCs) or lymphoid ontogenesis (CLPs) in normal mice. In cancer mice, cisplatin induced similar results and inhibited multipotent progenitors and myeloid ontogenesis of GMPs, and thus validate our models to recapitulate leukopenia as shown in cancer patients (62). However, cisplatin did not inhibit megakaryocytic/erythroid progenitors in cancer mice because the lung carcinoma cells already prevent MEPs as compared to normal mice. These results concur with the peripheral blood counts as cisplatin inhibits myeloid ontogenesis and therefore neutrophils and monocytes in normal and cancer mice. Cisplatin inhibits hematopoiesis by binding to the nuclear DNA of proliferative hematopoietic cells and inducing an in-chain DNA cross-linking that forms a ternary complex of DNA-platinated oligonucleotide-HMGB1 (high-mobility group Box one protein) that blocks DNA replication and cell proliferation (65, 66). Thus, cisplatin prevents the transition S to G_2/Mitosis phase as shown in normal mice, whereas higher concentrations in cancer mice were more effective at early stages and decrease cell counts in the S phase. This effect is also due to the potential of cisplatin to inhibit the expression of critical proteins related to the cell cycle. Our results show that cisplatin inhibited *Ccna2* expression of Cyclin A2, which is normally expressed in dividing somatic cells to control the G_1 to S transition as shown in

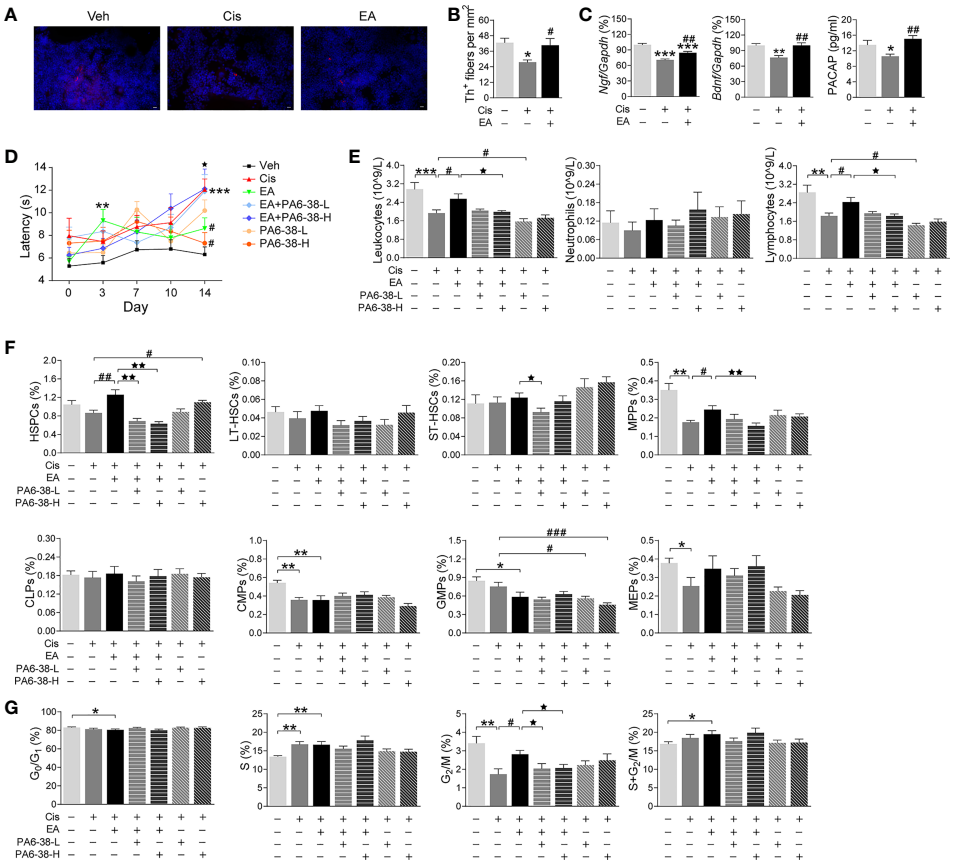

FIGURE 4 | Neurogenic PACAP mediated electroacupuncture-induced protection to cisplatin. **(A)** Representative immunofluorescence images (Scale bar=20.0 μm) and **(B)** Quantification of sympathetic Th^+ fibers (red) and nuclear (blue) in the BM of the experimental mice (n=4 per group). **(C)** Expression analyses of neurotrophic factors (*Ngf*, *Bndf*: Veh, n=7; Cis, n=6; EA, n=7. PACAP: Veh, n=5; Cis, n=6; EA, n=6). **(D)** Representation of the latency time (seconds) in hot-plate tests of mice treated with control (Veh), cisplatin (Cis; 3mg/kg), and cisplatin + electroacupuncture (EA) without or with PACAP6-38 (a blocker for PACAP receptor, PAC1) at low (10 μg/kg) or high (100 μg/kg) concentrations (Cis, n=7; other groups, n=8), P values were calculated using two-way repeated-measures ANOVA. **(E)** Peripheral blood counts of specific subpopulation of leukocytes (Veh, Cis, EA: n=6; other groups, n=7). **(F)** Analyses of hematopoietic BM subpopulation cells (Veh, Cis, EA, EA +PA6-38-L, EA+PA6-38-H: n=7; PA6-38-L, n=8, PA6-38-H, n=6). **(G)** Quantification of PI nuclear staining of BM cells (Veh, n=8; Cis, n=7; EA, n=8; EA+PA6-38-L, n=8; EA+PA6-38-H, n=7; PA6-38-L, n=8; PA6-38-H, n=7). Data are mean ± SEM $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Veh; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* Cis; $^*P < 0.05$, $^{**}P < 0.01$ *vs* EA.

our results with cancer mice. These results reveal the detrimental side effects of cisplatin in hematopoiesis during chemotherapy and the clinical need to develop safe complementary treatments to prevent immunosuppression in cancer patients.

Multiple clinical studies have confirmed the potential of acupuncture to treat anemia and leukopenia during chemotherapy (17, 20), but the use and efficacy of EA are still moot because of the weak response in many patients. The mechanism of EA is still unknown and thus why it is effective in many patients but not in others with similar symptoms. According to traditional Chinese medicine, acupuncture at ST36 and SP6 have the effect of tonifying blood. Several studies show that stimulation of these two acupoints protects against chemotherapy induced anemia, leukopenia, and other peripheral neuropathies (67–71). Our results show that EA ST36 and SP6 inhibited the most detrimental effects of cisplatin in normal and cancer mice. EA preserved normal peripheral counts of all leukocytes, and BM counts all hematopoietic cells (HSPCs, MPPs, CMPs, and MEPs) but not GMPs in normal mice. In cancer mice, EA halted leukopenia and neutropenia and preserved normal counts of multipotent (MPPs) and GMPs. Actually, EA not only prevented the effects of cisplatin but also some of the effects of cancer on hematopoiesis. As LLC cells decreased BM counts of common myeloid and megakaryocytic/erythroid progenitors in cancer mice, EA restored normal counts of BM hematopoietic cells even if the treatment was started a week after the cancer onset.

Regarding the molecular mechanism of EA, gene chip results suggested that EA may modulate the BM extracellular matrix (ECM) and ribosome signaling pathway (cisplatin vs control, EA *vs* cisplatin, EA *vs* control). EA restored BM hematopoiesis despite the effects of cancer and chemotherapy by regulating type I collagen α1 chain (*Col1a1*). Actually, *Col1a1* is often increased in cancer patients and disrupts BM hematopoiesis and favors immunosuppression and tumor progression (72–74). Thus, the potential of EA to halt *Col1a1* and abnormal

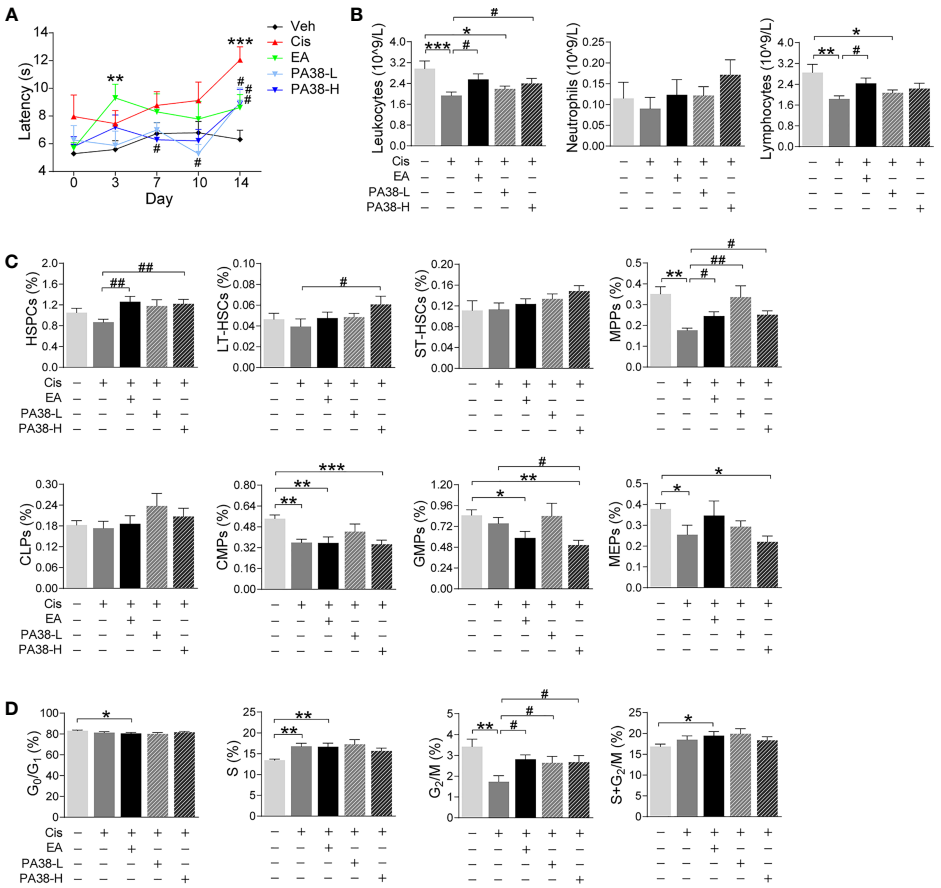

FIGURE 5 | PAC1-agonist mimics electroacupuncture-induced protection to cisplatin. **(A)** Representation of the latency time (seconds) in hot-plate tests of mice with control (Veh), cisplatin (Cis; 3 mg/kg), EA (cisplatin + electroacupuncture), cisplatin mice were treated with low (10 μg/kg) or high (50 μg/kg) concentrations PAC1-agonist, PACAP1-38 (Veh, *n*=8; Cis, *n*=7; EA, *n*=8; PA38-L, *n*=8; PA38-H, *n*=7), *P* values were calculated using two-way repeated-measures ANOVA. **(B)** Peripheral blood counts of specific subpopulation of leukocytes ((Veh, Cis, EA: *n*=6; other groups, *n*=7). **(C)** Analyses of hematopoietic BM cell subpopulation (Veh, Cis, EA: *n*=7; PA38-L, PA38-H: *n*=8). **(D)** Quantification of PI nuclear staining of BM cells (Veh, *n*=8; Cis, *n*=7; EA, *n*=8; PA38-L, *n*=8; PA38-H, *n*=7). Data are mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Veh; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Cis.

collagen production can explain its potential to restore hematopoiesis and ameliorate the cancer inhibition of common myeloid and megakaryocytic/erythroid progenitors as discussed above in cancer mice. These results may suggest that EA can be more effective than anticipated for cancer treatment and not only beneficial to patients with chemotherapy.

Furthermore, EA restores hematopoiesis by preserving normal hematopoietic cell proliferation and production critical factors regulating the cell cycle such as *Ccna2* expression of Cyclin A2. One significant advantage of EA is its potential to activate specific neuronal networks and induce local effects. Thus, EA preserved *Ccna2* expression and hematopoietic cell proliferation in the BM without enhancing tumor proliferation (75). In addition to *Ccna2*, EA also preserved the normal expression of *Ki67* for ribosomal RNA synthesis. These results concur with the KEGG and protein-protein interaction analyses showing the potential of EA to preserve multiple factors associated with ribosomal RNA synthesis. Ribosomes are critical intracellular translational machinery responsible for protein synthesis and cellular proliferation. Eukaryotic 80S ribosomes are composed of two subunits, a 40S decoding subunit, and a large 60S subunit that catalyzes the peptide bonds (76). Chemotherapy drugs inhibit ribosomes at different levels, whereas oxaliplatin induces DNA damage with nucleolar and ribosomal disruption as shown by proteomic profiling (77), cisplatin modifies ribosomal mRNA *via* 1xr1-TOR signaling pathway to prevent protein synthesis. Ixr1 is an HMGB protein that regulates the hypoxic regulon and controls the oxidative stress response or re-adaptation of catabolic and anabolic fluxes in hypoxia. Ixr1 binds with high affinity to cisplatin-DNA adducts and, thus, cisplatin treatment mimics IXR1 deletion, and prevents ribosome biogenesis. Ixr1 is critical to regulating multiple transcriptional factors that respond to nutrient availability and stress stimuli through the TOR and PKA pathways (78, 79). Our analyses showed cisplatin inhibiting multiple factors affecting both 40S and 60S ribosome subunits, whereas EA preserved their normal expression.

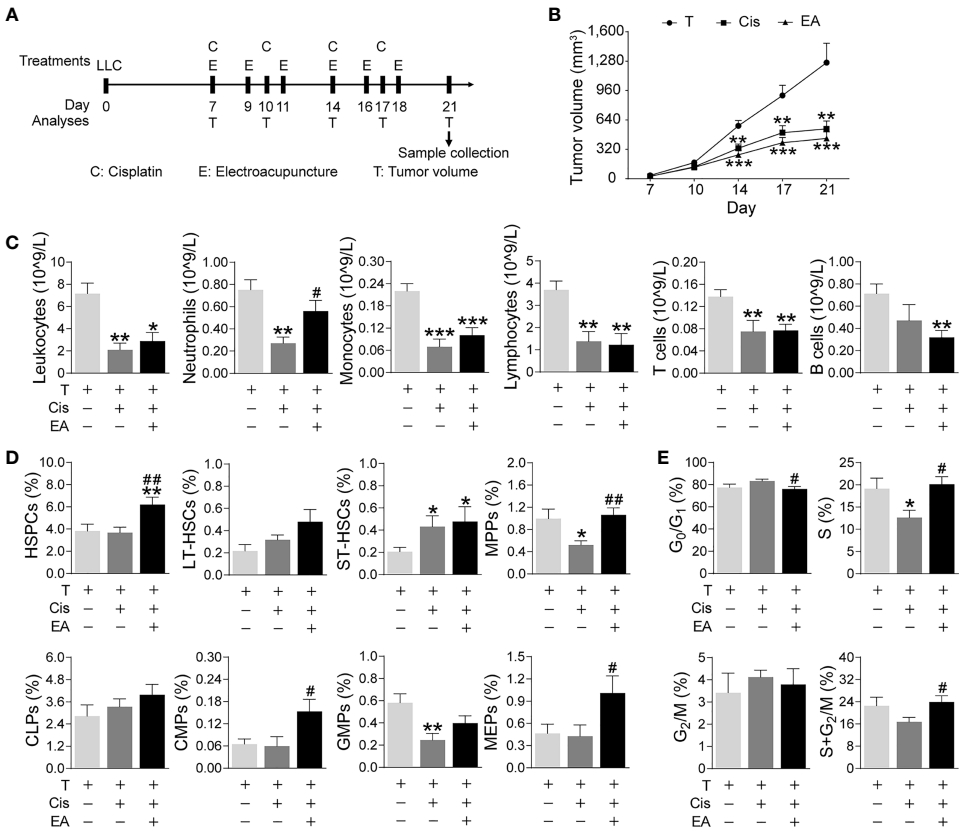

FIGURE 6 | Electroacupuncture restores hematopoiesis in cancer mice during cisplatin chemotherapy. **(A)** Experimental flowchart depicting the time of treatments of tumor (LLC) cells at day 0, cisplatin (C), electroacupuncture (E), and analyses of tumor volume (T) and sample collection. **(B)** Tumor growth curve (n=9 per group), P values were calculated using two-way repeated-measures ANOVA. **(C)** Peripheral blood counts of specific subpopulation of leukocytes (leukocytes, lymphocytes: T, n=8; Cis, n=6; EA, n=8. neutrophils, monocytes, T and B lymphocytes: T, n=8; Cis, n=6; EA, n=7). **(D)** Analyses of hematopoietic BM cell subpopulation (n =9 per group). **(E)** Quantification of PI nuclear staining of BM cells (n=9 per group). Data are mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* Veh; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Cis.

Our molecular analyses also show the potential of EA to modulate over 1,600 BM genes that are mainly related to extracellular matrix receptor interaction, B cell and toll-like receptors signaling, and the p53 and NF-kB pathways. Indeed, the extracellular matrix is critical to hematopoiesis and the response of hematopoietic cells to neurotransmitters and growth factors (24, 80). For instance, fibronectin is important for the adhesion and proliferation of hematopoietic and erythroid progenitors (81), whereas adiponectin can inhibit myelomonocytic cell expansion (82) and *Col1a1* and *Col1a2* are produced by BM stromal cells to define BM hematopoietic niche microenvironment (83, 84). Our RT-qPCR analyses showed that cisplatin activates *Col1a1 and Col1a2*, and EA preserved normal *Col1a1* production. The potential of EA to modulate *Col1a1* may be more significant than anticipate and not only beneficial to patients with chemotherapy. The control of *Col1a1* by EA can explain its potential to restore hematopoiesis and ameliorate the cancer inhibition of common myeloid and megakaryocytic/erythroid progenitors as discussed above in cancer mice. Our results warrant future studies to determine the role of this mechanism in hematopoietic cell translocation and egress and their clinical implications in cancer progression.

The main effects of EA are mediated by the nervous system, which is critical to coordinate BM hematopoiesis for physiological homeostasis. Many studies have shown that chemotherapy drugs such as cisplatin are neurotoxic and damage BM autonomic nerves compromising hematopoiesis (45). Thus, ablation of sensory nerves with capsaicin also reduces BM cellularity and causes leukopenia (85). Our results show that cisplatin induced neurotoxicity and inhibited the production of multiple neurogenic factors such as *Ngf*, *Bdnf*, and PACAP, whereas EA induced sympathetic neuroprotection and preserved the production of these factors. Of note, previous studies reported that the 28-38 tail of PACAP is important for blood transportation, BBB crossing, and degradation by plasma endopeptidases (31, 33). Furthermore, PACAP has two isoforms, PACAP27 and PACAP38, with the latter being the dominant in mammalian tissue at normal physiological conditions. However, their respective levels change in different physiological and pathological conditions. For instance, PACAP27 and PACAP38 levels were lower in lung cancer samples than in

healthy tissue. Likewise, our present study shows lower PACAP levels during cisplatin chemotherapy. Given that our PACAP ELISA kit recognizes both PACAP27 and PACAP38, future detailed studies will be required to determine the differential role of PACAP27 and PACAP38 in chemotherapy, neuromodulation of bone marrow hematopoiesis, and electroacupuncture.

Our previous studies showed that PACAP-specific receptor (PAC1) is strongly expressed on HSPCs of murine BM, and adcyap1$^{-/-}$ mice exhibited lower MPP populations and cell frequency in the S-phase of the cell cycle. Exogenous PACAP38 increased the numbers of colony forming unit-granulocyte/macrophage progenitor cells (CFU-GM) derived from HPSCs, and increased Cyclin D1 and Ki67 expression, and these effects were prevented by the PAC1 antagonist. Of note, the direct sympathetic regulation of HSPCs proliferation is also evidence by the fact that PACAP is not produced by BM cells, but secreted from the sympathetic terminals (42). In this study, our results showed PACAP is a critical neurogenic factor mediating the protective effects of EA during chemotherapy. We showed that EA-induced PACAP expression in BM is critical to sympathetic nerve neuroprotection during cisplatin chemotherapy, and neurogenic PACAP derived from BM sympathetic nerve terminals mediated the protective effects of EA in cisplatin chemotherapy.

Inhibition of PACAP receptor PAC1 with high dose of PACAP6-38, abrogated the potential of EA to preserve thermal nociception, BM hematopoiesis, hematopoietic cell proliferation, and peripheral leukopenia. Conversely, pharmacologic activation of PAC1-agonist, with high dose of PACAP1-38 mimics EA-induced neuroprotection and preserved thermal nociception in cisplatin-treated mice. PACAP1-38 also preserved BM hematopoiesis, hematopoietic cell proliferation, and peripheral leukocyte levels. Furthermore, PACAP6-38 treatment can decrease the hematopoiesis in cisplatin-treated mice, probably by blocking the hematopoiesis promoting effect of the remaining PACAP secreted from injured sympathetic nerve terminal in BM. As shown in previous studies, activated PAC1 can interact with Gαs stimulating adenylyl cyclase leading to elevated cAMP, protein kinase A activation to promote neuronal survival in cerebellar granule neurons (86). Meanwhile, PAC1 signaling also stimulated the proliferation of adult mouse neural progenitor cells through PKC-dependent pathway (31, 87). The potential pathway maybe involve that activated PAC1 can interact with Gαq stimulating PLC causing phosphatidyl inositol turnover. The diacylglycerol activates protein kinase C leading to Src phosphorylation to activate matrix metalloprotease metabolizing transforming growth factor-α (TGF-α) from inactive precursors, leading to the tyrosine phosphorylation of the epidermal growth factor receptor to activate Ras and Raf, resulting in the tyrosine phosphorylation of mitogen/extracellular signal-regulated kinase and extracellular signal-regulated kinase to increase cellular proliferation (31, 88). The effects and molecular mechanism of PAC1 receptor in mediating preservation of BM hematopoiesis in lung carcinoma mice by EA needs further investigation. In conclusion, our results indicate that PAC1 signaling may be one of the mechanisms induced by EA to protect against cisplatin-induced neurotoxicity and immunosuppression in cancer patients, and PAC1-agonists may provide therapeutic advantages to treat patients with advanced neurotoxicity or neuropathies limiting EA efficacy.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI BioProject, accession no: PRJNA687726.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Tianjin University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

ZX and LU conceived the project. SLi, JH, JW, SLu, BW, YGong, SQ, SW, YF and SZ performed the experiments. YL, SLi, YMG and JH performed the data analysis. ZX and YGuo provided administrative, technical, or material support. SLi and JH wrote the initial manuscript draft. ZX and LU analyzed data, organized data presentation, and completed manuscript writing and preparation. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by the National Natural Science Foundation of China (grant numbers 81704146, 82030125, 82074534). Research Project of Tianjin Municipal Health Commission on Traditional Chinese Medicine and Integrative Medicine (grant number 2019140), Graduate Research Innovation Project of Tianjin University of Traditional Chinese Medicine (grant numbers YJSKC-20201009, YJSKC-20201029), and LU are supported by the NIH R01-GM114180.

ACKNOWLEDGMENTS

We would like to thank Dr. Yinli Yang from Tianjin's Clinical Research Center for Cancer for providing LLC cells, and thank Dr. Hongzhe Sun and Lei Guo from TissueGnostics Asia Pacific Limited for their technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.714244/full#supplementary-material

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frontiers in Immunology

ORIGINAL RESEARCH
published: 11 November 2021
doi: 10.3389/fimmu.2021.706150

Solid Tumor Microenvironment Can Harbor and Support Functional Properties of Memory T Cells

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Robust T cell responses are crucial for effective anti-tumor responses and often dictate patient survival. However, in the context of solid tumors, both endogenous T cell responses and current adoptive T cell therapies are impeded by the immunosuppressive tumor microenvironment (TME). A multitude of inhibitory signals, suppressive immune cells, metabolites, hypoxic conditions and limiting nutrients are believed to render the TME non-conducive to sustaining productive T cell responses. In this study we conducted an in-depth phenotypic and functional comparison of tumor-specific T cells and tumor-nonspecific bystander memory T cells within the same TME. Using two distinct TCR transgenic and solid-tumor models, our data demonstrate that despite exposure to the same cell-extrinsic factors of the TME, the tumor-nonspecific bystander CD8 T cells retain the complete panoply of memory markers, and do not share the same exhaustive phenotype as tumor-reactive T cells. Compared to tumor-specific T cells, bystander memory CD8 T cells in the TME also retain functional effector cytokine production capabilities in response to *ex vivo* cognate antigenic stimulation. Consistent with these results, bystander memory T cells isolated from tumors showed enhanced recall responses to secondary bacterial challenge in a T cell transplant model. Importantly, the tumor-resident bystander memory cells could also efficiently utilize the available resources within the TME to elaborate *in situ* recall effector functions following intra-tumoral peptide antigen injection. Additionally, CRISPR-Cas9 gene deletion studies showed that CXCR3 was critical for the trafficking of both tumor antigen-specific and bystander memory T cells to solid tumors. Collectively, these findings that T cells can persist and retain their functionality in distinct solid tumor environments in the absence of cognate antigenic stimulation, support the notion that persistent antigenic signaling is the central driver of T cell exhaustion within the TME. These studies bear implications for programming more efficacious TCR- and CAR-T cells with augmented therapeutic efficacy and longevity through regulation of antigen and chemokine receptors.

Keywords: bystander memory anti-tumor immunity, CAR T therapy, tumor microenvironment, chemokines, CXCR3, antigen

OPEN ACCESS

Edited by:
Xi Wang,
Capital Medical University, China

Reviewed by:
Brian S. Sheridan,
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Nevil Singh,
University of Maryland, Baltimore, United States

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Specialty section:
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology

Received: *06 May 2021*
Accepted: *25 October 2021*
Published: *11 November 2021*

Citation:
Sullivan PM, Reed SJ, Kalia V and Sarkar S (2021) Solid Tumor Microenvironment Can Harbor and Support Functional Properties of Memory T Cells. Front. Immunol. 12:706150. doi: 10.3389/fimmu.2021.706150

INTRODUCTION

The limited success of adoptive T cell immunotherapy against solid tumors has been attributed to a multitude of variables including the trafficking of infused T cells to solid tumors and subsequent penetration and infiltration into the tumor microenvironment (TME) (1–3). In addition to chronic antigenic signaling, the TME harbors a multitude of inhibitory signals (i.e. PD-L1, IL-10, TGF-β), suppressive immune cells (i.e. regulatory T cells, (T_{reg}); monocyte derived suppressor cells, MDSC), metabolites (i.e. kynurenine metabolites), hypoxic conditions and limiting nutrients, which are believed to render the TME non-conducive to sustaining productive T cell responses (4–7). Consequently, most tumor-reactive T cells develop a hallmark exhaustive state characterized by loss of functionality and impaired memory differentiation, thus compromising anti-tumor immunity (2).

Current strategies to prevent T cell exhaustion and prolong T cell function within the TME are largely focused on targeting immune checkpoint molecules such as PD-1/PD-L1 and CTLA4 (8–11). However, only a fraction of patients receiving T cell immunotherapy for solid tumors are responsive to immune checkpoint blockade (ICB) (8), and responsiveness appears to depend on the retention of a stem-cell like phenotype by T cells which is sequentially lost as T cells become terminally exhausted (12–17). Hence, developing alternative strategies to combat T cell exhaustion and dysfunction in the TME will be instrumental in enhancing future adoptive T cell therapies against solid tumors, and expanding the reach of current ICB combination therapies to more patients. To develop such strategies, a greater understanding of the contributions of the individual immunosuppressive TME factors on T cell exhaustion, stemness, and responsiveness to ICB must be established.

Recent studies have identified non-tumor-antigen specific, or bystander T cells, within the TME (18–21). Bystander memory T Cells within the TME appear to retain their functionality, as the activation of bystander memory T cells within the TME has been shown to enhance the general anti-tumor response (18, 19) by inducing a local pro-inflammatory environment and production of effector cytokines such as IL-2 (22). Collectively, these findings suggest that T cell dysfunction in the TME is not a result of immunosuppressive factors alone but occurs in combination with chronic antigenic stimulation.

Multiple types of tumors have been shown to harbor bystander cells even when tumor antigen-specific cells are not detectable (20, 23, 24). These intriguing observations raise the question whether bystander memory T cells display superior trafficking to solid tumors compared to naïve tumor-specific T cells. Determining the mechanisms behind the migration of bystander memory T cells to solid tumors may guide immunotherapeutic approaches for both tumor-reactive T cells and harnessing the potential of bystander memory T cell activation in the TME to augment the anti-tumor response.

Here, we focus on ascertaining the in-depth phenotype, function, and memory recall potential of bystander memory T cells by comparing tumor-specific T cells and tumor-nonspecific bystander memory T cells within the same TME, using two distinct solid tumor models. We demonstrate that while tumor-specific T cells developed a characteristic exhaustive state within the TME (4–7), bystander memory T cells in the same tumors retained their expression of markers associated with canonical memory T cells … Further studies on T cell functionality showed that bystander memory T cells isolated from solid tumors retained their capacity for rapid effector cytokine production upon restimulation both *ex vivo* and *in situ*, and generated canonical recall responses to viral infection. Similar to reports of antigen-specific T cells migration to solid tumors (25, 26), the trafficking of bystander memory T cells to solid tumors was found to be largely dependent on CXCR3. Finally, we extend our findings to show that tumor-resident bystander memory T cells show similar resistance to exhaustion in a murine model of CAR T cell immunotherapy.

Collectively, the results from this study reveal a mechanism for antigen-independent trafficking of T cells to solid tumors, and directly demonstrate the impact of antigenic signaling in driving T cell exhaustion within the TME. These findings highlight the potential for bioengineering strategies to enhance adoptive T cell therapy against solid tumors *via* increased T cell migration to solid tumors through chemokine receptor engineering (27), and combatting T cell exhaustion through tunable antigen receptor expression (28–34). Additionally, these studies support potential targeting of memory bystander T cells to augment the PD-1 checkpoint blockade responsiveness of adoptively transferred CAR T cells, as in the case of TCR transgenic T cell therapies (18–21).

METHODS AND MATERIALS

Animals

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). $Ly5.1^+$ $H\text{-}2K^b$ Ovalbumin-specific TCR transgenic OT-I mice were provided by Dr. Martin Prlic (Fred Hutchinson Cancer Resource Center). $Thy1.1^+$ $H\text{-}2D^b$ GP33-specific TCR-transgenic P14 mice were maintained in our colony. *Listeria monocytogenes* expressing the ovalbumin peptide (Lm-Ova) was used at $1x10^5$ CFU and injected intravenously and $LCMV_{Arm}$ was used at $2x10^5$ PFU and injected intraperitoneally. All procedures were approved by IACUC and conducted in accordance to institute guidelines.

Flow Cytometry

All antibodies were purchased from Biolegend (San Diego, CA, USA). Aqua fluorescent reactive dye was purchased from Invitrogen. $2x10^6$ cells were stained for surface or intracellular proteins by incubating cells with antibodies for 45 minutes on ice, fixed and permeabilized with 1x Cytofix/CytoPerm (BD Biosciences), then stained for 45 minutes for intracellular proteins with antibodies diluted in 1x Permwash, before being fixed in 2% PFA for 20 minutes as described previously (35–39). All samples were acquired on a LSRII Fortessa (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo V9 software.

Isolation, Adoptive Transfers, and Sorting of CD8 T Cells

CD8 T cells were isolated from spleens using MojoSort Mouse CD8 T Cell Isolation Kit (Biolegend). CD8 T cells were adoptively transferred intravenously at the indicated numbers. OT-I bystander memory cells were sorted on a FACSJazz (BD Biosciences) using antibodies specific to Ly5.1.

Intracellular Cytokine Staining

About $2x10^6$ lymphocytes were stimulated with 0.2 μg/ml GP33-41 peptide, 0.2μg/ml Ovalbumin peptide, or plate-coated αCD3/αCD28 for 5 hours in the presence of Brefeldin A (BFA), followed by surface staining and intracellular staining for IFN-γ, TNFα, and IL-2.

Intratumoral Cytokine Production

BFA, Ovalbumin peptide, and GP33-41 peptide in a total volume of 30 μl was injected directly into tumors. After 5 hours, the spleen and tumor were harvested and lymphocytes isolated. $2x10^6$ cells from each tissue were stained as described above.

Tumor Cells

MC38 and B16.F10 cell lines were obtained from ATCC. These lines were transduced with lentivirus to express EGFP, firefly luciferase, and the LCMV GP33-41 antigen. The lines were clonally selected and expanded. For tumor assays, $1x10^6$ tumor cells were injected subcutaneously on the right flank of the mouse. Tumor measurements began 7 days post tumor cell injection and were carried out every 2-3 days afterward. Tumor volume was calculated as length*(width^2)/2.

CRISPR/Cas9

The CXCR3 gene was edited for deletion using CRISPR/Cas9 with three guide RNAs targeting the CXCR3 gene simultaneously. Guide RNAs were designed and ordered from Integrated DNA Technologies (IDT). The RNA sequences used were 1. TCTGCGTGTACTGCAGCTAG, 2. TGAGGGCTACA CGTACCCGG, and 3. AGTTAACACCAGCAGAACAT. The RNP complex was produced using Alt-R s.p. Cas9 Nuclease V3 protein (IDT), Alt-R CRISPR-Cas9 tracrRNA with ATTO550 (IDT), and Alt-R CRISPR-Cas9 sgRNA targeted to CXCR3 (IDT). The RNP complex was introduced using the Neon Transfection System (ThermoFisher Scientific). Uptake of the RNP complex was verified by ATTO550 staining using flow cytometry and CXCR3 knockout was confirmed by antibody staining and flow cytometry.

CAR T Cell Design and Transduction

An αCD19 CAR based on published methods was constructed in a MP71 vector (Chen et al, 2019). Retrovirus was produced by transient transfection of Plat E cells (Cell Bio Labs). CD8 T cells were isolated using the MojoSort Mouse CD8 T Cell Isolation kit (Biolegend). Cells were activated by plate bound αCD3/αCD28 for 24 hours then spinoculated by centrifuging at 2000x*g* for 60 minutes at 32°C. Cells were then adoptively transferred into day 1 $LCMV_{Arm}$ infection matched mice.

Statistical Analysis

Paired or unpaired Student's t-tests as appropriate were used to evaluate differences between samples. ANOVA with multiple comparisons was used to evaluate statistical significance between three or more groups. All analysis was performed using Graphpad Prism. P values of statistical significance are indicated with an asterisk: *p<0.05, **p<0.01, ***p<0.001. p>0.05 were considered non-significant (ns).

RESULTS

Bystander Memory CD8 T Cells Infiltrate Established Solid Tumors

Solid tumors have been recently reported to harbor bystander memory T cells (18–20), however the origin of these cells has not been studied extensively. To examine the role of antigen-specificity in CD8 T cell trafficking to the TME, we compared the ability of bystander memory T cells and tumor-antigen-specific (tumor-specific) T cells to traffic to tumors following adoptive co-transfer into mice. Briefly, naïve OT-1 T cells were transferred into naïve C57Bl/6 mice which were then infected with LM-Ova to generate Ova-specific OT-I (bystander) memory CD8 T cells (**Figure S1A**). About 30 days after infection, naive TCR transgenic P14 CD8 T cells specific for the LCMV GP33 epitope were transferred into the OT-1 memory mice, which were subsequently inoculated with GP33-expressing MC38 colon carcinoma or B16.F10 melanoma tumors (**Figure S1A**). By 21 days post-tumor inoculation, the tumors were well established (**Figure S1B**) and both the bystander memory and tumor-specific donor cells were detectable in the spleen, inguinal (tumor-proximal) and brachial (tumor-distal) lymph nodes, liver, lung, and tumor sites (**Figures 1A, B** and **S1C**). In the non-tumor bearing mice, bystander and tumor-specific populations of cells showed largely similar distribution patterns across the various tissues (**Figure 1B, C**). However, in tumor-bearing mice, the tumor-specific CD8 T cells were redistributed from spleens to the tumor sites as indicated by decrease in absolute donor cell numbers, as well as percent localization when compared to non-tumor bearing control mice (**Figures 1B, C** and **S1D**). These results suggest that, while T cells may traffic to solid tumors from all tissues examined, the spleen acts as the primary reservoir for cells recruited to solid tumors (**Figures 1C** and **S1D**). Somewhat unexpectedly, a significantly greater proportion of the bystander memory cell population was found in both the MC38 and B16.F10 tumors compared to the naïve tumor-specific cells (**Figures 1C** and **S1D**). These data demonstrate that bystander memory cells are effectively recruited to solid tumors in an antigen-independent manner.

Bystander Memory CD8 T Cells Maintain a Quiescent Phenotype in the TME

The rapid exhaustion of tumor-Ag sp. T cells in the TME has been attributed to chronic antigenic stimulation in combination with a multitude of cell extrinsic variables. Such factors include inhibitory receptor ligands and cytokines found on immunosuppressive cells

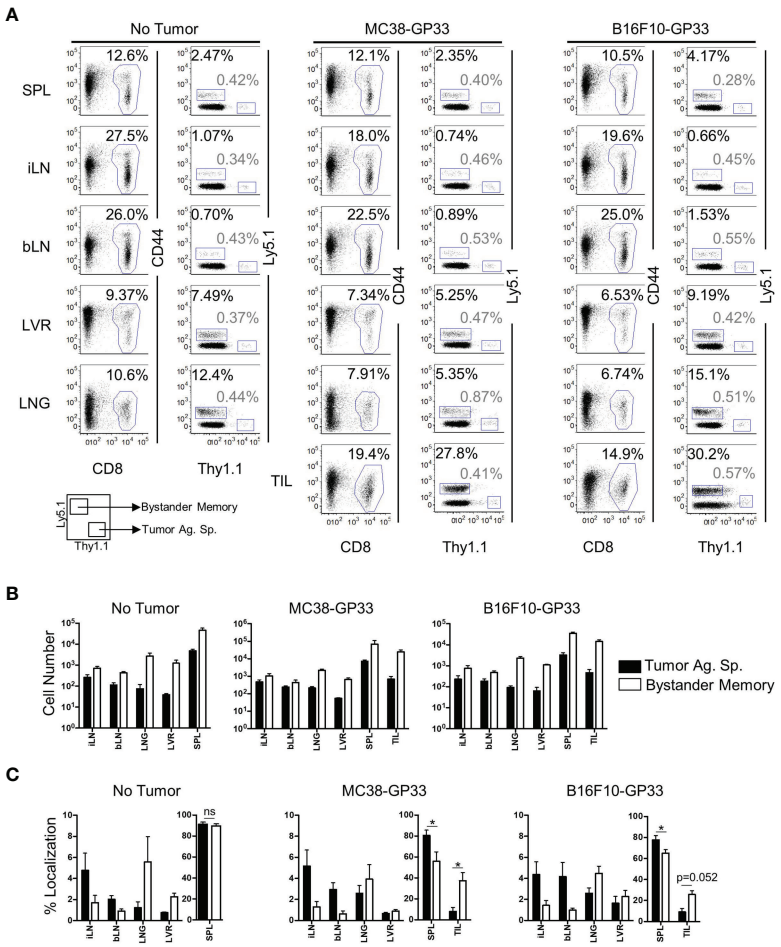

FIGURE 1 | Bystander Memory T Cells infiltrate into established solid tumors. WT OT-I CD8 T cells were adoptively transferred into C57BL6 mice and infected with LM-Ova. Following memory differentiation (>day 30 post infection), naïve P14 CD8 T cells were transferred into the mice. The mice were then subcutaneously injected with MC38-GP33 or B16.F10-GP33 tumor cells. **(A)** FACS plots of CD8 T cells in spleen (SPL), brachial lymph node (bLN), inguinal lymph node, (iLN), liver (LVR), lung (LNG), and tumor infiltrating lymphocytes (TIL) show the frequency of donor CD8 T cells of total CD8 T cells or the frequency of bystander memory OT-I donors (black) and tumor antigen specific P14 donors (gray) of total CD8 T cells at day 21 post tumor injection. Bar graphs show **(B)** the total number of CD8 T cells, bystander memory, and tumor antigen specific T cells in each tissue and **(C)** the percent localization of bystander memory cells and tumor antigen specific CD8 T cells in each tissue. Percent localization was calculated as total number of specified cell population in a given tissue divided by sum of that cell population identified in all the tissues collected. Representative plots are shown from N=5 mice. Significance was determined by paired T-test. *p < 005. Differences were non-significant if not otherwise indicated. Data is representative of 3 separate experiments. ns, non-significant.

in the TME and tumor cells, nutrient deprivation, and a hypoxic microenvironment (4, 5, 40, 41). In comparison to how these factors influence responding T cells, even less is known about their influence on T cell programming and function in the absence of antigenic signaling. To independently evaluate roles of antigenic signaling *vs* cell-extrinsic variables on CD8 T cell exhaustion in the TME, we compared the phenotype of bystander memory and tumor-specific T cells isolated from tumors and spleens of mice, as in **Figure 1**. Consistent with T cell phenotypes in an antigen-free environment, both the bystander memory and tumor-specific cells isolated from the spleens of naïve mice showed a quiescent phenotype as elucidated by low levels of expression of GzmB, the exhaustion markers PD-1 and TIM-3, and high expression levels of the pro-survival marker Bcl-2 and lymph node homing marker L-selectin (CD62L) (**Figure 2A**). Bystander and tumor-specific T cells isolated from the spleens of tumor bearing mice displayed a similar phenotype to those from the non-tumor bearing controls, suggesting that negligible amounts of tumor-Ag were present in the spleens of tumor-bearing mice (**Figure 2A**). In contrast, striking phenotypic differences between bystander and tumor-specific T cells were observed in tumor infiltrating lymphocytes (TIL) isolated from both MC38 and B16.F10 tumors. While the tumor-specific T cells isolated from the tumors displayed a phenotype characteristic of strong antigenic signaling and possible exhaustion, the bystander memory cells largely retained a phenotype similar to those isolated from the spleen (**Figure 2A**). Compared to bystander memory T cells, the tumor-specific TIL exhibited significantly higher expression levels

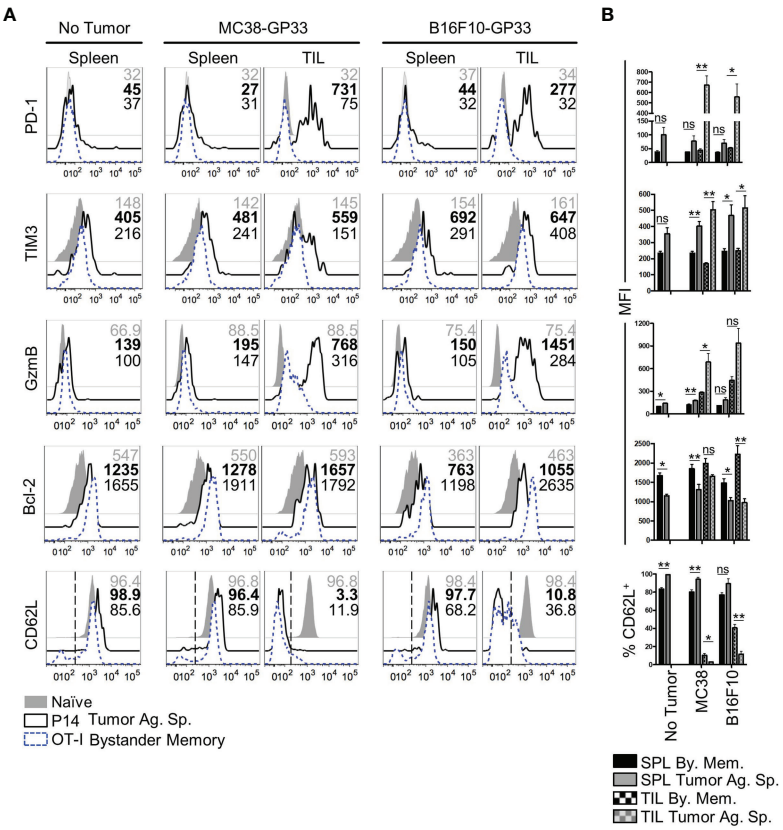

FIGURE 2 | Phenotype of bystander memory CD8 T cells in tumor microenvironment. **(A)** Histograms are gated on CD8 T cells and show the respective markers in spleen of naïve (gray), tumor antigen specific (solid, black), or bystander memory (dashed, blue) CD8 T cells. Numbers show MFI of given markers for naïve (gray), tumor antigen specific (bold), and bystander memory (black) T cells for spleen and TIL taken from B6 mice with no tumor, MC38-GP33 tumor, or B16.F10-GP33 tumor. Bar charts to the right **(B)** show the average MFI or average percent positive with SEM for each population of CD8 T cells. Representative plots are shown from N=5 mice per group. *p < 0.05. **p < 0.01 as determine by paired T-test. Data is representative of 3 independent experiments. ns, non-significant.

of GzmB, PD-1, TIM-3, and significantly lower expression levels of Bcl-2, and significantly reduced proportions of $CD62L^+$ cells (**Figure 2B**). The bystander memory TIL population also contained a significantly lower frequency of CD38 and CD101 double-positive cells compared to the tumor-specific T cells, thus indicating that bystander memory cells in the TME are resistant to terminal differentiation. (**Figure S2A**). Hence, despite exposure to the harsh and immunosuppressive cell extrinsic variables in the TME, the bystander cells evidently retained a quiescent, and largely undifferentiated state. These results strongly support the notion that chronic antigenic signaling is the predominant factor driving an exhausted phenotype in the tumor-specific T cells within the TME, and mere exposure to environmental factors in the TME does not result in T cell exhaustion.

Bystander Memory T Cells Maintain Functionality in the Tumor Microenvironment

T cell dysfunction in the TME can result from T cell exhaustion, anergy, or senescence (42, 43). While induction of inhibitory receptors (such as PD-1 and TIM-3) is a key phenotype of exhausted CD8 T cells, PD-1 is also induced during early stages of activation in acute infections (44). Therefore, we next confirmed whether the bystander memory TILs retained their functionality in the TME, consistent with their lack of an exhausted phenotype. To assess the functionality of bystander memory and tumor-specific T cells, we first evaluated the cytokine production of each population following direct *ex vivo* stimulation with plate-bound αCD3/αCD28. Consistent with the phenotypes observed in cells recovered from spleen and TILs in **Figure 2**, the bystander memory T cells from both spleen and tumor showed strong cytokine production following restimulation (**Figures S3A**). Of the cells isolated from MC38 tumors, the bystander memory cells showed superior cytokine production to the tumor-specific T cells and contained on average 3.7-fold more IFN-γ^+ TNFα^+ cells, and nearly 9-fold more IFN-γ^+ IL-2^+ cells than tumor-specific T cells (**Figures 3A, B**). Similar patterns were observed in TILs from B16.F10 tumors, where the bystander cells contained 1.5-fold more IFN-γ^+ TNFα^+, and 3.4-fold more IFN-γ^+ IL-2^+ cells compared to tumor-Ag sp. T cells (**Figures 3C, D**). To further evaluate the functional *vs* exhaustive phenotype of the bystander memory T cells, we compared the

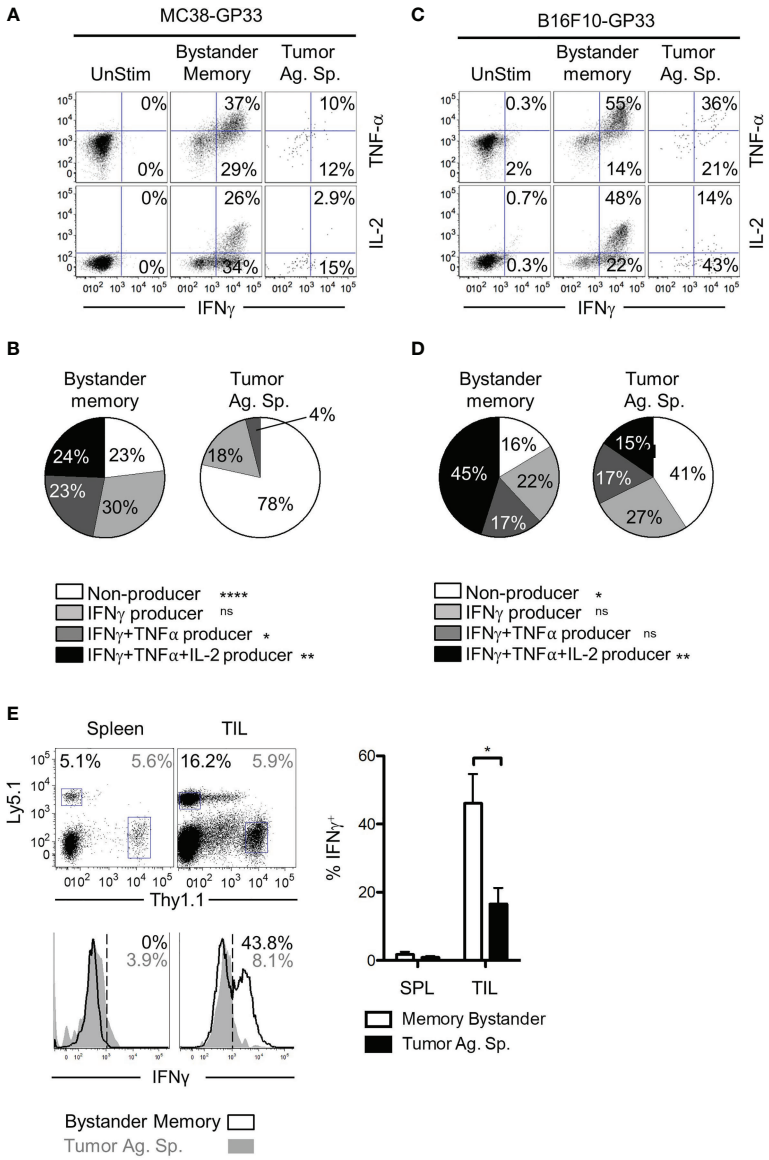

FIGURE 3 | Polyfunctionality of bystander memory T cells in tumor microenvironment. CD8 T cells from spleen and tumor of mice bearing MC38-GP33 tumors **(A, B)** or B16.F10-GP33 tumors **(C, D)** were stimulated with αCD3/αCD28 for 5 hours in the presence of BFA and cytokine production was assessed. **(A, C)**. FACS plots are gated on total donor population (unstim), OT-I donors (bystander memory) or P14 donors (tumor ag-sp) cells from tumors. FACS plots show % of IFN-γ/TNF-α double positive or % IFN-γ/IL-2 double positive of each population. **(B, D)**. To assess the degree of polyfunctionality of each population of cells, the proportion of nonproducing (white), IFN-γ^+ (light gray), IFN-γ^+ TNFα^+ (dark gray) and IFN-γ^+ TNFα^+ IL-2^+ (black) donor CD8 T cells were plotted in pie charts. **(E)**. Established B16.F10-GP33 tumors were injected intratumorally with 30 ul PBS containing Ova peptide, GP33 peptide, and BFA to assess *in situ* cytokine response. Five hours later, the spleen and tumors were collected, and cells were assessed for IFN-γ production. Gating of donor population is shown in the top panel, with Ly5.1^+ memory bystanders and Thy1.1^+ tumor antigen specific CD8 T cells. Bottom panel show histograms for IFN-γ in the spleen (left) and tumor (right). Percentage of IFN-γ positive cells is shown in the upper right corner and is plotted in the bar chart to the right. Representative plots are shown from N=5 mice per group. Statistical significance was determined by ANOVA with multiple comparisons **(B, D)** or paired t-test **(E)** *$p < 0.05$, Data is representative of 3 independent experiments. **$p < 0.01$, ****$p < 0.0001$, ns, non-significant.

polyfunctionality of the bystander memory and tumor-specific T cells following stimulation. Consistent with the exhausted phenotype displayed by the tumor-specific TILs (**Figures 2A** and **S3D, E**), IFN-γ^+ TNFα^+ IL-2^+ tumor-specific T cells were undetectable in MC38 tumors, and on average made up only 15% of tumor-specific T cells isolated from B16.F10 tumors. In contrast, the bystander memory population isolated from the MC38 and B16.F10 tumors contained an average of 24% and 45% of IFN-γ^+ TNFα^+ IL-2^+ T cells, respectively, following restimulation (**Figures 3B, D**). These results are consistent with

the phenotypes observed in **Figure 2**, and indicate that despite residency in the distinct TME, bystander memory T cells retain functionality.

The results from the *ex vivo* restimulation demonstrate that compared to tumor-specific CD8 T cells, the bystander memory T cells retain their polyfunctionality following exposure to the TME. However, *in vitro* conditions do not recapitulate the immunosuppressive environment of the TME. To test whether the bystander memory T cells were capable of elaborating effector cytokine production within the TME, T cells in B16.F10-GP33 tumors were directly restimulated *in vivo* through intratumoral injection of GP33 and OVA peptides, and Brefeldin A (BFA). Five hours after peptide injection, tumor-Ag sp. and bystander memory T cells were isolated from tumors and spleens, then immediately examined for intracellular IFN-γ production (**Figure S3F**). As expected, neither the tumor-specific nor bystander memory T cells isolated from the spleens expressed IFN-γ, due to the localized administration of peptide-Ag inside the tumors (**Figure 3E**). Meanwhile, amongst the TILs, there were significantly more IFN-γ-producing bystander memory T cells (44% avg) compared to the tumor-specific T cells (17.5% avg) (**Figure 3E**). The IFN-γ expression patterns observed in the TILs were reflective of their exhausted state as determined by PD-1 expression (**Figure S3G**). In addition, a greater frequency of the bystander memory cells with intermediate and high levels of PD-1 expression were IFN-γ^+ compared to tumor-specific counterparts (**Figure S3G**). Collectively, using both *ex vivo* and *in situ* stimulation with cognate peptide antigens, these studies confirm that bystander memory T cells in solid tumors maintain their functionality as exemplified by their rapid and robust cytokine production, even within the immunosuppressive TME.

Memory Bystander T Cells Retain Their Recall Potential Following Residency in the TME

The results thus far demonstrated that the cell-extrinsic variables in the TME had little effect on bystander memory exhaustion and polyfunctionality. We next investigated the impact of immunosuppressive factors within the TME on bystander memory T cells' recall expansion potential – another hallmark functional property of robust memory CD8 T cells. To test this, OT-I bystander memory T cells were isolated from the spleens and tumors of B16.F10-GP33 and MC38-GP33 bearing mice 21 days after tumor inoculation, (**Figure S4A**). Equal numbers of bystander memory CD8 T cells from tumors and spleens were then transferred separately into naive B6 mice which were subsequently challenged with LM-Ova. T cell expansion kinetics were used to evaluate memory responses. Nearly identical expansion and contraction dynamics were observed between OT-I donors originating from the spleen and tumors of both MC38 and B16.F10 recipients (**Figures 4A, B**). Analysis of the spleen, liver, and lymph nodes on D28 post-infection (P.I.) showed similar OT-I cell numbers between spleen and tumor-derived donors (**Figures 4C, D**). Phenotypically, the spleen and tumor-derived donors expressed similar levels of Bcl-2, TIM3, CXCR3, PD-1, and had undergone similar patterns for memory *vs* effector differentiation as determined by CD62L, CD127, and KLRG-1 (**Figures 4E, F**, and **S4B, C**). Furthermore, the spleen and tumor-derived donors were equally functional when stimulated *ex vivo* with αCD3/αCD28 or with Ova peptide (**Figures S4D, E**). Collectively, these data demonstrate that the residency of bystander memory T cells in the TME does not impair CD8 T cell memory differentiation with respect to recall expansion potential and are consistent with our data showing retention of polyfunctionality of bystander memory T cells in tumor and secondary lymphoid sites alike.

CXCR3 Is Critical for Bystander Cell Localization to Solid Tumors

Despite the lack of an antigenic target, the bystander memory cells displayed efficient trafficking to solid tumors (**Figure 1**). While the chemokine receptor CXCR3 has been shown to be critical for tumor-specific T cell migration to solid tumors, it is unknown whether the trafficking of bystander memory T cells to the tumors in absence of cognate Ag is also dependent on CXCR3. To directly test this, CRISPR-Cas9 was used to remove *cxcr3* from naïve P14 CD8 T cells (**Figure 5A**) which were then adoptively co-transferred into mice along with ATTO-only CRISPR-Cas9 (WT) controls (**Figure 5B**). The recipient mice were infected with $LCMV_{Arm}$ (**Figure S5A**). After the *cxcr3* WT and KO populations differentiated into memory CD8 T cells (~60d P.I.), *cxcr3* knockout was confirmed (**Figure 5C**), mice were inoculated subcutaneously with a parental line of B16.F10 tumors, which did not express the GP33 epitope. Twenty-one days post-tumor inoculation, tissues were collected as in **Figure 1** and analyzed for donor populations. CXCR3 expression remained at high levels on greater than 90% of the WT cells isolated from the spleen and lymph nodes but was downregulated in the TILs (**Figure 5D**). As expected, expression of CXCR3 was absent on the vast majority (>90-85%) of *cxcr3* KO cells across all tissues examined (**Figure 5D**). Aside from the expression of CXCR3, the WT and KO cells isolated from the tumors and spleens were found to be similarly quiescent as determined by high expression of CD62L and CD127, and low expression of PD-1 and KLRG1 (**Figure S5B**). In addition, *cxcr3* KO cells largely retained the functional ability to express IFN-γ and TNFα following *ex vivo* restimulation with αCD3/αCD28, albeit to slightly lower levels than WT cells as reported previously (45–48) (**Figure S5C**). Interestingly, despite the lack of an antigenic target on the tumors for both the WT and *cxcr3* KO cells, a clear dissimilarity was observed in the anatomical distribution of the two populations. Although roughly equal numbers of the WT and *cxcr3* KO cells were transferred prior to tumor inoculation (46.6% *cxcr3* KO and 51.4% WT) (**Figure 5B**), of the donor cells, a skewed ratio of *cxcr3* KO : WT (~70% *cxcr3* KO and ~30% WT) cells was detected in the spleens, and lymph nodes of mice following tumor inoculation (**Figure 5D**). However, of the TILs, the frequency of donor cells that were WT increased to an average of 45% (**Figure 5D**). Furthermore, in the context of the total number of respective donor cells recovered from all analyzed

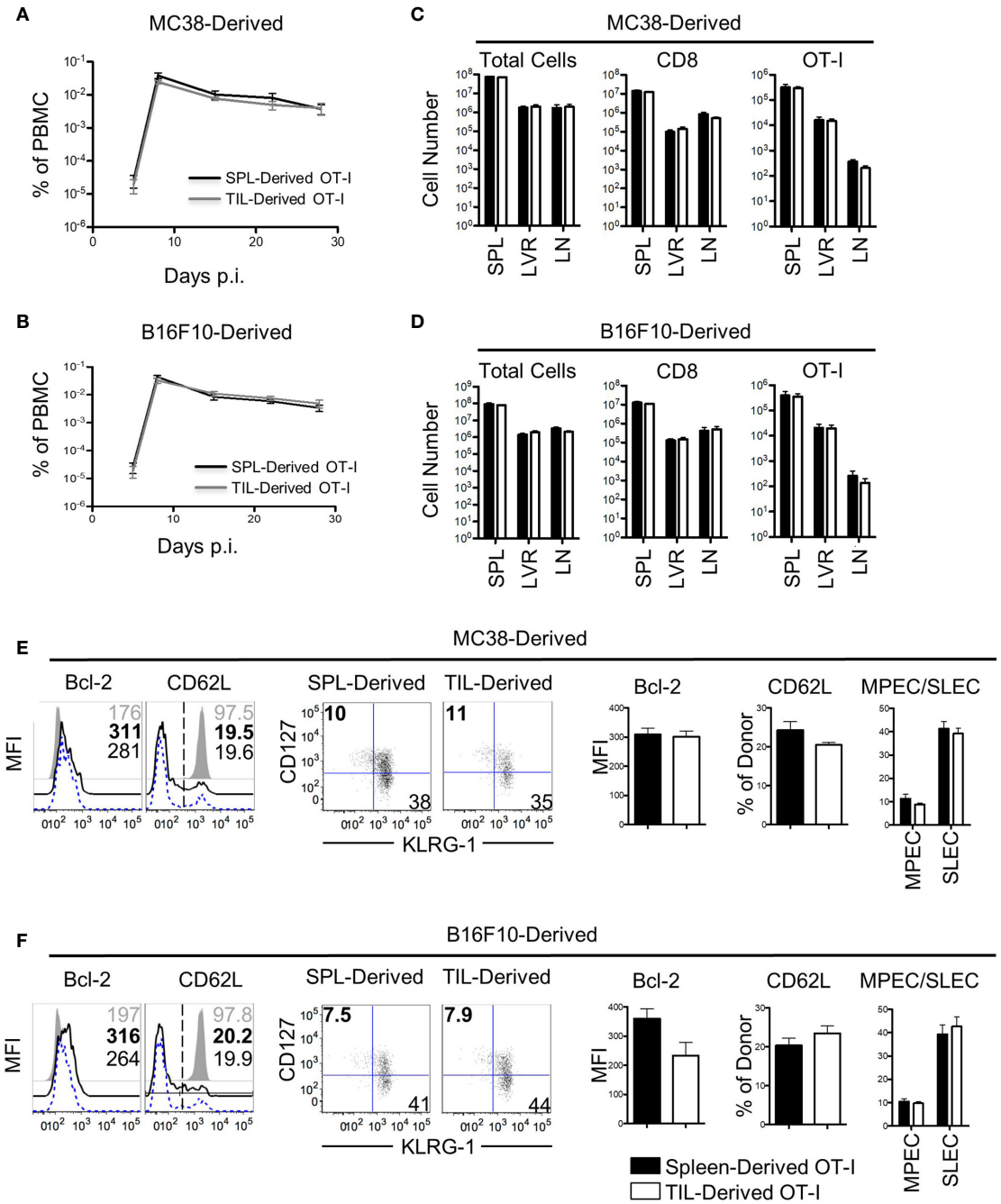

FIGURE 4 | Bystander memory CD8 T cells from the tumor microenvironment retain their recall ability. Bystander memory CD8 T cells were isolated from TILs or spleens of mice bearing MC38-GP33 tumors and were sorted by FACS. 4000 OT-I bystander memory cells were adoptively transferred in naïve B6 mice and subsequently infected with 15k CFU of Lm-Ova. **(A, C)**. Mice were bled to follow donor expansion and contraction. Spleen (SPL)-derived (black) and TIL-derived (gray) bystander memory cells are plotted as percent of total PBMC. Bystander memory isolated from MC38-GP33 bearing mice are shown in **(A)** and bystander memory from B16.F10-GP33 bearing mice in **(C, D)**. At day 28, tissues were collected from these mice and donor cells from SPL, liver (LVR) and lymph node (LN) were analyzed. The total number of cells, total number of CD8 T cells, and OT-I donor cells are quantified from spleen-derived donors (black bars) and tumor-derived donors (white bars). Bystander memory isolated from MC38-GP33 bearing mice are shown in **(B)** and bystander memory from B16.F10-Gp33 bearing mice in **(D, E)** Spleens samples of mice that received donors originating from MC38-GP33 bearing mice **(E)** or from B16.F10-GP33 **(F)** were stained for phenotypic markers. Histograms depict the mean fluorescence intensity (MFI) or percent of cells expressing the given marker. Quantification of each marker is shown to the right. Short-live effector cells (SLEC) were gated on KLRG1+CD127- populations and memory precursor effector cells (MPEC) were gated on KLRG1-CD127+ populations. Unpaired T-tests were run to compare the SPL-derived donors to the TIL-derived donors with no significant differences found between any groups. N=5 mice per group. Data is representative of 2 independent experiments.

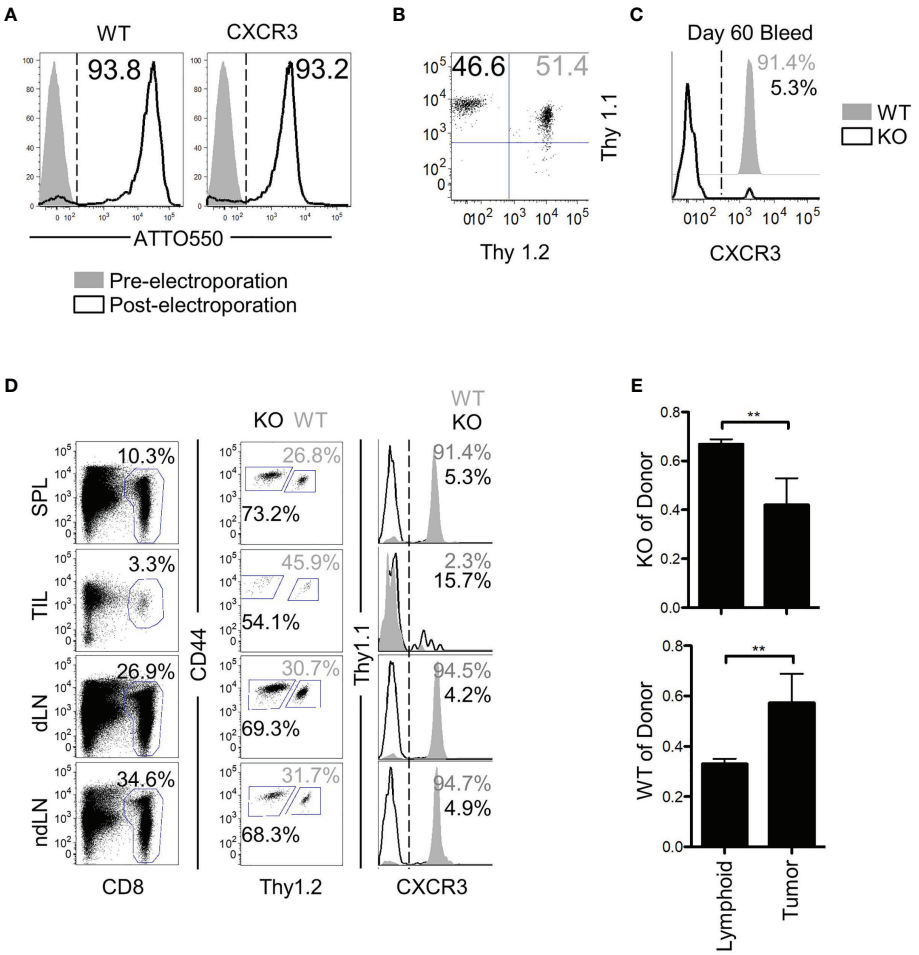

FIGURE 5 | CXCR3 is necessary for proper localization of bystander memory to the tumor. **(A)** Histograms of CD8 T cells pre- and post-Neon electroporation to show percent of cells that acquired ATTO550-labeled CRISPR-Cas9 RNP complex. Percent WT of donor is shown in solid grey, KO in black. **(B)** Cells were mixed 1:1 before adoptive transfer into B6 mice. **(C)** Confirmation of loss of CXCR3 protein in the knockout population at memory. **(D)** Representative plots of total CD8 and of donor populations in each tissue collected. Percent of CD8 is shown above the CD8 gate. Percent of *cxcr3* WT (gray) and KO (black) out of total donor CD8 cells is shown adjacent to each gate (middle column). Confirmation of *cxcr3* KO in each tissue is shown by histogram for CXCR3 expression. Percent WT of donor is shown in solid grey, KO in black. **(E)** Bar graphs showing the proportion of *cxcr3* KO (top) or WT (bottom) CD8 T cells to total donor cells present in either lymphoid tissues (Spleen and Lymph Nodes combined) or in tumors. **p < 0.01 as determined by paired T-test. Representative plots are shown from N=5 mice per group. Data is representative of 2 independent experiments.

tissues, a significantly greater proportion of the WT donor cells localized to the tumors compared to the *cxcr3* KO cells (as determined by the observed change in ratio of *cxcr3* KO donor cells to total donor cells) (**Figure 5E**). These findings demonstrate that similar to CXCR3-dependent trafficking of Ag-specific T cells to solid tumors (25, 26), CXCR3 plays a major role in the antigen-independent migration of bystander memory CD8 T cells to solid tumors.

Bystander CD8 T Cells Are Functional in a CAR T Therapy Mouse Model

Finally, we asked whether the characteristics of bystander memory CD8 T cells were translatable to a CAR T cell therapy model. To test this, we retrovirally transduced P14 CD8 T cells with an anti-CD19 CAR construct and adoptively transferred a mixed population of transduced (CAR^+) and non-transduced (CAR^-) T cells into naïve C57Bl/6 mice (**Figure S6A**). The mice were then infected with $LCMV_{Arm}$ to expand both populations of cells using the TCR. Having established the localization and functional competence of tumor non-reactive bystander memory cells in two distinct solid tumor-types, we next sought to determine if CAR-T cells that are nonreactive to tumor antigens also localize to tumors and retain functionality. Since *in vivo* expansion in response to cognate antigen on tumors is essential for CAR-T cell detection, we engaged the strategy of LCMV infection to expand CAR T cells generated using P14 T cells through the $H2D^b$: GP33-specific TCR six days after infection, mice were inoculated subcutaneously with MC38

tumors expressing truncated hCD19 (hCD19t) antigen as a model tumor-associated antigen (**Figure S6A**). The CAR$^+$ and CAR$^-$ T cells were detectable in roughly similar proportions in both the spleen and tumor sites 25 days after transfer (**Figure 6A**). Phenotypic analysis of the CAR$^+$ and CAR$^-$ donors showed no significant differences in the cells isolated from the spleen (**Figure 6B, D**). Similar to the TCR-based models of the bystander donor population that infiltrated the tumors, the CAR$^-$ T cells, which acted as bystanders in this model, did not express effector protein GzmB, or exhaustion markers PD-1 and CD38 (**Figures 6B–E**). In contrast, majority of the CAR$^+$ T cells isolated from the tumors exhibited increased expression of GzmB, PD-1, and CD38 compared to the CAR$^+$ cells from the spleen (**Figures 6B–E**). Furthermore, compared to the CAR$^+$ cells, the bystander CAR$^-$ TILs exhibited memory phenotype, based on increased expression of CD127 (**Figure 6B, D**), similar to the data in **Figure 1**. Finally, compared to the CAR$^+$ cells, a greater frequency of CAR$^-$ T cells retained polyfunctionality following residency in the TME as determined by expression of both IFN-γ and TNFα following *in vitro* restimulation (**Figure S6B**). Tumor cell expression of hCD19t was confirmed at the experimental end-point, thus indicating that the CAR T cells in the tumors had persisted under chronic antigenic conditions (**Figure S6D**). Collectively, these data mirror the results from the bystander memory cells in the TCR-based models, and further support that chronic antigenic signaling through CARs in the TME is the central driver of CAR T cell exhaustion in solid tumors. Importantly, these data also provide evidence that bystander

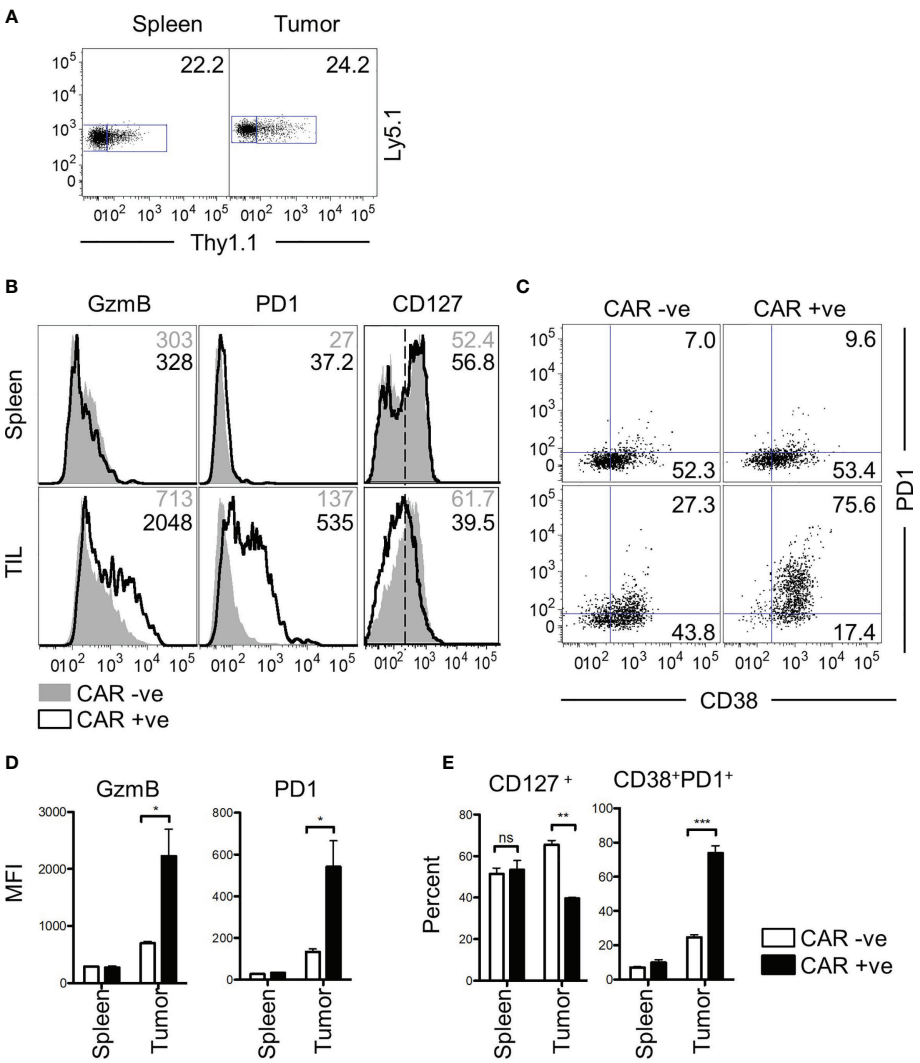

FIGURE 6 | Bystander CD8 T cells are functional in a CAR T therapy mouse model. **(A)** CD8 T cells transduced with a CD19 CAR and Thy1.1 transduction marker are observed in both spleen and tumor of mice at day 25 post transfer. Donor cells express Ly5.1 and CAR transduced cells express Thy1.1. **(B)** Phenotypic markers GzmB, PD-1, CD127, and **(C)** CD127 vs CD38 were assessed by flow cytometry. Values for GzmB and PD-1 show MFI, whereas the numbers for CD127 and CD38 v PD-1 show percent gated positive or double positive. GzmB and PD1 markers are graphed below in **(D)** CD127+ and CD38+PD1+ are graphed in **(E)** All data is representative of two independent repeats with N=3 mice per group. ns, non-significant. *p < 0.05, **p < 0.01, ***p < 0.001 as determined by paired t-test.

memory T cells display a similar phenotype, and functionality in both TCR and CAR-based models.

DISCUSSION

The limited success of current adoptive T cell therapies against solid tumors has widely been attributed to the milieu of immunosuppressive factors present within the TME (4–7). While these variables almost certainly contribute to tumor-specific T cell exhaustion and subsequent loss of function, the results from this study suggest that extended exposure to these extrinsic factors alone are not sufficient to drive terminal exhaustion in T cells. In the context of TCR and CAR-based models of T cell immunity against solid tumors, here we show that in the absence of antigenic signaling, bystander memory T cells retain a quiescent phenotype and functional potency in both immunogenically hot MC38 carcinoma, and cold B16.F10 melanoma tumors (49–51). These findings are consistent with similar reports in distinct murine and human tumors as well (19, 20, 24, 52). The differences in functionality observed between the bystander memory cells recovered from spleens, and TILs, or between TILs recovered from MC38 and B16.F10 tumors were not found to be statistically significant (**Figure 3B**). However, the differences observed were consistent within experimental groups and raise the possibility that tumor-specific factors such as the composition of cytokines (immunosuppressive *vs* pro-inflammatory), costimulatory or inhibitory molecule signaling on tumor or immune cells, and access to nutrients and metabolites may impact bystander memory functionality. The antigenic encounter history of memory T cells is also likely to impact the functionality of bystander memory cells in the TME. For example, primary, secondary, tertiary and quaternary bystander memory T cells are expected to show progressively higher functionality in the TME, as suggested by recent report of increased responsiveness to inflammatory signaling and tumor control by memory cells that have encountered multiple rounds of antigenic restimulation (53). In rigorous functional tests, our studies show that primary bystander memory CD8 T cells retained robust polyfunctionality when restimulated with cognate antigen even *in situ* in the immunosuppressive tumor microenvironment. Thus, our findings are consistent with those of Rosato et al. who demonstrated that the activation of bystander memory T cells *via* antigenic signaling can augment the anti-tumor response (19). Tumor-derived bystander memory cells further exhibited robust recall expansion potential *in vivo* and were capable of undergoing potent expansion and effector differentiation upon rechallenge, thus further supporting the notion that they did not adopt a terminally differentiated state. Due to the largely isolated nature of solid tumor Ag to the tumors, our T cell transplant results demonstrate that functional T cells can persist and function as long-lived memory T cells in extra-tumoral sites reinforce the hypothesis that chronic antigenic signaling is the driving force behind T cell exhaustion and subsequent dysfunction in the TME.

CXCR3 has been identified as a key chemokine receptor for efficient localization of CD8 T cells to solid tumors (54–56). Loss of CXCR3 or its ligands, CXCL9 or CXCL10, has been shown to disrupt the migration of adoptively transferred T cells to solid tumors resulting in impaired anti-tumor responses (25, 26, 57). That the bystander memory T cells showed a dependency on CXCR3 for effective tumor migration is consistent with these results and reinforces that CXCR3-mediated T cell trafficking to tumors can occur in an antigen-independent manner (46). As CXCR3 is upregulated following CD8 T cell activation and is maintained on effector and memory cells independently of continued antigenic stimulation (47), the superior trafficking of the bystander memory T cells compared to the naïve tumor-specific T cells in this study was likely attributed to their previously-activated state.

Although CXCR3 appeared to play a significant role in T cell trafficking to solid tumors, a notable number of *cxcr3* KO cells were present in the TILs. Interpretation of these results is complicated due to both antigen specific and bystander memory cell downregulation of CXCR3 expression within the TME. The loss of CXCR3 expression has been observed in multiple solid tumor types and is likely attributed to cell-extrinsic variables within the TME such as inhibitory receptor signaling and TGF-β secreted by tumor cells (58). In light of these observations, it is possible that the CXCR3 KO donor TILs stemmed from the minority population that did not successfully ablate CXCR3 expression, but downregulated CXCR3 expression after reaching the tumor sites. It is also possible that the trafficking of *cxcr3* KO T cells to the tumors was facilitated by other redundant chemokines that assume a dominant role in the absence of CXCR3. A recent study showed that CXCR3 was critical for responsiveness to checkpoint blockade immunotherapy by increasing T cell proximity to intratumoral $CD103^+$ DCs in the TME (45). Consistent with this report, our studies suggest that CXCR3 overexpression may be exploited in adoptive T cell immunotherapy to drive the trafficking of tumor-reactive T cells to tumor-sites and synergize with PD-1 checkpoint blockade immunotherapy. The multi-faceted role of CXCR3 expression in cancer biology warrants further investigation, especially in the context of diverse solid tumor-types and adoptive TCR and CAR T cell therapy.

Furthering the observations that bystander T cells are present in solid tumors, our studies provide deeper insight into the phenotype and functional capabilities of memory bystander T cells within the TME. Our findings that tumor infiltrating bystander CD8 T cells do not exhibit hallmarks of exhaustion, such as sustained inhibitory receptor expression, loss of cytokine production (4) and terminal differentiation (59), bear implications for future exploitation of bystander tumor-resident memory T cells for indirect augmentation of tumor-reactive T cells during checkpoint blockade immunotherapy as suggested by Rosato et al. (19),. Importantly, our results directly show that without chronic antigenic signaling, T cells within the TME retain functionality and memory potential. As strategies emerge to control the expression of CARs (29, 34) as well as modulate TCR/CAR signaling (28, 60, 61), our findings reinforce the potential for strategies to mitigate T cell exhaustion in the TME by regulating TCR/CAR expression and/or signaling, thereby augmenting the therapeutic efficacy of adoptive T cell transfers against solid tumors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC, Seattle Children's Research institute.

AUTHOR CONTRIBUTIONS

PS and SJR conducted the experiments, analyzed the data, interpreted the results, and prepared the manuscript. VK and SS conceptualized the project, designed the experiments, supervised the work, analyzed the data, interpreted the results, and prepared the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by research funding from the American Cancer Society to SS, the Pediatric Cancer Research Foundation to SS, the Rachel Lynn Henley Foundation to VK, the National Institutes of Health (AI132819 to SS and AI103748 to SS; 5P30CA015704 and AI154363 to VK), and seed funds from the Seattle Children's Research Institute to SS and VK.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Shruti Bhise and Laura Arguedas for excellent technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.706150/full#supplementary-material

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