ROLES OF TUMOR-RECRUITED MYELOID CELLS IN IMMUNE EVASION IN CANCER

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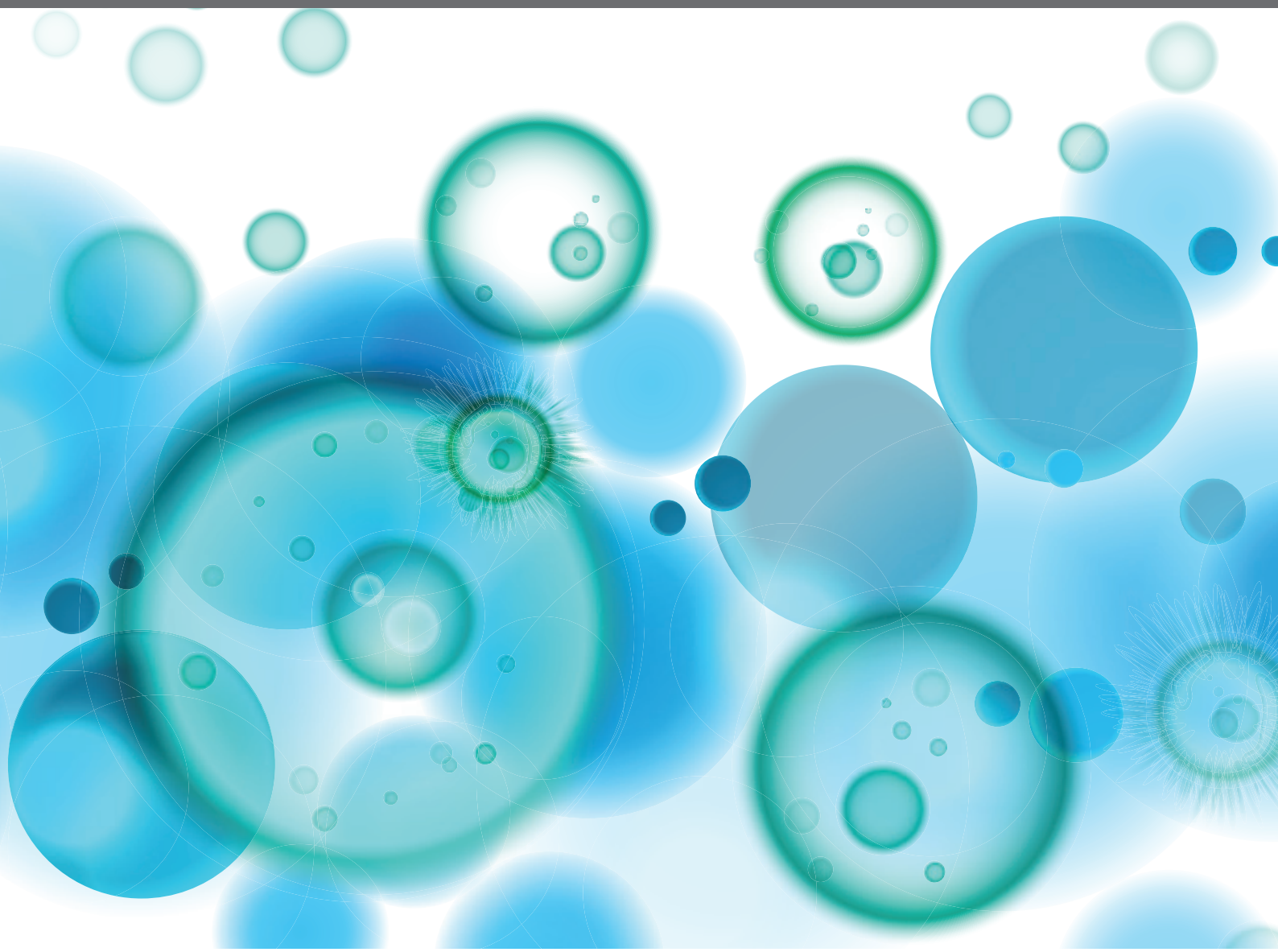

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ROLES OF TUMOR-RECRUITED MYELOID CELLS IN IMMUNE EVASION IN CANCER

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EDITORIAL
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Editorial: Roles of Tumor-Recruited Myeloid Cells in Immune Evasion in Cancer

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Keywords: tumor microenvironment, immune evasion, immune tolerance, MDSC (myeloid-derived suppressor cell), tumor-associated macrophage

Editorial on the Research Topic

Roles of Tumor-Recruited Myeloid Cells in Immune Evasion in Cancer

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During tumor formation and progression, tumors develop an immunosuppressive and tolerogenic microenvironment. Tumor-associated myeloid cells, including myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) represent one of the major immunosuppressive components in the tumor. Tumor-recruited myeloid cells contribute to tumor growth by exerting profound immunosuppressive effects through inhibition of adaptive and innate anti-tumor immune responses, stimulation of tumor angiogenesis, and remodeling extracellular matrix. This Research Topic aims to provide a comprehensive overview of the tumor-recruited myeloid cell subsets, describe the immune function of MDSCs, TAMs, tumor-associated neutrophils, and review the novel approaches targeting myeloid cells to enhance anti-tumor immunity and improve the efficacy of cancer immunotherapy. Our collection of 12 manuscripts consists of 2 Original Research papers, 3 Mini-Reviews, and 7 Reviews are summarized below. This Research Topic covers different aspects of MDSCs biology, including their generation and mobilization in cancer, mechanisms of tumor-associated immune suppression, and their contribution to resistance to immunotherapy. Also, it highlights the novel approaches for targeting MDSCs in cancer.

GENERATION AND MOBILIZATION OF MDSC IN CANCER

The increased accumulation of MDSCs in both peripheral blood and tumor tissues is very well documented for multiple cancer types in both experimental and clinical settings. Myeloid cell recruitment into tumors is associated with enhanced tumor-induced myelopoiesis and inflammation, which drives mobilization of myeloid cells to the tumor site. Tumors are inherently pro-inflammatory, with infiltrating myeloid cells thought to be critical for tumor development, maintenance, and progression. As demonstrated in experimental and clinical

studies, both chronic and acute inflammation can drive tumorigenesis of different origins (1–4). Despite our still limited knowledge of cancer-specific mechanisms of generation and tumor recruitment, it is clear that bone marrow and spleen are major sources of MDSCs. Wu et al. concisely reviewed experimental and clinical studies highlighting the mechanisms of tumor-mediated stimulation of myelopoiesis in tumor hosts with a major focus on the spleen, the major site of extramedullary hematopoiesis in the cancer setting. The authors summarize the distinct mechanisms, functional specialization, and clinical relevance of cancer-associated myeloid cell generation in the spleen and its potential as a novel therapeutic target. They also provide insight into mechanisms of tumor-dependent development of MDSCs from hematopoietic stem cells and myeloid cell progenitors and highlight the roles of specific hematopoietic cytokines and tumor-derived factors in this process. Karin reviewed the roles of specific chemokine receptors and their ligands in cancer-associated multistep MDSC mobilization. Although myelopoiesis is coordinated by multiple cytokines and transcription factors, mobilization is selectively directed by chemokine receptors and may differ between M-MDSC and PMN-MDSC. These myeloid cells may then undergo further expansion at these secondary lymphatic organs and then home to the tumor site. Thus, mobilization of MDSCs from bone marrow to the blood is directed by specific chemokine receptors such as CCR2 for monocytic MDSCs *via* CCR2-specific ligand CCL2, and CCR5 for the PMN-MDSCs *via* CLL3, CCL4, or CCL5 ligands. It should be noted that other chemokine-mediated signaling pathways including CXCL/CXCR2 also contribute to the tumor recruitment of myeloid cells and that signal integration between different chemokines may also play an important role in their differentiation and function.

MYELOID CELLS IN THE TUMOR MICROENVIRONMENT

While PMN-MDSCs are relatively short-living cells, monocytic MDSCs upon entering tumor tissue frequently differentiate into immunosuppressive tumor-associated macrophages (TAMs) and can function there for a long time. Wu and Zhang summarize the broad role of TAMs and TANs (tumor-associated neutrophils) in cancer, provide detailed information on how these cells contribute to the growth of primary and metastatic tumors, and discuss their clinical relevance. Davidov et al. review the recent advances in data science, including bioinformatics, single-cell RNA sequencing (scRNAseq) and mass cytometry, which have enabled the development of novel approaches to explore the myeloid cells in the tumor microenvironment. These methods may provide a greater understanding of the mechanisms of tumor-associated immune suppression and tolerance, differentiation and polarization of myeloid cells in the tumor microenvironment, and interaction of tumor-infiltrating myeloid cells with immune cells, tumor cells, and stromal compartment. Zwing et al. provide a detailed analysis of spatial organizational patterns of tumor-associated myeloid cells and T cells in tumor tissues obtained from 74 previously untreated patients with colorectal cancer. This study combined the digital image-based analysis, including cell density, cell-to-cell distance, and spatial overlap, with gene expression profiling to link the tumor spatial features with the biological function of tumor-infiltrating immune cells. In these patients, MDSCs seem to accumulate mostly at the invasive tumor edge and are strictly associated with CD8+T cells. This confirmed previous findings from Weed et al. in a limited number of patients with HNSCC and suggests that myeloid cells may provide a physical barrier to exclude CTL from the tumors.

Currently, patient stratification models focus mostly on the tumor-infiltrating $CD8^+$ T cells in tumor tissue. However, data provided by the authors, clearly suggest that both numbers of $CD8^+$ T cells and the spatial relationship between myeloid and T cells should be taken into account for the immune-based patient classification and stratification. Additionally, similar analyses should be performed after therapy to evaluate how anti-cancer treatment modulate MDSC and T cell infiltration.

MECHANISMS OF IMMUNE SUPPRESSION AND IMMUNE RESISTANCE MEDIATED BY MDSC

Several molecular mechanisms deployed by cancer-associated myeloid cells mediate the inhibition of innate and adaptive anti-tumor immune response, thus promoting immune evasion. The review by Grzywa et al. focused specifically on the role of myeloid cell-derived arginase (ARG) in the regulation of cancer immunity. ARG expression is substantially elevated in myeloid cells in cancer and mitigates antitumor response *via* multiple mechanisms. Authors provide detailed overview of the biochemistry and metabolism of arginase and its substrate L-arginine in tumors and in tumor-associated myeloid cells. ARG-expressing myeloid cells strongly inhibit T cell proliferation by impairing CD3 zeta chain expression, and this effect could be reversed by supplementation of L-arginine or by small molecule arginase inhibitors. Lebegge et al. focused on multiple innate immune mechanisms by myeloid cells that contribute to cancer immunotherapy resistance and promote tumor growth. The mechanisms include the release of pro-inflammatory mediators, neutrophil degranulation and respiratory burst, neutrophil extracellular trap formation, tissue pathogen, and damage recognition mechanisms, and others. Daveri et al. summarize various roles of microRNA in shaping myeloid cell-mediated resistance to the cancer immunotherapy. Small non-coding RNA molecules, the microRNAs (miR) contribute to myeloid cell regulation at different levels, including cell metabolism and immune function, as well as affecting MDSC differentiation and skewing their phenotype. MiR expression in myeloid cells can be indirectly induced by tumor-derived factors or through direct miR import *via* extracellular vesicles. Indeed, extracellular vesicles are becoming important factors that regulate both MDSC differentiation and function.

MDSC AS BIOMARKERS FOR RESPONSE TO THE CANCER IMMUNOTHERAPY

The increased presence of immunosuppressive cells in patient's peripheral blood and tumor tissue may affect the response of cancer patients to immunotherapy. Peranzoni et al. reviewed the existing evidence of the relation between myeloid cell subsets and the response of cancer patients to the treatment with immune checkpoint inhibitors. The authors propose that circulating and tumor-infiltrating myeloid cell populations can be used as predictive biomarkers for immune checkpoint inhibitors in different human cancers, both at baseline and on treatment. Thus, in patients with advanced melanoma, treated with a combination of anti-PD1 antibody and a multi-peptide vaccine, the M-MDSC expansion in peripheral blood was associated with poorer response to the immunotherapy. The authors also note that given the plasticity of myeloid cells and the differences in the microenvironment among tumors, the phenotype of this lineage can greatly vary. Therefore, the simple abundance of CD68 cells, classically considered to represent macrophages, is thus rarely informative, while the functional orientation of myeloid cells by multi-parameter IHC, flow cytometry, or RNA sequencing allows defining a clearer relationship between the distinct subsets and the clinical outcome. This review also highlights myeloid cell plasticity and how treatments can induce the appearance of different immune evasion mechanisms.

TARGETING MDSC IN CANCER

Recent years have been marked by significant progress in developing, clinical testing, and validation of new immunotherapeutic agents for cancer therapy, including immune checkpoint inhibitors, engineered immune cells, and novel cancer vaccines. However, the clinical efficacy of cancer immunotherapy is limited due to tumor-associated immune suppression and immune tolerance. Therefore, targeting MDSCs holds a great potential to boost the anti-tumor immune response and produce a more powerful therapeutic effect than immunotherapy alone. de Cicco et al. reviewed recent progress in the development of novel strategies for targeting MDSCs in cancer such as: (i) depletion of MDSC populations; (ii) prevention of MDSCs recruitment and/or migration to the tumor site; (iii) attenuation of immune suppression in cancer by targeting specific molecular pathways that are involved in MDSC-mediated inhibition of anti-tumor immune response; iv) promoting the differentiation of MDSCs into mature non-suppressive myeloid cells like M1-macrophages or dendritic cells. The heterogeneity of these myeloid cells makes their identification in human cancer very challenging. The authors suggest that since phenotype and mechanisms of action of MDSCs appear to be tumor-dependent, it is important to accurately characterize the precise MDSC subsets that have clinical relevance in each tumor environment to more efficiently target them. Also, the authors provide the specific characteristics of immunosuppressive myeloid cell subsets detected in several human cancers including melanoma, breast cancer, prostate cancer, colorectal cancer, hepatocellular carcinoma, and lung cancer. Alban et al. demonstrated that monocytic MDSCs in glioblastoma express high levels of CD74, which serves as a cognate receptor macrophage migration inhibitory factor (MIF). Targeting of MDSCs with ibudilast, a MIF-CD74 interaction inhibitor, resulted in a reduction of the production of monocyte chemoattractant protein 1 (MCP1, CCL2) and stimulated the expansion of CD8 T cells. However, using ibudilast as a single agent for the treatment of the experimental model of glioblastoma did not improve the survival rate. Sieminska and Baran reviewed targeting MDSCs in colorectal cancer. Since survival and expansion of MDSCs is regulated by PGE2, administration of COX2 or MPGES1 inhibitors in animals with experimental tumors results in a reduction of MDSCs and inhibition of tumor growth.

In conclusion, our Research Topic underscores the diverse roles of tumor-associated myeloid cell subsets in in immune evasion and the active suppression of anticancer immune responses. At the same time, this manuscript collection clearly indicates that there is still much to discover about the novel MDSC markers, cancer-specific mechanisms of MDSCs generation, and molecular pathways that control differentiation and polarization of recruited myeloid cells in tumor tissue.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Myeloid Cell-Derived Arginase in Cancer Immune Response

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Amino acid metabolism is a critical regulator of the immune response, and its modulating becomes a promising approach in various forms of immunotherapy. Insufficient concentrations of essential amino acids restrict T-cells activation and proliferation. However, only arginases, that degrade L-arginine, as well as enzymes that hydrolyze L-tryptophan are substantially increased in cancer. Two arginase isoforms, ARG1 and ARG2, have been found to be present in tumors and their increased activity usually correlates with more advanced disease and worse clinical prognosis. Nearly all types of myeloid cells were reported to produce arginases and the increased numbers of various populations of myeloid-derived suppressor cells and macrophages correlate with inferior clinical outcomes of cancer patients. Here, we describe the role of arginases produced by myeloid cells in regulating various populations of immune cells, discuss molecular mechanisms of immunoregulatory processes involving L-arginine metabolism and outline therapeutic approaches to mitigate the negative effects of arginases on antitumor immune response. Development of potent arginase inhibitors, with improved pharmacokinetic properties, may lead to the elaboration of novel therapeutic strategies based on targeting immunoregulatory pathways controlled by L-arginine degradation.

Keywords: arginase, arginine, immunosuppression, tumor immunology, immunotherapy, T lymphocyte, T-cell metabolism

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INTRODUCTION

The idea that the immune system can be harnessed to destroy tumors has been pursued for over a century (1). However, for decades the efforts have mainly focused on stimulating the immune system with recombinant cytokines, immune adjuvants, or co-stimulatory agonists that seemed critical for the induction of potent and sustained immune responses (1, 2). The rationale was that the immune system in cancer patients lacks sufficient power to mount anti-tumor response. It now seems however, that the interference with pathways dampening lymphocyte reactivity appears to be more effective in cancer patients than over-stimulation of effector mechanisms of immune system.

The most successful approaches to impair tumor-elicited immunosuppressive mechanisms turned out to be monoclonal antibodies (referred to as immune checkpoint inhibitors) interfering with co-inhibitory molecules or their ligands, such as CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), PD-1 (programmed cell death protein 1), or PD-L1 (programmed death-ligand 1). The spectacular therapeutic effects with unexpected ability to induce long-term tumor control led to clinical approval of checkpoint inhibitors (3–5).

Despite unprecedented antitumor efficacy, checkpoint inhibitors are effective in a minority of cancer patients, however. Thus, identification of response biomarkers as well as resistance mechanisms has become a priority for cancer researchers. A number of molecular mechanisms involved in the evasion of the anti-tumor immunity have been characterized in recent years (6). Central among them is the development of chronic inflammation (7, 8). Epidemiological data indicate that chronic inflammation is associated with poor prognosis (9). Mounting evidence indicates that the tumor microenvironment alters lymphoid and myeloid cells and converts them into potent immunosuppressive cells. It has become clear that tumor microenvironment, rich in inflammatory cells, is an indispensable component in the neoplastic process fostering proliferation, survival, and invasiveness of tumor cells (7). Chronic inflammation also triggers multiple regulatory pathways aimed at dampening immunity. The evolutionary rationale for this is to mitigate tissue damage and fibrosis. Coincidentally, the regulatory pathways impair development and/or activity of adaptive immune mechanisms that could be involved in eradication of tumor cells (8). Simultaneously, tumor cells frequently co-opt some of the signaling molecules participating in inflammation, such as adhesion molecules, cytokines, and growth factors for migration, invasion, and metastasis. Although there are many phenotypical and functional changes in different myeloid cell subpopulations, their precise role in the development of cancer resistance to immunotherapy is still not well-understood. This review will address the role of arginases (ARG), enzymes produced by tumor-infiltrating myeloid cells. The role of L-arginine (L-arg) metabolism in the regulation of immune response was of great interest in the 1980s and 1990s. However, further studies were focused mainly on L-arg-derived nitric oxide (NO) and its antimicrobial activity (10, 11), rather than immunosuppressive effects of L-arg deprivation. It is currently experiencing a renaissance due to increased awareness of the role of metabolic pathways in the regulation of immune cells function as well as due to the development of selective arginase inhibitors with improved pharmacokinetic properties. Novel tools and experimental models allowed to more precisely and comprehensively address the critical metabolic adaptations to microenvironmental changes experienced by immune cells. This is a clearly arginase-centered review, and it should be kept in mind that there are multiple other independent mechanisms of tumor immune evasion, including those affecting amino acids metabolism.

ARGININE AND ARGINASES—BASIC BIOCHEMISTRY

L-arginine is a dibasic cationic amino acid participating in a variety of metabolic pathways (**Figure 1**) (12). There are three major sources of L-arg in the body—dietary intake, endogenous *de novo* production from L-citrulline or recycling, i.e., retrieval from degraded proteins. Under pathological conditions (bleeding, sepsis, trauma, cancer, or chronic inflammation) endogenous sources of L-arg become insufficient (13). Thus, L-arg is considered to be a semi-essential or conditionally-essential amino acid that in stressful conditions must be supplied in diet. Most of the endogenous L-arg synthesis is carried out in the kidney proximal tubules from intestinal L-citrulline (14). L-Arg plasma concentrations range between 50 and 250 μM (15–18) and are much lower than those in subcellular compartments (up to 1 mM) (19). In mammalian cells, L-arg transport through the plasma membrane is mediated by at least eight transporters (20). The uptake of L-arg occurs mainly via cationic amino acid transporters (CAT-1, CAT-2A, CAT-2B, and CAT-3, SLC7A1-3) (21). In human T-cells L-arg transport is mediated mainly by CAT-1 (22), while in myeloid cells by CAT-2 (23). Moreover, L-arg is transported through the plasma membrane by $b^{0,+}$ AT (SLC7A9) and $ATB^{0,+}$ (SLC6A14) that also transport neutral amino acids (20, 24, 25). L-type amino acid transporters γ^+LAT1 (SLC7A7) and γ^+LAT2 (SLC7A6) mediate mostly arginine export from the cells (20, 24). L-arg is metabolized in animal cells by four groups of enzymes, some of which exist in various isoforms. These include arginases, nitric oxide synthases (NOS), arginine decarboxylase (ADC), and arginine:glycine amidinotransferase (AGAT). Moreover, arginine deiminase (ADI) that hydrolyzes L-arg to L-citrulline and ammonia is expressed by some bacteria (26, 27). It is the first enzyme of the arginine dihydrolase system (ADS) that generates alkali and ATP for growth (28). These enzymes are encoded by arginine catabolic mobile element (ACME) (29) that was detected in *Staphylococcus aureus* and *Staphylococcus epidermidis* (30). L-arg metabolism by ADS enables survival in acidic environments, including human skin, disrupts host arginine metabolism, and contributes to the success of community-associated methicillin-resistant *S. aureus* (CA-MRSA) (31).

Arginases are manganese-containing enzymes that hydrolyze L-arg to L-ornithine and urea in the liver urea cycle (32). This is the most important pathway responsible for the conversion of highly toxic ammonia to excretable urea (33). L-Ornithine is a substrate for ornithine decarboxylase (ODC) that initiates polyamines synthesis, or it is metabolized by ornithine aminotransferase (OAT) to proline. Polyamines, such as putrescine, spermine, or spermidine are necessary for cell proliferation, while proline is necessary for collagen synthesis. Initially, it was thought that arginase is expressed only in the liver. However, further studies revealed that arginase is ubiquitously expressed in many types of cells (33), and that there are two different isoforms of this enzyme that catalyze the same biochemical reaction, but are expressed by different cells and are located in different cellular compartments. Human arginase 1

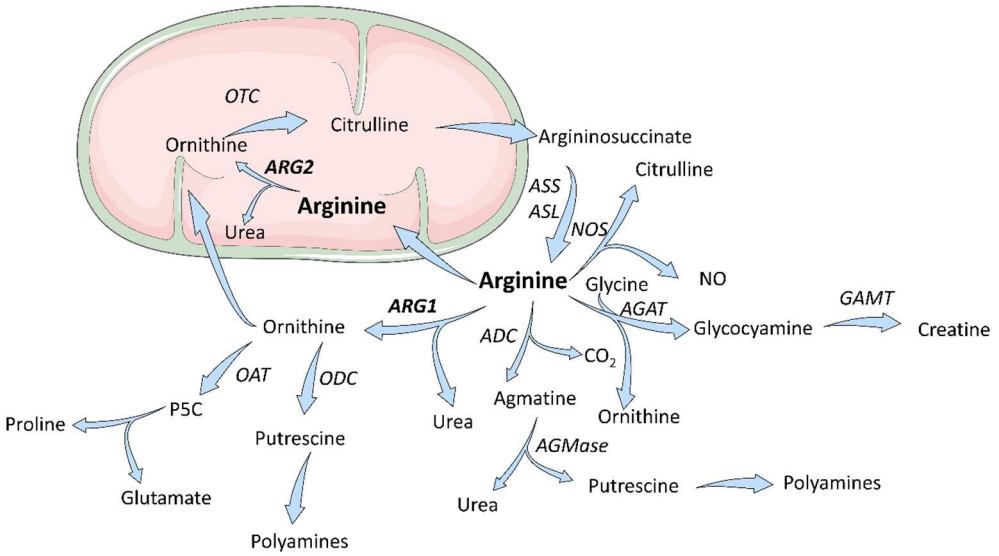

FIGURE 1 | Scheme for arginine metabolism. In mammalian cells, L-Arginine is a substrate for four enzymes: ARG, NOS, ADC, AGAT. L-Arginine downstream metabolites are components of multiple metabolic pathways and are necessary for cells proliferation and collagen synthesis. ADC, arginine decarboxylase; AGAT, arginine:glycine amidinotransferase; AGMase, agmatinase; ARG, arginase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; GAMT, guanidinoacetate N-methyltransferase; NOS, nitric oxide synthase; OAT; ornithine aminotransferase; OTC, ornithine transcarbamylase; P5C, pyrroline-5-carboxylic acid. Figure was modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License. http://smart.servier.com/.

(ARG1) has 322 amino acids and is a cytosolic protein expressed primarily in the liver cells (34) as well as in the cells of the myeloid lineage (35). Human arginase 2 (ARG2) consists of 354 amino acids and can be found in mitochondria (36). It has ubiquitous expression, but usually at a lower level than ARG1. ARG2 has 58% sequence identity to ARG1 (37), but both enzymes are nearly identical within the catalytic region. There are also types of cells, such as endothelial cells, which have relatively high expression of both isoenzymes (38). The summary of the most important information on the two isoforms of arginase is presented in **Table 1**.

An important metabolic pathway of L-arg involves the activity of NOS. There are three isoforms of this enzyme—neuronal (nNOS or NOS1), inducible (iNOS or NOS2) and endothelial (eNOS or NOS3). NOS2 can be induced in many types of cells, but when present in activated myeloid cells it produces NO at a very high rate. There are multiple layers of competition between NOS2 and ARG1 in myeloid cells and both enzymes are induced by cytokines regulating different types of the immune response. NOS2 in myeloid cells is induced by type 1 cytokines (mainly IFN-γ), while ARG1 expression is regulated by IL-4 and IL-13. Considering that K_m of ARG1 is ∼1,000-fold higher than that of NOS2, the intracellular L-arg could be expected to be mainly metabolized to NO, rather than to L-ornithine and urea. However, V_{max} of NOS is three orders of magnitude slower than that of ARG1 (53, 54). Thus, both enzymes compete for the same substrate. Intriguingly, insufficient L-arg concentrations lead to NOS uncoupling, whereby rather than NO these enzymes generate superoxide anions. Superoxide then rapidly reacts with any available NO molecules to form peroxinitrites that further decrease NOS activity by oxidizing tetrahydrobiopterin (BH_4) (54). Moreover, induction of ARG1 that limits L-arg availability is involved in the regulation of NOS2 expression as L-arg is necessary for the translation of NOS2-encoding mRNA (55).

During acute wound healing resident myeloid cells express high levels of NADPH oxidase (NOX2) and NOS2, which participate in normal antimicrobial defense mechanisms by producing superoxide anion and NO, respectively. Then, after 3–5 days, a repair phase is initiated, which is associated with the appearance of $ARG1^+$ macrophages. L-arg degradation produces L-ornithine that is converted by OAT to L-proline used as a substrate in collagen synthesis (56). ODC converts L-ornithine to polyamines that stimulate cell proliferation. This highly regulated process is perpetuated in tumors that are frequently described as wounds that never heal (57).

ARGININE AND ARGINASE IN TUMORS

Tumor progression is associated with alterations in metabolic pathways in tumor cells as well as in the cells forming the tumor microenvironment. Altered metabolic phenotype of tumors includes changes in L-arg concentrations. For example, the concentration of L-arg in the core regions of solid tumors is about 5 times lower as compared with tumor periphery and this difference turned out to be the highest among all of the measured amino acids (58). Quantification of interstitial fluid metabolites in murine tumors has also revealed that L-arg is the most strongly depleted amino acid in the tumor microenvironment (59). The mechanisms of L-arg depletion are incompletely elucidated. On the one hand, L-arg can be consumed by tumor cells that have increased metabolic demands and use it for protein synthesis, but it can also be used by enzymes such as arginases or NOS. Many

TABLE 1 | Properties of the two arginase isoforms.

Enzyme	Arginase 1	Arginase 2
Genomic location in mouse	10; 10 A4	12; 12 C3
Number of amino acids	323	354
Genomic location in human	6q23	14q24.1
Number of amino acids	322	354
Sequence identity	ARG2 has 58% sequence identity to ARG1	
Structure	Homotrimer	
Catalyzed reaction	L-arg → urea + L-ornithine	
Localization	Cytosol	Mitochondrion
Tissue specificity	Liver, to a lesser extent kidney	Expressed ubiquitously, mainly kidney and prostate
Phenotype of knockout (KO) mice	Lethal, death occurs typically by postnatal day 17 (39). In conditional knockouts, death of adult mice occurs typically after 21 days of KO induction (40)	KO viable and apparently indistinguishable from wild-type mice (41, 42)
Phenotype of deficiency in humans	Urea cycle disorder, hyperargininemia, progressive neurologic impairment (43)	Defects not described. ARG2 level is increased in ARG1-deficient patients (43, 44)
Effect of ARG on immune response	Immunosuppression (45)	Unclear - immunosuppression (46–49), but also expressed by proinflammatory cells (50–52)

studies reported that arginases can be produced by tumor cells (46, 60, 61), but even larger number of reports indicate that the major L-arg-metabolizing cells are found in the tumor stroma. It has not been studied in sufficient detail as to which cells in the tumor environment are mainly responsible for L-arg depletion. It is also entirely possible that this process is highly variable and changes in the course of tumor progression, with tumor cells or stromal cells predominating in L-arg metabolism at various stages of neoplastic disease.

Arginase and Tumor Prognosis

High ARG expression and activity have been reported in many types of human cancers, but its role as a prognosis factor remains vastly undetermined and usually studied on small populations of patients. Moreover, drawing conclusions from the limited number of studies is further complicated by a lack of standardized criteria for ARG measurements. For example, different cutoff criteria were applied to groups of patients with "low arginase" and "high arginase" expressing tumors, or studying either ARG1 or ARG2 expression profiles. Nonetheless, increasing evidence shows that overexpression of ARG1/2 (with or without subsequent decline in serum L-arg concentrations) should be perceived as a poor prognostic factor in a wide variety of cancer types including head and neck cancer (62), neuroblastoma (46), acute myeloid leukemia (AML) (61), pancreatic ductal carcinoma (63), ovarian carcinoma (64), or colorectal cancer (65). High expression of ARG1 in hepatocellular carcinoma also seems to play a role as a negative predictive factor that correlates with shorter median time to recurrence (66) and more aggressive tumors (67), but further evidence is required to support these observations as a contradictory report exists (68).

Although a number of studies provide strong evidence for increased ARG activity in both tissue (69) and blood (70–72) obtained from patients with breast cancer, so far no study was conducted to establish the role of ARG activity in determining the prognosis of breast cancer patients. Notably, contradictory reports exist that show a decrease in blood plasma ARG activity in breast cancer patients, however, these are based on very limited number of enrolled patients (73, 74). Similarly to breast cancer, increased ARG activity was found in skin (75), cervical (76), thyroid follicular (77), thyroid papillary and follicular variant of papillary (77), gastric, bile duct (78), and esophageal (79) cancers. However, again no study exists in these types of cancers that would demonstrate the impact of ARG activity/abundance on patients' prognosis.

Finally, there are tumors such as prostate (80–82) and lung cancer (83) as well as tumors that are auxotrophic for L-arg (these are not capable of re-synthesis of L-arg from citrulline due to the lack of expression of argininosuccinate synthetase-1, ASS-1), such as melanoma (84) and renal carcinoma (85, 86), where no correlation between ARG levels and survival has been found.

A critical question arises whether ARG in tumors is produced by tumor cells or by tumor-associated stromal cells that include mesenchymal as well as immune cells, among which myeloid cells seem to be the main source of the enzyme. Regrettably, no studies have been conducted that would directly address this issue and whether this is of any significance for cancer patients survival, whether ARG is expressed by tumor or tumor-infiltrating myeloid cells.

ARGINASE IN TUMOR-INFILTRATING MYELOID CELLS

Myeloid cells are major contributors to immune defense against pathogens and play an important role in tissue remodeling. During acute infections GM-CSF drives myelopoiesis in the bone marrow, and G-CSF as well as M-CSF induce further differentiation of granulocytes and macrophages, respectively (87). Some tissue macrophages develop from embryonic precursors that directly home to peripheral tissues and become a self-renewing population (88). Mature myeloid cells are

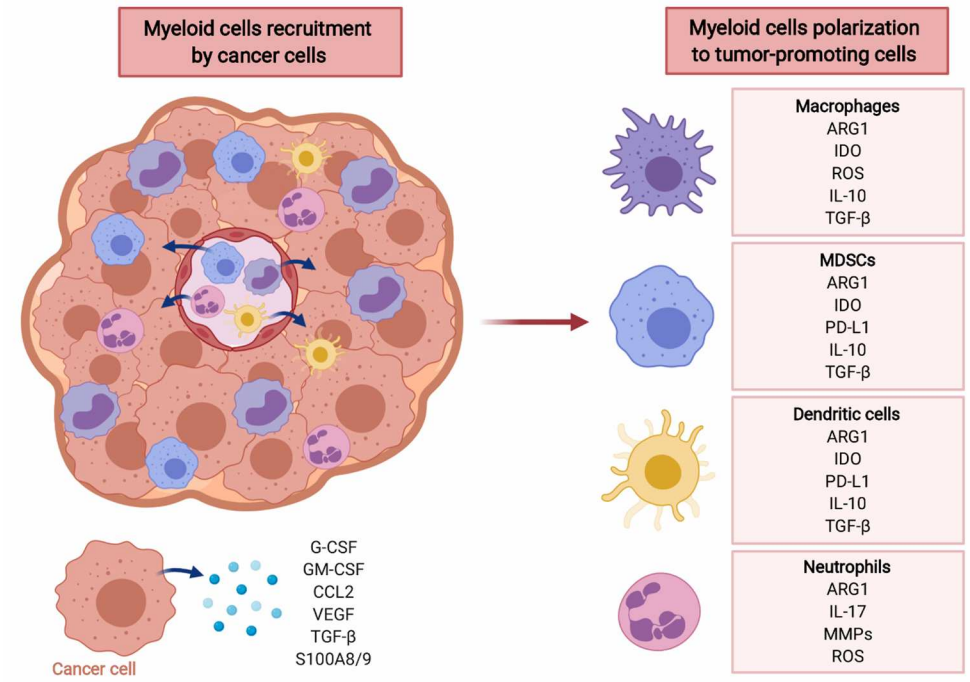

FIGURE 2 | Cancer cells recruit myeloid cells to tumor microenvironment (TME) and induce their polarization to immunosuppressive phenotype. Myeloid cells, including macrophages, MDSC, dendritic cells, and neutrophils create tumor-promoting, immunosuppressive TME via multiple factors including reactive oxygen species (ROS), cytokines (IL-10, TGF-β), PD-L1, as well as ARG1. Created with BioRender.

specialized in killing infectious microorganisms and play an important role in promoting development of adaptive immunity. However, in cancer and other chronic inflammatory conditions constant production of low concentrations of myeloid growth factors and various inflammatory mediators dysregulate myeloid cells differentiation (**Figure 2**) (89–94). It is currently not well-understood what events trigger this disturbed myelopoiesis, but it must be emphasized that this process evolves over many years of tumor development and likely involves multiple independent mechanisms. Some of these might be completely stochastic, but in the course of tumor progression become promoted in a trial-and-error process that selects for mechanisms that best fit the demands of growing tumors.

Myeloid cells, especially tumor-infiltrating myeloid cells (TIMs), are a highly heterogeneous population (95). TIMs include monocytes, macrophages, dendritic cells, granulocytes, mast cells, as well as their immature precursors that have not completed their differentiation processes. The latter cells are normally found in the bone marrow, but in the course of tumor development they frequently expand and relocate to the spleen, lymph nodes and the tumor itself, and can be found at increased numbers in the peripheral blood (96, 97). These cells express immune checkpoint molecules, deplete essential metabolites, release immunosuppressive adenosine and its metabolites, produce reactive oxygen species, secrete immunoregulatory cytokines, growth-promoting, and proangiogenic factors (**Figure 2**). Moreover, they induce various populations of regulatory T-cells that impair antitumor immune response (98). Due to their strong immunosuppressive functions these cells have been termed myeloid-derived suppressor cells (MDSCs). There are two major subsets of MDSCs—monocytic (M-MDSC) and granulocytic (polymorphonuclear, PMN-MDSC) (99). Both have been associated with dysregulation of immune response in murine cancer models and in cancer patients, although still the majority of studies report the suppressive potential of total MDSCs (100). In mouse tumor models that mostly involve transplantation of tumor cells, the expansion of MDSCs is very rapid. This is in contrast to slow-growing tumors, including diethylnitrosoamine (DEN)-induced or MYC-expressing hepatocellular carcinoma, that in terms of the rate of tumor progression more accurately reflect human cancer (101). In many types of humans tumors, including lung, colon, uterus, cervix, bladder, or thyroid gland cancers, the increased numbers of M-MDSCs in peripheral blood correlate with worse clinical outcomes (102). In melanoma or liver cancer, however the increased numbers of both PMN-MDSCs and M-MDSCs were associated with poorer outcomes (102), while in renal cell carcinoma PMN-MDSCs seem to predominate (103). Importantly, increased numbers of MDSCs are observed also in patients with pancreatic premalignancy—intraductal papillary mucinous neoplasm (IPMN), and in patients with colon adenomas, as compared with healthy controls (97).

TABLE 2 | Differences in arginases expression in myeloid cells between mouse and human.

Type of cell	Arginase 1				Arginase 2			
	Mouse		Human		Mouse		Human	
Monocytes	+	(106)	–[c]	(107, 108)	+	(109)	+	(50, 110)
Macrophages	+[a]	(111)	–[c]	(105)	+[b,c,d]	(36, 112)	+	(113)
M2 macrophages	+[a]	(114)	–[c]	(108)	–[d,e]	(51)	–[e]	(50)
TAMs	+	(115)	–[f]	(116)	+	(117, 118)	+	(63)
MDSCs	+[a]	(103)	+	(103)	+ –	(119, 120)	+	(121)
Neutrophils	+	(122)	+	(105, 123)	+	(117, 124)	+	(113)
Dendritic cells	+[a]	(112)	–[c]	(105)	+ –	(112, 125)	+	(126)

[a]Induced by Th2 cytokines.[b]Not significantly modulated by Th1 cytokines.[c]Not significantly modulated by Th2 cytokines. [d]Induced by LPS.[e]ARG2 is proposed as a marker of proinflammatory M1 macrophages (50–52, 127). [f]ARG1 expression in human TAMs was minimal and on the same level as in control tissue-resident macrophages (116). +, expression; –, undetectable or very low expression; TAMs, tumor associated macrophages; MDSCs, myeloid-derived suppressor cells.

Nearly all myeloid cells have been shown to produce ARG1 in mice (**Figure 2**). However, there are substantial differences in the expression of arginases by myeloid cells between mice and men (104). In humans, arginase is produced mainly by granulocytes and no arginase activity is detectable in monocytes, macrophages nor dendritic cells (105). The differences in expression of both isoforms of arginase by myeloid cells in mice and humans is summarized in **Table 2**.

The first report linking immunosuppression with arginase activity in macrophages was published over 40 years ago (128). However, the concept that L-arg metabolism is associated with regulation of the immune response did not gain much attention initially. It was suggested that suppressive effect of arginase may be just an interesting problem of *in vitro* culture (129). However, soon other studies described depletion of L-arg by macrophages expressing arginase both *in vitro* (130) and *in vivo* in tumor-bearing mice (131). The authors hypothesized that arginase may be an effector mechanism of macrophages against infectious microorganisms and tumor cells (131). After over 30 years we know that arginase plays an opposite role in immune response and is one of the main mechanism of immunosuppression.

L-arg depletion by suppressive myeloid cells in the tumor microenvironment can occur by increased L-arg uptake by CAT-2B transporters (132), which is followed by arginase-mediated hydrolysis (**Figure 3**). Myeloid cells also secrete arginase to the microenvironment (133), where it acts mainly locally due to short circulating half-life (134). Murine MDSCs deplete L-arg by increased uptake and intracellular degradation, in contrast to human MDSCs that mainly release arginase into the circulation (103). ARG1 may also be secreted in extracellular vesicles (EVs) by MDSCs (135). In EVs, arginase remains stable and may exert greater than local effects, for instance in draining lymph nodes (64).

Arginase in Myeloid-Derived Suppressor Cells

MDSCs have been the most intensively studied cells in terms of L-arg metabolism. Bronte et al. were the first to show that myeloid cells accumulating in the spleens of tumor-bearing mice express ARG1 and suppress the proliferation of allogeneic T-cells (141). Liu et al. showed that myeloid cells in the tumor microenvironment express arginase and suppress cytotoxic T lymphocyte (CTL) activity in NO-independent manner (142). Since then, many other studies confirmed that immature tumor myeloid cells express ARG1 in mice and humans with cancer and that the activity of this enzyme is involved in suppression of T-cell response (132, 143–146). The majority of studies indicate that arginase plays a more important role in PMN-MDSC rather than M-MDSC (103, 147–149). However, the role of this enzyme in the regulatory activities of the latter cells should not be completely dismissed. For example, iNOS inhibitor together with ARG inhibitor diminished the suppression driven by M-MDSC, with no effect on PMN-MDSC (150).

In humans, PMN-MDSCs store ARG1 in granules and release it to the extracellular milieu (103). It leads to the depletion of L-arg and suppression of anti-tumor response. In patients with pancreatic ductal adenocarcinoma $CD13^{hi}$ PMN-MDSCs were identified that produce ARG1 and suppress alloreactive T-cell responses in ARG1-dependent manner. Patients with more $CD13^{hi}$ PMN-MDSCs had significantly shorter survival than those with predominant $CD13^{low}$ PMN-MDSCs in the tumor infiltrates (149). Similarly, ARG1-producing MDSCs in patients with renal cell carcinoma turned out to be of granulocytic lineage (103). Interestingly, treatment of patients with IL-2 increased the number of these cells in peripheral blood, as well as in the plasma concentrations of ARG1 (103). Whole mount labeling and clearing followed by three-dimensional light sheet microscopy of head and neck carcinomas identified intratumoral hotspots of PMN-MDSCs that co-localized with T-cells. Those T-cells that were in close proximity to ARG1-positive PMN-MDSCs had strongly reduced expression of granzyme B (serpin participating in cytotoxic effects of T-cells) and Ki67 (a proliferation marker) (151). In multiple myeloma IL-18 was shown to induce $ARG1^{+}$ PMN-MDSCs that suppress immune response (152). In $KRAS^{G12D}$ genetically engineered mice that develop lung tumors resembling NSCLC, PMN-MDSCs were observed to cause T-cell suppression by L-arg depletion. Arginase inhibitor has not only restored T-cell function, but caused significant regressions of tumors in these mice (117). Arginase-expressing MDSCs were also shown to induce Tregs in murine tumor models (153) as well as in cancer patients (154). In some of these studies, this effect was abrogated by arginase inhibitor (153) indicating a specific role of this enzyme in Treg development (see below).

Arginase expression in tumor MDSCs is increased as compared with the cells of the same phenotype isolated from spleen (155). Both inflammatory and tumor-derived factors are involved in the regulation of ARG1 expression in MDSCs (**Figure 4**). For example, tumor-infiltrating MDSCs stimulated with TGF-β and IL-10 demonstrated high ARG1

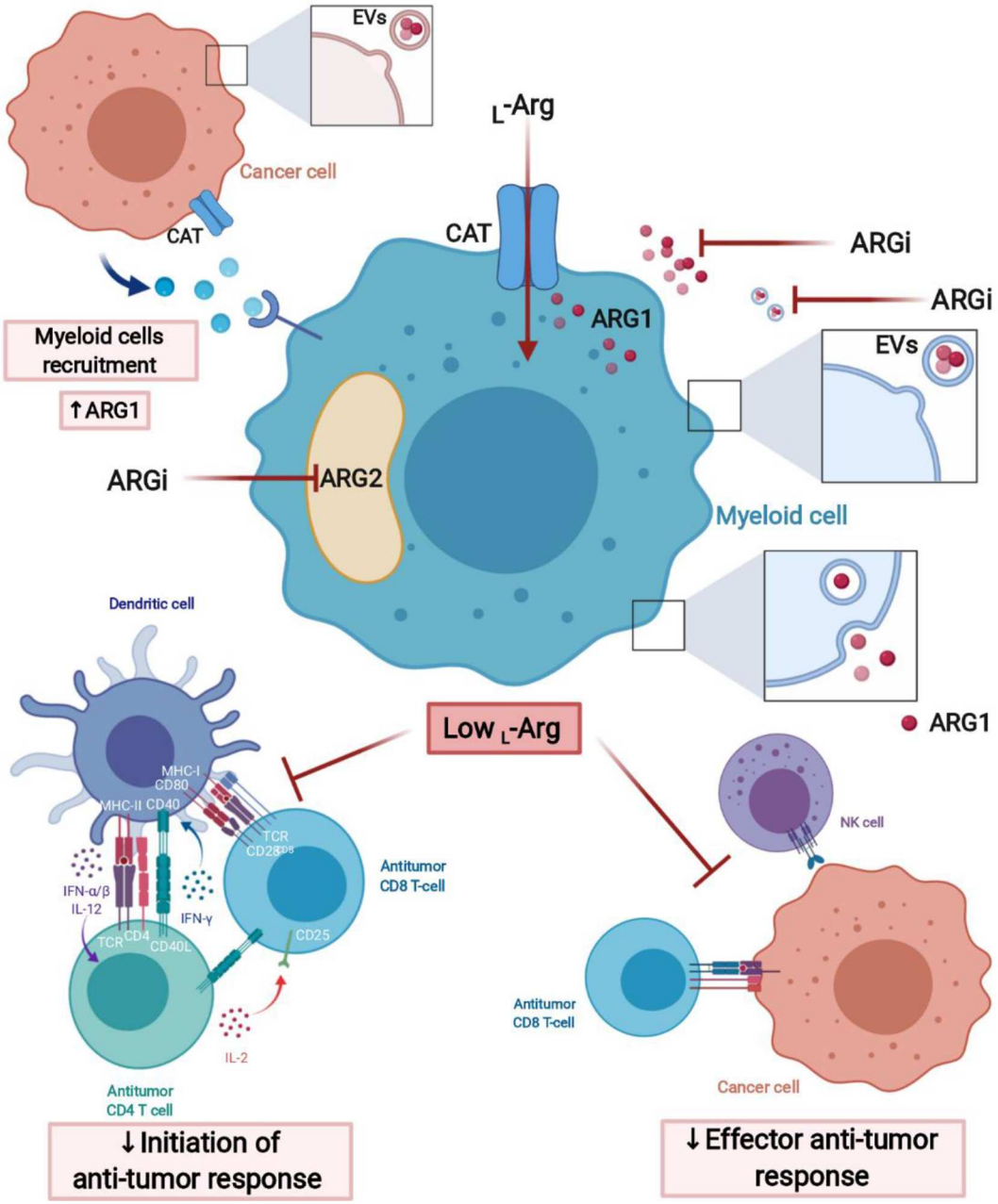

FIGURE 3 | L-Arginine-depleting arginases lead to the impaired anti-tumor response. Arginases may act intracellularly (cytoplasmic ARG1 and mitochondrial ARG2) and extracellularly (secreted ARG1) leading to the local depletion of L-arginine in tumor microenvironment (TME). Moreover, ARG1 may have effects in sites distant from the TME, when packed into extracellular vesicles (EVs), transported over long distance and internalized by myeloid cells, for instance, in tumor-draining lymph node. Arginase inhibitors (ARGi) should target both isoforms (ARG1 and ARG2) and easily penetrate the cell membrane to block extracellular and intracellular arginases, as well as arginase in EVs. Created with BioRender.

activity (156). One mechanism involves stress sensor C/EBP-homologous protein (CHOP), which directly activates ARG1 gene through inhibition of LIP transcription suppressor. CHOP expression in MDSCs is induced by ROS and further by the activating-transcription factor-4 (ATF-4) (157). Intriguingly, diminished L-arg concentrations have been shown to induce accumulation of arginase-expressing MDSCs in the tumors after administration of pegylated recombinant ARG1 to tumor-bearing mice (158) indicating potential threats associated with L-arg-depleting therapeutic strategies for cancer. ARG1 levels in MDSCs from patients with head and neck cancer were regulated by STAT3 signaling (159). Accordingly, STAT3 silencing in MDSCs from prostate cancer patients abrogated their immunosuppressive activity (160). Chronic stress, which

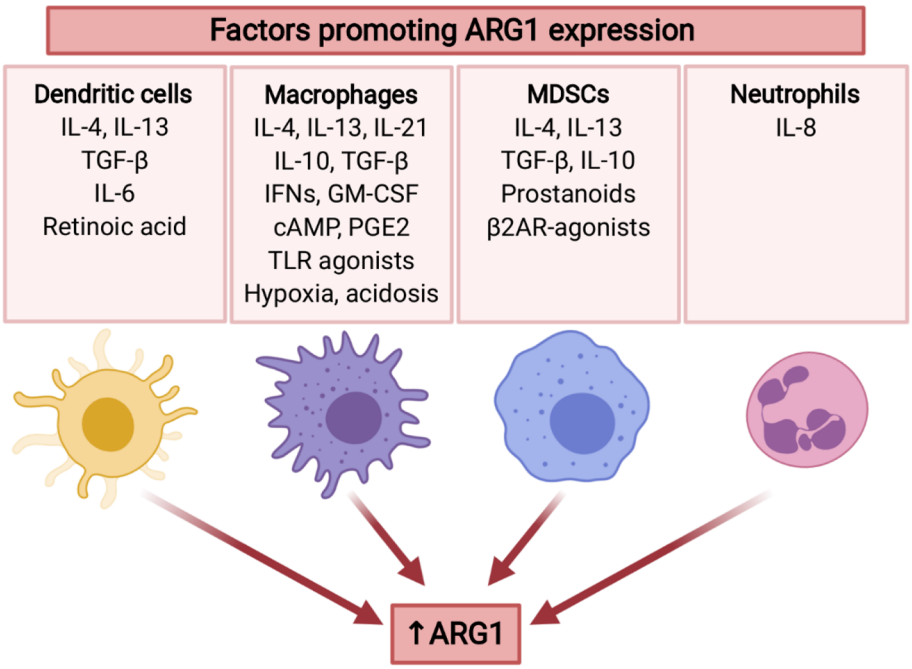

FIGURE 4 | Several tumor-associated factors promote ARG1 expression in myeloid cells. ARG1 expression is mainly induced by type 2 cytokines (IL-4, IL-13), as well as immunosuppressive cytokines (TGF-β, IL-10). Moreover, it may be promoted by TME factors including hypoxia and acidosis, as well as stress mediators. ARG1 expression is also induced by GM-CSF (136), TLR agonists (137), and cAMP (138). IL-10 and IL-21 increase IL-4-induced ARG1 expression (139, 140). Created with BioRender.

frequently accompanies cancer, was reported to increase the generation of $ARG1^+$ MDSCs in mice and humans, through catecholamines stimulating β2 adrenergic receptors (β2AR). Induction of ARG1 by isoproterenol (a β2AR agonist) was associated with STAT3 phosphorylation in MDSCs (161). It was found that prostanoids produced by COX2 are responsible for mediating ARG1 overexpression in MDSCs by lung cancer cells in *in vitro* and *in vivo* models (162). The mechanism of ARG1 upregulation in MDSC is probably controlled by EP4 receptor for PGE_2. Those findings were confirmed in other tumors (144, 163). MDSC not only infiltrate tumor and its environment, they were also found in peripheral blood. (103). MDSC abundance in blood correlated with staging in HNSCC patients. Moreover, MDSC in HNSCC have high level of pSTAT3 and ARG1 and potently inhibit T-cells proliferation (159).

Macrophages

Macrophages are the main phagocytic population of cells within tumors (47). However, contrary to their natural role in promoting immunity against infectious microorganisms, tumor-associated macrophages (TAMs) are involved in promoting tumor progression, partly through creating an immunosuppressive microenvironment (47). The majority of reports on macrophages in cancers describe their function in the context of *in vitro* polarization into M1 or M2 subsets (164). This classification is currently not recommended as TAMs are represented by a continuum of phenotypic variants (47), but will be incidentally used hereafter considering that the existing literature specifically refers to M1 and M2 macrophage subsets. It must be underscored however, that TAMs are highly diverse and form a wide range of populations with various functional roles (165). Additionally, these cells do not form a stable population, but are highly variable both in time and location within the tumor milieu (165, 166). So called M1 macrophages are induced by lipopolysaccharide (LPS) and type 1 cytokines (mainly IFN-γ), express high levels of tumor necrosis factor (TNF), IL-12, iNOS, and MHC class II molecules and are considered to participate in anti-tumor immunity (47). M2 macrophages are induced by type 2 cytokines and express ARG1, IL-4, IL-13, IL-10, and CD206 (47, 167). Cytokines, especially those associated with type 2 immune response (IL-4 and IL-13) that activate the transcription factors STAT6, PU.1, and CCAAT/enhancer binding protein β (C/EBPβ) were shown to directly induce signaling pathways leading to increased production of ARG1 in macrophages (168). IL-4- and IL-13-activated STAT6 with STAT3 and C/EBPβ bind to an enhancer in the ARG1 locus (169). Some cytokines, including IL-10 and IL-21, upregulate the expression of IL-4Rα and IL-13Rα1, leading to the increased IL-4-induced ARG1 expression (139, 140). M2 macrophages are the most abundant population of myeloid cells in tumors, and their presence is usually associated with poor prognosis, tumor cell invasion, metastasis, and neovascularization (170, 171). Importantly, TAMs are considered to be of either embryonic origin or to derive from hematopoietic stem cells (HSCs) (172–175). Both populations are found in the tumors in approximately similar ratio, but it seems that it is mainly the latter population

that includes cells with immunosuppressive properties (47). HSC-derived macrophages in a tumor microenvironment sense local physicochemical conditions that are different than in many normal tissues and include hypoxia, acidosis, changes in the composition of extracellular matrix proteins (that affects rigidity of the tumor tissue), nutrient insufficiency, different cellular metabolites, various growth factors and inflammatory mediators (prostanoids, cytokines, etc.) (165). Necrosis and other forms of cell death lead to appearance of cell debris as well as cell death-associated molecular patterns [CDAMPs, also known as death-associated molecular patterns—DAMPs (176)] that additionally affect differentiation of macrophages. Many of these environmental conditions have been shown to induce ARG1 in TAMs including hypoxia via hypoxia-inducible factors (HIFs) (177), lactic acid (in a HIF-1α-dependent mechanism) (178), or COX2 via prostaglandin E_2 (162) (**Figure 4**). Even local acidosis might be involved in ARG1 induction as resting macrophages at pH of 6.1 were observed to induce expression of VEGF, HIF-1α and ARG1 (179), and induction of ARG1 by IL-4 was stronger at pH of 6.8 (180). Cancer-associated fibroblasts (CAFs) have been shown to regulate macrophage differentiation and confer these immunosuppressive cells with the ability to secrete high levels of IL-6 and to produce collagen that leads to the development of tumor desmoplasia (181). Collagen forms a scaffold for many secreted mediators including TGF-β. The number of ARG1 positive macrophages was decreased in Mer tyrosine protein kinase (MERTK) knock-out mice (182). MERTK is involved in signaling triggered by recognition of apoptotic cells. Quite unexpectedly, a recent study revealed that type I interferons (IFNs) inhibit monocyte to macrophage differentiation within tumor and induce strong expression of ARG1 (183).

Macrophages are the main source of ARG1 within tumors in a murine model of colon adenocarcinoma (115). *In vivo* imaging of tumor macrophages revealed that in contrast to tumor periphery these cells are highly mobile within the tumor microenvironment, exhibit structural diversity and gene expression profile that includes increased ARG1. The number of these ARG^+ macrophages significantly decreased after anti-PD-1 monoclonal antibody treatment (115). TAMs in lung cancer and melanoma also express more ARG1 than all other cells within tumor combined (178) and have over 20 times higher expression of ARG1 as compared with peritoneal macrophages (184).

Arginase production by macrophages not only leads to the inhibition of anti-tumor response *via* L-arg degradation, but also increases the proliferation of tumor cells, which is associated with the production of L-ornithine and then a polyamine—putrescine that promote tumor cells proliferation (185). Moreover, L-arg depletion in the tumor microenvironment attenuates NO production and reduces its cytotoxic effects on tumor cells (185). Several studies also indicate that arginase activity might be associated with delivery of additional metabolites with immunosuppressive properties. For example, inhibition of polyamines synthesis together with blocking of dietary polyamine transport was shown to exert antitumor effects that were associated with decreased numbers of intratumoral MDSCs and increased numbers of T-cells (186). Similar approach was shown to increase in granzyme $B^+IFN\text{-}\gamma^+CD8^+$ T-cells and a decrease in immunosuppressive tumor-infiltrating cells including PMN-MDSCs, Tregs, and M2 macrophages (187).

Neutrophils

Neutrophils are the most abundant leukocytes in peripheral blood and are produced in the bone marrow at a prodigious rate of 1×10^{11} cells per day (188). These cells constitute a rapidly reacting part of innate immune response, playing important role in defense against bacteria and fungi. Despite their important role in host defense, the increased numbers of neutrophils in blood of cancer patients correlate with poor prognosis (189). These cells can also be found in tumors, but their role in tumor has been largely neglected, mainly due to the belief that their life-span is one of the shortest among all leukocytes. However, tumor-associated neutrophils (TANs) persist in tumor microenvironment for extended time in response to GM-CSF and TGF-β (126). TANs are divided into two subtypes: N1 and N2, with anti-tumor and protumorigenic phenotype, respectively, but to date no specific molecular surface markers have been identified to distinguish them. Nonetheless, N2 neutrophils are characterized by high arginase expression (132, 190). ARG1 is in fact constitutively expressed in human neutrophils. However, these cells do not metabolize L-arg (123) possibly due to the confinement of ARG1 in gelatinase granules (191). Neutrophils can release ARG1 leading to the suppression of T-cells function (192). This process requires simultaneous exocytosis of ARG1-containg gelatinase granules and azurophil granules (192). It was assessed that 1×10^6 of neutrophils secrete ARG1 at amounts sufficient to catabolize all the L-arg contained in 5 ml of blood in 1 h (193). At least in some tumors $ARG1^+$ neutrophils are quite abundant and the presence of $ARG1^+$ neutrophils correlates with suppressed T-cell functions (193, 194). Intriguingly, in non-small cell lung cancers despite high arginase activity in tumor microenvironment, most of the TANs display low or no ARG1 expression, in contrast to neutrophils in peritumoral tissue that strongly stain for ARG1 (193). It turned out that tumor cells release IL-8 that induces ARG1 exocytosis from neutrophils into extracellular milieu (193) (**Figure 4**). Degranulated neutrophils are also expanded in peripheral circulation of cancer patients, and ARG1 released from these cells strongly contributes to general suppression of T-cell functions (195). ARG1 released from neutrophils has also been shown to inhibit the proliferation of NK cells and IL-12/IL-18-induced production of IFN-γ (196). Zoledronic acid, a bisphosphonate used in the treatment of osteoporosis has been shown to induce ARG1 in neutrophils that suppress the activity of γδ T-cells (197). All these observations indicate that $ARG1^+$ neutrophils seem to play a detrimental role in tumor progression, mainly due to immunosuppressive effects. Notably however, a recent study indicated that high intratumoral neutrophil numbers expressing ARG1 correlate with better survival of patients with colorectal cancer (198).

Dendritic Cells

Dendritic cells (DCs) are classically described as professional antigen-presenting cells that produce cytokines and provide co-stimulatory molecules, leading to naïve T-cells activation and

differentiation into effector cells (199). There are conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (MoDCs) that have different origin and differ in function. Within cDCs there are additional subsets both in mice and in humans that are referred to as cDC1 and cDC2. cDCs1 are presumed to be primarily involved in cross-presentation of antigens to $CD8^+$ T-cells, while cDCs2 seem to be largely associated with stimulating $CD4^+$ T-cells (200). Another layer of subdivision into migratory and lymph node (LN)-resident DCs reflects location and the mechanisms of antigen acquisition by these cells. Migratory $CD103^+$ DCs take up antigens in non-lymphoid tissues (including tumors) and traffic through lymphatic vessels into LNs. LN-resident $CD8\alpha\alpha^+$ DCs enter the LNs from the blood and acquire antigens draining through the lymphatics or transported to LNs by other cells (200).

Tumors are frequently infiltrated by various populations of DCs. During infections DCs acquire, process and present antigen in association with MHC molecules, deliver co-stimulatory signals and release cytokines that shape T-cell responses. The same role is expected to be played by DCs in tumors. However, the stimulatory activity of these cells is often compromised and tumor DCs often drive tolerance rather than immunity in cancer patients (201). The mechanisms of tumor-infiltrating DCs that hamper development of antitumor immune response include decrease in MHC class I and II levels as well as in co-stimulatory molecules (CD40, CD80, CD86), rise in co-inhibitory molecules (such as PD-L1, PD-L2, VISTA), increased tryptophan degradation by indoleamine 2,3-dioxygenase (IDO1), decreased release of IL-12, but increased secretion of IL-10 and TGF-β, among others (201). Arginases can be added to this expanding list, based on numerous reports.

Lung cancer cells isolated from murine tumors induced DCs to differentiate into regulatory cells that suppressed T-cell response through ARG1 (202). In another study tumor-infiltrating DCs were observed to decrease the expression of CD3ζ in T-cells in ARG1-dependent manner and induced anergy in naïve $CD8^+$ T-cells (203). ARG1 produced by DCs promotes the generation of $FoxP3^+$ Tregs (204, 205). Not only ARG1 was shown to be expressed by DCs. Human fetal cDC2 cells uniquely express constitutively high levels of ARG2, through which these cells inhibit T-cell activation and TNF-α release (206).

The expression of ARG1 in DCs is regulated by a number of cytokines and tissue factors (**Figure 4**). As in other myeloid cells, ARG1 is induced by type 2 cytokines, including IL-4 and IL-13. Tregs were reported to induce ARG1 in DCs in a TGF-β-dependent mechanism (207). Supernatants from tumor cells experiencing endoplasmic reticulum (ER) stress and unfolded protein response (UPR) was shown to induce ARG1 in DCs (208). Retinoic acid was also shown to be a key mediator regulating expression of ARG1 in DCs, mediated by retinoic acid-responsive elements in the 5′ non-coding region of the ARG1 gene. Blockade of retinoic acid receptors makes DCs less responsive to IL-4 and GM-CSF (205).

MECHANISMS OF IMMUNOREGULATORY FUNCTION OF ARGINASE

An obvious question in understanding the role of amino acid-degrading enzymes in the regulation of the immune response is why do myeloid cells degrade L-arg Perhaps the best answers come from studies in mice with targeted deficiency of ARG1 in myeloid cells and the regulation of immune response and inflammation triggered by infectious microorganisms. ARG1 induced in macrophages during *Schistosoma mansoni* infection prevented cachexia, neutrophilia, and endotoxemia during acute schistosomiasis. Moreover, $ARG1^+$ macrophages promoted TGF-β production and Foxp3 expression, suppressed antigen-specific T-cell proliferation, and limited Th17 differentiation. In mice with deficiency of ARG1 in myeloid cells infection with *Schistosoma mansoni* triggered a lethal T-cell-dependent immunopathology with non-resolving inflammation (209). On the other side, ARG deficiency in myeloid cells results in substantially decreased tumor growth (210) and increased $CD8^+$ T-cells numbers and activity as compared with wild-type mice (211).

Effects on Effector Functions in T-Cells

Lack of any single essential amino acids restricts T-cells activation and proliferation and this phenomenon is not specific to L-arg. Depletion of L-histidine, L-leucine, L-lysine, L-phenylalanine, L-threonine, and L-valine inhibited the proliferation of T-cells to a similar extent as L-arg depletion (207). Of importance, however, only arginases as well as IDO that hydrolyzes L-tryptophan (212, 213) are substantially increased in cancer.

Role of L-arg in T-Cell Proliferation

One of the hallmarks of ARG activity in the immune system is impaired T-cell proliferation (**Figure 5**). Proliferation of both human and murine T-cells is completely inhibited in L-arg-free medium after stimulation with anti-CD3- and anti-CD28-coupled beads or different types of mitogens. A similar inhibition of the T-cells proliferation is also triggered by ARG-producing cells, and this effect is restored by L-arg supplementation or arginase inhibitors (123, 132, 203, 214, 215). It is of note that T-cells remain viable in L-arg-depleted medium (123) and resume proliferation as soon as L-arg is added to the culture medium. The minimum L-arg concentration in cell culture medium necessary for one division of murine T-cell was determined to be 23 μM (216). Upon activation, when large amounts of L-arg are needed, T-cells rely mainly on the extracellular L-arg transport. A potent increase in the expression of cationic amino acid transporter-1 (CAT-1) is observed in both naïve and memory $CD4^+$ and $CD8^+$ T-cells after activation (22). Silencing of CAT-1 expression leads to the inhibition of T-cell proliferation, but not impaired TNF-α, IFN-γ, IL-2, IL-6 production (22).

Role of L-arg in T-Cell Cytokine Production

Secretion of several cytokines that play a critical role in T-cell differentiation and effector functions is also diminished in L-arg-starved cells (**Figure 5**). Conspicuously, this especially

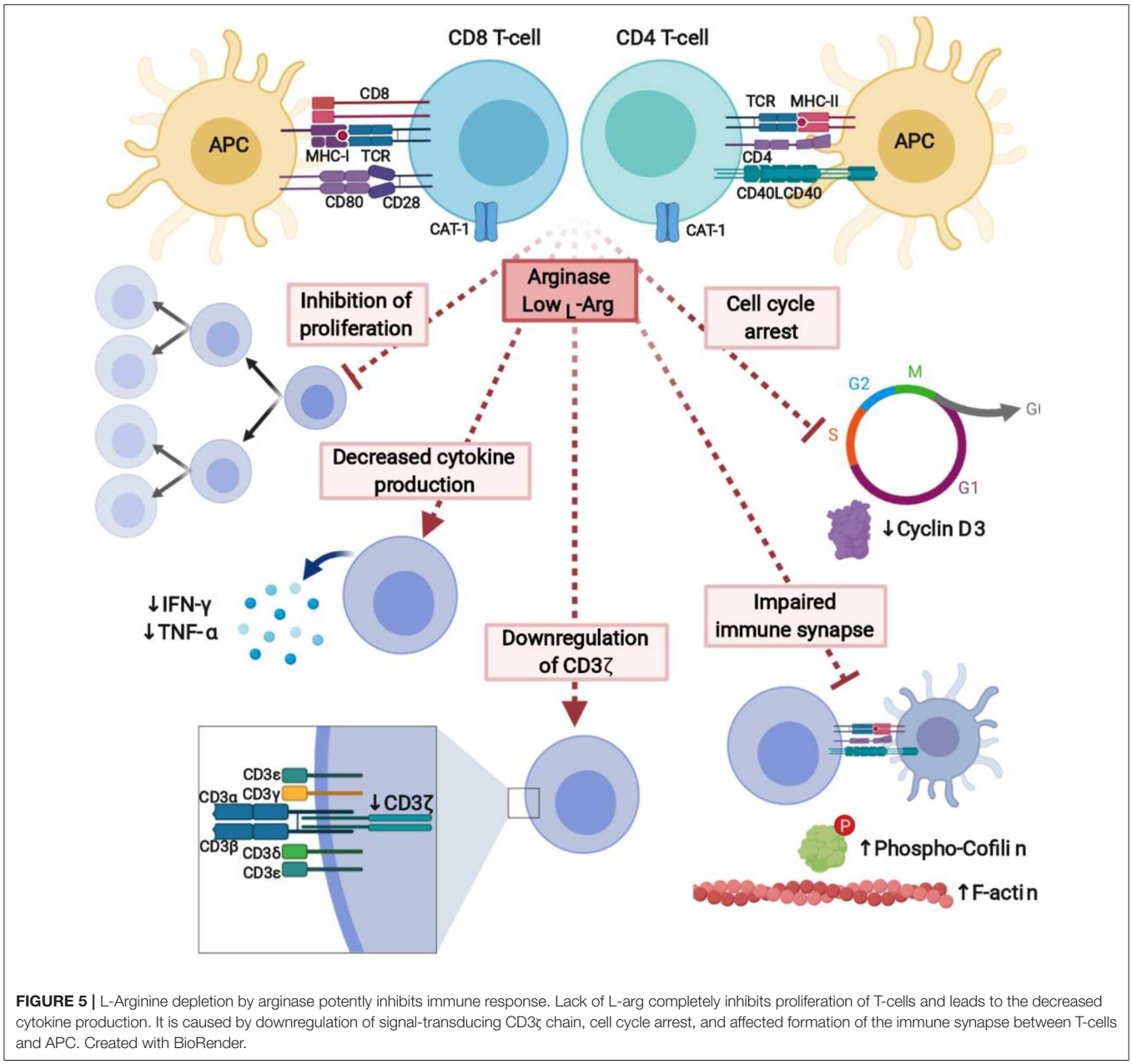

FIGURE 5 | L-Arginine depletion by arginase potently inhibits immune response. Lack of L-arg completely inhibits proliferation of T-cells and leads to the decreased cytokine production. It is caused by downregulation of signal-transducing CD3ζ chain, cell cycle arrest, and affected formation of the immune synapse between T-cells and APC. Created with BioRender.

refers to the secretion of Th1 cytokines, including IFN-γ and tumor necrosis factor β (TNF-β) (123, 214, 217), although T-cells cultured in L-arg-free medium also secrete lower amounts of IL-5, and IL-10 as compared with T-cells cultured in complete medium (218). The decrease in IFN-γ secretion is also induced by ARG^+ tumor-infiltrating DCs (203) and ARG inhibitors administered *in vivo* increase IFN-γ secretion (219). On the contrary, the synthesis of IL-2, IL-6, and IL-8 seems to be unaffected by the absence of L-arg (217), although in another study PMN-MDSCs were shown to suppress IL-2 production from T-cells and this effect was restored by ARG inhibitor (220).

Role of L-arg in T-Cell Differentiation

Upon antigen recognition naïve T-cells proliferate and acquire effector functions that are dependent on multiple additional signals delivered in the microenvironment of secondary lymphoid organs. The signals include various cytokines, growth factors, and surface-associated molecules (including co-stimulatory and adhesion molecules) (221, 222). Accumulating evidence indicates that L-arg metabolism plays an important role in regulating T-cell differentiation. For example, oral administration of L-arg in a mouse model of breast cancer increased the levels of T-bet, a transcription factor associated with Th1 cells (223). Moreover, it increased the frequency of

$CD8^+$ T-cells, and mRNA levels of granzyme B and IFN-γ in the tumor (223). High extracellular L-arg increased the survival of T-cells stimulated with IL-2 after cytokine withdrawal and favored the formation of central memory T-cells (224). Inhibition of L-arg transport into the cell decreased T-cell longevity further confirming the role of L-arg in human T-cells survival (224).

Somewhat enigmatic and to some extent contradictory reports refer to L-arg metabolism and Treg cells development. In an interesting study $FoxP3^+$ Tregs were shown to induce ARG1 (as well as other enzymes involved in amino acid metabolism) in DCs, thereby increasing amino acid consumption in local microenvironment. This reduced mTOR signaling and favored development of additional Tregs (207). Inhibition of mTOR signaling by rapamycin or amino acid depletion was shown to induce FoxP3, but L-arg deficiency was effective only when TGF-β was added (207). Moreover, ARG2 was found in Tregs from normal skin and its expression increased in metastatic melanoma (225). ARG2 in Tregs was demonstrated to attenuate mTOR activity and conferred Tregs with enhanced suppressive activity (225) suggesting that low intracellular L-arg concentrations may facilitate Tregs development. Consistently, with these findings, T-cells from mTOR-deficient mice preferentially become regulatory, but not effector T-cells (226).

However, another study showed that mice fed with L-arg-deficient diet had modestly reduced number of peripheral effector Tregs and these cells had reduced expression of ICOS and CTLA4. L-arg turned out to be essential for sustaining mTORC1 activity, functional programming, and Treg cell-mediated immunosuppression (227). Moreover, disruption of mTORC1 in $FoxP3^+$ T-cells caused a loss of Treg suppressive activity *in vivo* and led to development of systemic immunopathology in mice indicating that Treg cell responses are critically dependent on mTORC1 signaling (228). Clearly, the effects of L-arg metabolism on T-cell differentiation are very complex and require further studies, especially that still another report indicated that ARG1 in MDSCs is participating in promotion of Th17 differentiation (229).

Molecular Mechanisms of Immunoregulatory Effects Associated With L-arg Metabolism

The exact molecular mechanisms of L-arg starvation-mediated immunosuppression still remain to a large extent enigmatic. Up to now, L-arg starvation was shown to affect T-cell antigen receptor ζ chain (CD3ζ) expression (230) and phosphorylation of other signal-transducing proteins (231), and therefore to impair transduction of activation signal, cell cycle progression (232), as well as formation of the immune synapse (231) (**Figure 5**).

Downregulation of the CD3ζ and Impaired Signal Transduction

The main mechanism by which L-arg starvation inhibits T-cells proliferation is through downregulation of the CD3ζ chain (230, 233). CD3ζ is a critical component of the TCR complex that couples antigen recognition to the intracellular signaling pathways (234). After T-cells stimulation, TCR proteins including CD3ζ undergo internalization followed by re-expression, externalization, or sorting to lysosomes for degradation (235, 236). A common finding in cancer patients is a marked decrease in the expression of CD3ζ in T-cells (143, 237).

Many studies reported that L-arg depletion in culture medium leads to a rapid decrease of CD3ζ levels (132, 230, 238). Of note, the changes in TCR receptor subunits expression during L-arg starvation are observed only in stimulated T-cells (218). This decrease is specific to L-arg-starvation, since lack of glutamine or leucine (233) as well as glycine or lysine (218) did not change the levels of CD3ζ. However, a decrease in CD3ζ was also reported to be caused by hydrogen peroxide secreted from tumor macrophages (239). The decrease in CD3ζ is completely reversed by L-arg supplementation in cell medium (230) or ARG inhibition when co-culture with ARG-producing cells is used (132). A similar downregulation of CD3ζ and CD3ε levels is induced by tumor-associated myeloid cells, which express ARG1 (132). This effect is prevented by the addition of ARG inhibitor (N-hydroxy-nor-L-arg) or L-arg supplementation, but not by the catalase, a hydrogen peroxide scavenger (132), as suggested before (239).

How L-arg starvation selectively impairs CD3ζ expression still remains unclear. L-arg starvation of human T-cells did not affect the degradation of CD3ζ in proteasome or lysosomes (218). Therefore, it was suggested that L-arg depletion may impair CD3ζ synthesis (218) or the stability of mRNA for CD3ζ (230).

Cell Cycle Arrest

Another defined mechanisms by which L-arg starvation restricts T-cells activation and proliferation is the regulation of cell cycle progression (232) *via* modulation of cyclin D3 mRNA stability (240). Cyclins, including cyclin D3 (241), are critical regulators of the cell cycle, immune cells development and proliferation (242). L-arg starvation arrests human T-cells in G_0-G_1 phase (232). The levels of cyclin D3 as well as CDK4 significantly increase after T-cells activation, however, not in absence of L-arg. Moreover, silencing of cyclin D3 in Jurkat cells reproduces effects induced by L-arg starvation (232). Cyclin D3 was shown to be regulated by L-arg through transcriptional, posttranscriptional, and translational mechanisms (232). In the absence of L-arg human T-cells have decreased phosphorylation of the retinoblastoma protein (Rb), which is the major substrate for the cyclin D/cyclin-dependent kinase complex, as well as decreased levels of E2F-1, which is crucial for the initiation of the transcription of genes involved in the G_2/S transition (232). In the absence of L-arg there is a global arrest in *de novo* protein synthesis. L-arg starvation also affects the expression of HuR, RNA binding protein, that stabilizes mRNA of cyclin D3 by the binding to the 3'-untranslated region (UTR) and shuttles its transport to the cytoplasm. Silencing of HuR exerts similar effect on T-cells proliferation as L-arg starvation (240).

Changes in the Immune Synapse Between APC and T-Cells

Proliferation of T-cells after antigen presenting cells (APC)-based cellular activation is also completely inhibited in the absence of L-arg (231). The formation of immune synapse between

T-cell and APC is critical for the activation of effector cell (243). In L-arg-depleted medium, the formation of the immune synapse is impaired. T-cells activated in the absence of L-arg have increased F-actin concentration, which may be caused by impaired cofilin dephosphorylation (231). Cofilin is a small actin-remodeling protein that couples T-cell activation via the TCR and co-stimulatory receptors in the immune synapse (231, 244). Phosphorylation of ERK1/2 is significantly reduced in L-arg absence, however, the phosphorylation of AKT is increased to the higher level compared to the cells activated in L-arg-containing medium (231). It leads to the impaired dephosphorylation of cofilin that results in impair immune synapse formation. Impaired dephosphorylation of cofilin in human T-cells was also induced by cell-free human pus supernatant, which is known to contain high arginase activity (123). This effect may be prevented by arginase inhibitor (231).

L-arg in Metabolic Regulation of T-Cells

Proliferation and differentiation of T-cells can occur only if sufficient access to metabolites and nutrients is ensured (245). A recent metabolomic analysis of activated T-cells revealed that out of 429 measured metabolites only 14 were less abundant in activated T-cells, and L-arg was the only protein amino acid among them (224). A drop in intracellular L-arg levels was observed despite induction of CAT-1 transporters. Interestingly, the intracellular levels of L-glutamine, which is also intensively metabolized in activated cells, remained high. Along with CAT-1 induction, T-cell activation was associated with increased expression of L-arg metabolism-related enzymes including ARG2, OAT, and spermidine synthase (SRM). Once entering the cell, L-arg turned out to be rapidly converted into L-ornithine, agmatine, and putrescine. Importantly, increasing L-arg concentration in the culture medium upregulated gluconeogenesis-related genes, serine biosynthesis pathway, and mitochondrial tricarboxylic acid cycle, while downregulating glucose transporter and glycolytic enzymes. These changes promoted mitochondrial OXPHOS in activated T-cells, while downregulating glycolysis (224). Global analysis of T-cells proteome changes in response to high L-arg concentration revealed several proteins that are responsible for increased T-cells survival. These can be assigned into four functional groups, including mRNA splicing, DNA repair mechanisms, regulation of the cytoskeleton and the ribosome (224).

Oral supplementation of L-arg that increased its serum concentration over 4-fold allowed more robust induction of antigen-specific T-cell proliferation in mice. Moreover, T-cells from $ARG2^{-/-}$ mice, incubated with supplemental L-arg or treated with ARG inhibitor reveled much better survival after cytokine withdrawal (224). In a complementary study, $CD8^{+}$ T-cells from ARG2-deficient mice showed markedly superior antitumor activity in mice and turned out to respond stronger to PD-1 blockade as compared with $ARG2^{+}$ T-cells (246). Moreover, ARG2-deficient T-cells were characterized by faster acquisition of effector functions, increased persistence and enhanced differentiation into memory cells.

Altogether, these studies indicate that ARG2 might be a metabolic gatekeeper in T-cells. In activated T-cells ARG2 degrades L-arg and generates agmatine and polyamines. In case of accessible L-arg in the extracellular environment the intracellular pool of this amino acid can be replenished. However, at sites, where extracellular L-arg is depleted (by tumor cells or tumor-infiltrating myeloid cells) the intracellular pool cannot be restored leading to T-cell suppression (224).

Mechanisms of arginine-starvation sensing in immune cells are still unclear. It is suggested that mTOR together with GCN2 kinase regulate amino acid metabolism and response to arginine starvation (207, 227, 232, 247, 248), however, the exact mechanism is unknown and requires further investigation.

B Cells and L-arg

The role of L-arg in B-cells functions was much less investigated and is poorly understood. It was shown that L-arg deficiency due to high ARG1 activity in F/A-$2^{+/+}$ transgenic mice, that overexpress arginase in enterocytes, potently impairs early B cell maturation with no major impact on T-cells (249). F/A-$2^{+/+}$ mice have reduced number of B cells, decreased serum IgM concentration and hampered B cell maturation in the early pre-B cell stage (249). L-Arg-free diet fed mice which have significantly lower concentration of plasma L-arg compared to L-arg-supplemented diet had also impaired antigen-specific mucosal immune response against tetanus toxoid (TT). After oral administration, no TT-specific fecal IgA antibodies were detected in L-arg-free diet fed mice (250). Both PMN-MDSCs and M-MDSCs were shown to regulate key B-cell functions, particularly B-cell proliferation and antibody production. PMN-MDSC-mediated B-cell suppression turned out to be cell contact dependent and involved ARG1 (251). A recent study from the same group indicated that M-MDSCs suppress B-cell proliferation, and downregulate IgM, HLA-DR, CD80, CD86, TACI, and CD95 in contact independent, but ARG1 and iNOS-dependent mechanism (252).

Myeloid Cells and L-arg

The role of L-arg in differentiation of myeloid cells is poorly investigated. Most of the studies focused on the role of ARG1 produced by myeloid cells rather than the dependence of these cells on L-arg. Individual results *in vitro* show no influence of L-arg on macrophages differentiation, maturation, and effector functions. In the absence of L-arg, maturation of macrophages into classically activated macrophages (M1) and alternatively activated macrophages (M2) was unaffected (253). Moreover, the production of cytokines by both macrophage subtypes was unimpaired under L-arg-starvation (253). Likewise, the expression of iNOS by M1 cells as well as the expression of ARG by M2 cells turned out to be independent from the L-arg concentration (253). However, ARG1 expression was essential for monocytic DC differentiation (254). ARG1 was also recently shown to be crucial in efferocytic clearance of apoptotic cells by macrophages (255).

In vivo however, L-arg supplementation was shown to promote Gr-$1^{+}CD11b^{-}F4/80^{+}$, but suppressed Gr-$1^{+}CD11b^{+}F4/80^{+}$ macrophages in a murine model of breast

cancer (223). However, these effects might not be caused directly, but rather result from the effects on T-cells activation. Another study showed that L-arg starvation promotes tumor G-MDSC accumulation, which further suppress T-cells anti-tumor response (158). Similar results were obtained with PEG-asparaginase administration, suggesting that generally amino acid starvation results in MDSC accumulation. PEG-ARG1-induced MDSC accumulation was found to be regulated by GCN2, since the accumulation of MDSCs in GCN2-deficient mice treated with PEG-ARG1 was negligible (158). Importantly, MDSCs isolated from GCN2-deficient mice had similar immunosuppressive properties as compared with MDSCs isolated from wild-type mice, which suggests that GCN2 is involved in the accumulation of MDSC, but not in their effector functions. Moreover, it was observed that ARG2-releasing AML blasts as well as ARG2-rich plasma of patients with AML promotes the differentiation of monocytes toward M2 macrophages. These effects were diminished by L-arg supplementation or arginase inhibitors (61).

NK Cells and L-arg

NK cells are less sensitive to low L-arg concentrations as compared with T-cells however, L-arg starvation affects the main effector functions of NK cells (196). L-arg starvation decreases NK cells proliferation and viability, as well as cytotoxic activity (210, 256). Depletion of L-arg leads to the reduction in the expression of NKp46 and NKp30 activating receptors, as well as the NK cell ζ chain expression in the FcγRIIIA, similar to the CD3ζ chain in T-cells. Moreover, in the absence of L-arg the production of IFN-γ by NK cells is significantly decreased (256). Similar effect is exert by arginase from human neutrophils (196). However, NK cell degranulation and cytotoxicity seems to be unaffected by L-arg depletion (196).

L-arg Metabolites and Immune Response

L-arg is in the center of many metabolic pathways. Arginase not only depletes L-arg, but also creates multiple downstream metabolites including L-ornithine and urea, as well as L-proline, glutamate, agmatine, putrescine, L-citrulline, and polyamines.

Ornithine

L-arg is degraded by arginase to L-ornithine and urea. While the concentration of L-arg substantially decreases in cancer, the concentration of L-ornithine increases (59, 75, 257). High concentration of L-ornithine in tumor interstitial fluid may inhibit anti-tumor cytotoxic response of $CD8^+$ T-cells (258, 259), and together with L-arg depletion, that affects T-cells properties but not cytotoxicity (214), provide effective tumor evasion of the immune system. Reversible inhibition of cytotoxicity of T-cells in the presence of L-ornithine is independent from the type of stimulation and it seems that it affects early stages of CTL activation (258). However, L-ornithine did not impair mitogenic response to the stimulation (258, 259), as well as IL-2 and IL-3 production (258). ODC catalyzes the conversion of L-ornithine to polyamines.

Polyamines

A diamine putrescine, triamine spermidine, and tetraamine spermine are ubiquitous L-ornithine metabolites associated with important cellular processes. Polyamines are essential for cell growth and proliferation during development, wound healing, and tissue regeneration. ODC catalyzes the conversion of L-ornithine into putrescine, which is then metabolized to spermidine by spermidine synthase and spermine by spermine synthase (260). At physiological pH polyamines are positively charged and bind to acidic sites in DNA and RNA, controlling gene expression (261). Moreover, polyamines have antioxidative properties, bind to K^+ channels, NMDA receptors, and modulate the activity of various enzymes (261).

Growth promoting functions of polyamines are best described in tumors. However, it seems that polyamines are also important in T-cell clonal expansion. It has been suggested that the synthesis of polyamines in T-cells is under the direction of Myc, as Myc-deficient T-cells fail to induce ODC and other genes involved in polyamine synthesis, leading to decreased polyamine production (262). Spermidine is also a precursor of hypusine, which post-translationally binds to eukaryotic initiation factor 5a (eIF5a). Intriguingly, eIF5a, which prevents ribosomal stalling during translation of certain mRNAs, is one of the most strongly expressed proteins in activated T-cells (263).

Polyamines were reported to exert anti-inflammatory effects in macrophages by restraining activation of M1 while promoting differentiation of M2 subtype. For example, LPS-induced expression of TNF, IL-1, IL-6, IL-12, iNOS, and CD80 was suppressed by polyamines (264–266). Polyamines also modulate immunoregulatory activities of DCs. IDO1 activity in TGF-β-treated DCs requires ARG-1-dependent spermidine synthesis that activates Src tyrosine kinase, which participates in IDO1 phosphorylation (267).

ARGINASE INHIBITORS

Expanding knowledge on the biological role of arginases prompts the idea of therapeutic inhibition of these enzymes. The interplay between ARG and NOS resulting mainly from the competition for the common substrate L-arg makes ARG inhibition an attractive approach in the treatment of cardiovascular and inflammatory conditions (such as asthma, diabetes, hypertension, atherosclerosis, coronary artery disease, heart failure or erectile dysfunctions). Furthermore, inhibition of immunosuppressive functions of arginases is being explored in the treatment of cancer. Modulation of L-arg metabolism is also being explored as a therapeutic strategy in Alzheimer's disease (268).

As many pathogenic bacteria (such as *Helicobacter*, *Mycobacterium*, *Salmonella*), fungi (*Candida*) and parasites (*Trypanosoma*, *Leishmania*, *Schistosoma*) express species-specific isoforms of ARG to facilitate their survival in the host, finding pathogen-ARG-specific inhibitors emerges as a timely approach in the antibiotic-resistance era. Interestingly, *Leishmania* parasites induce ARG1 expression in infected macrophages to decrease L-arg availability for iNOS and thus to avoid NO toxicity

(269). The latter observation further supports the potential use of ARG inhibitors in the treatment of infectious diseases.

Currently, almost all ARG inhibitors being developed as drug candidates are competitive inhibitors of both isoenzymes (ARG1 and ARG2) and in vast majority are L-arg analogs (270). Finding an isoform-specific ARG inhibitor is challenging as 100% homology exists in the active site between human ARG1 and ARG2. As the results of the preclinical, mainly *in vitro*, testing of ARG inhibitors have been extensively reviewed elsewhere (270, 271), here we just briefly summarize the data on *in vivo* and clinical activity of selected ARG inhibitors.

So called first generation of ARG inhibitors such as N-hydroxy-nor-L-arginine (nor-NOHA) (272), (S)-2-amino-6-boronohexanoic acid (ABH) (273) and (S)-(2-boronoethyl)-L-cysteine (BEC) (274) are reversible, modest inhibitors of ARG1 and ARG2 enzymatic activity with either poor pharmacokinetic properties or insufficient penetration through the plasma membrane. In mouse models nor-NOHA has been shown to inhibit local tumor growth in B- and T-cells-dependent manner as well as to reduce metastatic burden (132, 178, 275). Second generation compounds are characterized by better pharmacokinetic and pharmacodynamic properties. As an example, so called compound 9 [(R)-2-amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic acid] has been recently showed to decrease the growth of KRAS mutated murine lung tumors via inhibition of ARG activity in tumor-infiltrating myeloid cells (117).

Up to date, there are only two ARG inhibitors being tested in clinical trials. Both drug candidates have been developed by Calithera Biosciences and are orally available small-molecule compounds. INCB001158 (CB-1158) is being evaluated in Phase 2 as a single agent and in combination with immune checkpoint inhibitors in cancer (both solid tumors and multiple myeloma), while CB-280 in Phase 1 in cystic fibrosis, exploiting the novel idea of increasing NO production to improve lung function. CB-1158 has been shown *ex vivo* to reverse human T-cell immunosuppression mediated by ARG1 produced by neutrophils as well as MDSCs (210). It also exerts immune-based antitumor effects in syngeneic mouse tumor models *in vivo* as a single agent as well as in combination with the immune checkpoint inhibitors (210). An interesting ARG inhibitor to watch is OATD-02 (276), a compound being developed by Oncoarendi Therapeutics. In preclinical models it has been shown to delay ovarian cancer progression and to revert ARG1-mediated inhibition of antigen-specific T-cells proliferation and to restore their CD3ζ levels (64). Moreover, in syngeneic mouse tumors it potentiated the antitumor efficacy of immune checkpoint inhibitors (277). The company claims OATD-02 Phase 1 trial in cancer patients to begin in 2020-2021.

Arginase inhibition cannot be replaced, however, by chronic L-arg supplementation. Dietary intake of L-arg results only in a transient increase of L-arg plasma concentration (278). Moreover, if arginases are active in blood or body tissues, it is very likely that they easily degrade the excessive amounts of this amino acid.

Global ARG1 inhibition rises significant safety concerns. ARG1 gene knockout mice die 10–14 days post-birth (39). Similarly, induction of whole body *Arg1* KO in adult "floxed" *Arg1 CreET*^{T2} transgenic mice leads to the animals death in up to 2 weeks post-tamoxifen administration (279). The major cause of death in *Arg1* KO mice is hyperammonemia resulting from the defect of the liver urea cycle. It is the lack of *Arg1* expression in the liver that is fatal, as hepatocyte-specific knockout of *Arg1* mimics the whole body deficiency of this enzyme (280). Lack of *Arg1* expression leads to altered hepatocytes morphology, significantly increased plasma L-arg and L-citrulline concentrations accompanied by decreased plasma L-ornithine and L-proline concentrations (39). Interestingly, *Arg2* knockout mice are viable and do not have a disabling phenotype apart from high plasma L-arg concentrations and decreased male fertility. Moreover, *Arg2* KO mice have significantly extended lifespan, indicating some role of this enzyme in aging (41). Double *Arg1* and *Arg2* KO mice show the same phenotype as *Arg1*-lacking animals. Unexpectedly, in *Arg1* KO mouse embryo no compensatory *Arg2* expression was observed (281), suggesting non-overlapping role of both arginase isoenzymes in murine embryonal development. In humans, ARG1 deficiency is a rare autosomal recessive disorder, resulting from over 40 reported mutations in *ARG1*. In the most severe form ARG1 deficiency results in hyperargininemia, neurological impairment and eventually fatal episodes of hyperammonemia (282). ARG1 deficiency is frequently accompanied by a compensatory increase in ARG2 activity in the kidney, ameliorating metabolic disturbances (44). The latter observation encourages a still very challenging attempt to develop ARG1-specific inhibitors.

Animal studies confirmed that there is a safe therapeutic window for tested ARG inhibitors. In both mice and rats, over 2-months long daily systemic administration of nor-NOHA did not result in detectable toxicity. It is likely, that due to the quantitative differences in ARG1 expression between the liver and other tissues way lower ARG inhibitors concentrations are needed to exert immunomodulatory and/or vascular effects than to block the Krebs cycle in hepatocytes (270).

Initial results of the investigational trial of the oral ARG inhibitor INCB001158 in colon cancer patients proved acceptable safety profile of this drug candidate. A maximum tolerated dose was not reached even for the twice daily total dose of 150 mg. Moreover, clinically significant urea cycle inhibition was not observed. In microsatellite stable (MSS) colorectal cancer patients involved in this study, 7 and 6% of partial responses to the INCB001158 and pembrolizumab (anti-PD1 monoclonal antibody) combination or INCB001158 monotherapy, respectively, were reported. Importantly, objective pharmacodynamic parameters such as an increase in the intratumoral CD8$^+$ T-cells as well as dose-related increase in plasma L-arg were achieved in the treated individuals (283).

To evaluate the clinical efficacy of ARG inhibition in a comprehensive way we need much more data. Nonetheless, existing preclinical and initial clinical evidence seems to support the idea that therapeutic targeting of the immunomodulatory ARG might serve as a potent addition to the other immunotherapeutic strategies rather than as an effective single agent treatment. Moreover, it would be crucial not only to evaluate proper dosing, timing and treatment

duration but also to find reliable biomarkers predicting desirable clinical effects.

Although recent data support the idea that ARG overexpression correlates with poor prognosis, a number of studies indicate that arginine depletion may also be beneficial for subgroups of patients, especially those with inactivation of ASS1 in cancer cells that leads to the dependence on exogenous L-arg (284). L-Arg deprivation by ADI conjugated with polyethylene glycol (ADI-PEG) (84, 285) as well as pegylated recombinant human ARG (rhARG-PEG) (286, 287) were applied to the treatment of arginine-auxotrophic tumors and showed potent anticancer effects [reviewed in (288)].

Noteworthy, L-arg-restriction as the regulation of immune response is not specific to the cancer. It was shown that *Helicobacter pylori* by arginase not only produces urea which can be used to CO_2 and NH_3 production by urease to support acid tolerance (289). *H. pylori* using ARG also depletes L-arg which leads to the downregulation of CD3ζ and inhibition of T-cells proliferation during infection (290). T-cells response is also suppressed *via* ARG by human embryonic stem cells (291). ARG also mediates T-cells hyporesponsiveness in human pregnancy (292), post-stroke immunosuppression (293), as well as in the control of autoimmunity (294). Moreover, *H. pylori* induces ARG2 expression in macrophages contributing to the immune evasion by limiting production of antimicrobial NO (48). Crucial role of ARG in the regulation of immune response by impairing NO production was also described in the model of cutaneous contact hypersensitivity (295) as well as in immune response to *Leishmania major* infections (296). Importantly, some intracellular pathogens induce expression of ARG1 in macrophages that hampers effective immune response (137). A recent study revealed that increased ARG levels may play a role in fatigue intensification in cancer patients undergoing external-beam radiation therapy (297)

FINAL REMARKS

ARG expression is substantially elevated in myeloid cells in cancer and mitigate antitumor response via multiple mechanisms. Intriguingly, cytotoxic effects of T-cells are unaffected by a lack of L-arg, despite the fact that CD3ζ and CD3ε are downregulated and thus TCR signal transduction should be inhibited. In contrast, T-cell proliferation is strongly suppressed, but it must be emphasized that T-cells proliferate extensively in tumor-draining lymph nodes, and not in the tumor. L-arg concentrations in tumor-draining LN have not been measured so far. It would also be interesting to see whether increased ARG activity contributes to fibrotic processes leading to desmoplastic changes in some types of tumors, such as pancreatic cancer. Increased activity of arginases could limit L-arg availability to NOS—could it be responsible for vascular abnormalities frequently described in tumorsxx Altogether, increasing evidence indicates that arginases become potentially important targets for therapeutic interventions that might improve the efficacy of immunotherapy, decrease infectious complications and improve quality of life of cancer patients.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Generation of Myeloid Cells in Cancer: The Spleen Matters

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Myeloid cells are key components of the tumor microenvironment and critical regulators of disease progression. These innate immune cells are usually short-lived and require constant replenishment. Emerging evidence indicates that tumors alter the host hematopoietic system and induce the biased differentiation of myeloid cells to tip the balance of the systemic immune activities toward tumor-promoting functions. Altered myelopoiesis is not restricted to the bone marrow and also occurs in extramedullary organs. In this review, we outline the recent advances in the field of cancer-associated myelopoiesis, with a focus on the spleen, the major site of extramedullary hematopoiesis in the cancer setting. We discuss the functional specialization, distinct mechanisms, and clinical relevance of cancer-associated myeloid cell generation from early progenitors in the spleen and its potential as a novel therapeutic target.

Keywords: cancer, myeloid cell, spleen, hematopoietic stem/progenitor cell, myelopoiesis

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INTRODUCTION

Cancer is now viewed as an ecological disease in which interactions between neoplastic, stromal, and infiltrating immune cells profoundly regulate disease progression. Myeloid cells are major components of this ecosystem. These cells belong to the innate immune system and comprise various mononuclear and polymorphonuclear phagocytes and precursors, including monocytes/macrophages (Mos/Mϕs), dendritic cells (DCs), granulocytes, and myeloid-derived suppressor cells (MDSCs). Over the past two decades, a wealth of studies has revealed the crucial roles that myeloid cells play in many, if not all, steps of tumor initiation, progression and metastasis (1–6). The importance of myeloid cells has been further underlined by identifying the broad involvement of myeloid cells in regulating treatment responses and has thereby spurred interest in therapeutically targeting these cells (7–12).

In addition to directly modulating myeloid cells in tumor tissues using small molecules (13–16), antibodies (17–19), and nanoparticles (20–24), a novel myeloid cell-targeting strategy is now emerging into the research spotlight. The idea is to limit the tumor-supporting myeloid cell response at its root by restraining tumor-associated myelopoiesis. Tumor progression often parallels a coordinated expansion and continuous accumulation of myeloid cells such as tumor-associated macrophages (TAMs) (11, 25–28), neutrophils (TANs) (5, 15, 29–31), and MDSCs (3, 32, 33). Considering that cells of the myeloid compartment are generally short-lived, this growing and fast-turnover pool of tumor-associated myeloid cells needs to be promptly and constantly regenerated from hematopoietic stem and progenitor cells (HSCs and HPCs, or HSPCs combined). Therefore, tumors interfere with host hematopoiesis and skew the process toward the generation of myeloid cells with tumor-promoting properties. The generality and importance of hematopoietic deviation in cancers are supported by evidence from both human and mouse

studies (34–38). Notably, hematopoietic alteration is not restricted to the bone marrow (BM), the primary hematopoiesis site for adults, but has also been observed in multiple extramedullary organs. However, our knowledge about the nature and properties of cancer-induced myelopoiesis, in particular the necessity and advantages of extramedullary hematopoiesis, is still limited. In this review, we briefly introduce myelopoiesis in different sites discovered to date in the context of solid tumors and then focus on the spleen, the major site of extramedullary myelopoiesis. The expansion of downstream immature [e.g., MDSCs (2, 39, 40)] or mature myeloid cells [e.g., TAMs (41)] has been well-summarized in several recent reviews; thus, here, we focus on the role of early HSPCs in cancer-associated myelopoiesis.

SITES OF MYELOPOIESIS IN CANCERS

At steady state, HSPCs reside primarily in the BM and generate cells of the blood and immune systems (42, 43), with a small subpopulation constantly recirculating between the BM and blood (44, 45). These peripheral HSPCs survey extramedullary tissues and respond rapidly to danger signals to resolve hematopoietic/immunological stress conditions (46). In recent years, the paradigm that HSPCs divide in response to peripheral cytopenia has given way to one in which HSPCs can sense environmental stimuli and pro-inflammatory cytokines directly and thus can actively serve as a foundation for the immune response (47, 48). These mechanisms operating in "emergency" myelopoiesis are hijacked by cancers, which instruct HSPC activity, at least in part, through the constant and progressive release of cytokines, chemokines, and metabolites (49, 50). Here, we summarize the recent discoveries in cancer-associated myeloid cell generation taking place in the BM and extramedullary sites.

Bone Marrow

In the BM, the binding of stromal-cell-derived factor-1 (SDF-1, also known as CXCL12) to its receptor CXCR4 represents a critical axis in the BM retention and homing of HSPCs (45, 51–53). Granulocyte colony-stimulating factor (G-CSF) is known to antagonize this SDF-1/CXCR4 axis, modulate BM HSPC mobilization, and direct hematopoietic differentiation. A recent study using a mouse model of breast cancer showed that tumor-derived G-CSF induces the expansion and differentiation of HSPCs to skew hematopoiesis toward the myeloid lineage. Myeloid-biased hematopoiesis results in the systemic expansion of myeloid suppressors with the distinguishing characteristics of tumor-induced immunosuppressive neutrophils (36). These results are consistent with previous findings showing that the BM $CD11b^{+}Gr1^{+}$ myeloid cell compartment expands in response to tumor-derived G-CSF and is functionally altered before these cells are mobilized into the circulation (54, 55), via the activation of the retinoic-acid-related orphan receptor (RORC1/RORγ) and CCAAT/enhancer-binding protein β (C/EBPβ) pathways (56).

In addition, other hematopoietic cytokines, such as macrophage colony-stimulating factor (M-CSF) (57), granulocyte/macrophage colony-stimulating factor (GM-CSF) (58), vascular endothelial growth factor A (VEGF-A) (59, 60), placental growth factor (PlGF) (59, 61), osteopontin (62, 63), transforming growth factor-β (TGF-β) (60), and tumor necrosis factor-α (TNF-α) (60, 64), are known to influence hematopoiesis and are secreted by a variety of solid cancers to affect the BM (65). Although the precise effect and mechanisms are not yet fully elucidated, these cytokines may also impact the differentiation pattern of HSPCs and regulate tumor-promoting myeloid cell responses.

Primary Tumor and Pre-metastatic Sites

In the context of cancer, we have found that circulating HSPCs from patients with various types of solid tumor, including hepatocellular, breast, cervical, esophageal, gastrointestinal, lung, and ovarian tumors, exhibit a generalized myeloid bias that skews toward granulocytic differentiation (35). Whether these trafficking HSPCs have a preset destination other than returning to the BM remains unclear. One possible extramedullary site for HSPC residence and function is the tumor. BM-derived HSPCs have been observed within the stroma of primary tumors and are thought to promote tumor progression (59, 62, 63, 66). In support of these findings, we have found that there is significant infiltration of CD133-expressing precursor cells with multipotent colony-formation capabilities in human colon cancer tissues (35, 67). These HSPCs give rise to immature myeloid cells with a potent immunosuppressive function in a glutamine metabolism-dependent manner (67). Recent studies have demonstrated that in addition to homing to the primary tumor, a distinct subset of HSPCs that express vascular endothelial growth factor receptor 1 (VEGFR1; also known as Flt1) can home to tumor-specific pre-metastatic sites. These HSPCs express necessary adhesion molecules and growth factors and differentiate into immunosuppressive MDSCs to form a permissive niche for incoming tumor cells (61, 68, 69).

In contrast to the above findings, there are some reports based on transplant-treatment models showing that the transfer of BM-derived HSPCs can enhance adoptive T cell immunotherapy (ACT) in mouse melanoma (70) and glioma models (71), thus arguing that HSPCs can play an antitumor role in ACT. Wildes et al. reported that the combination of ACT and HSPC transfer could lead to HSPC differentiation into immune-stimulating DCs in mouse glioma. The treatment began with a sublethal- or lethal-dose total body irradiation, followed by adoptive transfer of autologous HSPCs and tumor-reactive T cells. These T cells released IFN-γ in the brain tumor microenvironment to augment HSPC differentiation into potent DCs, which in turn further activated tumor-reactive cytotoxic T lymphocytes (CTLs) in a positive feedback manner (72). Such treatments, involving total body irradiation, may raise concerns regarding the translational value, but these studies did provide hints of the potential mechanisms by which altering the tumor microenvironment/hematopoietic niche may reprogram the typical immunosuppressive myelopoiesis and function of HSPCs. Thus, current evidence suggests that the existence, biological nature, and clinical relevance of myelopoiesis in primary

tumors and pre-metastatic sites are highly heterogeneous and tumor-dependent.

Spleen

The spleen is now viewed as the prominent site of extramedullary hematopoiesis (EMH) in cancers. The spleen, which is located in the abdominal cavity, right beneath the diaphragm and connected to the stomach, is the largest secondary lymphoid organ in the body. The spleen plays a crucial role in filtering antigenic particles and abnormal cells from the blood, destroying aged erythrocytes, and recycling iron and is an important organ for the differentiation and activation of T and B cells and production of antibodies (73–75). In hematology, the spleen serves as an important reservoir of monocytes (76, 77), platelets (78, 79), and memory B cells (80). The spleen is also a significant site of hematopoiesis throughout vertebrate evolution and during fetal development in humans (81). Although the contribution of splenic EMH in steady-state adults seems trivial, a vast spectrum of hematopoietic stresses, including myelofibrosis (82), anemia (83), pregnancy (84), infection (85, 86), myeloablation (87), myocardial infarction (88, 89), diabetes (90), atherosclerosis (91, 92), colitis (93), and spondyloarthritis (94), can induce profound EMH in the spleen. Splenic EMH also occurs in the context of cancer. In addition to reports on the expansion of myeloid precursors in the spleen (95–97), Cortez-Retamozo et al. found that the spleen of hosts bearing lung adenocarcinomas accommodates a large number of HSPCs, including HSCs and granulocyte/macrophage progenitors (GMPs), that are phenotypically and functionally analogous to their BM counterparts. These splenic HSPCs give rise to myeloid descendants, such as monocytes and neutrophils, that subsequently migrate to the tumor and exert tumor-promoting functions (34, 98). Consistently, in various mouse models with transplanted, genetically engineered, or chemically induced malignancies and in patients with hepatocellular, gastric, renal, or pancreatic cancers, the spleen accommodates a profound expansion of early HSPCs and supports myeloid-biased myelopoiesis, suggesting the generality of splenic myelopoiesis in various types of solid tumors (37). It is also noteworthy that cancer-induced EMH does not produce only myeloid cells; in late-stage cancers, the spleen generates unique erythrocytic cell populations to further alleviate the disease (99–101).

To evaluate the significance of splenic myelopoiesis in cancers, two central questions need to be addressed: (1) What is the relative contribution of splenic myelopoiesis, compared with that of the BM and other extramedullary tissues, to cancer-associated myeloid cells? (2) Is splenic myelopoiesis a mere complement to BM hematopoiesis or does it play a unique role in generating particular myeloid subsets? To date, the relative contribution of splenic myelopoiesis is controversial. Current evidence suggests that this depends on the type of cancer and the settings of the tumor model. In some experiments, splenectomy causes a significant decrease in the tumor-infiltrating myeloid population and restricts tumor growth (34, 102–104), whereas in other settings, these effects seem marginal (37, 38, 105). Beyond the comparison of production capacity, we recently found that although splenectomy does not change the frequency or distribution of tumor myeloid cells in a hepatoma model, the abrogation of splenic EMH reduces the expression of arginase 1 (Arg1) and abolishes the suppressive activity of tumor $CD11b^{+}Ly6G^{+}Ly6C^{low}$ granulocytic MDSCs, the major MDSC subset in that tumor (37). Thus, emerging studies suggest that splenic myelopoiesis is more than a complement to BM myelopoiesis and may represent myeloid cell biogenesis that is functionally and mechanistically different from its BM counterpart. This mechanism is important for systemic tumor-promoting myeloid cell responses. Therefore, a systematic understanding of cancer-induced splenic EMH (myelopoiesis) is critical for guiding the development of novel therapeutic strategies targeting myeloid cell responses.

Other Extramedullary Organs

Hematopoiesis can take place in many tissues (106–108). Although EMH plays a physiological role during fetal development, its occurrence after birth is typically abnormal, usually associated with inflammation or hematological diseases such as myelofibrosis, leukemia, and hemolytic anemia. In cases of malignant solid tumors, this process seems to rarely develop in organs other than the spleen. The liver is an important hematopoietic organ during the fetal stage, but liver hematopoiesis in solid cancers has only been reported in patients undergoing liver transplantation (109, 110). Even in the context of hepatoma, there is no detectable HSPC accumulation in the non-cancerous livers of mice bearing orthotopic hepatic tumors or in the tumor stroma of patients with hepatocellular carcinoma (37). Similarly, a recent study revealed that the lung is a reservoir for HSPCs and an important site of platelet biogenesis in adults (111). However, reports on lung hematopoiesis in cancers are still rare (112).

MECHANISMS REGULATING SPLENIC MYELOPOIESIS

Splenic EMH is a highly flexible and adaptable response that differs in scale and output in homeostasis, under physiological stress conditions, and in various disease states. How splenic HSPC activity and the EMH niche are shaped to adapt to the organismal environment is incompletely understood, but it may involve at least two essential mechanisms: the selective recruitment of HSPCs and dynamic HSPC-niche interactions (**Figure 1**). Below, we discuss the potential mechanisms by which splenic EMH is induced and regulated in the context of cancer.

Stromal and Endothelial Cells

The structure and fundamental functions of the spleen have been thoroughly described in recent reviews (73–75). The spleen is organized in regions called the red pulp and white pulp. During EMH, HSCs are found mainly around sinusoids in the red pulp. Stem cell factor (SCF, also known as kit ligand) and SDF-1 are key factors in the BM niche of HSCs (42, 51–53). Based on the similarities between splenic EMH and normal BM hematopoiesis under physiological stress conditions such as myeloablation, blood loss, and pregnancy, the

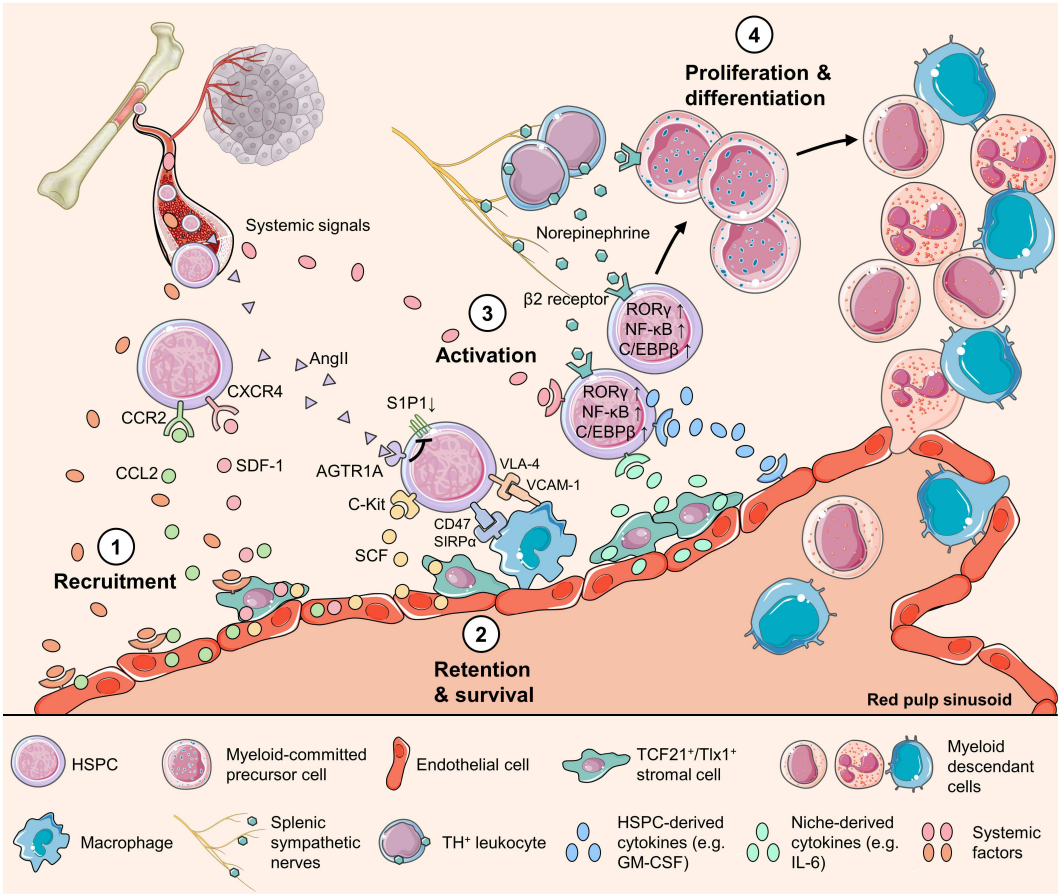

FIGURE 1 | Mechanisms regulating HSPC activity in the spleen. Schematic representation of the HSPC behavior during splenic myelopoiesis, showing multiple cell types and factors of various origins that directly or indirectly regulate HSPC activity. The splenic HSPC response is initiated with (1) increased production of chemokines, such as SDF-1 and CCL2, by endothelial cells and stromal cells around sinusoids. This change of chemokine production might be triggered by systemic factors that convey organismal stress messages. (2) HSPC survival is supported with the key niche-derived cytokine SCF and HSPCs express CD47 to avoid being engulfed by splenic macrophages. In addition, HSPCs express VLA-4 and downregulate S1P1 to maintain in the splenic niche. (3) Activated by systemic, niche-derived, and neural signals, splenic HSPCs upregulate transcription factors including RORC1/RORγ and C/EBPβ to direct myeloid-biased differentiation. Emerging evidence highlights the roles of the HSPC endogenous cytokines such as GM-CSF, and the transcription factor NF-κB that drives the production of cytokines in HSPC, as key regulators of HSPC behavior. (4) HSPCs proliferate and differentiate into different myeloid cell populations to respond to the body's or, unfortunately, the tumor's call. AGTE1A, type1A angiotensin II receptor; AngII, angiotensin II; C/EBPβ, CCAAT/enhancer-binding protein β; CCL2, C-C motif chemokine ligand 2; CCR2, C-C motif chemokine receptor 5; CXCR4, C-X-C motif chemokine receptor 4; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RORγ, related orphan receptor γ; S1P1, sphingosine-1-phosphate receptor 1; SCF, stem cell factor; SDF-1, stromal-cell-derived factor-1; SIRPα, signal regulatory protein α; Tlx1, T-cell leukemia homeobox protein 1; VCAM-1, vascular cell adhesion molecule-1; VLA4, very late antigen-4.

splenic EMH niche components are thought to be analogous to those in the BM. Indeed, murine splenic stromal cells ($PDGFR\beta^+TCF21^+$ and $Tlx1^+$) and endothelial cells have been found to be the major source of SCF, whereas a fraction of the non-endothelial SCF-expressing stromal cells are the source of SDF-1. EMH induction significantly expands the SCF-expressing endothelial and stromal cell populations to which most splenic HSPCs are found to be adjacent (113, 114). However, it should be noted that the structure of the human spleen is different from that in mice in many aspects (74, 115), and this may also be true regarding the EMH niche components. For example, SDF-1 expression has been detected in humans (116) but not mouse (113) splenic endothelial cells. A detailed depiction of the EMH niche in the human spleen is still lacking.

Although the splenic EMH niche is poorly understood, growing evidence indicates that tumor-induced splenic EMH may not entirely mimic BM EMH. In hepatoma-bearing mice, SDF-1 expression in the spleen is markedly decreased, rather than increased, at both the RNA and protein levels. In contrast, the CCR2 ligand CCL2, mainly expressed by $VE\text{-}cadherin^+$ stromal/endothelial cells, has been found to profoundly increase as tumor grows (37). CCR2 is expressed on a subset of the highly active HSPC population in the circulation. Peripheral $CCR2^+$ HSPCs are armed with pattern recognition receptors (PRRs) such as TLR4 and TLR2 and

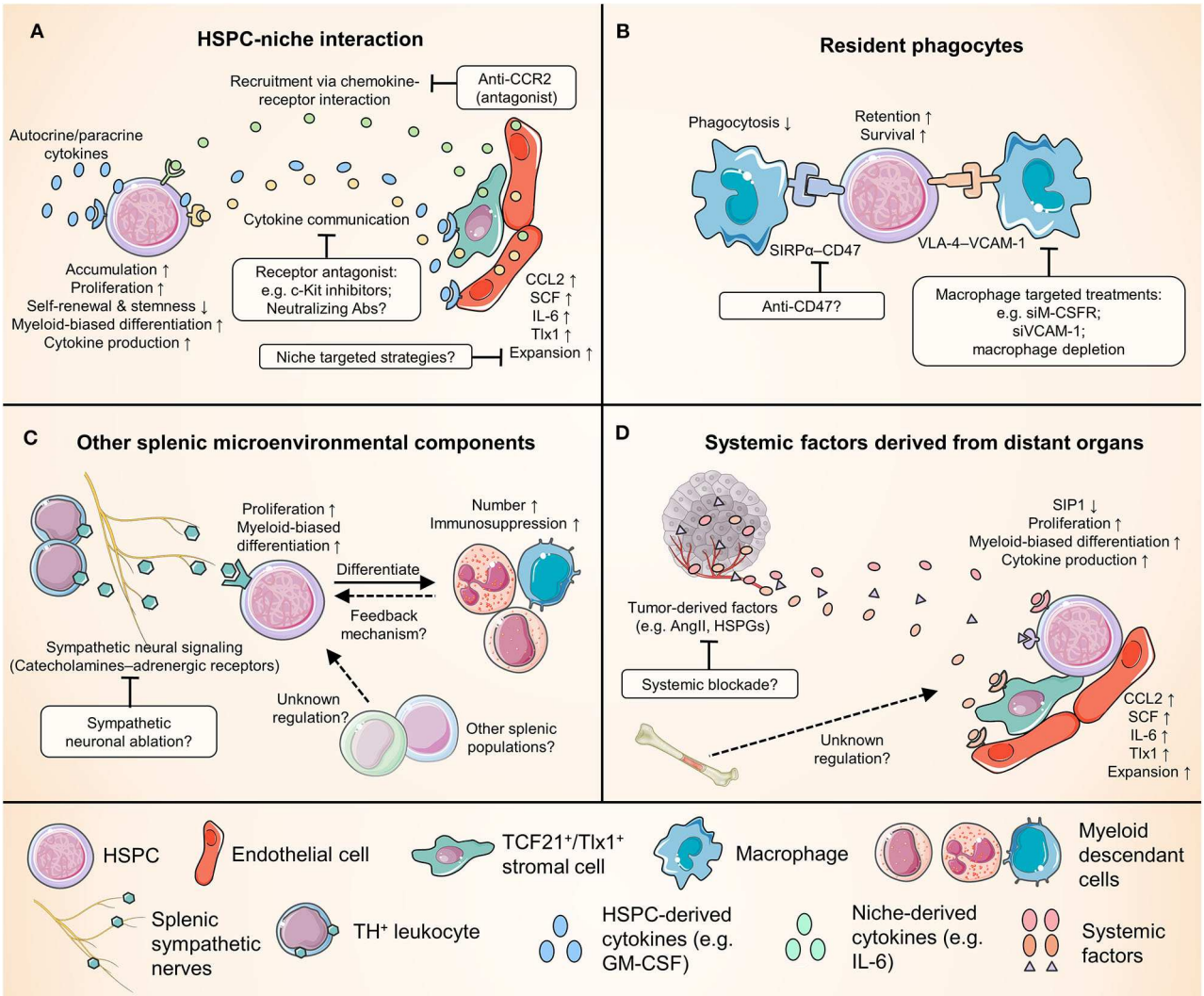

FIGURE 2 | Crucial relationships for splenic HSPCs in cancer and potential therapeutic targets. Numerous cell types and factors come into play in regulating the cancer-induced splenic HSPC activity, providing a wide range of potential therapeutic targets. This figure categorizes these interplays into four groups, and highlights examples of some potential therapies. **(A)** The complex reciprocal interplay between HSPCs and niche cells. **(B)** The interaction between HSPCs and splenic macrophages. Note that macrophages could play dual roles in modulating splenic EMH. **(C)** The regulation of splenic HSPC response by other splenic microenvironmental components, e.g., the sympathetic neurons and leukocytes that produce catecholamines. **(D)** The remote control of splenic myelopoiesis by tumor and possibly other distant organs such as the bone marrow. ACE, angiotensin-converting enzyme; AngII, angiotensin II; CCL2, C-C motif chemokine ligand 2; CCR2, C-C chemokine receptor 2; HSPGs, heparan sulfate proteoglycans; IL-6, interleukin-6; M-CSFR, macrophage colony-stimulating factor receptor; S1P1, sphingosine-1-phosphate receptor 1; SCF, stem cell factor; SIRPα, signal regulatory protein α; Tlx1, T-cell leukemia homeobox protein 1; VCAM-1, vascular cell adhesion molecule-1; VLA4, very late antigen-4.

preferentially differentiate into reparative myeloid cells, such as M2 macrophages, representing the most upstream point of increased local myelopoiesis after aseptic inflammation, liver injury, and myocardial infarction (117, 118). The CCL2/CCR2 axis is employed to mediate the splenic recruitment of HSPCs in tumor-bearing mice. A lack of CCR2 expression on HSPCs reduces splenic myelopoiesis, impairs the suppression activity of tumor MDSCs, allows an increase in the number of tumor-infiltrating $IFN\gamma^{+}CD3^{+}CD8^{+}$ CTLs, and enhances immunotherapy efficacy (37, 38). Thus, this selective recruitment mechanism may in part account for splenic immunosuppressive myelopoiesis in cancer.

Endogenous HSPC Signals and the HSPC-Niche Interplay

In addition to niche signaling, it is well-accepted that HSPCs themselves can secrete a long list of cytokines that modulate their own function in an autocrine or paracrine manner in response to stimuli (86). Although the contribution of these endogenous

signals to the inflammatory response in inflamed tissues remains doubtful, HSPC-derived pro-inflammatory factors may play a significant role in the hematopoietic niche. We recently identified a subset of GM-CSF-expressing HSPCs found exclusively in the spleens of mice bearing different types of solid tumors but not in the BM, control mouse spleen, or spleens of mice with EMH induced by repeated bleeding (37). GM-CSF, as shown in other studies (93, 94, 119), can direct HSPC proliferation and myeloid differentiation. More surprisingly, GM-CSF-expressing splenic HSPCs, but not BM HSPCs, can readily generate myeloid suppressors independent of the presence of tumors when transferred into tumor-free mice (37). These findings represent the tip of a far larger iceberg. It is logical to assume that under pathological conditions, a considerable proportion of HSPCs may produce a broad spectrum of cytokines in the splenic niche to direct splenic EMH. Moreover, one may infer that these cytokines would also affect the dynamic niche. If so, the HSPC-niche cell interplay would be reciprocal. Understanding how the unique combination of HSPC-derived and niche factors orchestrate HSPC activity to regulate the output in the spleen of a tumor-bearing host will certainly advance our understanding of cancer-induced splenic myelopoiesis.

Macrophages as Double-Edged Swords in Regulating Splenic EMH

Splenic red pulp macrophages also play an important role in regulating splenic EMH. On the one hand, macrophages retain HSPCs in the splenic red pulp by providing adhesion via vascular cell adhesion molecule-1 (VCAM-1) and may thus promote splenic EMH. Hindering macrophage maturation using *in vivo* RNAi silencing, depleting splenic macrophages, or silencing VCAM-1 in macrophages releases HSCs from the spleen and compromises splenic EMH (120). On the other hand, macrophages can regulate splenic EMH by phagocytosing redundant HSPCs in the spleen. According to an early study, the phagocytosis of HSPCs by the numerous active macrophages present in the cords of the red pulp results in limited EMH in human spleens (121), suggesting that phagocytosis is a key mechanism regulating splenic HSPC activity. CD47 is a "don't eat me" signal that inhibits phagocytosis by binding to its receptor signal regulatory protein α (SIRPα), which is expressed on phagocytes. HSPCs upregulate CD47 expression just before and during their migration to the periphery to avoid inappropriate phagocytosis (122). Thus, the downregulation of CD47 expression might lead to the clearance of splenic HSPCs as they age or become dysfunctional. Therefore, macrophages could play dual roles in modulating splenic EMH. However, the roles that splenic macrophages play in regulating cancer-induced splenic EMH during cancer development and the relationship between these functions are still largely unknown. Since therapies targeting macrophages (21, 32, 123) and anti-CD47 treatment (122, 124, 125) are emerging as novel anti-tumor strategies, a deeper understanding of these issues may reveal the impact of these treatments on splenic EMH.

The Nervous System and Neural Signal-Expressing Cells

Recent studies have revealed an intricate, panicle-shaped sympathetic architecture in the spleen (126). Most detectable nerves entering the spleen arise from the nerve plexus that surrounds branches of the splenic artery and are catecholaminergic (127). Such sympathetic architecture is absent in the other classic lymphoid organs, but whether and how this unique innervation of the spleen contributes to the distinct EMH remains largely unclear. A recent study showed that in liver cancer models, blocking β-adrenergic signaling could prevent the redistribution of splenic myeloid cells and inhibit tumor growth induced by restraint stress (128). In addition, immune cells such as macrophages and T cells can also produce catecholamines (129, 130). Although data from cancer models are limited, in hyperglycemic conditions, the spleens of diabetic patients and mice harbor increased numbers of tyrosine hydroxylase (TH)-expressing leukocytes that produce catecholamines, and GMPs that are actively proliferating. These two events are closely linked, as the interaction of catecholamine and β2 adrenergic receptors expressed on splenic GMPs mediates GMP proliferation and myeloid cell production. Moreover, TH^+ leukocytes are located close to splenic nerves and express high levels of neuropeptide Y receptors, suggesting that these cells are involved in neuroimmune communication (90). These mechanisms may also exist in cancer-bearing hosts. Future studies are required to identify the roles of the nervous system and neural signal-expressing cells in regulating cancer-induced myelopoiesis.

Signals From Distant Organs

Although it is almost certain that tumors can profoundly affect splenic myelopoiesis, either directly or indirectly, as the tumor influences the BM (65), the molecular mechanisms remain largely undetermined. In the scenario of cancers expressing high levels of CSFs, these cytokines may be the major cause of HSPC mobilization, splenomegaly, and vigorous splenic myelopoiesis (36, 97, 131, 132). In addition to hematopoietic cytokines, other tumor-derived factors, e.g., peptides and carbohydrates, can also impact on HSPC behaviors. Cortez-Retamozo et al. showed angiotensin II (AngII), a peptide hormone that belongs to the renin-angiotensin system, may also play a significant role in HSPC retention (98). They found that the expression of angiotensinogen, the AngII precursor, was upregulated in a mouse model of lung adenocarcinoma as well as in human lung cancer stroma. AngII could directly induce HSPC amplification in the splenic red pulp, suppressing the signaling between sphingosine-1-phosphate receptor 1 (S1P1) and sphingosine-1-phosphate and thus sequestrating HSPCs in the spleen. A 3 week treatment with the angiotensin-converting enzyme inhibitor enalapril suppressed the expansion of HSPCs in the spleen but not in the BM and reduced the amplification of monocytes in the spleen and macrophage accumulation in the lungs (98). Heparan sulfate proteoglycans (HSPGs) represent another class of potential factors that tumors may exploit to impact on host hematopoiesis. These molecules are composed of a core protein

to which chains of the glycosaminoglycan, heparan sulfate (HS), are covalently bound. HSPGs are wildly expressed and released by most types of tumor cells (133) and have known essential effect on furnishing the myelopoiesis microenvironment (134). Early studies have implicated that these structures may play an important role in regulating splenic EMH in tumor conditions (135, 136), but the exact mechanism remains to be further explored and validated. Nevertheless, these potential mechanisms exemplify how the tumor remotely expands the splenic HSPC response and regulates splenic myelopoiesis.

To date, we have limited information about the mechanism by which tumors systemically modulate the scale, functional characteristics, and output of splenic HSPC responses. Several important questions warrant investigation. For example, do the systemic factors derived from the tumor qualitatively and quantitatively affect splenic EMH and myelopoiesis to the same extent as they impact BM hematopoiesis? In addition, although splenic EMH is myeloid-biased in early stages, cancer-induced EMH also generates unique tumor-promoting cells of the erythrocytic lineage in late-stage cancers (99–101); what tumor-derived signals through which mechanism mediates this functional shift of splenic EMH? A better understanding of these issues is crucial to delineate cancer-associated myelopoiesis and myeloid cell responses and pave the way to developing novel strategies for cancer immunotherapy (**Figure 2**).

CLINICAL RELEVANCE OF SPLENIC MYELOPOIESIS IN CANCER

Splenic EMH in Humans

Although the role of splenic EMH in tumor-induced myelopoiesis and disease progression is increasingly being appreciated in animal models, it remains largely unknown whether the same is true in cancer patients. Previous studies in human subjects suggested that there is very limited hematopoiesis in the fetal spleen (81, 121) and that adult spleens from individuals without EMH (exemplified by increased circulating HSPC numbers) do not contain committed hematopoietic progenitors (137). Thus, it has been speculated that the human spleen may not function as an EMH site for altered myelopoiesis. However, this view has been challenged by a growing body of more recent data. First, a study using functional identification assays demonstrated that although the frequency of early colony-forming units (CFUs) in the spleen of healthy adults was significantly lower than that in the BM, the frequency of cobblestone area-forming cells in long-term stromal cultures and the frequency of secondary CFUs in long-term culture-initiating cells (both assays determine the long-term HSCs) were comparable in the spleen and BM (138). These results suggest that the human spleen is an important reservoir of dormant early HPCs or even HSCs at steady state. Second, the significant role splenic EMH plays in human pathology is now emerging. The expansion of splenic HSPCs has been observed in patients with osteopetrosis (137), myelofibrosis (139), and acute myocardial infarction (88), supporting the hypothesis that the spleen is the preferred site for extramedullary "emergent" hematopoiesis in a wide spectrum of pathological conditions.

We found that in cancer, in addition to the generalized myeloid bias in the circulating HSPC compartment from various patients with solid tumors, there is a positive correlation between the levels of circulating GMPs and clinical stages in patients with hepatocellular (HCC), cervical and colorectal carcinomas. Moreover, within a small group of HCC patients, Kaplan-Meier analysis revealed that the frequency of GMPs was negatively correlated with the time to progression (35). Accordingly, elevated proportions of HSPCs in the circulation were also found in newly diagnosed cancer patients with rhabdomyosarcoma and breast cancer and correlated with an increased risk for metastatic relapse (69). These data indicate that there is a correlation between heightened EMH and the progression of human cancer. Moreover, the spleen has been reported to be a site of cancer-related EMH in metastatic carcinomas of different origins, including lung, breast, prostate, and kidney (140). We and others have confirmed and extended this observation by showing the splenic accumulation of HSPCs and myeloid cells in patients with different types of solid tumor (34, 37). In a cohort of patients with gastric cancers, the accumulation of HSPCs was inversely correlated with reduced overall survival after surgery (34, 37). However, larger-scale studies are required to confirm the clinical relevance of splenic EMH in cancer and to test the utility of HSPC number and phenotype in circulation as biomarkers to predict disease progression and the therapeutic response in cancer patients.

Impact of Splenectomy on Malignancy

To date, most clinical data regarding the impact of spleen function on malignancy come from studies on splenectomized patients. These studies relate to issues in two categories: (1) whether splenectomy predisposes one to increased or reduced risk of tumorigenesis and (2) the effect of splenectomy on tumor growth, progression, and relapse. For the first issue, epidemiological studies have observed that splenectomy is followed by increased risk for a large array of solid tumors and hematological malignancies (141–143). This finding is supported by a recent population-based cohort study demonstrating that people with splenectomy have an increased risk of developing overall cancer, as well as certain site-specific cancers, especially patients with non-traumatic conditions (144). These results suggest that the normal spleen plays an immune surveillance role, protecting against tumor development.

For the second issue, the effect of splenectomy pertaining to cancer progression has also been studied, but the evidence remains inconclusive. Studies on concomitant splenectomy in patients with gastric, colon, liver, and pancreatic cancers have shown marginal, if any, effects on the disease-free and overall survival of patients (145). Among these data, it may be of particular interest to look at the results from liver cancer because the so-called "liver-spleen axis" in liver disease is now gaining increasing attention (146–149). Liver transplantation (LT) has been established as a standard treatment for patients with HCC who meet the Milan criteria. Splenectomized LT patients benefit from increased platelet counts, but they suffer

risks, including increased operation time, intraoperative blood loss, intraoperative red blood cell transfusion, and postoperative complications (150). Splenectomy improves patient prognosis but only in a subgroup of patients with an increased neutrophil-lymphocyte ratio (NLR) and increased infiltration of $CD163^+$ TAMs in the tumor stroma, both of which are indicative of enhanced myelopoiesis (151). However, whether the abolishment of splenic myelopoiesis is directly involved in the therapeutic effect of splenectomy and the mechanisms by which splenic EMH, or lack thereof, may influence cancer progression and treatment are yet to be elucidated.

TARGETING CANCER-INDUCED SPLENIC MYELOPOIESIS

One explanation for the modest effect of splenectomy on tumor progression in both patients and mice is that the spleen is a multifunctional organ. As noted before, the spleen is an important organ for blood homeostasis and is a reservoir of various immune and blood cell populations that have differential impacts on tumor progression via diverse mechanisms. The ultimate impact of splenectomy on cancer patients is determined by the net balance of these known or still unknown factors, dependent on the individual's status. Therefore, an enhanced strategy is to seek a selective treatment modality that specifically targets protumoral splenic EMH while maintaining the normal physiological and antitumoral immune functions of the spleen (**Figure 2**).

In this context, Ugel et al. evaluated a large panel of conventional chemotherapeutic agents for their ability to eliminate splenic committed myeloid precursors. Low-dose 5-fluorouracil (5-FU) treatment, for example, could reduce the splenic (but not BM) expansion of committed precursors with high proliferative potential, restore antitumor immunity, and enhance the efficacy of ACT, recapitulating the effect of splenectomy (38). We recently found that a low-dose c-Kit inhibitor inhibits proliferation, induces apoptosis, and thus reduces the total number of upstream early HSPCs in the spleen but has a much smaller effect on those in the BM. Moreover, low-dose c-Kit inhibitor treatment attenuates endogenous GM-CSF expression in splenic HSPCs, inhibits the suppressive functions of tumor PMN-MDSCs, and synergistically increases the efficacy of immune checkpoint blockade (37). Why splenic HSPCs and committed myeloid precursors are more sensitive than their BM counterparts to such treatments is presently unclear. One possibility might be due to the anatomical structure and physiological function of the spleen, which often causes drug retention. Another possibility for the differential effects could be the distinct cellular characteristics of the BM and splenic HSPCs in tumor-bearing hosts. If so, a better understanding of the biological features of splenic HSPCs and myeloid precursors may provide a molecular basis for the development of novel therapeutic strategies to selectively target splenic myelopoiesis.

In addition to the regulation of splenic HSPC proliferation and survival, the specific abrogation of cancer-induced myelopoiesis could also be achieved by targeting the recruitment and retention of splenic HSPCs. In this scenario, the CCL2/CCR2 axis is attracting particular interest and plays multiple important roles in systemic tumor-associated myeloid cell responses. This axis mediates the migration of BM monocytes into the bloodstream (152), guides monocytes to the marginal zone of the spleen (38), and directs the infiltration of monocytes in the tumor (34, 153, 154). Moreover, as noted before, CCR2 expression identifies an upstream subset of circulating HSPCs that can respond to splenic CCL2 and home to the splenic niche (38). Thus, CCR2-specific antagonists may act as multivalent inhibitors targeting multiple events of cancer-induced myeloid cell responses. Currently, a number of clinical trials have been established to investigate the safety and efficacy of CCR2 inhibitors, including CCX872-B, PF-04136309, MLN1202, and BMS-813160, for the treatment of solid tumors [reviewed in (155)]. In addition, CD47 and AngII have been revealed as critical mediators of splenic HSPC retention and expansion. Blocking these signaling pathways may inhibit tumor-promoting splenic myelopoiesis, as shown in mouse models (75, 122). Nevertheless, the translational values of these findings need to be further investigated in cancer patients to validate whether the blockade of these signals will be effective and beneficial and, importantly, whether the therapeutic effects rely on the impact on altered myelopoiesis in the spleen.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The emerging field of cancer-induced hematopoiesis, EMH in particular, complements and completes our knowledge of tumor-associated myeloid responses. The spleen, as the main EMH site in tumor-bearing hosts, generates significant amounts of myeloid cells that continuously replenish the large and rapidly turned over pool but is functionally and mechanistically different from that in the BM. The understanding of the unique splenic myelopoiesis opens a new avenue of myeloid cell-targeting strategies, which pursue the goal of restraining systemic tumor-promoting myeloid responses at their source.

From the therapeutic perspective, splenic myelopoiesis may be the "weakest link" in the chain of myeloid cell reactions because the spleen is a rather pharmacodynamically favorable organ due to its anatomical structure and the large blood flow (75). In addition, splenic HSPCs, partially due to their highly proliferative nature and residence in a less protective niche, are more vulnerable to targeted drugs than their BM counterparts and downstream myeloid descendants (37, 38). Therefore, targeting splenic myelopoiesis holds real potential to restrain tumor-promoting myeloid cell responses and to tip the balance toward tumor suppression. A better understanding of the functional specialization and regulatory mechanism of splenic myelopoiesis will provide the keys to controlling myeloid cell responses at the source.

Finally, more human data are needed to demonstrate the clinical relevance of splenic myelopoiesis in cancer patients. Studies on cancer-induced splenic myelopoiesis in humans are hampered by the limited availability of spleen samples, the poorly

defined phenotypes and functions of the highly heterogeneous circulating HSPC subsets, and the unclear nature of the splenic niche constitution. *In situ* studies using novel multiplex staining and detection methods, lineage-tracing and imaging techniques, and informative tools and statistical modeling would be invaluable for identifying disease-specific splenic myelopoiesis patterns. Single-cell analyses, such as cytometry by time of flight (CyTOF) and single-cell RNA sequencing, can help to reveal the heterogeneity of splenic HSPC populations in different conditions. Dynamic modeling using *in vitro* experiments will be crucial to identify key regulatory pathways and search for checkpoints that are susceptible to therapy. These advanced methodologies and experimental models will not only facilitate human studies but also facilitate the translation of clinical insights back to improvements in mouse models, which may produce applicable and precise therapeutics. Such parallel studies may provide a long sought-after means to reshape the tumor immune micro- and macroenvironment by rerouting myeloid cell responses.

AUTHOR CONTRIBUTIONS

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

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Glioblastoma Myeloid-Derived Suppressor Cell Subsets Express Differential Macrophage Migration Inhibitory Factor Receptor Profiles That Can Be Targeted to Reduce Immune Suppression

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The application of tumor immunotherapy to glioblastoma (GBM) is limited by an unprecedented degree of immune suppression due to factors that include high numbers of immune suppressive myeloid cells, the blood brain barrier, and T cell sequestration to the bone marrow. We previously identified an increase in immune suppressive myeloid-derived suppressor cells (MDSCs) in GBM patients, which correlated with poor prognosis and was dependent on macrophage migration inhibitory factor (MIF). Here we examine the MIF signaling axis in detail in murine MDSC models, GBM-educated MDSCs and human GBM. We found that the monocytic subset of MDSCs (M-MDSCs) expressed high levels of the MIF cognate receptor CD74 and was localized in the tumor microenvironment. In contrast, granulocytic MDSCs (G-MDSCs) expressed high levels of the MIF non-cognate receptor CXCR2 and showed minimal accumulation in the tumor microenvironment. Furthermore, targeting M-MDSCs with Ibudilast, a brain penetrant MIF-CD74 interaction inhibitor, reduced MDSC function and enhanced CD8 T cell activity in the tumor microenvironment. These findings demonstrate the MDSC subsets differentially express MIF receptors and may be leveraged for specific MDSC targeting.

Keywords: MDSC, glioma, MIF–macrophage migration inhibitory factor, immunetherapy, immunesuppresion

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INTRODUCTION

Glioblastoma (GBM) is the most prevalent primary malignant brain tumor and remains uniformly fatal despite aggressive therapies including surgery, radiation, and chemotherapy (1, 2). With limited treatment options, the success of immunotherapies in other advanced cancers, including melanoma and non-small cell lung cancer, has inspired investigation of immune based therapies in GBM (3–6). However, early clinical trials of immune checkpoint therapies in GBM have demonstrated limited response, if any, and despite some evidence of immune cell accumulation, GBM growth persists (7, 8). One explanation for these failures could be the potent immunosuppressive factors present in GBM, including the high tumor content of myeloid-derived suppressor cell (MDSC) (9–12). MDSCs are a heterogeneous population of bone marrow-derived cells consisting of monocytic (M-MDSC) and granulocytic (G-MDSC) subsets that accumulate in the tumor, spleen, and peripheral blood of GBM patients, where they exert immune suppression by dampening the function of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) (13–18).

Recent work from our laboratory and others identified an increase in circulating M-MDSCs in the peripheral blood of GBM patients compared to benign and grade I/II glioma patients (9, 19). However, this difference was not observed for other immunosuppressive cell populations, such as macrophages or T-regulatory cells, which were not different between patients of different glioma grades. In addition, MDSCs in the peripheral circulation and infiltrating in the GBM microenvironment correlated with poor prognosis (9, 19). Based on these observations in GBM and other cancers, attempts to target MDSCs using multiple approaches, including low-dose chemotherapy in a recent GBM trial are in clinical evaluation (20). Notably, these approaches use non-specific strategies that attenuate MDSCs, as opposed to targeted approaches that are MDSC-specific and may have a higher therapeutic utility.

In seeking to develop MDSC targeted therapies to reduce immune suppression, we focused our attention on macrophage migration inhibitory factor (MIF). MIF is highly conserved in mammals, exhibiting approximately 90% homology across species, and interestingly can also be found in parasites, plants and cyanobacteria, possibly indicating its importance in basic biological functions (21). MIF has also been shown to be produced by many immune cells including T cells, monocytes, macrophages, and neutrophils and has been shown to be expressed in multiple cancers including GBM, lung cancer, and breast cancer (21–23). In its secreted form, MIF is a homotrimer and contains an enzymatic pocket at the interface of two monomers (22). While the enzymatic pocket has been clearly identified, there is no known natural substrate. There is tautomerase activity with the substrate p-hydroxyphenylpyruvic acid, although the Km/kcat is not in a physiologic range (24, 25). MIF has been associated with multiple inflammatory pathogeneses including sepsis, asthma, arthritis, inflammatory bowel disease, malaria, and atherosclerosis (26, 27). Perhaps one of the best examples of how MIF can alter the immune response is that of sepsis, where inhibition of MIF has been demonstrated to inhibit the inflammatory cascade induced by LPS that would typically result in death (27). These early studies of MIF also demonstrated that it is crucial for macrophage response to pathogens, ultimately resulting in its name, macrophage migration inhibitory factor (28, 29). In relation to GBM patients, it is important to note that glucocorticoids, such as those used to treat edema, induce MIF expression and that MIF is highly expressed by GBM cells (30). Furthermore, MIF expression is increased with glioma grade, and high levels of MIF in The Cancer Genome Atlas (TCGA) datasets correlate with a poor prognosis.

Targeting MIF is of interest due to our previous work where we observed that MIF derived from GBM cells, specifically therapeutically resistant cancer stem cells (CSCs), was necessary for MDSC survival and function (31). Moreover, reducing MIF levels in GBM cells did not alter their proliferation, but when transplanted into an immune competent orthotopic model, resulted in increased host survival and an increase in the number of CD8 T cells in the tumor microenvironment. MIF has also been shown by other groups to enhance the immune suppressive capacity of myeloid cells (32, 33); for instance, MIF downregulation was demonstrated to aid in the resistance of anti-VEGF therapies (34). In seeking to understand exactly how MIF effects the immune response in GBM one must consider that it has been shown to be highly context specific, exerting both inflammatory and anti-inflammatory effects depending on the disease and tissue (21, 31, 33, 35–37). MIF signals through a variety of receptors, including via its cognate receptor CD74, and by non-cognate interactions with CXCR2, CXCR4, CXCR7. CD74 is the cell surface form of the Class II invariant chain, but is expressed independently of Class II to mediate MIF signal transduction (38–40). MIF binding to CD74 leads to the recruitment of CD44 as a signaling co-receptor, leading to downstream Src/MAPK signaling. By contrast, MIF signaling through CXCR2 primarily through PI3K/Akt-dependent signaling with Ca transients (41). The pharmacologic targeting of MIF has also been of great interest in a variety of inflammatory conditions including multiple sclerosis, systemic lupus erythrematosus, rheumatoid arthritis, inflammatory bowel disease, and other inflammatory disorders (32, 42–49). Additionally, clinically approved MIF inhibitors have been developed that could potentially be repurposed for GBM (42). To gain a more mechanistic understanding into the MIF signaling axis in MDSCs for potential targeting in GBM, we examined the expression and function of MIF receptors in MDSCs derived from mouse and human GBM models.

METHODS

Co-culture Assay

Co-culture induction of MDSCs was adapted from previously described work in melanoma (33). At day zero bone marrow (BM) was freshly isolated from the tibias and femurs of male 000664-C57BL/6J. To obtain BM derived MDSCs, the freshly isolated BM was incubated for 3 days in a medium consisting of 50% conditioned medium from a 24 h GL261 (glioma) cell line

culture and fresh RPMI medium with 10% FBS. Additionally, this medium was supplemented with GM-CSF (40 ng/mL, Biolegend Catalog # 575906), and IL-13 (80 ng/mL, Biolegend Catalog # 576306), which have been shown to increase MDSC expansion and activity. BM was cultured in this medium in 6 well plates at a density of 2,000,000 cells per well as previously described and utilized for analysis on day 3 post initiation (33).

Flow Cytometry of Co-culture

At day 3 of the co-culture cells were extracted from the wells using gentle washing with RPMI medium, blocked in FcReceptor block (Miltenyi Biotec 130-092-575) and then stained live on ice. Samples were then fixed using eBioscience fixation buffer before analysis. Gating for MDSCs was performed using FlowJO V10, and M-MDSCs were identified by (Singlets/Live/CD45+/CD11b+/CD68-/IAIE-/Ly6G-/LyC+) and G-MDSCs by (Singlets/Live/CD45+/CD11b+/CD68-/IAIE-/Ly6C-/Ly6G+). Antibodies were obtained from Biolegend (San Diego, CA) for analysis of mouse immune profile Fluorophore-conjugated anti-Ly6C (Clone HK1.4, Catalog # 128024), anti-Ly6G (Clone A8, Catalog # 127618), anti-CD11b (Clone M1/70, Catalog # 101212), anti-CD68 (Clone FA-11, Catalog # 137024), anti-I-A/I-E (Clone M5/114.15.2, Catalog # 107606), anti-CD11c (Clone N418, Catalog # 117330), anti-Ki-67 (Clone 16A8, Catalog # 652404), anti-CD45 (Clone 30-F11, Catalog # 103132), anti-CD74 (Clone IN-1 Catalog # 740385), anti-P2Ry12 (Clone S16007D, Catalog # 848004), anti-CXCR2 (Clone SA044G4, Catalog # 149313), anti-CXCR4 (Clone L276F12, Catalog # 146506), anti-CXCR7 (Clone 8F11-M16, Catalog # 331115), anti-CD44 (Clone IM7, Catalog # 103039). Antibody compensation was performed using AbC Total Antibody Compensation Bead Kit (Catalog # A10497).

Flow Cytometry Patient Tumor Samples

Flow cytometry data was utilized from Peereboom et al. (20). Tumor tissue was received from recurrent GBM patients undergoing treatment in clinical trial NCT02669173. Tissue was digested in collagenase IV (STEMCELL Technologies) for 1 h at 37 degrees Celsius and then mechanical dissociated via 40-uM filter. Dissociated tumors were then washed in RPMI medium before being viably frozen for flow cytometry analysis. MDSC panel consisted of CD11b (Catalog # CD11b29), HLA-DR (Catalog # 559866), CD14 (Catalog # 560180), CD15 (Catalog # 555400), CD33 (Catalog # 555450), CXCR2 (Catalog # 551126), CD74 (Catalog # 555538 with Lightning-Link PE-Cy7 Catalog # 762-9902). Staining and analysis were performed using standard protocols previously described, with MDSCs marked by CD11b+, CD33+, and HLA-DR–/lo and then further subdivided into granulocytic MDSCs (CD15+) and monocytic MDSCs (CD14+) (9, 20, 50). After gating for MDSC populations the MFI of CXCR2 and CD74 was analyzed using FlowJo V10 for each sample.

T Cell Suppression Assay

At day 3 post MDSC co-culture, T cell suppression was assessed. Splenocytes were freshly isolated from male 000664-C57BL/6J mice using sterile techniques. Post isolation the red blood cells were lysed using RBC lysis buffer (Biolegend Catalog # 420301) before being magnetically sorted using the (Pan T cell isolation kit Catalog # 130-095-130, Miltenyi Biotec). Isolated T cells were then stained using CFSE Cell Division Tracker Kit (Biolegend Catalog # 23801). CFSE stained T cells were then collected and distributed into round bottom 96 well plates at 100,000 cells per well in IL-2(30 IU) as unstimulated control. Stimulated controls additionally contained CD3/CD28 mAb-coated beads (ThermoFisher Scientific) at a ratio of 3:1. T-cell activation was measured by flow cytometry with the controls consisting of CFSE labeled T cells alone and CFSE labeled T cells with beads. Co-culture derived MDSCs, isolated by magnetic sorting (MACS Miltenyi MDSC isolation kit Catalog # 130-094-538), were seeded with T cells at a concentration of 1:2 (1 MDSC for every 2 T cells).

Quantitative PCR

Quantitative PCR was performed for MDSC markers and immune suppressive genes

Arg1 (Forward: AAGAATGGAAGAGTCAGTGTGG, Reverse: GGGAGTGTTGATGTCAGTGTG) ,

iNOS (Forward: TGTGCTTTGATGGAGATGAGG, Reverse: CAAAGTTGTCTCTGAGGTCTGG),

Ly6G (Forward:TTGTATTGGGGTCCCACCTG, Reverse: CCAGAGCAACGCAAAATCCA),

CXCR2 (Forward: TCTTCCAGTTCAACCAGCC, Reverse: ATCCACCTTGAATTCTCCCATC),

CD74 (Forward: ATGGCGTGAACTGGAAGATC, Reverse: CAGGGATGTGGCTGACTTC),

MCP-1 (Forward: GTCCCTGTCATGCTTCTGG, Reverse: GCTCTCCAGCCTACTCATTG).

RNA was isolated using Qiagen RNeasy Mini Kit and cDNA was generated using aScript cDNA SuperMix (Quantabio). After cDNA generation qPCR was performed using the Fast SYBR™ Green Master Mix (ThermoFisher Scientific).

GBM-Seq Database Mining

Darmanis et al. data was utilized in this analysis where normalized count data was acquired from http://www.gbmseq.org/ (51). Subsequently, CD74 and other MIF receptor expression levels were graphed for the myeloid populations and other immune populations as characterized by Darmanis et al. in their supplemental data. All populations' names were kept the same as previously published and identified.

MIF Inhibitor Screen

The co-culture system was utilized to screen inhibitors of MIF and MIF/CD74 interaction by dosing inhibitors at day zero when the co-culture was initiated and then reading out % MDSCS of live cells by flow cytometry. The same gating strategy as in the co-culture methods section was used to determine if the MDSC population was shifting. Screens were performed in biological replicates of 3 on two separate experiments for a total of 6 biological replicates. The studied MIF inhibitors were anti-MIF mAb (IIID.9), 4-IPP (Tocris Catalog # 3429) (52), Ibudilast (gift of Medicinova) (53–55), ISO-1 (Tocris Catalog # 4288) (52), MIF098 (56–58), AV1013(gift of Medicinova) (55), and the PDE4 inhibitor was Rolipram (Tocris Catalog # 0905).

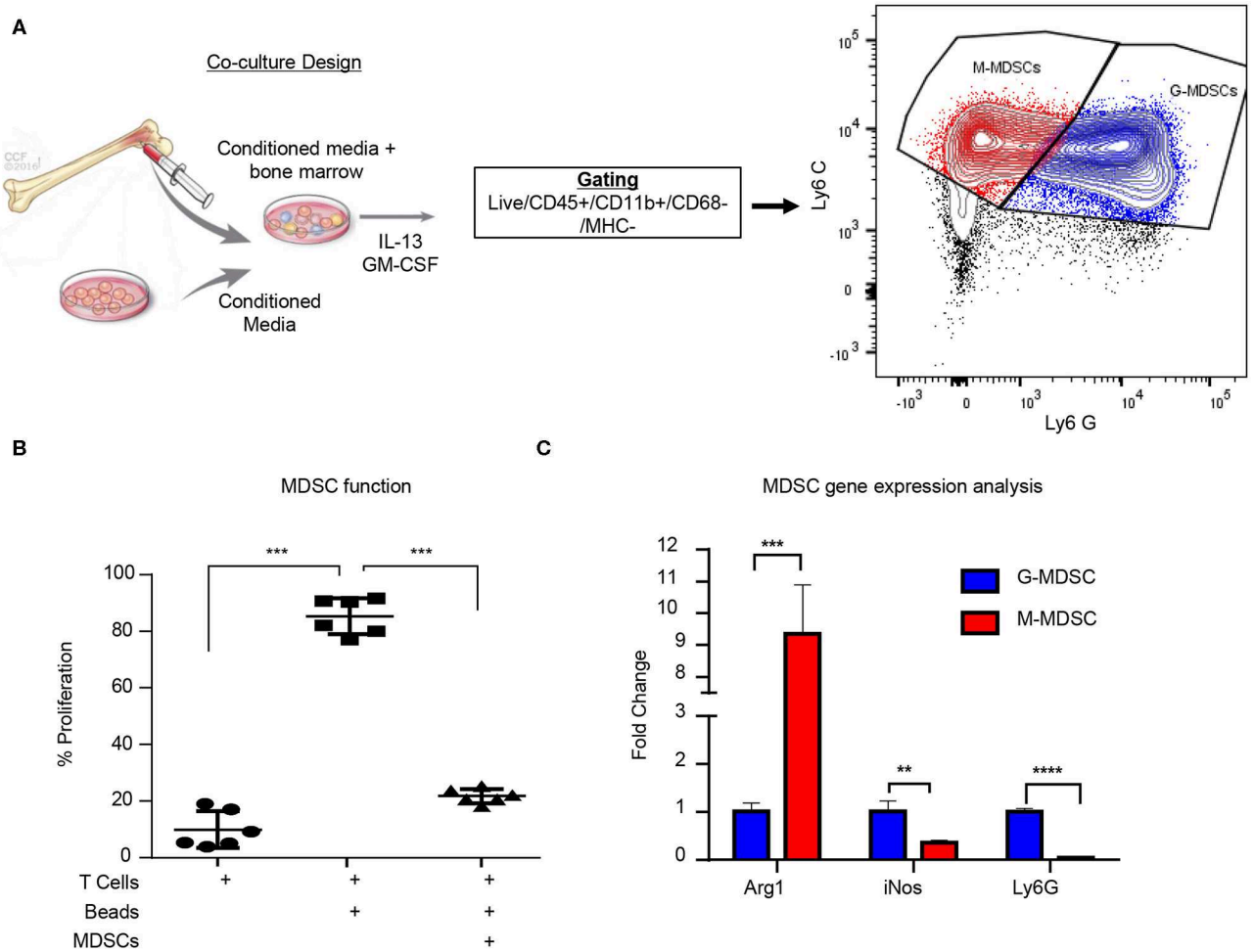


FIGURE 1 | Glioma educated MDSCS can be generated *in vitro.* MDSCs are induced using freshly isolated bone marrow cultured with 50:50 mix of fresh media and conditioned media from a 24-h culture of GL261 cells with the addition of IL-13 and GM-CSF over 3 days **(A)**. M-MDSCs were gated by Live/CD45$^+$/*CD11b*$^+$/*CD68*$^-$/*MHC*$^-$/*Ly6C*$^+$/*Ly6G*$^-$ while G-MDSCs were gated by Live/CD45$^+$/*CD11b*$^+$/*CD68*$^-$/*MHC*$^-$/*Ly6C*$^+$/*Ly6G*$^+$. Co-cultured MDSCs from $n = 6$ mice were generated over 3 days and then isolated by magnetic bead sorting and subsequently used for T cell suppression assay where the controls were T cells alone unstimulated without CD3/CD28 activation beads and T cells with CD3/CD28 activation beads **(B)**. FACs sorting of M-MDSCs and G-MDSCs from 3 day old co-cultures of $n = 3$ mice was used to isolate RNA and perform qPCR for Arginase (Arg1), Nitric oxide synthase (iNOS), and Ly6G **(C)**. Two-Tailed *T*-Test was performed for comparisons in **(B,C)** *<0.05, **<0.01, ***<0.001.

In vivo Syngeneic Glioma Model

Ibudilast treatment was assessed in two cohorts using the syngeneic mouse model of glioma GL261 acquired from the NCI. Six-week-old aged-matched male 000664-C57BL/6J mice were anesthetized using isoflurane and then intracranially injected in the left cerebral hemisphere with 20,000 GL261 cells in 5 μl of RPMI medium using a stereotactic frame. This model has been established in the laboratory with neurological symptoms as an indicating endpoint and a median survival time of ∼20 days (31). Ibudilast treatment was via intraperitoneal injection of 50 mg/kg 2x weekly starting day 5 post tumor implantation. Ibudilast was suspended in a mixture of 50 μl PEG400 and 50 μl PBS for 100 μl injections as previously reported (54). Flow cytometry was performed on mechanically dissociated tumors isolated from the left hemisphere from sacrificed animals at day 18 post implantation, and a terminal cardiac bleed was analyzed for MDSC and T cell levels using the myeloid panel: live/deadUV, CD45, CD11b, CD11C, IA/E, CD74, Ly6G, Ly6C, CD68, and the lymphoid panel: live/deadUV, CD45, CD3, CD4, CD8, LPD1, NK1.1, CD107a. Antibodies were obtained from Biolegend (San Diego, CA) for analysis of mouse immune profile Fluorophore-conjugated anti-Ly6C (Clone HK1.4, Catalog # 128024), anti-Ly6G (Clone A8, Catalog # 127618), anti-CD11b (Clone M1/70, Catalog # 101212), anti-CD68 (Clone FA-11, Catalog # 137024), anti-I-A/I-E (Clone M5/114.15.2, Catalog # 107606), anti-CD11c (Clone N418, Catalog # 117330), anti-CD3 (Clone 145-2C11, Catalog # 100330), anti-CD4 (Clone GK1.5, Catalog # 100422), anti-CD8 (Clone 53-6.7, Catalog # 100712), anti-NK1.1 (Clone PK136, Catalog # 108741), anti-CD45 (Clone 30-F11, Catalog # 103132). An initial study included 10 vehicles

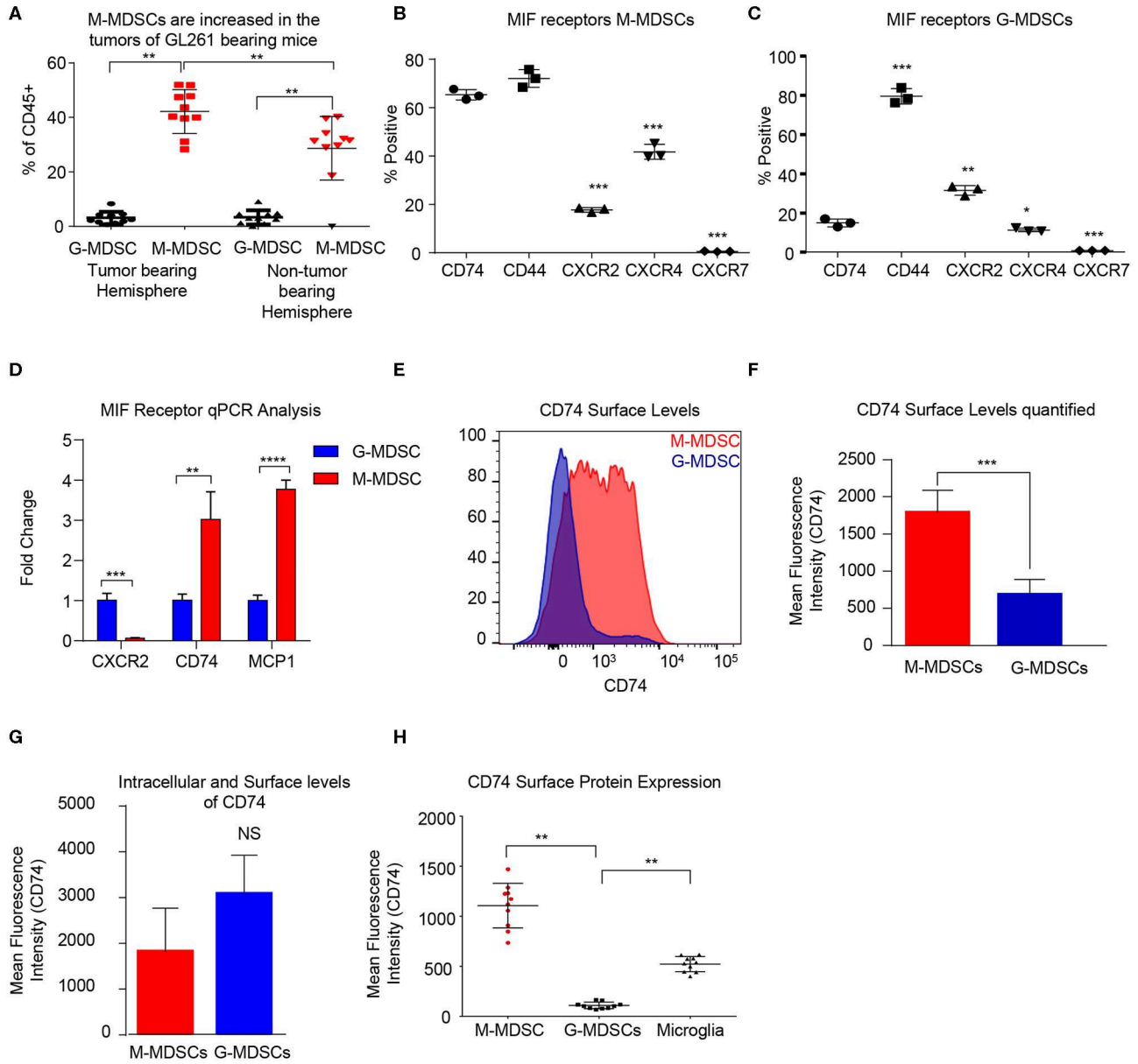
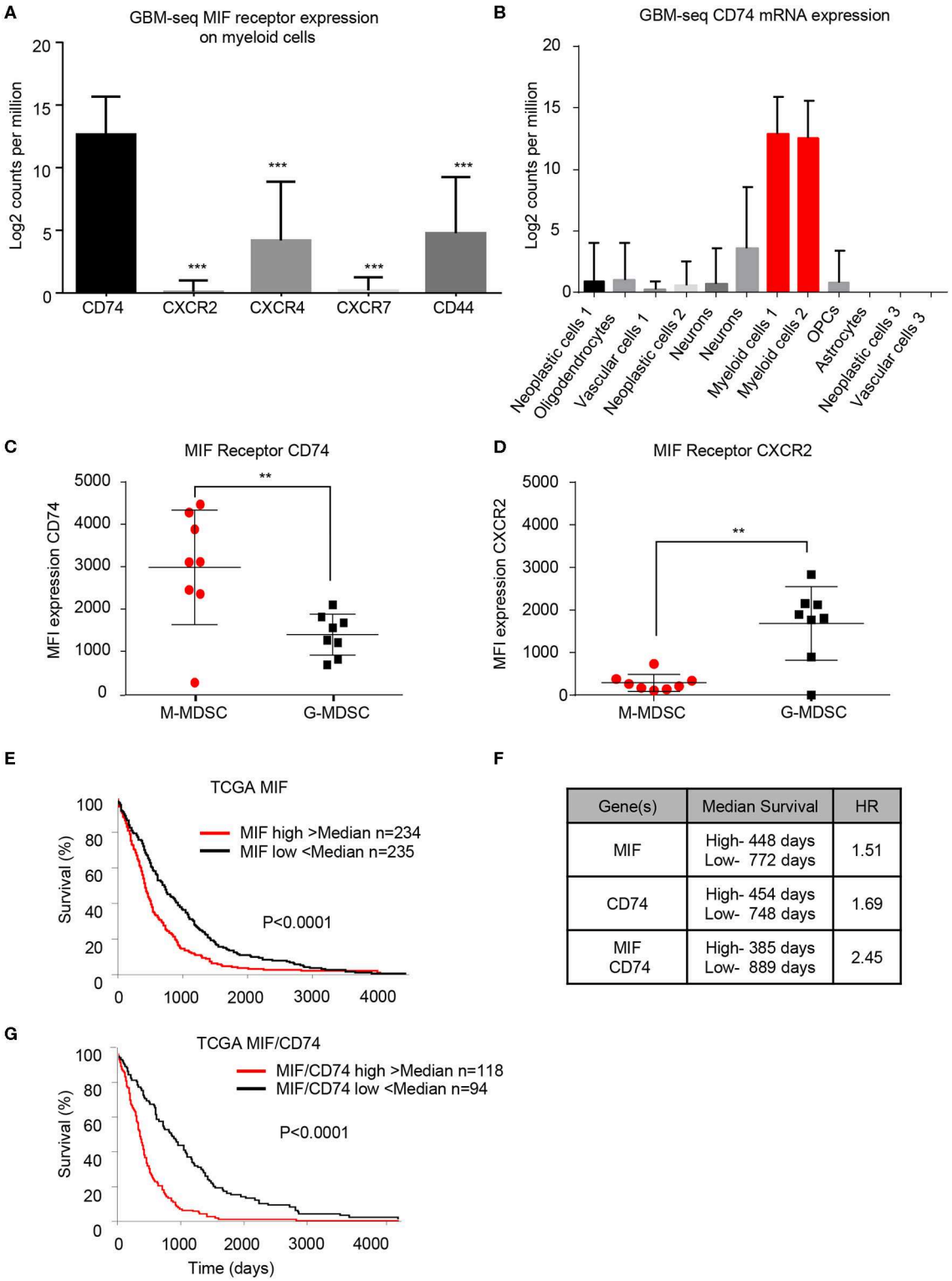


FIGURE 2 | Murine M-MDSCs express the MIF receptor CD74. $n = 10$ mice were intracranially injected with the syngeneic mouse glioma cell line GL261 at day 0 and then at Day 18 post injection the tumor bearing and non-tumor bearing hemispheres were resected, dissociated, and analyzed by flow cytometry **(A)**. M-MDSCs Live/CD45$^+$/*CD*11*b*$^+$/*CD*68$^-$/*P2Ry*12 − /*MHC*$^-$/*Ly*6*C*$^+$/*Ly*6*G*$^-$, and G-MDSCs Live/CD45$^+$/*CD*11*b*$^+$/*CD*68$^-$/*P2Ry*12 − /*MHC*$^-$/*Ly*6*C*$^+$/*Ly*6*G*$^+$. $n = 3$ mice were used for co-culture induction of MDSCs and at day 3 M-MDSCs and G-MDSCs were analyzed for the MIF receptors CD74, CD44, CXCR2 CXCR4, and CXCR7 by flow cytometry and gated for the % positive in each group **(B, C)**. FACs sorting of G-MDSCs and M-MDSCs from co-cultures of $n = 3$ mice were performed and then RNA isolated for qPCR analysis of the expression of MIF receptors (CXCR2, and CD74) as well as MCP-1, the CD74 downstream activation product **(D)**. CD74 expression was assessed by flow cytometry using flow cytometry staining of co-cultures where the histogram demonstrates the expression level of CD74 on M-MDSCs compared to G-MDSCs **(E)**. Quantification of $n = 3$ co-culture derived M-MDSCs and G-MDSCs CD74 Mean Fluorescence intensity shows higher levels of CD74 on M-MDSCs **(F)**. Intracellular staining post permiablization of the same cohort of M-MDSCs and G-MDSs from **(F)** shows that CD74 levels were not significantly different when staining internally **(G)**. *In vivo*, the tumor bearing mice that were evaluated for MDSC levels in **(A)** were also evaluated for CD74 expression on the surface of M-MDSCS, G-MDSCS and Microglia **(H)**. Two-Tailed *T*-Test was performed for comparisons in **(A, D, F, G, H)**. *<0.05, **<0.01, ***<0.001.

and 10 Ibudilast treated animals, but at day 18, the 2 vehicle treated animals demonstrated neurological symptoms and were euthanized prior to analysis time-point. Additionally, tumor could not be identified visually at day 18 in 3 ibudilast treated mice and 2 vehicle treated mice, so their matched non-tumor bearing tissue was not included in analysis.



Gene(s)	Median Survival	HR
MIF	High- 448 days Low- 772 days	1.51
CD74	High- 454 days Low- 748 days	1.69
MIF CD74	High- 385 days Low- 889 days	2.45

FIGURE 3 | Human derived M-MDSCs express the MIF receptor CD74. Data mining of the GBM-seq database from Darmanis et al. (51), was used to analyze the myeloid cell expression of the MIF receptors CD74, CXCR2, CXCR4, CXCR7 and CD44 showing that CD74 expressed by the myeloid populations in GBM tumor

(Continued)

FIGURE 3 | single cell sequencing **(A)**. Further analysis was performed separating the single cell populations into the previously published cell identities **(B)**. Using a previously published cohort of GBM patient tumors (20) $n = 8$ GBM patients the MIF receptors CD74 and CXCR2 were assessed on M-MDSCs and G-MDSCs (M-MDSCs: $CD11b^+/HLA-DR^-/CD33^+/CD14^+/CD15^-$, G-MDSCs: $CD11b^+/HLA-DR^-/CD33^+/CD14^-/CD15^+$) **(C,D)**. TCGA data analysis of GBMLGG cohort identified MIF expression and CD74 expression levels correlating with survival with a similar hazard ratio (HR) **(E,F)**. When a signature for both MIF and CD74 is created where samples that were above the median for both MIF and CD74 expression compared to those below the median for both MIF and CD74 further separates survival **(F,G)**. Two-Tailed *T*-Test was performed for comparisons in **(A,C,D)** *<0.05, **<0.01, ***<0.001. Survival curve analysis was performed in GraphPad Prism using Log-rank (Mantel-Cox) test for *p*-value and hazard ratio log rank was computed on the same data using GraphPad Prism.

Nanostring Analysis

RNA was isolated using RNeasy mini kit (Qiagen) and then the nCounter® Mouse Myeloid Innate Immunity Panel v2 was used to analyze the RNA expression of tumors isolated from 6 endpoint vehicle tumors and 6 endpoint Ibudilast treated animals.

Immunohistochemically Analysis

At endpoint, vehicle and ibudilast treated animals were perfused with 4%PFA before removing the brain and fixing in PFA overnight at 4°C. Post Fixed brains were cryopreserved in sucrose and embedded in O.C.T compound (Fisher Healthcare) to make frozen sections (10 μm thick). Endogenous peroxide activity was quenched by 3% H2O2 incubation and blocked in 5% normal goat serum/0.2%Triton in PBS for 30 min before primary antibodies were added. Phospho-Histone3 (1:500, catalog # 06-570, MillopreSigma) and Ki67 (1:1,000, catalog # ab15580, Abcam) antibodies were allowed to bind overnight at 4°C. After rinsing with 1xPBS, biotinylated secondary antibodies (1:500, Invitrogen) were added and incubated at RT for 1 h. Signal was amplified using avidin-biotin complex staining (30 min) before DAB substrate was used to visualize the signal (Vector Laboratories). Hematoxylin was used for counterstain. After washing in PBS, the slides were dehydrated through alcohol series and mounted with Permount (Fisher Chemical).

MCP-1 ELISA

R&D systems Mouse CCL2/MCP-1 DuoSet ELISA catalog# DY479 was used to analyze MCP-1 *in vitro* from conditioned media isolated at day 4 post treatment at varying doses 0–10 μM and *in vivo* from serum of $n = 3$ vehicle and $n = 3$ Ibudilast treated mice at day 18 post tumor implantation following the timeline for Ibudilast treatment described in the *in vivo* syngeneic glioma model section.

Statistical Analysis

Graph-Pad Prism was utilized for statistical analysis of survival curves for log-rank tests and also for *T*-tests throughout the manuscript. * <0.05, ** <0.01, *** <0.001. Nanostring statistics were performed within nSolver software supplied by Nanostring and the advanced analyzer V 4.0.

RESULTS

Development of MDSC Co-culture to Study the MIF Signaling Axis

While MDSCs have been linked to GBM prognosis and progression, technical hurdles including the inability for their long-term expansion have been a challenge for mechanistic insight and functional assessment (9, 19, 59). Our group previously identified that MIF is secreted by GBM CSCs and driving MDSCs, however the mechanism by which MIF increased MDSC function remains unclear (31, 32). Initially we sought to determine if the survival extension we previously observed with MIF knockdown GBM cells was solely due to an immunologic event. We performed the same studies in immune compromised NSG mice and found that there was no survival benefit when the adaptive immune response was absent (**Supplemental Figures 1A,B**). Furthermore, when MIF was depleted using an established neutralizing anti-MIF antibody 5-days post tumor implantation there was no survival benefit (**Supplemental Figure 1C**). These findings confirm our previous observations that MIF likely acts on the immune system, as opposed to acting on GBM cells in an autocrine manner. To further understand how GBM-derived MDSCs function, we adapted a co-culture system previously developed in a melanoma model (**Figure 1A**) (33). The co-culture utilizes freshly-isolated bone marrow combined culture in 50% conditioned media from a 24-hour culture of the mouse glioma cell line GL261 and supplemented with GM-CSF and IL-13 to generate M-MDSCs and G-MDSCs over a 3-day period. Day 3 was chosen for MDSC generation assays based on a flow cytometry longitudinal study of the culture showing a steep decline in viable CD45+ cells after day 4 (**Supplemental Figure 1D**). At day 3 of co-culture, the numbers of M- and G-MDSCs were determined by flow cytometry analysis where M-MDSCs were gated by Singlets/Live/$CD45^+$/$CD11b^+$/$CD68^-$/$IAIE^-$/$Ly6G^-$/$Ly6C^+$ and G-MDSCs by Singlets/Live/$CD45^+$/$CD11b^+$/CD68-/$IAIE^-$/$Ly6C^-$/$Ly6G^+$. Furthermore, co-culture generated MDSC function was determined by T cell suppression assay. In this assay, CFSE labeled T cells which were activated by CD3/CD28 mAb coated beads, were suppressed by MDSCs at a ratio of 1 MDSC to 2 T cells (**Figure 1B**). Furthermore, FACs sorted M-MDSCs and G-MDSCs were analyzed by QPCR for Arginase-1, iNOS, and Ly6G to validate the subsets, and G-MDSCs were observed to have increased Ly6G and iNOS, while M-MDSCs highly expressed Arginase-1 (**Figure 1C**). These data validate a model system for generating functional GBM-educated MDSCs as a platform for functional assessment and inhibitor studies.

In vivo and *in vitro* Analysis Demonstrate M-MDSCs With Surface Expression of the MIF Receptor CD74

In order to determine the MDSC subset driving immune suppression GBM, we used a syngeneic model of glioma GL261,

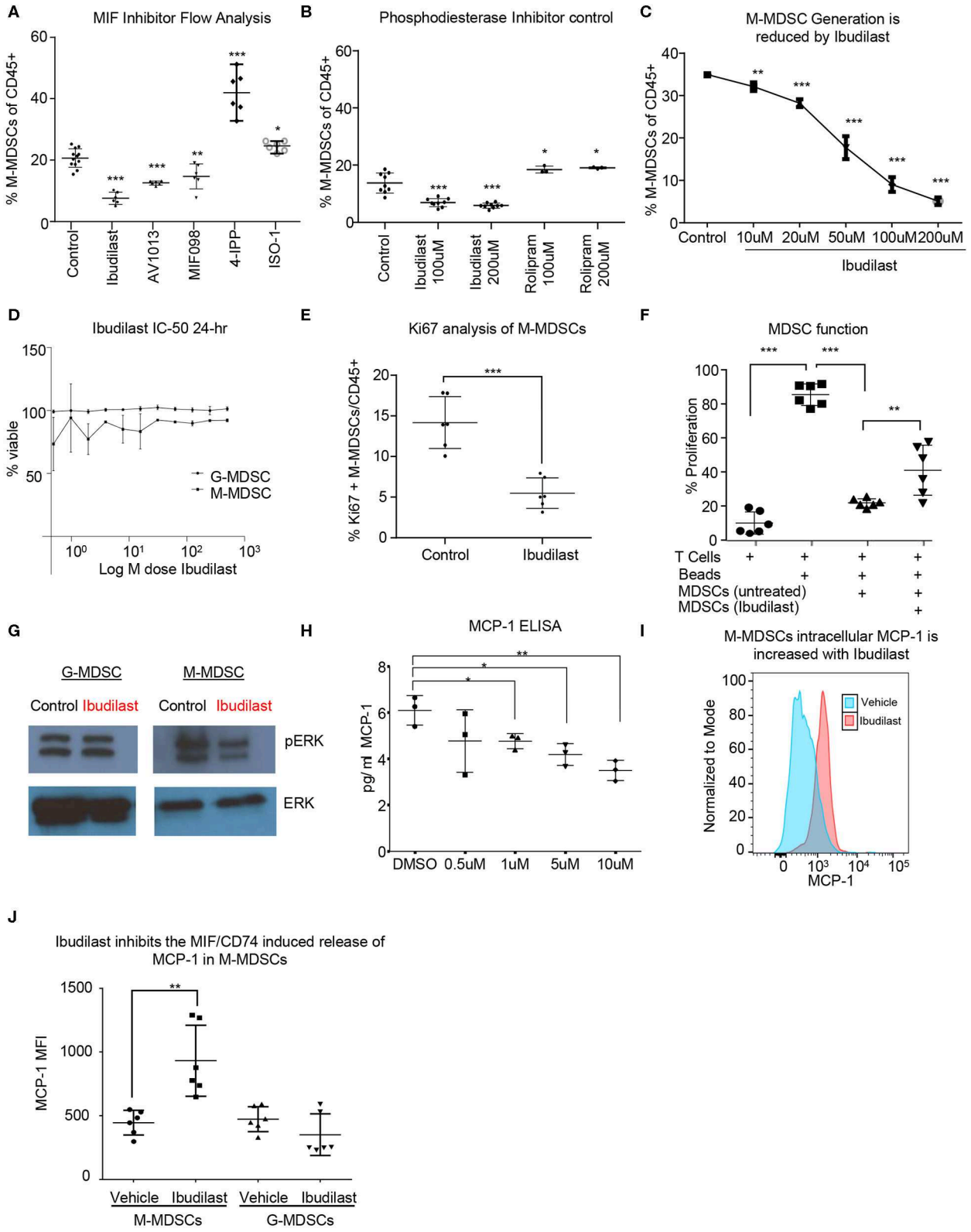


FIGURE 4 | Ibudilast inhibits the MIF disrupting M-MDSC generation *in vitro*. Utilizing the co-culture system described in **Figure 1** MIF inhibitors were assessed for their ability to inhibit MDSC generation **(A)**. Inhibitors were added at 200 μM at day 0 during the co-culture initiation and then assessed at day 3 for the % of

(Continued)

FIGURE 4 | M-MDSCs of CD45+ cells **(A)** $n = 6$ mice from $n = 2$ separate experiments. As a control for Ibudilast off target effects on phosphodiesterase Ibudilast was directly compared to Rolipram at 100 and 200 μM doses $n = 6$ control and Ibudilast treated co-cultures from $n = 6$ mice and $n = 3$ Rolipram treated co-cultures **(B)**. $n = 3$ mice per co-culture were used and ibudilast evaluated at 10, 20, 50, 100, and 200 μM and then assessed by flow cytometry at day 3 **(C)**. To determine if ibudilast was killing the M-MDSCs or G-MDSCs we isolated M-MDSCs and G-MDSCs from untreated co-cultures at day 3 from $n = 3$ mice by FACs sorting and then treated them for 24 h with Ibudilast as an IC50 using celltiterglo as a readout for viability **(D)**. Flow cytometry Ki67 staining of M-MDSCs at day 4 post treatment from co-culture generation in $n = 6$ biological replicates **(E)**. Shows The function of MDSCs treated with ibudilast was assessed by generating MDSCs in the presence of ibudilast and then magnetically sorting for MDSCs comparing untreated and Ibudilast treated MDSCs **(F)**. To assess the disruption of the MIF/CD74 signaling mechanism M-MDSCs and G-MDSCs were FACs sorted from day 3 co-cultures and then subsequently 50 ng/ml MIF was added to each well containing 500,000 cells and then treated with Ibudilast at 200 μM for 24 h prior to lysing the cells and performing western blot analysis for pERK and total ERK **(G)**. MCP-1 ELISA was performed on conditioned media from Co-cultures at day 4 post initiation, treated with Ibudilast ranging from 0 to 10 μM, $n = 3$ biological replicates. **(H)** Representative MCP-1 levels, y-axis normalized to mode and graphed in FlowJo using histogram plot comparing vehicle and Ibudilast treated M-MDSCs from co-cultures treated with 200 μM Ibudilast at day 4. **(I)** Quantification of $n = 6$ replicates from the experiment performed in **(H)**, briefly, live M-MDSCs and G-MDSCs were gated and then the mean fluorescent intensity of internally stained MCP-1 was measured and graphed for each replicate. T between Two-Tailed *T*-Test was performed for comparisons in **(A,B,D,E,H,J)** *<0.05, **<0.01, ***<0.001.

which was intracranially implanted to generate syngeneic tumors. At day 18 post implantation the tumor bearing (left) and non-tumor bearing (right) hemispheres were removed and analyzed by flow cytometry for MDSC subpopulations using the same gating strategy as in the co-culture system with the addition of pP2RY12 to exclude microglia. Analysis identified higher levels of M-MDSCs in the tumor bearing and non-tumor bearing hemispheres of the brain compared to G-MDSCs (**Figure 2A**). In order to determine the MIF receptor profiles, flow cytometry of the MIF receptors CD74, CXCR2, CXCR4, and CXCR7 was performed 3-days post co-culture initiation (**Figures 2B,C**). The percent positive for each receptor was analyzed by flow cytometry, which identified M-MDSC as having high expression of CD74 and its co-receptor CD44, while G-MDSCS primarily expressed CXCR2 (**Figures 2B,C**). FACs sorted M-MDSCs and G-MDSCS from co-cultures confirmed these findings, showing CXCR2 expression in G-MDSCs, and CD74 with the downstream effector MCP-1 as being highly expressed, suggesting activation through MIF/CD74 signaling axis (**Figure 2D**) (60). Furthermore, the analysis of M-MDSCs by flow cytometry showed high levels of CD74 expression compared to G-MDSCs (**Figure 2E**), and when quantified significantly higher than in G-MDSCs (**Figure 2F**). Interestingly, when MDSCs were permeabilized and stained for intracellular CD74 there was no difference between G- and M-MDSCs in the intracellular amounts of CD74 (**Figure 2G**). *In vivo* analysis of M-MDSCs in the tumor microenvironment using the syngeneic glioma model further supports these findings by showing the mean fluorescence intensity (MFI) of CD74 as higher on M-MDSCs compared to G-MDSCs or microglia of the tumor bearing hemisphere (**Figure 2H**). Taken together, these data demonstrate differential MIF receptor expression in MDSC subsets in mouse models.

GBM Patient Derived Specimens Show the MIF Receptor CD74 Expressed on MDSCs and Associate With Poor Prognosis

To determine if the findings in the mouse glioma model are recapitulated in the tumor microenvironment of human GBM patients, we utilized bioinformatics analysis of previously published single-cell sequencing datasets and flow cytometry analysis of GBM tumor specimens. The GBMseq dataset provides single cell sequencing of 3,589 cells from a cohort of 4 GBM patients annotated for their population names (51). Utilizing this dataset, we isolated the log2 counts for the myeloid populations identified and looked at the MIF receptor expression of CXCR2, CXCR4, CXCR7, CD74, and CD44 (**Figure 3A**) (40). Statistical analysis revealed that CD74 was most highly expressed in the myeloid populations. Furthermore, using the annotated populations, the level of CD74 expression was compared across all populations in the GBMseq dataset, which revealed highest levels on the myeloid cells (**Figure 3B**). To validate these findings, a separate cohort of 8 GBM tumors were analyzed by flow cytometry using a human panel previously validated, where M-MDSCs were identified by the following gating strategy singlets/live/HLA-DR$^-$/CD33$^+$/CD11b$^+$/CD14$^+$/CD15$^-$ and G-MDSCs by singlets/live/HLA-DR$^-$/CD33$^+$/CD11b$^+$/CD14$^-$/CD15$^+$. The expression of CD74 and CXCR2 were analyzed on each subpopulation by MFI, where CD74 was shown to be more highly expressed on M-MDSCs, while CXCR2 was more highly expressed on G-MDSCs (**Figures 3C,D**). Based on these findings, we tested the hypothesis that MIF and CD74 are signaling together and driving GBM immune suppression. We analyzed the cancer genome atlas (TCGA) GBMLGG database for survival and MIF expression and CD74 expression and the combination (**Figures 3E–G**). These data demonstrate that MIF and CD74 expression individually predict a poor prognosis, but when combined into MIF and CD74 double high as defined by greater than median expression of MIF and CD74, then the prognosis becomes poorer as demonstrated by hazard ratios MIF alone HR: 1.51, CD74 alone HR: 1.69, MIF/CD74 HR:2.45 (**Figure 3G**). These data demonstrate that human GBM specimens' express the MIF receptor CD74 on M-MDSCs in the tumor microenvironment and align with the murine models used in these studies.

MIF Inhibitor Screening Identified the MIF/CD74 Interaction Inhibitor Ibudilast

In order to identify a potential targeted therapy that acts on the MIF/CD74 signaling axis and neutralizes M-MDSCs, we utilized the *in vitro* co-culture system to generate glioma educated MDSCS in the presence of different small molecule

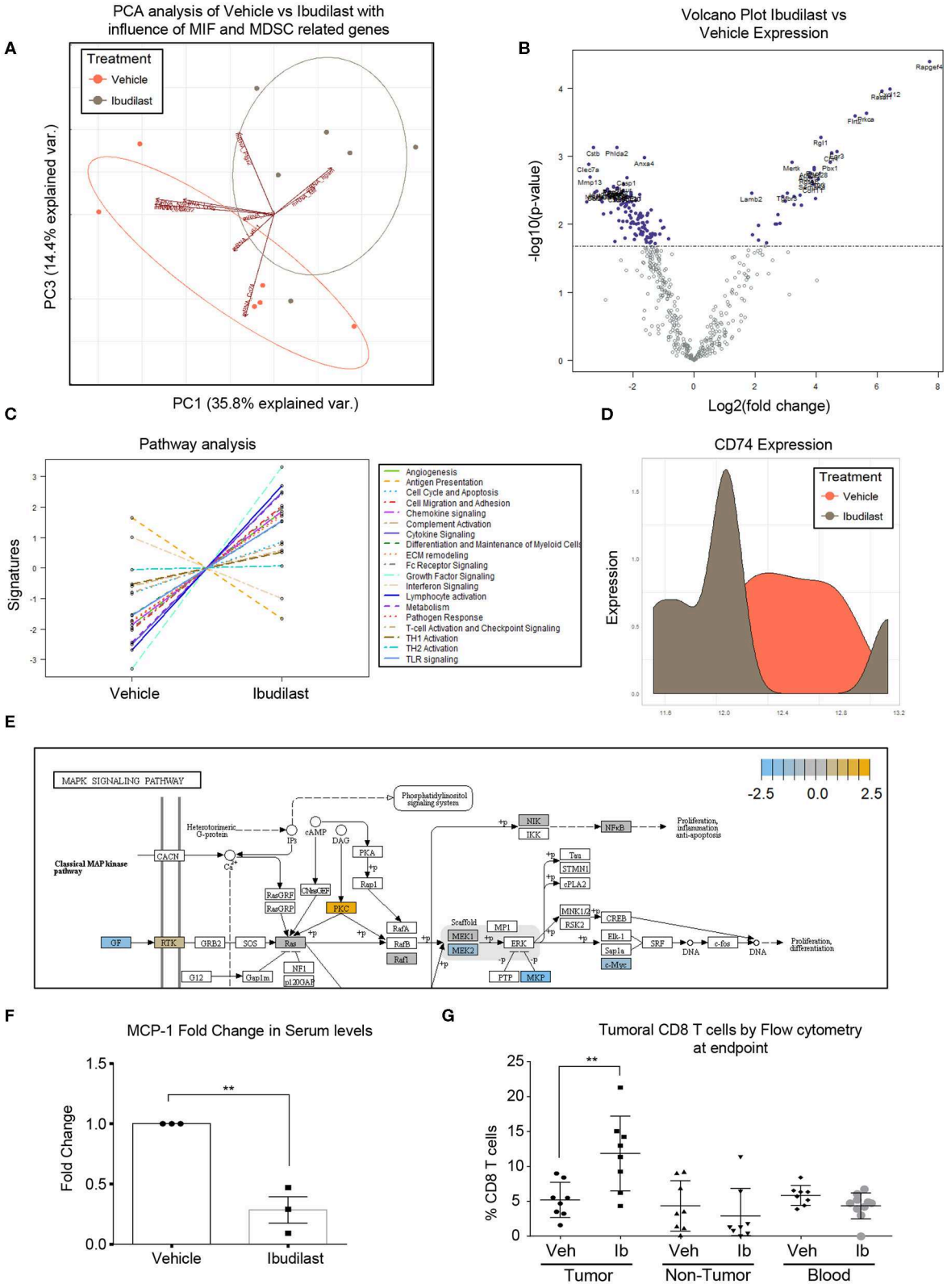


FIGURE 5 | Ibudilast inhibits the MIF disrupting M-MDSC generation *in vitro*. $n = 6$ vehicle and $n = 6$ Ibudilast treated mice (50 mg/kg 2x weekly starting day 5 post tumor implantation) were sacrificed at endpoint and tumors were dissected from the brain for RNA isolation. RNA from isolated tumors of vehicle and ibudilast treated

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FIGURE 5 | mice was analyzed via Nanostring murine myeloid panel and PCA was performed showing separation of ibudilast vs vehicle **(A)**. Volcano plot comparing log2fold change in genes between Ibudilast and vehicle demonstrates significant changes in the myeloid populations between vehicle and ibudilast treated tumors **(B)**. Pathway analysis of Ibudilast treated tumors shows increased activation of many immune pathways including lymphocyte activation while there is a reduction in antigen presentation **(C)**. Summary of CD74 expression in histogram format comparing all Vehicle and all Ibudilast treated samples **(D)**. Pathway analysis of Nanostring data identifies the MAPK signaling pathway in Ibudilast treated tumors with a reduction in MEK2 **(E)**. $n = 3$ mice treated with vehicle of 50 mg/kg 2x per week Ibudilast were sacrificed at day 18 post tumor initiation and serum was isolated from their blood and measured MCP-1 by ELISA **(F)**. A cohort of $n = 8$ vehicle and $n = 8$ Ibudilast treated mice were sacrificed at day 18 post injection and tumor, non-tumor tissue, and blood were analyzed by flow cytometry for immune populations where CD8 T cells were shown to be significantly increased in the tumors of Ibudilast treated mice **(G)**. Two-Tailed *T*-Test was performed for comparisons in **(F)** * <0.05, ** <0.01, *** <0.001. All other statistics were performed in Nanostring Nsolver software including the PCA and volcano plot differential gene expression and pathway analysis.

MIF inhibitors. In this system the generation of M-MDSCs was monitored at day 3 post co-culture in the presence of various MIF inhibitors at 200 μM, a concentration achieved in circulation with Ibudilast, a primary drug of interest due to its known toxicity profile and ability to penetrate the blood brain barrier (**Figure 4A**) (54, 61). Other MIF inhibitors previously identified as either MIF tautomerase inhibitors (4-IPP, ISO-1, AV1013, MIF098), or MIF/CD74 interaction inhibitors (Ibudilast, MIF098), were compared to Ibudilast at similar 200 μM concentrations to determine the specificity of Ibudilast in reducing M-MDSCs (52, 55, 58). While Ibudilast has been studied in different concentrations, it has recently been used at a similar dose in a patient derived xenograft model of glioma so we began with 200 μM (43). For comparison, 4-IPP has been used at 100 μM in lung cancer studies along with ISO-1, and MIF098 has been shown effective at 10 μM (62–64). The MIF/CD74 interaction inhibitor Ibudilast demonstrated an effective reduction in M-MDSC generation (**Figure 4A**). This reduction in M-MDSCs was not a result of a major change in cell viability as assessed by live/dead staining. Additionally, the MIF inhibitor 4-IPP, which does disrupt the interaction of MIF with CD74 showed no efficacy (**Figure 4A**) (52). While Ibudilast has been shown to inhibit the interaction of MIF and CD74, it was first discovered as a phosphodiesterase inhibitor (65, 66). To assess specificity, we compared Ibudilast at 100 and 200 μM to Rolipram, which is a known specific and potent phosphodiesterase inhibitor at the same concentrations (**Figure 4B**) (67). Rolipram was unable to alter the generation of M-MDSCs and thus the reduction of M-MDSCs is likely not due to the ability of ibudilast to inhibit PDE activity. The reduction in M-MDSC generation was not a result of a major change in cell viability as assessed by live dead staining. M-MDSC generation was reduced by ibudilast in a dose dependent manner treating co-cultures at 10 μM, 20 μM, 50 μM, 100 μM, and 200 μM Ibudilast (**Figure 4C**). Also, to determine if MDSCs could be killed by Ibudilast an IC-50 was performed using FACs sorted M-MDSCS and G-MDSCs increasing doses of Ibudilast were added to cultures for 24 h before being analyzed. No change in viability of M- or G-MDSCs was detected, however the flow cytometry analysis of Ki-67 on M-MDSCs treated with Ibudilast demonstrated a reduction in proliferation (**Figures 4D,E**). The function of MDSCs generated in co-culture with Ibudilast was analyzed using the T cell suppression assay, and identified as a reduction in the ability of MDSCs to suppress T cell proliferation (**Figure 4F**). Additionally, untreated M-MDSCs and G-MDSCs were isolated by FACs sorting and then treated for 24 h with Ibudilast before western blot analysis for pERK, a proximal downstream target of MIF/CD74 signaling (60). This revealed a specific reduction of pERK signaling compared to total ERK expression in M-MDSCs and not in G-MDSCs, showing specific MIF/CD74 inhibition by ibudilast in M-MDSCs (**Figure 4G**). Downstream of MIF/CD74 signaling, we analyzed secretion of MCP-1 by ELISA. In these studies, conditioned media from *in vitro* MDSC generation assays were used at day 4 post initiation, with Ibudilast ranging from 0 to 10 μM (**Figure 4H**). MCP-1 secretion was demonstrated to be dose dependent on Ibudilast within this assay (**Figure 4H**). To ensure that MCP-1 secretion was inhibited in Ibudilast treated M-MDSCs, we performed intracellular MCP-1 staining with vehicle and Ibudilast treated groups (**Figures 4I,J**). In this assay MCP-1 was shown to be increased intracellularly in M-MDSCs treated with Ibudilast, compared to the vehicle control (**Figure 4J**). In contrast G-MDSCs, which lack the MIF/CD74 signaling axis, had no change the intracellular storage of MCP-1 (**Figure 4J**). Internal accumulation of MCP-1 in Ibudilast treated M-MDSCs also aligns with ELISA data showing reduced MCP-1 in the media of Ibudilast tread co-cultures (**Figure 4H**). Taken together, these data demonstrate that M-MDSC expansion and function can be disrupted by pharmacologic a MIF/CD74 inhibition.

Ibudilast Treatment Reduced MIF/CD74 Signaling in a Syngeneic Model

To determine the *in vivo* effects of Ibudilast treatment, a cohort of tumor bearing animals were treated 5 days post tumor implantation [at 50 mg/kg 2x weekly based on previous experiments and the known effect dose effect of Ibudilast in a murine model (54)]. Daily dosing has been demonstrated in rodents to increase CYP enzymes and degrade ibudilast, reducing the bioavailability (54), and thus high doses of bi-weekly ibudilast was chosen for this treatment. Animals were analyzed at endpoint and tumors were dissected from the brain for RNA analyses by Nanostring Ncounter myeloid panel. Initial analysis by principal component analysis revealed that vehicle tumors and ibudilast tumors separate and the separation is driven by the vectors of MIF, CD74, PTGS2, Arg1, CXCR2 (**Figure 5A**). A volcano plot comparing the significantly differentially expressed genes between vehicle and ibudilast treated tumors showed significant change in immune genes upon treatment (**Figure 5B**). Pathway analysis between vehicle and Ibudilast treated tumors showed reduced antigen presentation, which coincides with reduced CD74 and MHC expression following the hypothesis that

Ibudilast is targeting CD74 *in vivo* as well as *in vitro* (**Figure 5C**). Pathway analysis also demonstrated increased Lymphocyte activation upon treatment showing possibly increased immune response (**Figure 5C**) and CD74 expression was reduced upon treatment (**Figure 5D**). Furthermore, analysis of Nanostring data also revealed a predicted reduction of MEK2 expression, which is downstream of MIF/CD74 signaling, but upstream of the pERK reduction that we initially analyzed by western blot *in vitro* (**Figure 5E**) and consistent with the western blot findings of reduced pERK signaling upon Ibudilast treatment. Additionally, MCP-1 was analyzed by ELISA in the serum of mice treated with Ibudilast and identified a reduction of MCP-1 upon treatment (**Figure 5F**). Flow cytometry analysis of tumor, non-tumor, and blood from this cohort at day 18 post injection tumors, 14 days of Ibudilast treatment, identified an increase in CD8 T cells specific to the tumor, while other immune cell populations were unchanged (**Figure 5G**, **Supplemental Figures 2, 3**). Additionally, immunohistochemistry staining identified a reduction of proliferation in Ibudilast treated tumors via reduced p-Histone3 and ki-67 staining (**Supplemental Figure 4**). Importantly, we saw no changes in other T cell or myeloid cell populations, including the overall number of CD45+ cells (**Supplemental Figures 2, 3**). Taken together, these data reveal that CD74/MIF inhibition via Ibudilast can

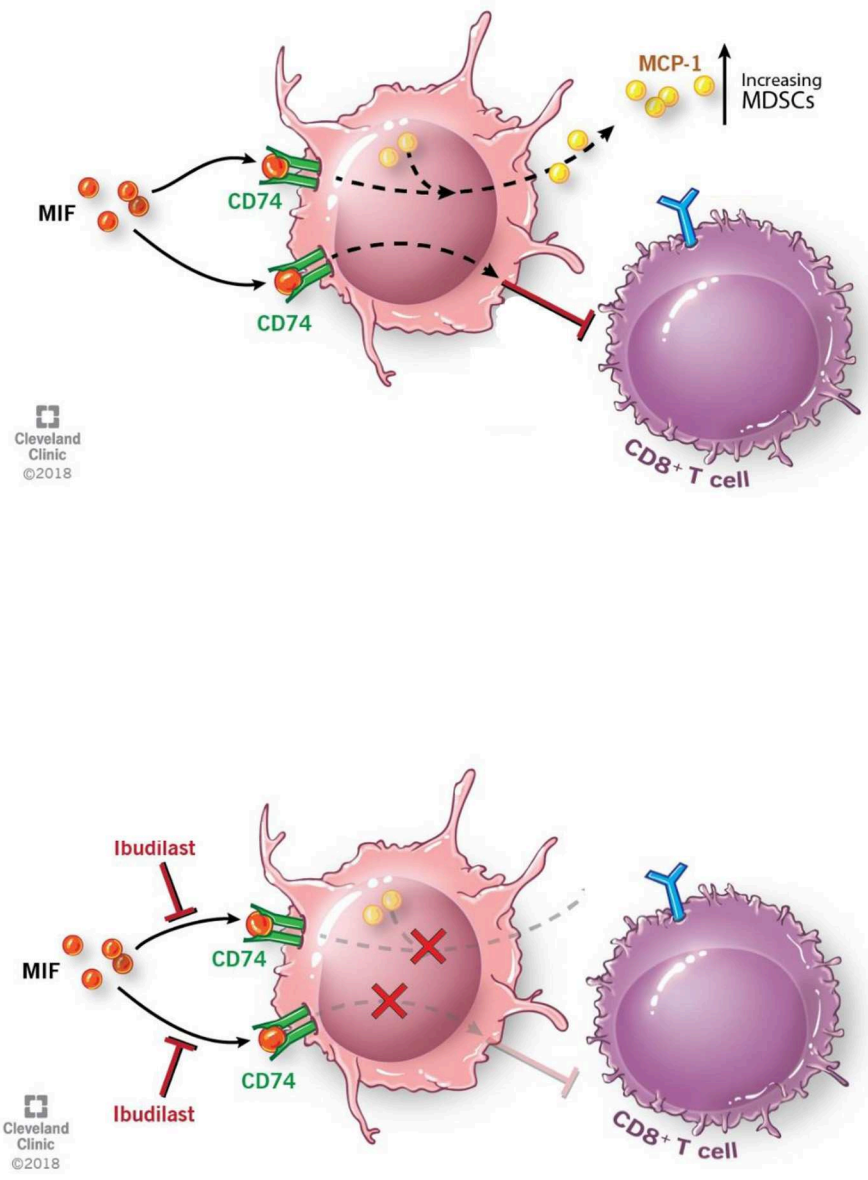

FIGURE 6 | Schematic depicting pathway described where MIF binds CD74 on M-MDSCs enhancing their activity to inhibit CD8 T cells and also produce the downstream target MCP-1. With the addition of Ibudilast to inhibit this process we show a reduction of the MDSCs generation and function removing the inhibitor effect from CD8 T cells.

reduce MDSCs *in vivo* and increase immune activation in the tumor microenvironment.

DISCUSSION

While multiple groups including our own have identified MDSCs as being increased in GBM and other cancers (9, 11, 12, 19, 31), our understanding of the factors driving these cells has been lacking and strategies to target these cells has not matured. Here we focused our efforts on MIF as a driver of MDSCs based on our previous work showing that MIF depletion could reduce MDSC function (31). Additionally, multiple groups have indicated a link between MIF and MDSCs (32, 33, 68). Here we found that the receptor CD74 may play a greater role in GBM MDSC biology because the subset of MDSCs primarily found in the tumor microenvironment were M-MDSCs, which predominantly express CD74 as a MIF receptor. This is in contrast to metastatic breast cancer models that show G-MDSCs infiltrating tumors and driving metastasis (69, 70); where in those cases we would hypothesize that CXCR2 or another MIF receptor may play a more vital role. While our previous work focused on the MIF/CXCR2 signaling pathway in GBM and MDSCs the entirety of that was focused on G-MDSCs, marked by CD244.2 positivity. These studies instead focus on the majority population of MDSCs in our GBM mouse models, M-MDSCs, and targeting their signaling pathway with MIF/CD74. In relation we believe these pathways could be intertwined based the data presented here showing that permeabilized G-MDSCs contained CD74 levels similar to M-MDSCs. We hypothesize this could be due to the known ability of M-MDSCs to differentiate into G-MDSCs and during this process and maintain intracellular stores of CD74 (18, 71). Further studies should be performed analyzing the intracellular stores of CD74 during the differentiation process to determine how this phenomenon occurs.

In seeking to target the interaction of MIF and CD74 on MDSCs we identified Ibudilast as an agent of interest, and were able to treat mice to reduce CD74 expression and increase CD8 T cells in the tumor. Importantly this inhibitor is blood brain barrier penetrant, which overcomes one of the major therapeutic obstacles in the treatment of brain tumors (54). One difficulty in using Ibudilast in mouse models is the drug passage effect, where daily treatment increases CYP enzymes leading to rapid degradation (54). However, in humans the drug is stable in the circulation and accumulates in the CNS with repeated exposure such as daily dosing (53, 61). For these reasons in the mouse model we settled on a 2x weekly dose of Ibudilast to minimize the drug passage effect, but believe that Ibudilast may be more efficacious in humans than in mouse models. Efforts are currently underway to evaluate Ibudilast in GBM in a clinical trial (NCT03782415) (43) and will likely provide more insight into how this drug effects the anti-tumor immune response. Additionally, Ibudilast recently demonstrated promising results in a phase 2 clinical trial of multiple sclerosis, where it is thought to have a protective effect by reducing brain atrophy, as compared to anti-inflammatory drugs commonly used to treat multiple sclerosis (44).

In summary we believe that the M-MDSCs driven by GBM secreted MIF is signaling through the MIF receptor CD74 (**Figure 6**). Inhibition of the interaction between MIF and CD74 via ibudilast treatment results in reduced downstream signaling of MCP-1, which has been shown to be MIF-dependent in studies of autoimmunity (Leng et al., SLE study), and further drives monocyte and MDSC recruitment to the microenvironment and enhancing the expansion of M-MDSCs (**Figure 6**) (57, 60, 72, 73). The importance of MCP-1 in glioma MDSC recruitment has recently been highlighted, where loss of CCR2, the MCP-1 receptor, demonstrated a reduction of MDSCs in the tumor and bone marrow of glioma bearing mice. (73) While our data demonstrates these phenomena, we did not readily observe enhanced survival in our model that involved the use of Ibudilast as a single agent. Nonetheless, we observed that Ibudilast produced an expansion of CD8 T cells and Nanostring analysis predicted an increase multiple pathways including lymphocyte activation. These findings support an interpretation that inhibition of immune suppression, alone, will not be sufficient to produce an anti-tumor immune response. This interpretation mirrors the clinical trial results to date that indicate that treatment with an immune stimulatory therapy alone has been an ineffective strategy. Instead, we hypothesize that better clinical outcomes will be seen when the reversal of tumor-induced immune suppression associated with Ibudilast is combined with an immune stimulatory therapy.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB 2559 and clinical trial NCT02669173. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Cleveland Clinic IACUC.

AUTHOR'S NOTE

This manuscript has been released as a Pre-Print at BioRxiv (74).

AUTHOR CONTRIBUTIONS

TA, DB, BO, KM, KY, RB, MV, and JL provided conceptualization and design. TA, DB, AR, GR, LJ-S, AL, DP, MA, and AMM performed the experiments. TA, DB, BO, LJ-S, GR, KY, RB, AAM, MV, and JL analyzed the data. TA, DB, RB, BO, DP, MA, MV, and JL wrote the manuscript. RB, MV, and JL provided financial support. All authors provided final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01191/full#supplementary-material

Supplemental Figure 1 | shRNA knockdown of MIF in GL261 was performed using 2 separate shRNA's which were the top targets from previously published work from our group to generate stable knockdown cell lines of GL261 **(A)**. Comparing the survival of intracranially implanted tumors in NSG immune incompetent mice demonstrate no survival difference in NSG mice **(B)**. Treating $n = 10$ GL261 tumor bearing mice 2x weekly with anti-MIF antibody (gifted from Dr. Richard Bucala) vs. $n = 10$ IgG control treated mice demonstrated no survival benefit **(C)**. MDSC co-culture dynamics over time analyzing $n = 3$ mice in separate co-cultures where one well was used each day over 7 days to check the number of CD45+ cells by flow cytometry **(D)**. Survival curve analysis was performed in GraphPad Prism using Log-rank (Mantel-Cox) test for p value and hazard ratio log rank was computed on the same data using GraphPad Prism.

Supplemental Figure 2 | Intracrainially injected tumors vehicle vs ibudilast treated tumors, non-tumor tissue, and blood analysis from **Figure 5** demonstrate no significant difference in M-MDSCs, G-MDSCs, Macrophages, or Microglia **(A–D)**. Two-Tailed *T*-Test was performed for comparing vehicle vs. ibudilast in each compartment *<0.05, **<0.01, ***<0.001.

Supplemental Figure 3 | Intracrainially injected tumors vehicle vs ibudilast treated tumors, non-tumor tissue, and blood analysis from **Figure 5** demonstrate no significant difference in total CD45+ cells, CD4 T cells, ratio of CD8/ CD4 T cells, or NK cells **(A–D)**. CD74 expression was analyzed on vehicle and Ibudilast treated M-MDSCs demonstrating an increase in CD74 protein expression on the cell surface of M-MDSCs upon treatment in the tumor compartment only **(E)**. Two-Tailed *T*-Test was performed for comparing vehicle vs. ibudilast in each compartment *<0.05, **<0.01, ***<0.001.

Supplemental Figure 4 | Intracrainially injected tumors vehicle vs ibudilast treated mice were perfused at endpoint and tissue was paraffin embedded for IHC analysis. Staining for p-Histone3 and Ki67 demonstrated a reduction in proliferation in the ibudilast treated tumors. Two-Tailed *T*-Test was performed for comparing vehicle (mock) vs. ibudilast treated tumors *<0.05, **<0.01, ***<0.001.

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Conflict of Interest: RB is a co-inventor on patents describing the therapeutic potential of anti-MIF and MIF098.

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.

frontiers in Immunology

REVIEW
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Innate Immune Defense Mechanisms by Myeloid Cells That Hamper Cancer Immunotherapy

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Over the past decade, cancer immunotherapy has been steering immune responses toward cancer cell eradication. However, these immunotherapeutic approaches are hampered by the tumor-promoting nature of myeloid cells, including monocytes, macrophages, and neutrophils. Despite the arsenal of defense strategies against foreign invaders, myeloid cells succumb to the instructions of an established tumor. Interestingly, the most primordial defense responses employed by myeloid cells against pathogens, such as complement activation, antibody-dependent cell cytotoxicity and phagocytosis, actually seem to favor cancer progression. In this review, we discuss how rudimentary defense mechanisms deployed by myeloid cells can promote tumor progression.

Keywords: cancer immunotherapy, tumor-associated myeloid cells, tumor microenvironment, innate immune response, immune suppression, immunotherapy resistance

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INTRODUCTION

Immune cells abundantly infiltrate tumors, creating a complex environment mediated by repetitive cycles of antitumor responses and immune evasion (1). Myeloid innate immune cells, such as granulocytes, monocytes, macrophages and dendritic cells (DCs), play an important role in cancer-cell recognition, initiation of inflammation and antitumor responses (2). Chronic inflammation, however, can initiate tumorigenesis and can drive cancer progression in some cancer types (3, 4). Hence, myeloid cells play a dual role in cancer as they can initiate antitumor responses and communicate with cells of the adaptive immune system, but also promote local inflammation leading to chronic cancer-associated inflammation (5, 6).

In the tumor microenvironment, tumor-associated macrophages (TAMs) display an array of phenotypes beyond the M1/M2 paradigm, ranging from antitumoral to immunosuppressive, proangiogenic, immunomodulatory and tissue-remodeling phenotypes (7–9). The presence of TAMs in most solid tumors is correlated with poor prognosis and overall survival of patients (10). In addition to TAMs, solid tumors are also infiltrated by immunosuppressive, immature myeloid progenitor cells, commonly referred to as monocytic or polymorphonuclear myeloid-derived suppressor cells (M/PMN-MDSC) (11–13). Similarly, an increased infiltration of MDSCs has been associated with poor prognosis for a variety of cancer types (14). Neutrophils also contribute to tumor progression, yet establishing the difference between PMN-MDSCs and tumor-associated neutrophils (TAN) remains challenging (11, 15, 16). Although tumor-promoting functions have been attributed to other granulocytes, like

eosinophils (17), basophils (18) and mast cells (19), further research is required to fully elucidate their role in cancer, as antitumoral roles have also been described (20, 21). Another myeloid population in the tumor microenvironment (TME) are DCs, that originate from different precursors and display various phenotypes, ranging from immunosuppressive monocyte-derived DCs (Mo-DCs) to immunocompetent cDC1 and cDC2 subsets (22). Altogether, the myeloid compartment in the TME is heterogenous and varies across tumor types, individuals and tumor stage (23). Nevertheless, the majority of scientific discoveries points toward a more tumor-supporting role for myeloid cells in the TME.

RUDIMENTARY MYELOID DEFENSE STRATEGIES AS TUMOR PROMOTERS

The innate immune response by myeloid cells occurs as a succession of events starting at signaling through cytosolic or surface PRRs, followed by effector responses including the release of cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), antibacterial peptides and degranulation (**Figure 1**). PRR on myeloid cells can be triggered by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), but also indirectly by secondary mechanisms such as complement activation and circulating antibodies (Abs), resulting in cytolytic and phagocytic effector mechanisms. Pathogen clearance is mediated by mechanisms such as phagocytosis, respiratory burst with the production of ROS and RNS and release of bacteriostatic peptides, but also through the cell-extrinsic initiation of inflammation via the release of proinflammatory cytokines and chemokines (24). However, this succession of events does not always appear to be a linear cascade, as feedforward loops and interactions exist between different effector mechanisms (**Figure 2**). Yet, even such early, innate effector mechanisms performed by myeloid cells surprisingly seem capable of promoting tumor progression.

Pathogen and Tissue Damage Recognition Mechanisms as Tumor Promoters

Toll-like receptors (TLRs), C-type lectin receptors (CLRs), the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) are PRR families expressed by macrophages and DCs, but also by non-immune cells, such as endothelial cells and fibroblasts (25). Based on current literature, it seems that PRR signaling can both contribute to cancer progression and is capable of steering antitumor responses. Here, we will focus on the tumor-promoting role of PRRs, where TLR signaling and inflammasome activation will serve as an example to demonstrate the effect of PRR signaling in tumor-infiltrating myeloid cells.

In response to the tumor-derived proteoglycan versican V1, TLR2- and TLR6-signaling in TAMs induces the expression of cathelicidin (hCAP18/LL-37), an antimicrobial peptide which in turn promotes the proliferation of human ovarian cancer cells *in vitro* (26). When a TLR2-agonist, lipoprotein Pam2CSK4, is administered intravenously, TLR2-expressing PMN-MDSCs accumulate and proliferate systemically in EG7 lymphoma-bearing mice (27). Moreover, Pam2CSK4-mediated TLR2 signaling promotes the survival of M-MDSCs and mediates the differentiation of M-MDSCs into macrophages. These macrophages are capable of presenting tumor antigens to CTLs, resulting in interferon gamma (IFNγ) release upon T-cell activation and the subsequent expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) release by surrounding macrophages, which in turn leads to T-cell suppression (28). In the presence of bacterial lipopolysaccharides in the colonic lumen, TLR4 signaling in TAMs promotes chronic inflammation through increased production of cyclo-oxygenase 2 (COX2) and prostaglandin E_2 (PGE_2) (29). Damage-associated high mobility group box-1 protein (HMGB1), released from necrotic keratinocytes in the skin upon irradiation, interacts with TLR4 on bone marrow-derived immune cells (30). The resulting signaling facilitates papilloma progression through an increase in the recruitment of proinflammatory immune cells (30). Moreover, HMBG1-mediated TLR4 signaling causes an increased infiltration of radiation-resistant cells upon radiotherapy.

Upon intracellular PAMP or DAMP recognition by cytosolic sensors like NLRP3, inflammasomes are assembled, which results in the release of the proinflammatory cytokines IL-1ß and IL-18 and leads to a proinflammatory form of cell death, also referred to as pyroptosis (31). In different murine tumor models, NLRP3 plays a role in the migration of MDSCs to the TME, where MDSCs suppress antitumor CTL responses independent of NLRP3 and induce unresponsiveness to DC vaccination (32). The role of inflammasome activation in tumor progression is also demonstrated in obese mice, where obesity-associated NLRC4 inflammasome activation in tumor-infiltrating myeloid cells promotes breast cancer progression (33). Importantly, the release or administration of PRR agonists may give rise to therapy resistance in patients that underwent radiotherapy (34), chemotherapy (35, 36) or cancer vaccination (32). For example, myeloid Gr1-negative cells accumulate in murine B16 melanoma and CT26 colon adenocarcinoma tumors after local irradiation, where mitochondrial DNA of dead, irradiated cancer cells induces TLR9 signaling, which mediates revascularization and immune evasion in an interleukin (IL)-6- and STAT3-dependent manner (34, 37). Paclitaxel-induced TLR4 signaling in murine and human breast cancer cells results in the production of the proinflammatory cytokines IL-1ß and IL-6, which promotes the expansion of MDSCs in the bone marrow and spleen as well as their recruitment to the TME (36). In response to gemcitabine and 5-fluorouracil chemotherapy, cathepsin B is released in the cytosol of MDSCs which induces NLRP3-dependent IL-1ß release (35). In return, IL-1ß drives the polarization of CD4+ T cells into Th17 cells that promote tumor angiogenesis in the TME, which hampers the antitumor response of gemcitabine and 5-fluorouracil.

Altogether, it seems that the tumor microenvironment can be a source of PRR agonists, stimulating PRR signaling in myeloid cells that in turn perform tumor-promoting functions. Alternatively, PRR signaling can also directly affect cancer cells. TLR4 expression and signaling in gastric cancer cells results in mitochondrial ROS production, which induces secondary

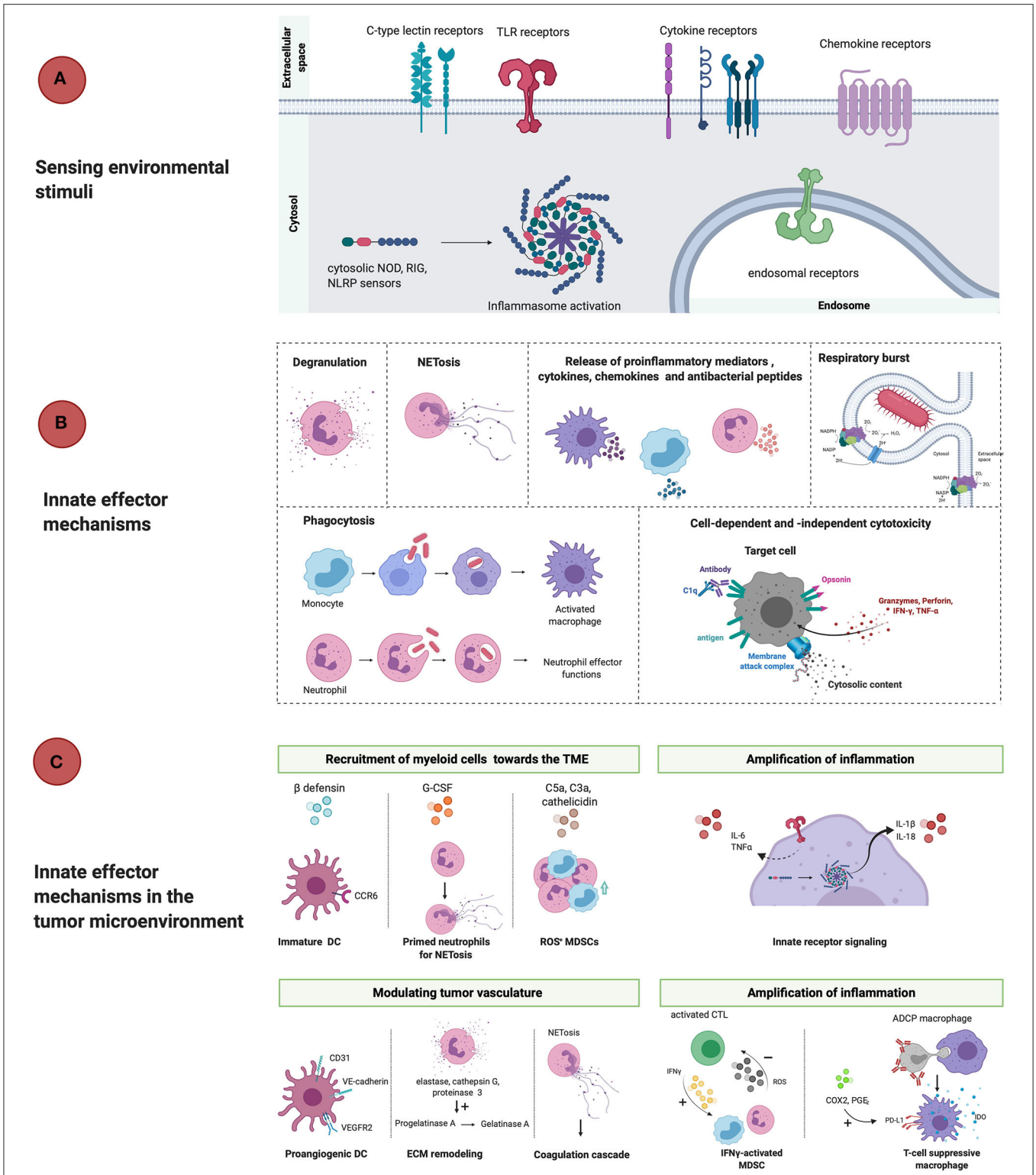

FIGURE 1 | Linear representation of classical innate immunity in response to threats and in the TME. **(A)** PAMPs and DAMPs are recognized by surface-expressed, endosomal and cytosolic pattern recognition receptors (TLR, CLR, cytokine, chemokine receptors, NLRP3) which results in phenotypical changes that counteract ongoing threats or tissue damage. **(B)** Effector mechanisms that take place during inflammation are degranulation, NETosis, release of proinflammatory mediators, respiratory burst, phagocytosis and cell-dependent and -independent cytotoxicity. The net result is the recruitment of immunocompetent cells that mount an inflammatory reaction and potentially resolve the infection. **(C)** However, in the tumor microenvironment innate myeloid cells promote tumor progression through active

(Continued)

FIGURE 1 | recruitment to the TME in response to ß-defensins, cathelicidin, G-CSF, complement factors and chemokines. Once arrived in the TME, myeloid cells are activated and release proinflammatory mediators, which empowers tumor-associated inflammation. Activation of myeloid cells also allows for remodeling of the tissue vasculature and extracellular matrix, which also allows for cancer-cell invasion and metastasis. Furthermore, myeloid cells contribute to immunosuppression once activated by for example, upregulation of PD-L1 and IDO release during antibody-dependent phagocytosis of target cells or stimulatory cytokines (IFNγ). DC, dendritic cell; ECM, extracellular matrix; VEGFR2, vascular endothelial growth factor receptor 2; IFNγ, interferon gamma; ROS, reactive oxygen species; MDSC, myeloid-derived suppressor cell; ADCP, antibody-dependent cell-mediated phagocytosis; IDO, indoleamine 2,3-dioxygenase; COX2, cyclooxygenase 2; PGE2, prostaglandin E2; TNFα, tumor necrosis factor alpha; G-CSF, granulocyte colony stimulating factor; NOD, nucleotide-binding oligomerization domain; RIG, retinoic acid-inducible gene; NLRP, nucleotide-binding oligomerization domain; leucine-rich repeat and pyrin domain containing.

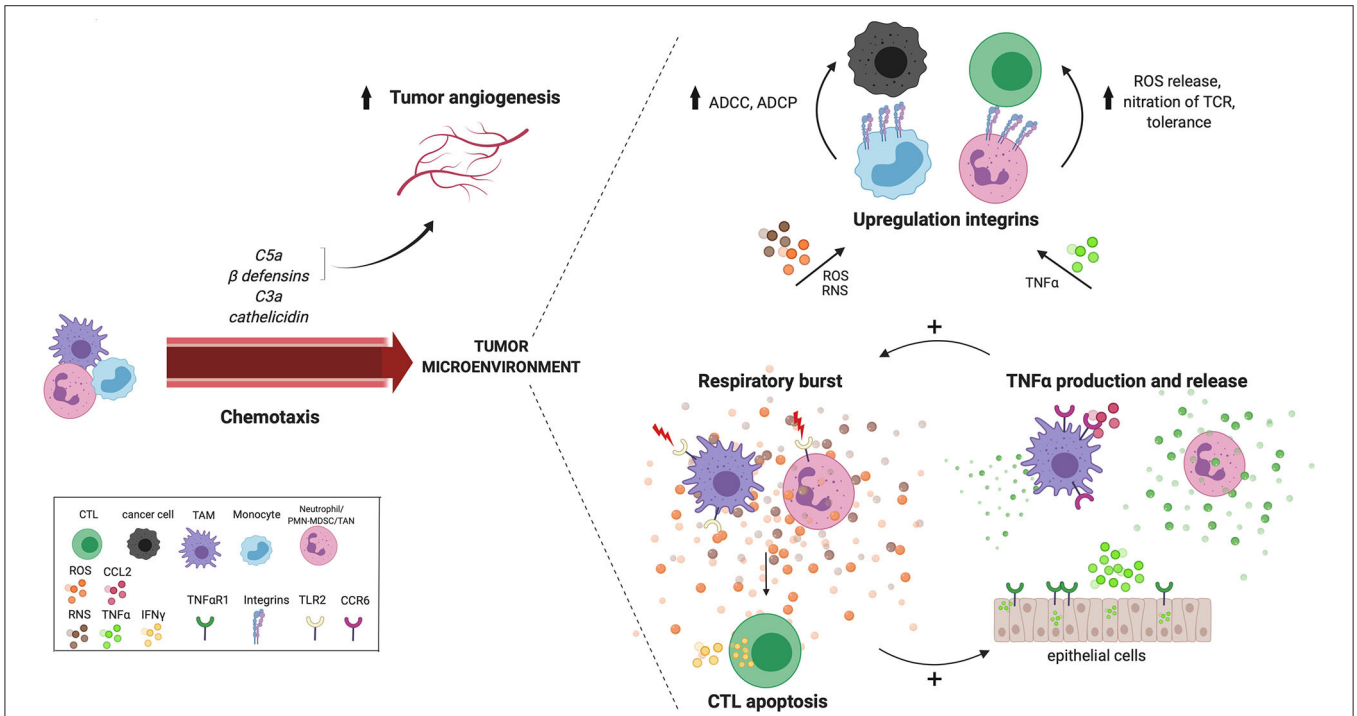

FIGURE 2 | Cross talk between reoccurring innate effector mechanisms in the TME. Tumor-derived chemokines that are produced as a result of innate effector mechanisms including C5a, C3a, cathelicidin and ß-defensin, recruit myeloid cells to the TME. Tissue vasculature during chronic inflammation is maintained by complement anaphylatoxin C5a and beta-defensins. Anaphylatoxin C5a also recruits MDSCs with increased ROS and NRS production in the TME. Many innate pathways converge at the production of ROS and NOS in the TME. For example, TLR2 signaling increases the antigen presenting capacity of TAMs, which activates CTLs resulting in IFNγ release and subsequent ROS and NO release by TAMs. Neutrophil-derived ROS induces CTL apoptosis, while hydrogen peroxide released by TAMs, induces the expression of TNFα and TNFαR1 in surrounding epithelial cells. A positive feedback loop seems to exist between the respiratory burst and TNFα release, creating a potential cross talk between TAMs, neutrophils and epithelial cells in the TME. Furthermore, both ROS and TNFα also increases the expression of integrins, which increases cell-cell contact and facilitates cell-mediated killing via ADCC and ADCP either by performed by monocytes to kill cancer cells, or by MDSCs to suppress CTLs. ADCC, antibody-dependent cell-mediated cytotoxicity; MDSC, myeloid-derived suppressor cell; ADCP, antibody-dependent cell-mediated phagocytosis; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNFα, tumor necrosis factor alpha; TNFαR1, tumor necrosis factor alpha receptor 1; CTL, cytotoxic T lymphocyte; IFNγ, interferon gamma; TCR, T-cell receptor; TAM, tumor-associated macrophage; CTL, cytotoxic T lymphocyte; TME, tumor microenvironment; TLR2, Toll-like receptor 2; TAM, tumor-associated macrophage.

signaling cascades in response to oxidative stress that may regulate cancer-cell survival (38). TLR4 signaling in colorectal cancer and breast cancer cells promotes invasion and metastasis of these cells (36, 39). Therefore, PRR signaling is not strictly a myeloid cell-restricted, tumor-promoting mechanism.

Release of Proinflammatory Mediators as Tumor Promoters

A common downstream effect of PRR signaling is the release of proinflammatory cytokines, like IL-12, IL-6, IL-1β and tumor necrosis factor alpha (TNFα). In the TME, cytokines like IL-10 and transforming growth factor beta (TGF-ß) play an important role in suppressing antitumor responses, so it is within expectation that strongly opposing, proinflammatory mediators would be capable of eliciting and sustaining antitumor responses. However, a number of key proinflammatory cytokines, such as IL-1β and IL-6, have been reported to promote tumor progression through the mobilization of MDSCs (40, 41), the contribution to chronic inflammation (40, 42) and the stimulation of angiogenesis (43, 44). For example, in murine models of pancreatic ductal adenocarcinoma, neutralization of tumor-derived IL-1β enhances CTL-infiltration and ameliorates

the response to anti-PD-1 immune checkpoint blockade (45). In accordance, IL-1β-blockade synergizes with anti-PD-1 immune checkpoint blockade in 4T1 breast cancers by restoring the cytotoxic capacity of CTLs without inducing systemic inflammation (46).

Other proinflammatory cytokines, such as TNFα and IFNγ, seem to have an ambiguous effect on cancer progression. For example, neutrophil-derived TNFα promotes the production of NO in an autocrine manner, which in turn induces apoptosis of non-activated CTLs in murine models of thoracic malignancies (47). Subcutaneous *Tnfr1*-deficient fibrosarcoma FB61 tumors are rejected in *Tnfr*-deficient mice, while tumor growth is reestablished via an adoptive transfer of *Tnfr1*-expressing MDSCs. Mechanistically, MDSCs of *Tnfr*-deficient mice displayed increased caspase-8 cleavage which induces apoptosis, and lower levels of c-FLIP, a natural caspase-8 inhibitor, which causes reduced accumulation of MDSCs in the TME along with a reduced tumor-suppressive capacity (48). These data suggest that endogenous and persistent TNFR signaling promotes tumor growth by maintaining survival of MDSCs (48). In accordance, a study by Sade-Feltman et al. demonstrated that TNFα is required to maintain the immature and immunosuppressive phenotype of MDSCs (49). Hence, TNFα blockade using Etanercept, a biological compound composed of the extracellular domain of TNFR fused to an IgG1 Fc fragment, restores NK-cell cytotoxicity and T-cell proliferation, reduces splenic MDSC accumulation and enhances the maturation of MDSC into $CD11b^+CD11c^+$ and $CD11b^+$ $F4/80^+$ cells (49). In addition, TNFα induced upon anti-PD-1 immune checkpoint blockade, increases PD-L1 and TIM-3 expression on tumor-infiltrating T cells and promotes their cell death upon TNFα binding to TNFRs (50). TNFα blockade increases the infiltration of tumor-specific CTLs, reduces the proliferation of immunosuppressive, regulatory T cells (Tregs) and minimizes toxicity of immune checkpoint blockade (51–53). These tumor-promoting effects of TNFα in the TME are in contrast to its inhibition of breast cancer-cell proliferation by blocking the G1/S phase transition of the cell cycle (54). Furthermore, TNFα may hamper the polarization and differentiation of monocytes into M2-like TAMs, instead steering the macrophage phenotype toward an anti-tumoral M1-like TAM in the TME (55). Altogether, TNFα also carries the potential to mount antitumoral responses in cancer therapy, as described elsewhere (56).

The role of the proinflammatory cytokine IFNγ in tumor progression appears to be concentration- (57, 58) and context-dependent (28, 59). He et al. demonstrated that, at low local levels, IFNγ promotes tumor progression of several murine tumor models, including hepatic, mammary and skin cancer, through increased gene expression of *Cd274* (PD-L1), *Ctla4* and *Foxp3*, whereas at higher levels, IFNγ reduces the gene expression of *Foxp3* and co-inhibitory molecules (58). If either TNFα or IFNγ signaling in tumor-infiltrating $CD4^+$ T cells is absent upon antigen recognition, tumor progression is stimulated, whereas combined TNFα and IFNγ signaling in $CD4^+$ T cells prevents tumor angiogenesis and tumor-cell proliferation (59). Hence, cytokines like IFNγ and TNFα can play dual roles in cancer progression and the internal complexity of combined receptor signaling strongly affects antitumor responses (59).

Besides cytokines, other inflammatory mediators influence tumor progression. Indeed, proinflammatory enzymes and products of the prostaglandin production pathway, including COX2 and PGE_2, have been associated with enhanced tumor progression, as they induce the expression of PD-L1 on macrophages and MDSCs (60). A tumor-promoting feedback loop has been discovered between MDSCs, colorectal cancer cells and T cells, that all release PGE_2 and express receptor-interacting protein kinase 3 (RIPK3) (61). PGE_2-induced RIPK3 signaling in MDSCs results in the expression of COX2 that catalyzes PGE_2 synthesis, which is then released in the TME. PGE_2 promotes proliferation of cancer cells and suppresses T-cell activation through RIPK3 signaling. Macrophage-derived IL-1β induces ROS-dependent COX2 production and activity in breast cancer cells, leading to PGE_2 release *in vitro* (62). Culturing blood-derived monocytes with PGE_2 induces the expression of COX2, which inhibits differentiation of monocytes into monocyte-derived DCs. Instead, the expression of indoleamine 2,3-dioxygenase (IDO), IL-4 receptor, iNOS and IL-10 is upregulated and drives the suppressive phenotype of M-MDSCs *in vitro* (63). Hence, PGE_2 contributes to polarizing the phenotype of myeloid cells in the TME.

In conclusion, two trends are observed regarding proinflammatory cytokines or mediators; (1) either their role in cancer progression is generally protumoral, such as IL-6, IL-1β, or PGE_2, or (2) their function in cancer progression is ambiguous, such as for TNFα and IFNγ. The severity of inflammation may play an important role here; to a certain extent, proinflammatory mediators are required to stimulate anti-tumoral T-cell responses, whereas prolonged exposure or exposure to high levels of inflammatory mediators can lead to unresponsiveness. In addition, it is not clear whether cancer cells or myeloid cells initiate the expression and release of tumor-promoting inflammatory mediators in the TME.

Respiratory Burst as Tumor Promoter

Upon PAMP recognition through PRR signaling, neutrophils and macrophages engulf pathogens via phagocytosis, which activates phagosome- and surface membrane-bound NADPH oxidase, resulting in the production of superoxide (O_2^-) and derivatives, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), through downstream processing by superoxide dismutase (SOD) and myeloperoxidase (MPO) (64–66). The release of ROS in phagosomes and the extracellular space is referred to as the respiratory burst, which is a primary antimicrobial and antifungal defense mechanism deployed by phagocytes (64). MDSCs are a major source of ROS in the TME, where ROS and peroxynitrite (HNO_3^-) abrogate antigen recognition by CTLs and instead induce tolerance (67, 68). This depends on direct contact between T cells and MDSCs, mediated by the integrins CD11b, CD18, and CD29 (68). Mechanistically, nitration and oxidation of amino acids in the T-cell receptor (TCR) and CD8 co-receptor molecules prevents interaction with major histocompatibility complex (MHC) molecules, which in turn induces tolerance (67). Constitutive upregulation of STAT3 in

MDSCs directly regulates the expression of NOX2 components necessary for the formation of the NADPH protein complex, which is followed by a subsequent increase in production and release of ROS (69). MDSCs are unable to suppress T cells in the absence of NOX2 activity, and instead differentiate into mature macrophages and DCs (69). ROS also mediate the polarization of macrophages, as inhibition of O_2^- impedes the differentiation of monocytes into M2 macrophages while differentiation into M1 macrophages remains unaltered (70, 71). Thus, while ROS production in MDSCs maintains their immature phenotype, MDSC-derived ROS in the TME mediates the differentiation of tumor-infiltrating monocytes. Furthermore, H_2O_2 released by macrophages and neutrophils induces the expression of *Tnfa* and *Tnfr1* in epithelial cells, that in turn release TNFα leading to the upregulation of other proinflammatory and angiogenic factors, hence, sustaining tumor progression in a paracrine loop (72). Aside from myeloid-derived ROS, Xia et al. demonstrated that ROS can also be produced by cancer cells themselves. They showed that ROS production by ovarian cancer cells promotes angiogenesis and tumor growth through *in vivo* transcriptional activation of *Vegf* and *Hif1a* (72, 73). The above-mentioned studies provide evidence for the protumoral role of ROS in tumor progression, by suppressing T-cell responses, supporting angiogenesis and maintaining the phenotypical identity of MDSCs, regardless of the strong pathogen-killing potential of the respiratory burst in mature myeloid cells.

Release of Antibacterial Peptides as Tumor Promoter

In addition to ROS, myeloid cells release a vast array of antimicrobial peptides such as defensins and cathelicidins, representing two major families of mammalian antibacterial peptides. In leukocytes, α- and ß-defensins are stored in cytoplasmic granules that fuse with the phagosome upon microbe phagocytosis, while epithelial cells can secrete defensins to maintain their barrier integrity (74). Yang et al. demonstrated that ß-defensins act as a chemoattractant for immature DCs and memory T cells by binding chemokine receptor CCR6, which bridges the innate recognition of microbes and the initiation of an adaptive immune response (75). As such, it is not surprising that in a similar fashion immature DCs are recruited to the TME in response to tumor-derived ß-defensins. Indeed, Conejo-Garcia et al. discovered a subset of immature DCs, that is recruited to murine and human ovarian tumors in response to ß-defensins through CCR6 signaling and that acquires epithelial features, including surface expression of CD31 and VE-cadherin. These cells support vasculogenesis in a VEGFR-2-dependent manner which leads to enhanced tumor progression (76). CCR6 signaling also promotes murine transplantable colon cancer by recruiting macrophages to the TME through a CCL2-CCR6 axis, which results in the release of IL-1β, IL-6, and TNFα, further enhancing tumor progression (77).

Holterman et al. reported that α-defensins overexpressed by cancer cells, stimulate the proliferation and migration of bladder cancer cell lines *in vitro*, most-likely in an autocrine and calcium-dependent manner (78). Similarly, Xu et al. showed that human ß-defensin 3 promoted *in vitro* proliferation, migration and invasion of cervical cancer through the NF-κB signaling pathway, demonstrating that cancer cells are also able to release defensins (79). It is important to note that defensin-secreting cancer cells are of epithelial origin, since epithelial cells are known to secrete defensins as part of their barrier function. In addition, it should be remarked that the role of defensins in tumor progression also seems ambiguous and may vary according to the cancer type or defensin molecule, as several studies showed a potential antitumoral role of defensins in cancer (80, 81).

The release of cathelicidins, human LL-37 and murine CRAMP, in the TME has been described in several studies, whereby macrophages and neutrophils are the main sources. Li et al. demonstrated that $CD68^+$ macrophages in tumor tissue of colorectal cancer patients stained positive for cathelicidin, whereas weak to unmeasurable signal was picked up for cathelicidins in colon epithelial cells (82). The importance of cathelicidins in tumor progression was demonstrated by a slower tumor growth in Lewis lung carcinoma-bearing, cathelicidin-deficient mice, along with a reduced infiltration of myeloid cells (83). These studies suggest that cathelicidins are chemoattractants that recruit myeloid cells to the TME (84), where, in turn, myeloid-derived cathelicidins directly enhance cancer-cell proliferation, creating a self-sustaining loop of cathelicidin production. In contrast, antitumoral roles of cathelicidins, independent of myeloid cells, have also been described. For example, cathelicidins could be involved in potentiating the cytotoxic capacity of tumor-infiltrating NK cells (85) and impairing the tumor-supportive role of cancer-associated fibroblasts (CAFs) in colon cancer (86).

Neutrophil Degranulation as Tumor Promoter

Neutrophils carry heterogenous primary, secondary and tertiary granules that contain different enzymes and modulatory proteins, such as elastase, gelatinase, MPO, cathepsins, ficolin-1, and lactoferrin (87). Neutrophil degranulation occurs in a calcium-dependent manner in response to proinflammatory mediators like TNFα (88), lipopolysaccharides (LPS) (89) and IL-8 (90). The majority of neutrophil-derived granule contents promote tumor progression, such as elastase, cathepsin D, cathepsin B, and proteinase 3.

Neutrophil-derived elastase hydrolyses insulin receptor substrate-1 (IRS1) in the cytosol of lung cancer cells, leading to an altered regulation of phosphoinositide 3-kinase (PI3K). IRS1 degradation indirectly increases the interaction between the p85 protein of PI3K and platelet-derived growth factor receptor (PDGFR), which enhances cancer-cell proliferation through signaling downstream of the PDGFR (91). Elastase released by PMN-MDSCs in lymphangioleiomyomatosis patients, a condition where estrogen-sensitive metastatic tumors grow in the lungs, stimulate the proliferation, migration and invasion of these tumor cells *in vitro* (92). Cathepsin-D stimulates cancer-cell proliferation as well, but also stimulates tumor angiogenesis and could protect cancer cells from apoptosis (93). Hepsin, a transmembrane serine protease involved in cell motility

and shape, is degraded by the proteasome through cathepsin D-stimulated ubiquitination (94). By downregulating hepsin, cathepsin D contributes to enhanced migration and invasion of breast cancer. Cathepsin B cleaves cell cycle inhibitor $p27^{Kip1}$ in the lysosomes of colorectal cancer cells, which contributes to tumorigenicity and metastasis of colorectal cancer cells (95). Extracellular matrix (ECM) and intracellular collagen IV can be degraded by cathepsin B, stimulating tumor invasion, metastasis, and the formation of vessel-like structures *in vivo* (96).

Although proteinase 3 can be secreted by myeloid cells, neutrophils carry a membrane-bound proteinase 3 that seems to play a role in cellular interactions. Neutrophils in acute myeloid leukemia inhibit T-cell proliferation in a contact-dependent manner. Antibody-based blockade of membrane-bound proteinase 3 on the surface of neutrophils partially restores proliferation of $CD4^+$ and $CD8^+$ T cells (97). The resulting signaling cascade caused by the interaction between membrane-bound proteinase 3 on neutrophils and receptor for advanced glycation end-products (RAGE) on prostate cancer cells promotes tumor-cell migration and metastasis to the bone barrow, independent of the proteolytic activity of proteinase 3 (98). Combined efforts of neutrophil elastase, cathepsin G and proteinase 3 activate progelatinase A, that degrades the extracellular matrix followed by the subsequent release of growth factors, tumor-cell invasion and angiogenesis in the TME (99). In conclusion, the majority of enzymes released or upregulated upon neutrophil degranulation can remodel the extracellular matrix, which stimulates tumor-cell invasion, metastasis and tumor growth, but also promotes tumor angiogenesis.

Neutrophil Extracellular Trap Formation (NETosis) as Tumor Promoter

Neutrophil extracellular traps (NETs) are extracellular strands composed of granule content and nuclear fragments that entrap and kill bacteria through granule proteases and DNA histones (100, 101). Various studies have demonstrated that the formation of NETs is ROS-dependent (64, 102, 103), but can also occur through CXCR2 signaling during chronic inflammation, and through TLR2 and C3 signaling (101, 104). A study unraveling the role of high sensitivity troponin T (hsTnT) plasma levels in the onset of ischemic stroke, revealed an unexpected high prevalence of cancer among patients with elevated hsTnT plasma levels in the post mortem analysis (105). In these patients, the elevated hsTnT plasma level was associated with an increased plasma level of NET-associated citrullinated histone H3, a marker for NETosis, as well as increased plasma levels of G-CSF and coagulation factors. This study demonstrates that NETosis can take place in cancer patients with elevated citrullinated histone H3 levels (105). In fact, tumor-derived G-CSF primes neutrophils to form NETs, which could also contribute to a systemic, prothrombic state in these cancer patients (105, 106).

Furthermore, a study by Miller-Ocuin et al. correlated circulating neutrophil DNA, resulting from NETosis, to the cancer stage of pancreatic ductal adenocarcinoma patients (107). They demonstrated that neutrophil DNA activates pancreatic stellate cells that support tumor progression, and propose that NET DNA acts as a DAMP capable of stimulating tumor progression (107). In patients that underwent major liver resection of metastatic colorectal cancer, in which ischemia and reperfusion is inevitable, NET formation was increased compared to cancer patients that underwent minor liver resection, in which ischemia and reperfusion is limited, demonstrating that surgery-induced stress promotes NET formation (108). These authors further demonstrated that NETs in the liver provide an anchoring site for circulating cancer cells, that supports metastases and cancer-cell growth after resection of the primary tumor. Hence, NETs may support tumor progression through various mechanisms.

Surrounding macrophages deal with the aftermath of NETosis by digesting cellular debris. Interestingly, M1-like macrophages have been shown to release uncoiled or uncondensed DNA upon interaction with NETs *in vitro*, suggesting a possible contribution to NETosis through their own form of extracellular trap formation (METosis) (109). It remains to be seen whether such a mechanism contributes to the tumor-promoting effects of macrophages.

Complement Activation as Tumor Promoter

Complement is an innate defense mechanism that detects and eliminates pathogens from the circulation and tissues, clears cellular debris and stimulates adaptive immunity.

Complement activation through the classical, alternative or lectin-mediated pathway ultimately results in the formation of a cytolytic membrane attack complex (MAC) in the membrane of target cells or microorganisms (110) and the production of anaphylatoxins C3a and C5a (111). Anaphylatoxins can be involved in T-cell homeostasis (112, 113) and in the recruitment of granulocytes (114–116), monocytes (117) and DCs (118) to the site of inflammation through chemotaxis via C3a and C5a receptors (C3aR, C5aR).

Aside from anaphylatoxin production during complement activation, opsonin C3b and its cleavage products (iC3b, C3c, C3d) are deposited on the surface of target cells or microorganisms, when C3 is cleaved by C3 convertase (119). Myeloid cells express complement receptors that bind C3-derivatives, leading to phagocytosis, cell-cell adhesion and adhesion to the extracellular matrix (120). Complement can also steer the adaptive immunity by activating B and T cells through combined engagement of complement receptors and the B-cell receptor or TCR, respectively (121).

Overall, the resulting effector mechanisms of complement activation are (1) cell-mediated phagocytosis (complement-dependent cellular phagocytosis or CDCP) and (2) cytotoxicity (complement-dependent cellular cytotoxicity or CDCC), initiated by the interaction between opsonized target cells or microbes and CR-expressing myeloid cells, as well as (3) complement-dependent cytotoxicity (CDC) through the formation of the MAC in the membrane of target cells or microorganisms, and (4) the recognition and clearance of dying cells (122) (**Figure 3**). However, distinguishing the different effector mechanisms that contribute to cancer-cell eradication as a result of complement activation remains challenging up to now. Furthermore, complement-induced cytolytic effector

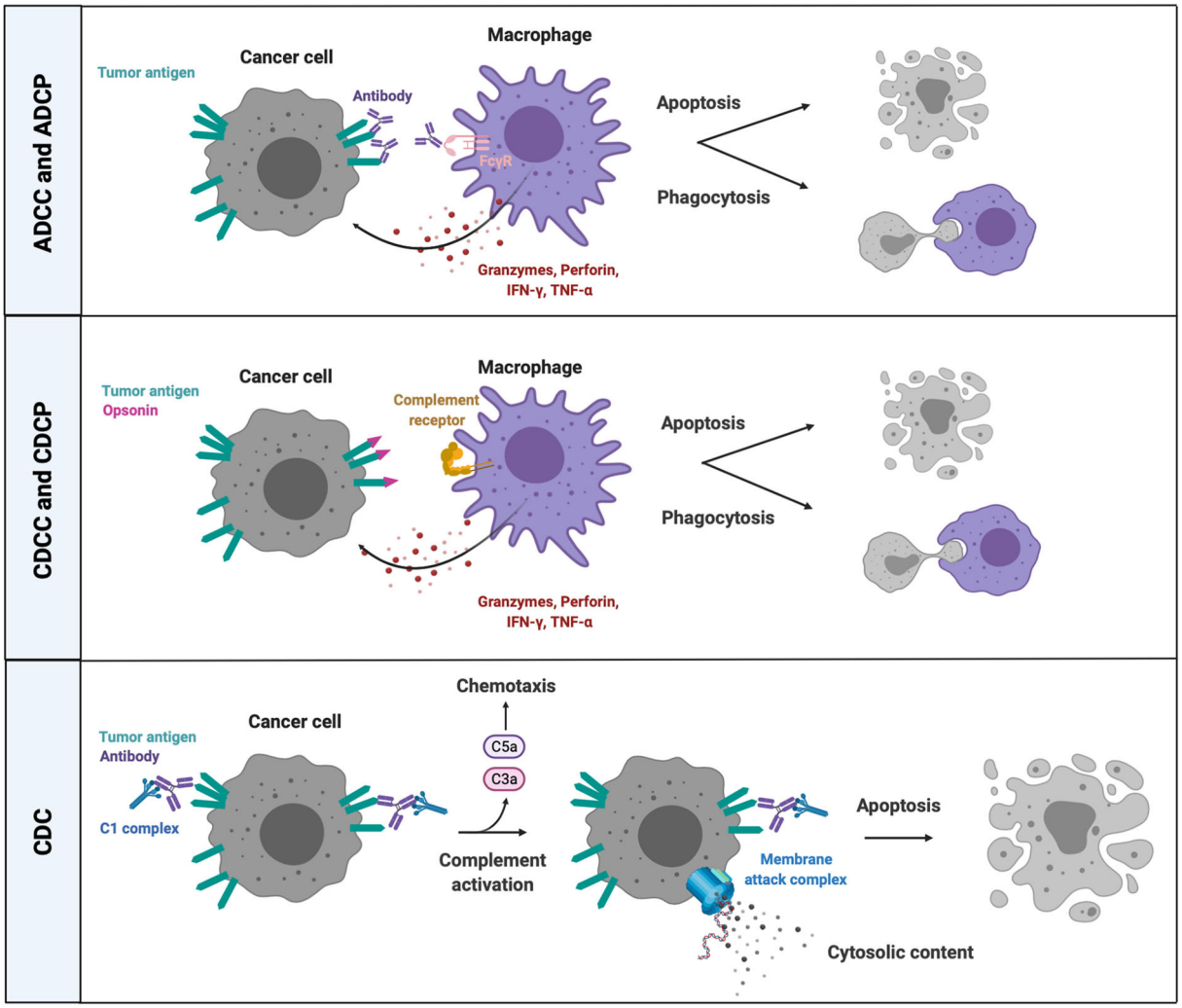

FIGURE 3 | Cell-dependent and -independent effector mechanisms of complement activation and FcR-mediated killing. Complement factor- and antibody-opsonized cancer cells can be eliminated through cell-dependent and cell-independent effector mechanisms. CRs and FcRs on phagocytes bind opsonins and antibodies, respectively, on the surface of targeted cancer cells, followed by phagocytosis and/or release of lytic enzymes (granzyme B, perforins) and proinflammatory mediators (TNFα, IFNγ). The classical pathway of complement activation mediates a cell-independent form of lytic cell death by introducing a MAC in the membrane of antibody opsonized target cells that are recognized by complement C1 complex. ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; CDC, complement-dependent cytotoxicity; CDCC, complement-dependent cell-mediated cytotoxicity; CDCP, complement-dependent cell-mediated phagocytosis; CRs, complement receptors; IFNγ, interferon gamma; TNFα, tumor necrosis factor alpha; C5a, complement factor 5a; C3a, complement factor C3a; FcγR, crystallizable fragment receptor gamma; C1, complement factor.

mechanisms on the surface of host cells is prevented through the expression of complement regulatory proteins (CRPs), such as CD46, CD55, CD59 and factor H. Several cancer types overexpress CRPs and make use of this defense mechanism against complement-induced cytolysis (123–125), whereas downregulation or blockade of CRPs sensitizes cancer cells to complement- and antibody-mediated cytotoxicity (126, 127).

Complement Anaphylatoxins as Tumor Promoters

Complement activation has been reported to promote tumor progression through the recruitment of immune suppressive macrophages, MDSCs and neutrophils, while on the other hand, there are also reports of its capacity to stimulate antitumoral T-cell responses and the recruitment of NK cells (128–130). Recruitment of MDSCs in response to anaphylatoxins has been demonstrated in several studies (130–132). Markiewski et al. revealed that aside from increased recruitment of MDSCs to the tumor in response to C5a, the latter also enhances the production of ROS and RNS in MDSCs via C5aR signaling (132). As mentioned earlier, ROS and RNS release by MDSCs in the TME abrogates antigen recognition by CTLs and instead induces tolerance (67, 68). Moreover, C5a is also implicated in the formation of new blood vessels. Corrales et al. demonstrated that human umbilical vein endothelial cells treated with C5a form vessel-like structures. They further elaborated on the vessel-like structures in a murine 3LL lung cancer model, where they

showed that the number of newly formed microvessels in the tumor is reduced upon C5aR antagonism (133). While the role of C5a in cancer progression has been extensively studied, less is known about the implication of C3a in cancer. In the absence of C3aR signaling, murine B16 melanoma tumor growth is reduced, along with an increased tumor infiltration of $CD4^+$ T cells and neutrophils (134). Similar results were observed in orthotopic mouse models of lung cancer (CMT167, LLC), where flow cytometry and immunohistochemistry analysis revealed an increased abundance of activated $CD4^+$ and $CD8^+$ T cells in tumors grown in C3-deficient mice (135). Interestingly, depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, restored tumor growth in C3-deficient mice.

Tumor-infiltrating macrophages and neutrophils also carry the potential to suppress the detrimental effects of complement activation through IL-1ß-induced expression of pentraxin 3 (PTX3) (136). Surface-expressed PTX3 recruits complement factor H that inhibits the C3 cleavage upstream of the complement cascade and prevents complement-induced inflammation and recruitment of immunosuppressive myeloid cells to the TME. However, *Ptx3* is epigenetically silenced at the gene level in murine and human colorectal cancer through hypermethylation (136). Altogether, the above-mentioned studies provide evidence for the role of complement in cancer that seems to promote tumor progression by recruiting MDSCs to the tumor, reducing the infiltration of activated $CD4^+$ T cells and stimulating new vessel formation. The effect of anaphylatoxins on tumor-infiltrating CTLs remains unresolved, whereas several studies highlight the importance of $CD4^+$ T cells in response to anaphylatoxins.

Complement in Cancer Immunotherapy

Despite the intrinsic protumoral functions of complement in cancer, it should not be forgotten that complement can be useful in the context of antibody-mediated cancer immunotherapy. Indeed, the classical pathway of complement activation, initiated by antibody-opsonized target cells, is one of the effector mechanisms of therapeutic monoclonal antibodies (mAb) (137, 138). This was demonstrated in a study by Lee et al., who designed therapeutic mAbs capable of discerning complement-mediated and Fc receptor (FcR)-mediated killing mechanisms (139). Aglycosylated, anti-CD20 IgG1 mAb, engineered with a C1q-selective Fc-part that does not bind FcRs, demonstrated similar potency in clearing $CD20^+$ Raji and Ramos lymphoblastic cells compared to antibodies that rely on FcR-mediated functions (139). Along the same line, the therapeutic anti-CD20 mAb Rituximab at least partially relies on the classical complement activation pathway for destruction of neoplastic $CD20^+$ B cells (140). However, the release of proinflammatory mediators (IL-6, TNFα) and degranulation by granulocytes in response to complement anaphylatoxins contribute to the toxic side effects of anti-CD20 therapy, such as fever, dyspnea, chills and flushes (141). Similarly, the *in vivo* effector functions of Cetuximab, an anti-EGFR mAb, have been attributed to complement activation in several murine models of non-small cell lung carcinoma (142). However, it should be remarked that the efficacy of mAb-mediated complement activation is likely to be cancer type-dependent and may be influenced by the characteristics of the cancer cells and/or factors present in the tumor microenvironment.

Moreover, the efficacy of antibody-based therapy, that relies on the cytotoxic effector mechanisms of complement and FcR-mediated cytotoxicity, is restricted by the limited availability of suitable antigens for therapeutic targeting. In addition, the dual role of complement in cancer must be taken into account when using complement as an effector mechanism of antibody-based therapy. It appears that complement can promote tumor growth through high C5a concentrations, sublytic MACs levels and high CRP levels on the surface of cancer cells, while intermediate concentrations of C5a, increased MAC formation in the membrane of cancer cells and low surface expression of CRPs could eliminate cancer cells (129, 143). Future therapeutic strategies should take this delicate balance between tumor promotion and tumor eradication into account.

FcR-Mediated Killing

When the Fc part of an antibody interacts with cognate surface-expressed FcRs, this may result in ADCC, ADCP, antigen presentation, degranulation and an altered cytokine production profile (**Figure 3**) (144). NK cells are thought to be the main effector cells of ADCC, yet studies have shown that antibody-based cellular destruction mechanisms can also take place in the absence of NK cells (145). The relevance for therapeutic mAbs is shown by mice deficient in the common gamma chain of the FcγR. These mice do not engage ADCC or ADCP in the presence of Trastuzumab and Rituximab (145, 146). Members of the mononuclear phagocyte system, including monocytes and macrophages, are responsible for the working mechanism of Rituximab (145). Indeed, CD20-targeted B-cell depletion seems to be dependent on FcγRI and FcγRIII expressed by monocytes and macrophages and is absent in colony stimulating factor 1-deficient mice, which lack tissue macrophage subsets (145). Biburger et al. discovered a murine subset of $Ly6C^{low}$ non-classical monocytes capable of autoantibody-mediated platelet depletion and antibody-dependent B-cell depletion via ADCC and ADCP mediated by FcγRIV, a low affinity FcγR that is not expressed by NK cells or tissue-resident macrophages (147). Human $CD16^+$ (FcγRI) monocytes similarly perform ADCC, almost as efficiently as NK cells. TNFα release by these $CD16^+$ monocytes upregulates type 2 beta integrins (CD11a, CD11b), which facilitate the interaction between $CD16^+$ monocytes and antibody-coated cancer cells (148). The number of murine B16 melanoma metastases in the lung of FcγRIIb-deficient mice significantly decreased when treated with a mAb targeting melanoma differentiation antigen gp75 (146). FcγRIIb is an inhibitory Fc receptor which is not expressed by NK cells. Therefore, an enhanced ADCC response cannot be attributed to increased NK-cell activation in FcγRIIb-deficient mice and is likely monocyte/macrophage-mediated. Moreover, a synergistic effect was observed when combining FcγRIIb deficiency and a therapeutic mAb against mouse and human HER2 (4D5, Trastuzumab) (146).

However, not all FcR-mediated effects are beneficial in the context of mAb-mediated therapy. For example, phagocytosis of

antibody-opsonized cancer cells by TAMs was shown to activate the inflammasome AIM2, which results in the subsequent release of IL-1ß, hence increasing PD-L1 surface expression and cytosolic IDO production in TAMs (149). As a result, TAMs that underwent ADCP display an immunosuppressive phenotype, which is relieved upon PD-L1 and IDO blockade (149). Furthermore, *in vivo* imaging by Arlauckas et al. (150) demonstrated that PD-1-negative TAMs take up anti-PD-1 antibodies that were initially bound to PD-1$^+$ CTLs, in an FcR-mediated way. Hence, TAMs could serve as a sink for anti-PD-1 antibodies and possibly also other mAbs, strongly diminishing the efficacy of mAb-dependent therapies such as immune checkpoint blockade (150).

ENTANGLED NETWORK OF INNATE RESPONSES

Innate immune responses are often regarded as the default first-line defense responses, that become less significant once a more complex, adaptive and antigen-directed response is initiated. With this review, we provide evidence for the detrimental effects of innate effector mechanisms performed by myeloid cells during cancer development and progression. Noteworthy, effector mechanisms that are initially deployed by innate myeloid cells, such as ROS production, release of inflammatory mediators and response to PRR signaling, can be adopted by cancer cells. However, contradicting literature studies are available on the role of several innate defense mechanisms in cancer, and this duality between tumor-promoting and -eradicating roles seems to be linked to the presence of persisting, tumor-associated inflammation. Inflammation is required to mount anti-tumor immune responses, while chronic tumor-associated inflammation promotes tumor progression. This duality can even be extended to the response of so called "hot tumors" and "cold tumors" to immunotherapy. Interestingly, mAb therapy targeting immune checkpoints seem to be effective in "hot tumors," abundantly infiltrated by T cells, whereas "cold tumors" that lack proper T-cell responses remain largely unresponsive to mAb therapy (151). Cold tumors, however, are still infiltrated by myeloid cells, that create an immune suppressive environment, which impedes T-cell infiltration and tumor eradication. Therefore, innate defense strategies might play a more important role in cancers with an inflammatory nature or origin, for example in organs like the liver, stomach, lungs and skin due to alcohol abuse, *H. pylori* infection, tobacco and asbestos, UV irradiation and even obesity. In any case, due to the abundance of tumor-infiltrating myeloid cells in multiple solid tumor types, their effector mechanisms should be investigated in depth and exploited in cancer therapy, perhaps alongside T-cell stimulatory immunotherapy to improve therapy outcome.

AUTHOR CONTRIBUTIONS

EL has conceptualized, written, reviewed, and edited the content of this review, with contributions from SA, PB, and MK. JV and GR contributed to the conceptualization and reviewing of this review. All authors contributed to the article and approved the submitted version.

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microRNAs Shape Myeloid Cell-Mediated Resistance to Cancer Immunotherapy

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Immunotherapy with immune checkpoint inhibitors can achieve long-term tumor control in subsets of patients. However, its effect can be blunted by myeloid-induced resistance mechanisms. Myeloid cells are highly plastic and physiologically devoted to wound healing and to immune homeostasis maintenance. In cancer, their physiological activities can be modulated, leading to an expansion of pro-inflammatory and immunosuppressive cells, the myeloid-derived suppressor cells (MDSCs), with detrimental consequences. The involvement of MDSCs in tumor development and progression has been widely investigated and MDSC-induced immunosuppression is acknowledged as a mechanism hindering effective immune checkpoint blockade. Small non-coding RNA molecules, the microRNAs (miRs), contribute to myeloid cell regulation at different levels, comprising metabolism and function, as well as their skewing to a MDSC phenotype. miR expression can be indirectly induced by cancer-derived factors or through direct miR import via extracellular vesicles. Due to their structural stability and their presence in body fluids miRs represent promising predictive biomarkers of resistance, as we recently found by investigating plasma samples of melanoma patients undergoing immune checkpoint blockade. Dissection of the miR-driven involved mechanisms would pave the way for the identification of new druggable targets. Here, we discuss the role of these miRs in shaping myeloid resistance to immunotherapy with a special focus on immunosuppression and immune escape.

Keywords: microRNAs, myeloid-derived suppressor cells, immunotherapy, immune checkpoints, therapy resistance, extracellular vesicles

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INTRODUCTION

Myeloid cells are involved in inflammatory processes, including cancer, and their accrual to the tumor microenvironment (TME) leads to immunosuppression and angiogenesis, thereby promoting tumor growth. Thanks to their plasticity, they are acknowledged cancer allies, negative prognostic factors, and pharmacological targets. Low/negative HLA-DR expression (1) defines monocytic myeloid-derived suppressor cells ($CD14^{+}HLA\text{-}DR^{low/neg}$; M-MDSCs), which influence cancer aggressiveness and resistance to immune checkpoint inhibitors (ICIs) (2). We focused on myeloid cells for more than a decade and first defined M-MDSCs in melanoma patients (3). We dissected underlying mechanisms via an *in vitro* tumor extracellular vesicle (EV)-healthy donor monocyte-MDSC model

and identified a set of causally involved microRNAs (miRs), the "MDSC-miRs." miRs are small non-coding RNAs of ∼22 nucleotides, which modulate biological processes by mostly interacting with the 3′-untranslated region (UTR) of the target messenger RNA (mRNA). An imperfect base-pair interaction induces translational repression, while a perfectly base-paired miR directly cleaves the mRNA (4, 5). However, some miRs can also bind the 5′-UTR of mRNA, upregulating its translation (6). We measured increased MDSC-miR levels in circulating $CD14^+$ cells and lesions of melanoma patients in association with myeloid infiltrates and peripheral blood MDSC accrual (7, 8). Matching of MDSC-miR predicted target genes with EV-MDSC transcriptional profile revealed miR involvement in chemotaxis, adhesion, and differentiation of myeloid cells. The upregulation of MDSC-miRs, including miR-146a, miR-146b, miR-155, miR-125b, miR-100, let-7e, miR-125a, and miR-99b, in baseline plasma predicted resistance to ICIs (8). *In vitro*, MDSC-miR antagonists relieved the suppressive potential of patients' monocytes leading to autologous T cell reactivation. Thus, MDSC-miRs could account for myeloid deregulation, implying an involvement of blood factors in the epigenetic control of MDSC functions. A higher MDSC frequency is associated with poor prognosis, even upon immunotherapy, anticipating a reduced treatment efficacy. Pharmacological MDSC reduction, inhibition of their suppressive activities or promotion of their differentiation are under testing at preclinical and clinical levels (9, 10). The functional roles of miR expression by immune cells remain controversial. In case of MDSC-miRs, their overexpression impacts myeloid cell differentiation and polarization by participating in immunosuppressive pathways. Like other miRs, also MDSC-miRs are detectable in EVs, whose size correlates with M-MDSC frequency (8). Tumor and immune cell EVs attracted interest as reservoirs of functional messages exchanged between adjacent cells in the TME and at a distance. EV membrane guarantees content integrity, enabling safe traveling of proteins, lipids, and genetic material to interaction-prone cells. Major efforts are dedicated to investigate EVs as biomarkers of response or drivers of resistance mechanisms to ICIs.

This review discusses the role of MDSC-miRs in shaping myeloid resistance to immunotherapy with a focus on immunosuppression and escape.

The Role of miRs in Cancer Therapy Resistance

As oncomiRs or tumor-suppressors, miRs can promote or inhibit cancer development. They directly target cell proliferation and apoptosis genes, thus being involved in chemotherapy resistance, drug target deregulation, and drug metabolism mechanisms (11). In immunotherapy, miRs can control the success of ICIs by targeting PD-1 and PD-L1, MHC-antigen presentation machinery, and TLR signaling (12). Among MDSC-miRs, miR-155 suppresses PD-L1 through directly binding the 3′-UTR of PD-L1 in human lymphatic endothelial cells (13). The reduction of PD-L1 expression and the consequent disruption of the PD-L1/PD-1 axis may contribute to sustaining T cell antitumor responses, thereby synergizing with ICIs to improve cancer immunotherapy outcome. This miR contributes essentially to mounting of $CD8^+$ T cell responses by restraining T cell senescence and exhaustion through epigenetic silencing of transcription factors determining their terminal differentiation (14). Moreover, miR-155 expression correlates with TCR stimulation of tumor-infiltrating T cells in melanoma patients (15). The MDSC-miR-146a, 146b, 155, and let-7, bind to the 3′-UTR of TLRs or TLR-associated genes resulting in post-translational TLR signaling repression and inflammatory response modulation (16). Similarly, the MDSC-miR-125a and let-7e regulate the inflammatory response and the IL-10-mediated tolerance to LPS, by targeting the TLR4 pathway in monocytes (17). TLR4 can promote expansion of $PD\text{-}L1^+$ MDSCs, an effect mediated via HSP86-TLR4 signaling pathway activation (18). Since specific miRs can directly activate TLRs expressed at endosomal level (19, 20), MDSC-miRs might target these proteins and contribute to expanding PD-L1 expressing MDSCs. Thus, targeting MDSC-miRs might potentiate ICI-based immunotherapy.

The development of therapeutic antagomiRs and miR mimics have entered phase I and II clinical studies (21). The DNA-single strand antagomiRs are usually designed on first-generation antisense oligonucleotides or modified with locked nucleic acids to reduce the oncomiR activity by competition with the native cancer-suppressing target transcripts (22). MiR mimics are double strand oligonucleotides that enter the native cellular process mimicking pre-miR duplex (23). Cobomarsen (MRG-106), a miR-155 inhibitor, has entered phase I trials to study safety and potential efficacy following local or intravenous administration in lymphoma and leukemia patients (24).

Despite the therapeutic potential of miRs, their delivery remains challenging, due to undesired off-target effects, hindered cell uptake, and short circulation half-life (25). Synthetic nanoparticles (NPs) mediate specific cell uptake and prevent miR clearance (26). In preclinical models, effective miR supply was obtained via neutral lipid emulsion-based approach for miRs of the let-7 family, as well as neutral liposomes and synthetic polyethylenimine-based nanocarriers for miR-145. Lastly, pH low insertion peptide-modified antagomiRs were able to inhibit the oncomiR miR-155 (27). Otherwise, miR-155-loaded NPs can repolarize tumor-associated macrophages (TAMs) from pro-tumorigenic M2 to anti-inflammatory M1-like phenotype, reversing the immunosuppressive TME (28). In clinical setting, NP-based miR manipulation comprises liposomal (DOPC)-encapsulated siRNAs targeting EphA2 in solid tumors (29), bacterial derived nanocells EDVs (EnGeneIC Delivery Vehicle), or TargomiRs, for miR-16 mimic delivery (30). EVs may be also suitable for miR delivery (31). Healthy donor plasma miR-loaded EVs promoted apoptosis in HCC cells (31), while miR-sponge engineered EVs reduced glioblastoma volume in rats (32). Finally, the natural exchange of endogenous miRs between immune cells, such as miR-155 and miR-146a carried by dendritic cell EVs, controls inflammatory gene expression or promotes

apoptotic cell clearance, as in case of endothelial cell EVs transferring miR-125a to macrophages (33).

Specific miR inhibition is accomplished by Small Molecule Inhibitors of miRs (SMIRs), which target synergistically tumor cells and oncomiRs, such as linifanib. This VEGF- and PDGF-receptor tyrosine kinase-inhibitor effectively inhibits the oncogenic function of miR-10b in preclinical cancer models (34). Finally, several miRs are related to tumor radioresistance management, where, thanks to the inhibition of ATM protein, they can modulate DNA damage response sensitizing tumor cells to radiotherapy (35).

Epigenetic Regulation of Immune Cell Functions by MDSC-miRs

The upregulation of miR-146a, miR-146b, miR-155, miR-125b, miR-100, let-7e, miR-125a, and miR-99b can skew immune cells into inhibitors of response to immune and other cancer therapies (**Table 1**). Of note, five miRs out of eight show a coordinated expression pattern due to their transcription as clusters. The miR-125a∼99b∼let-7e cluster is hosted in the first intron of the long non-coding RNA NCRNA00085, whereas miR-125b, miR-100, and let-7a are hosted in MIR100HG (17, 58). A clear association of miR-125a∼99b∼let-7e cluster and acquisition of a myeloid immunosuppressive phenotype has been demonstrated (17, 36, 59, 60). In particular, stimulation of monocytes with GM-CSF, IL-4, and R848 TLR7/8 agonist upregulates the miR-125a∼99b∼let-7e cluster, activates STAT3, and induces the acquisition of an immunosuppressive phenotype. Conversely, the depletion of the cluster reverses immunosuppressive functions and MDSC phenotype hallmarks, by downregulating PD-L1 and IDO, while increasing HLA-DR expression. This contributes to STAT3 stabilization through downregulation of TRIB2, a suppressor of MAPK signaling, and SOCS1, a key regulator of cytokine signaling and STAT3 inhibitor. The miR-125a∼99b∼let-7e cluster is negatively regulated by IFNγ, while it is induced by STAT3 and SMAD3, in turn activated by IL-10 and TGFβ immunoregulatory cytokines. miR-125a and let-7e also exert their anti-inflammatory activity by targeting the TLR signaling pathway molecules TLR4, CD14, and IRAK1, leading to decreased pro-inflammatory cytokine release by myeloid cells (17, 59).

MIR100HG and its encoded miR-125b and miR-100 are induced by TGFβ, the main cytokine released by M2 macrophages (61). TGFβ promotes cancer epithelial-to-mesenchymal transition (EMT) through MIR100HG induction and SMAD2/3 transcription factor activation. The dysregulation of this cluster is also causally linked with drug resistance in several tumor types (58, 62). In immune cells, miR-125b expression is usually linked to antitumor M1-like macrophages, whereas in T cells it inhibits CD4 T cell differentiation and γδ T cell activation (37). In contrast, little is known about miR-100 expression and function in immune cells. In regulatory T cells (Tregs) increased levels of the edited variant of miR-100 changes its target gene from MTOR to SMAD2, resulting in limited differentiation and increase of Treg plasticity (38).

MDSC-miRs and Response to Immunotherapy

Under physiological conditions the miR-146 family (miR-146a and miR-146b) and miR-155 actively control innate immunity, whereas in cancer these miRs have gained attention for their deregulation and acquisition of oncogenic roles. Both are transcriptionally regulated by NFkB, but with opposite functions: miR-146 represents the anti-inflammatory and miR-155 the pro-inflammatory counterpart. miR-146a/b act as negative feedback regulators of TLR signaling through inhibition of the NFkB pathway by downregulation of TRAF6 and IRAK1 (63), thereby dampening the production of pro-inflammatory mediators (64). On the other hand, miR-146b is also induced by TLR4 signaling via an IL-10-mediated STAT3-dependent loop (65), and it inhibits macrophage activation by targeting IRF5 (39). miR-146a is an essential regulator of immune cell activation and malignant transformation (64), and knockout mice are affected by chronic NFkB dysregulation and myeloid malignancies (40, 41). Several studies proposed miR-146a as an immunotherapeutic target: its overexpression reduces the metastatic potential of breast cancer (BC) cell lines through NFkB inhibition (42), whereas it supports the M2-like phenotype of TAMs in endometrial cancer (43). In a preclinical model of HCC, miR-146a inhibition alters the STAT3 activation-associated cytokine profile improving the anti-tumor effect of lymphocytes (44). Mastroianni et al. identified miR-146a as a central negative regulator of the STAT1/IFNγ axis, affecting migration, proliferation, and inducing PD-L1 expression. Combined PD-1 blockade and miR-146a antagomiR improve survival of melanoma-bearing mice (45). We found that high miR-146a levels, concomitantly with the other MDSC-miRs, are associated with MDSC induction and ICI resistance (8). In myeloid leukemia, miR-146a mimics can inhibit tumorigenic NFkB activity (46). Finally, miR-146a is also involved in ICI-mediated immune-related adverse events (irAEs), as shown by knockout mice exhibiting increased T cell activity and inflammation during ICI intake. These effects could be restrained by miR-146a mimics (47).

The pro-inflammatory miR-155 is induced upon TLR/IFNγ stimulation in monocyte/macrophages and drives their response by regulating mRNA targets with inhibitory effects on innate immune cell activation (66). In tumor cells, intrinsic miR-155 mediates pro- or anti-tumor effects (67). Similarly to miR-146a, miR-155 upregulation promotes cell proliferation, colony formation, and xenograft tumor growth in BC models by negative regulation of SOCS1 and SHIP1, leading to constitutive STAT3 activation and pro-tumor inflammation (48). Deficiency of miR-155 can also foster tumor growth through MDSC recruitment and potentiation of their tumor promoting functions, as demonstrated in BC. Here, miR-155 loss in myeloid cells impairs TAM activation, while in tumor cells it stimulates C/EBP-β-mediated cytokine production in turn stimulating tumor-infiltrating MDSCs (49, 50). Similar results were obtained in mouse models of melanoma and lung cancer (51). As for other miRs also miR-155 appears to cover apparently contradictory roles depending on the expressing cell or the setting. Li et al. showed that upregulated miR-155 together with

TABLE 1 | Role of MDSC-miRs in tumorigenesis and response to cancer therapies.

miR	Cells	Expression	Target genes/Pathways	Phenotype	References
miR-125a∼ 99b∼let-7e	Monocytes	↑	TRIB; SOCS1	Immunosuppressive properties mediated by STAT3 activation	(36)
miR-125a and let-7e	Monocytes	↑	TLR4; CD14; IRAK1	↓ Anti-inflammatory activity and cyto/chemokines	(17)
miR-125b	Macrophages	↑	IRF4	Acquisition of M1 phenotype	(37)
	T cells	↑	IFNG; IL10RA; IL2RB; PRDM1	Suppression of $CD4^+$ T cell differentiation	(37)
	T cells	↑	CD107a; TNFA; IFNG	Inhibition of γδ T cell activation	(37)
miR-100	Tregs	↑	SMAD2	↓ Treg differentiation and ↑ plasticity	(38)
miR-146b	Macrophages	↑	IRF5	↓ M1 macrophage and inflammation	(39)
miR-146a	Monocytes	↓	TRAF6; IRAK1	↑ chronic NFkB driving myeloid malignancy	(40, 41)
	Breast cancer	↓	TRAF6/IRAK1	↑ NFkB activity and metastasis	(42)
	Endometrial cancer	↑	NIFK-AS1	↑ M2-like phenotype of TAMs	(43)
	Hepatocellular carcinoma	↑	STAT3	Immunosuppression by ↑TGFβ, IL17, VEGF and ↓type I IFN	(44)
	Melanoma	↑	STAT1/IFNγ axis; PD-L1	Melanoma migration, MDSC promotion and resistance to ICIs	(8, 45)
	MDSCs	↑	NFkB	↓ NFkB-mediated inflammation	(46)
	T cells	↑	IFNγ and perforin	↓ ICI-mediated irAEs severity	(47)
miR-155	Breast cancer	↑	SOCS1/SHIP1	Activation of STAT3 signaling and pro-tumor inflammation	(48)
	Myeloid cells	↓	C/EBP-β	Breast tumor growth by MDSC infiltration and TAM tolerance	(49, 50)
	MDSCs	↓	HIF-1α	↑ MDSC recruitment and function, ↑ solid tumor growth	(51)
	MDSCs	↑	SHIP1	↑ STAT3 activation and expansion of functional MDSCs	(52)
	Colorectal cancer	↑	SOCS1	↑ MDSC activity and tumor growth	(53)
	T cells	↑	SHIP1	↑ IFNγ production, ↑ T cell-mediated antitumor immunity	(54)
	Melanoma	↑	ND	MDSC induction ↑ resistance to immunotherapy	(8)
	T cells	↑	T cell activation markers	↑ T cell response	(55)
	T cells	↑	PRC2/Phf19	↑ cancer immunotherapy by ↑ $CD8^+$ T cell function	(14)
	T cells	↑	TIM3	Cytolytic activity of $CD8^+$ T cells against HCC	(56)
	T cells	↑	ND	↑ antitumor activity of $CD8^+$ T cells	(57)

ND, not defined; ↑, increased; ↓, decreased.

miR-21 led to MDSC expansion, whereas their loss reversed this effect. In particular, by targeting SHIP1 and PTEN these miRs synergistically increase STAT3 activity, promoting MDSCs (52). In this line, loss of miR-155 can enhance antitumor T cell activity by reducing MDSC immunosuppression and tumor infiltration (53). In contrast, miR-155 expression by T cells promotes antitumor immunity and ICIs hinder miR-155-deficiency-induced immune escape (54). We found that miR-146a and miR-155 along with the other MDSC-miRs contribute to MDSC induction (8), suggesting that the expression levels of different miRs can influence the fine-tuning of pro- or anti-inflammatory pathways depending on the cell type. Interestingly, in tumors with high mutational burden, such as melanoma and lung cancer, miR-155 was associated with a strong immune signature and improved clinical outcomes (55). Likewise, miR-155 potentiates immunotherapy through epigenetic regulation of $CD8^+$ T cell differentiation via PRC2/Phf19 signaling (14). Yan and coworkers demonstrated that miR-155-induced downregulation of TIM3, a negative immune checkpoint, enhanced the cytolytic activity of anti-HCC $CD8^+$ T cells (56). Finally, miR-155 overexpression can optimize $CD8^+$ T cell antitumor activity and improve adoptive-transfer in low-affinity antigen tumors (57).

EVs as miR Shuttles and MDSC Modulators

All cell types release EVs, membrane-surrounded structures devoted to intercellular communication. EVs are present in body fluids including plasma, serum, lymph, urine, saliva, tears, and milk (68). Their content of proteins, nucleic acids, lipids, and their stability, make EVs potential biomarkers and therapeutic targets of disease (69, 70). Recent evidence shows the ability of tumor-derived EVs to blunt anti-tumor immunity at multiple levels (71). They can operate within the TME or at a distance by boosting angiogenesis, triggering tumor cell

EMT, activating cancer-associated fibroblasts, and shaping the immune environment toward a condition of immune escape. Tumor EVs can induce myeloid cell dysfunctions and increase MDSC expansion (7, 8, 18, 72, 73). Indeed, EVs derived from melanoma cell cultures as well as those from plasma of melanoma patients contain the MDSC-miRs and promote the acquisition of MDSC characteristics by healthy donors' monocytes (8). The potential of these miRs to induce such dramatic changes might depend on their integrity, protected by the EV membrane, as well as on their way of transfer. In fact, the internalization of whole EVs carrying different miRs or EV receptor-ligand interaction might account for diverse effects (74). In support of our findings, Gerloff et al. (75) found that miR-125b encapsulated in melanoma EVs promotes a TAM phenotype in macrophages through targeting of lysosomal acid lipase (LIPA). In fact, LIPA deficiency stimulates MDSC expansion in mice, and their tumor promoting functions are driven by mTOR pathway overactivation (76).

As a major obstacle to immunotherapy, it is crucial to study MDSC effects in the TME (77). Myeloid EVs may support immune activation or tolerance (78). Like other cells, also MDSCs release EVs, taking part in intercellular communication. Proteomics of MDSC EVs of BALB/c mice bearing 4T1 or 4T1-IL-1β^{+} mammary carcinoma showed a higher expression of 63 pro-inflammatory proteins in 4T1-IL-1β^{+} mice, due to a more inflammatory environment. The MDSC chemotactics S100A8 and S100A9 are abundant in MDSC EVs and polarize macrophages into M2 phenotype (79). These pro-inflammatory proteins are characterized by multiple ubiquitination sites, and MDSC EVs were identified as carriers of enzymes catalyzing ubiquitination (80). Interestingly, EVs from TME-resident MDSC display a stronger immunosuppressive potential than those deriving from spleen or bone marrow MDSCs, suggesting the existence of different phenotypes and functions (81). In contrast, the miR content of MDSC EVs is still elusive, but the dissection could contribute to targeting MDSC EVs release and spreading (82). EVs modulate innate and adaptive immune responses via ligand-receptor interaction or via miRs (83, 84). Indeed, a set of miRs including miR-155, regulate PD-L1 protein expression (85) and induce MDSCs if released via EVs by CLL cells (86). EVs also express immune checkpoints. Actually, PD-L1 carried by EVs was investigated for its role as biomarker and functional inducer of PD-1-mediated immunosuppression (87–90). TIM3 and GAL9 bound to EVs were proposed as prognostic biomarkers in NSCLC patients (91).

Translational Implications

Large scale profiling studies demonstrated the association of specific circulating miRs with certain types of human cancer, proposing miRs as biomarkers (92). Their detection could contribute to early cancer diagnosis, patient stratification, and evaluation of therapy outcomes (93). miRs can be found free or EV-bound in peripheral blood or other body fluids (94). Among

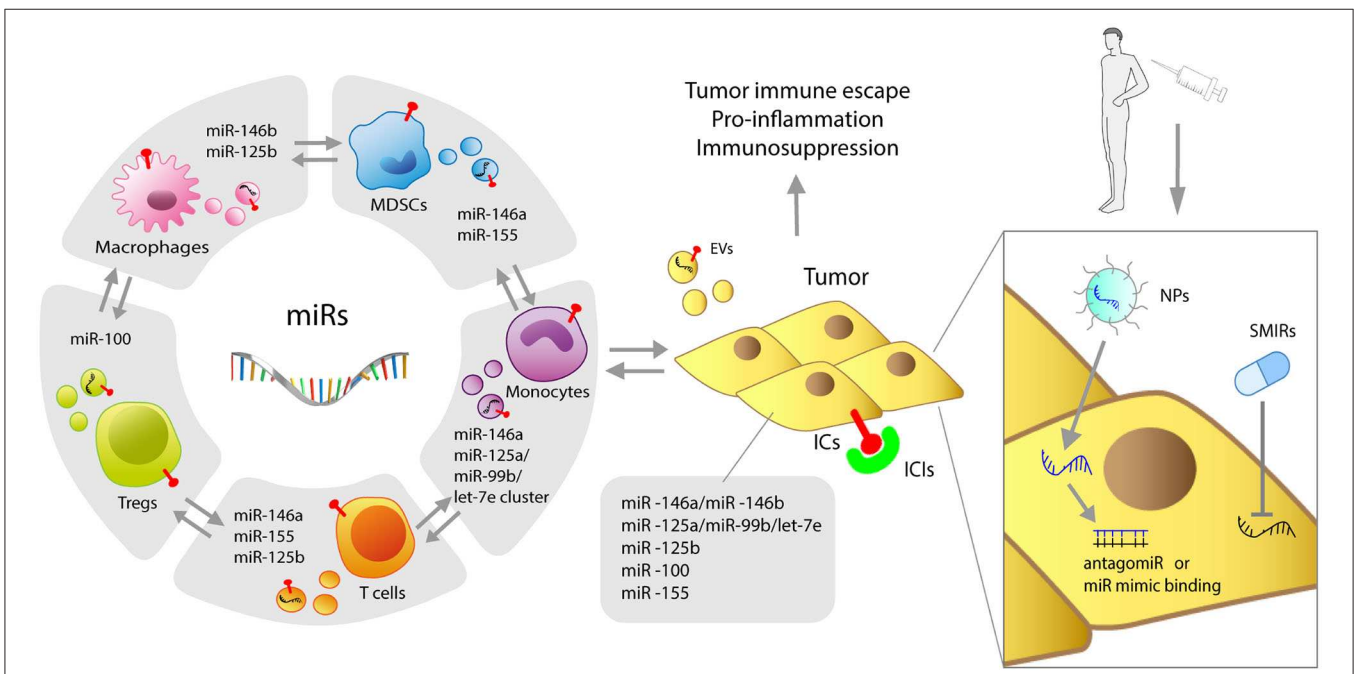

FIGURE 1 | Graphic representation of miRs shaping myeloid resistance to ICIs. Immune and tumor cells, both expressing miRs, mutually interact through miR-loaded EVs. MDSC-derived miRs, including miR-146a, miR-146b, miR-155, miR-125b, miR-100, let-7e, miR-125a, and miR-99b can intervene in cancer progression and interfere with the success of cancer immunotherapy by regulating immune checkpoints (ICs) and different molecular immune targets. Delivery of antagomiRs or miR mimics with NPs, as well as SMIR drugs, represents the current therapeutic strategies to overcome resistance to ICIs induced by miRs. NPs, nanoparticles; SMIR, small molecule inhibitor of miR; ICIs, immune checkpoint inhibitors; EVs, extracellular vesicles.

MDSC-miRs, the miR-125a∼99b∼let-7e cluster was identified as a potential diagnostic biomarker in many tumor types. Colorectal and ovarian cancer patients display lower levels of EV-bound miR-99b compared to healthy controls (95, 96). Dysregulated levels of free circulating let-7e were observed in retinoblastoma, papillary thyroid carcinoma, lung, and prostate cancer (97–100), whereas altered levels of EV-bound let-7 characterize esophageal adenocarcinoma and lung cancer patients (101, 102). miR-125a also represents a potential biomarker of treatment outcome for HCC patients (103) and altered levels of this miR were detected in certain blood malignancies (104, 105), where they predicted response to chemotherapy, as demonstrated in patients with myelodysplastic syndromes (106). In serum, increased miR-146b levels correlate with papillary thyroid carcinoma recurrence (92), while elevated miR-146a is associated with higher overall response rate and survival in NSCLC (107). Furthermore, lower EV-bound miR-146a levels correlate with cisplatin resistance and shorter progression-free survival in NSCLC patients (108). BC patients display high plasma miR-155 levels and in the absence of disease, its increase is associated with treatment failure (109). Interestingly, also urinary miR-155 can be correlated with BC development (93, 110). In NSCLC, an increase of miR-155 in plasma identifies stage I-II patients, implying this miR as diagnostic tool (93, 111), although it was not suitable as a prognostic biomarker (112). miR-155 expression is also related to risk of relapse in colorectal cancer patients and chemoresistance in pancreatic ductal adenocarcinoma, where anti-apoptotic mechanisms are driven by tumor cell exchange of miR-155 containing EVs (93, 113, 114). Concerning MIR100HG, reduced miR-100 expression coincides with diagnosis and prognosis of bladder cancer (115). In contrast, higher circulating miR-100 levels were found in HCC and esophageal squamous cell carcinoma patients and predicted poor survival (116, 117). Lastly, circulating miR-125b was identified as a biomarker of diagnosis and poor prognosis in NSCLC, BC, colorectal, and epithelial ovarian cancer patients, also during chemotherapy or after surgery (118–123).

CONCLUSION

Despite major advances, the role of miRs, including MDSC-miRs, expressed by immune cells remains controversial. For instance, both pro and antitumoral potentials are ascribed to miR-155, depending on its expression levels (124). Of interest is also their interplay: miR-146a$^{-/-}$ mice succumb to chronic inflammation and miR-155 expressed by T cells contributes to shortening lifespan by activating autoimmunity (125). The continuous technical improvement will facilitate in-depth investigations of the finely-tuned mechanisms governing the miR balance, expression levels, and consequent repression/overexpression of target genes to clarify the mechanisms governing myeloid cell dysfunctions and MDSC activity. This will be of major relevance also for cancer therapies. In fact, similarly to SMIRs, also ICIs may induce changes in myeloid MDSC-miR expression potentially related to clinical responses. The complex tumor-immune relationship regulated by miRs and the miR-based therapeutic approaches are summarized in **Figure 1**. Thus, the dissection of therapy-induced miR modulation in immune cells may contribute to decipher and antagonize resistance mechanisms.

AUTHOR CONTRIBUTIONS

All authors wrote and revised the manuscript. LB made the figure. All authors have read and agreed to the submitted version of the manuscript.

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REVIEW
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Myeloid Cells as Clinical Biomarkers for Immune Checkpoint Blockade

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Immune checkpoint inhibitors are becoming standard treatments in several cancer types, profoundly changing the prognosis of a fraction of patients. Currently, many efforts are being made to predict responders and to understand how to overcome resistance in non-responders. Given the crucial role of myeloid cells as modulators of T effector cell function in tumors, it is essential to understand their impact on the clinical outcome of immune checkpoint blockade and on the mechanisms of immune evasion. In this review we focus on the existing clinical evidence of the relation between the presence of myeloid cell subsets and the response to anti-PD(L)1 and anti-CTLA-4 treatment. We highlight how circulating and tumor-infiltrating myeloid populations can be used as predictive biomarkers for immune checkpoint inhibitors in different human cancers, both at baseline and on treatment. Moreover, we propose to follow the dynamics of myeloid cells during immunotherapy as pharmacodynamic biomarkers. Finally, we provide an overview of the current strategies tested in the clinic that use myeloid cell targeting together with immune checkpoint blockade with the aim of uncovering the most promising approaches for effective combinations.

Keywords: myeloid cells, predictive biomarkers, MDSC (myeloid-derived suppressor cell), TAM (tumor-associated macrophage), circulating biomarkers, resistance to immunotherapy, immune checkpoint inhibitors, tumor biomarkers

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INTRODUCTION

Immune checkpoint inhibitors (ICIs) have proven their efficacy in boosting the effector functions of tumor-reactive T lymphocytes against cancer cells. ICI activity is carried out through the specific targeting of negative immune checkpoint molecules or their ligands, expressed on either T cells or myeloid and tumor cells (1). Since 2011, the FDA has approved 7 ICIs (one anti-CTLA-4, three anti-PD-1, and three anti-PD-L1 antibodies) for several indications and many more drugs are in preclinical and clinical development. However, despite the exponential increase in the use of ICIs in the clinic, most patients with advanced cancers still do not respond to these treatments.

It is therefore imperative to understand the mechanisms of action of these drugs to better select responder patients before or during treatment, as well as to design new drugs or combinations that could increase the chances of clinical response and, at the same time, limit the exposure to adverse effects and ineffective therapies for non-responding patients. The importance of reliable biomarkers is progressively recognized for successful clinical trials and for the comprehension of ICI. Most of the understanding for the approved immune checkpoint blockers comes from preclinical experiments and still few clinically validated biomarkers are available.

Not surprisingly, biomarkers are currently mainly focused on T cells and tumor cells, but it is becoming clear that other cell types in the periphery and at the tumor site can impact the efficacy of

immunotherapy. Myeloid cells are among the "usual suspects," given their plasticity and their well-known role as immune modulators in tumor growth and metastasis (2–5). The modulation of ICI response by cells of the myeloid lineage is currently being examined, mainly at the preclinical level. Exploratory biomarkers in recent clinical trials have confirmed the necessity to take into account the presence of these cells for the selection of patients that could benefit from immune checkpoint blockade and the design of ICI combinations with myeloid-targeting agents (6–8).

Given the challenging translation of preclinical results into the clinical setting, especially for the phenotypic description of cell subsets, in this review we to focus on the clinical evidence of the predictive value of myeloid cells, both at baseline and during treatment, in response to the approved ICIs. An overview of the myeloid biomarkers that will be described can be found in **Figure 1** and **Supplementary Table 1**. In addition, we report some promising clinical results of ICI combinations with myeloid-targeting drugs, highlighting the importance of modulating these cell players for successful immunotherapy.

BIOMARKERS IN ICI THERAPY

Biomarkers are molecular or cellular parameters, measured in fluids and tissues, that give information about the disease, the condition of the host, the prognosis and the response to a treatment. In the context of a clinical trial, several types of biomarkers can be studied: *prognostic biomarkers*, that give information about the outcome of patients irrespective

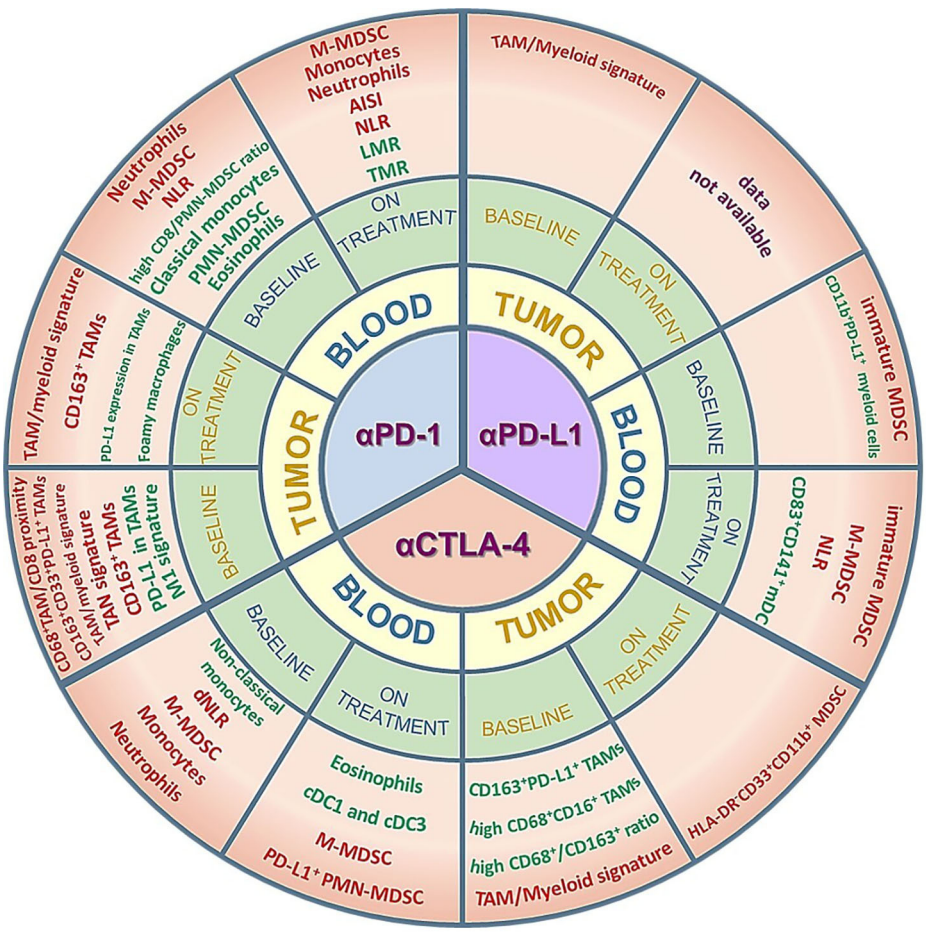

FIGURE 1 | Myeloid cell subsets as potential predictive biomarkers in ICI-treated patients. The figure summarizes the clinical data on circulating or tumor-infiltrating myeloid cells that are described as predictive of response/improved survival (green) or resistance/worse survival (red) in cohorts of patients treated with anti-PD-1, anti-PD-L1 or anti-CTLA-4 antibodies. *Positive predictors* (green) are myeloid subsets whose amounts are either higher than a specific cut-off value and associated to response/improved survival or lower than a specific cut-off value and associated to resistance/worse survival. Conversely, *negative predictors* (red) are myeloid subsets whose amounts are either higher than a specific cut-off value and associated to resistance/worse survival or lower than a specific cut-off value and associated to response/improved survival. The myeloid subsets are described in more detail in the main text and in the **Supplementary Table 1**. AISI, aggregate index of systemic inflammation = platelet count x AMC x NLR; NLR, neutrophil-to-lymphocyte ratio; dNLR, derived neutrophil-to-lymphocyte ratio; LMR, lymphocyte-to-monocyte ratio; TMR, Tregs to Lox-1+ PMN-MDSCs ratio; TAM, tumor-associated macrophages; TAN, tumor-associated neutrophils; M- or PMN-MDSC, monocytic- or polymorphonuclear-myeloid-derived suppressor cells; mDC, myeloid dendritic cells; cDC, conventional dendritic cells.

of the treatment, *predictive biomarkers*, that estimate whether an individual is likely to respond to a specific treatment, *pharmacodynamic biomarkers*, that evaluate the impact of a drug on its target and on disease progression, and *safety biomarkers,* that can rapidly alert on the toxicity of a therapy (9–11).

In this review we chose to focus on potential predictive and pharmacodynamic biomarkers, given their relevance for translational medicine. Predictive biomarkers can be measured at baseline or during treatment, helping in the selection of patients that can most benefit from a treatment or in the rapid adaptation of the therapy, respectively. This rational selection reduces the risk of exposing non-responding patients to adverse effects. Pharmacodynamic biomarkers allow to understand if patients are responding to the administered drug and to shed light on the mechanism of action of ICIs and their impact on the tumor microenvironment (TME) and the immune system. They thus help in a rapid assessment of response and can guide the choice of combinations.

However, as some authors have pointed out, the formal distinction between predictive and prognostic biomarkers requires randomized trials with two treatment arms, where the effect of a biomarker is evaluated in both the treatment and the control group (12). As many of the studies reviewed here comprise early phase clinical trials with only one arm of treated patients, we cannot exclude the possibility that the described predictive biomarkers might be instead prognostic or both predictive and prognostic. We thus propose to consider them as potentially predictive, unless otherwise specified, but we also recommend to formally confirm the predictive value of these biomarkers in ICI therapy through two-arm randomized trials.

Biomarkers can be broadly divided into *circulating* (non-invasive and measurable in the blood) and *tumor* biomarkers. Since immunotherapy can be accompanied by significant toxicities, high costs and the complexity of obtaining biopsies, the development of complementary approaches, like non-invasive biomarkers, is fundamental to maximize the therapeutic efficacy and the success of clinical trials. *Circulating biomarkers* ensure a finer follow-up of patients at baseline, during and after treatment, permitting the early detection of relapse or resistance and the rapid adjustment of therapy (13, 14). Different biomarkers, such as circulating tumor DNA, circulating tumor cells, cytokines, exosomes and factors such as lactate dehydrogenase (LDH) and C-reactive protein (CRP) can be analyzed using liquid biopsies (15–17). Additionally, investigating the presence and the dynamics of peripheral blood leukocytes may unveil important predictive and pharmacodynamic biomarkers.

In the context of ICIs, there are no validated circulating predictive biomarkers yet. Nonetheless, blood tumor mutational burden (bTMB) is gaining interest because it shows a good correlation with TMB in non-small cell lung cancer (NSCLC) and has thus the potential to become a useful non-invasive predictive biomarker (18). Regarding pharmacodynamic markers, several authors have observed an increase in Ki-67$^+$PD-1$^+$ T cells, representative of a reinvigoration of exhausted lymphocytes, as well as an expansion of tumor-specific T cell clones, in the circulation of responders to ICIs (19–21).

However, circulating cell subsets other than T lymphocytes might also be relevant in immunotherapy. In this regard, the accumulation of myeloid-derived suppressor cells (MDSCs) has been proven to impair the efficacy of anti-tumor therapies in human cancers (22).

MDSCs are cells of myeloid origin with systemic expansion in cancer that can be distinguished from mature, terminally differentiated myeloid cells for their phenotype and for their immune-suppressive functions. Before the definition of standards for their identification in humans by a group of experts in the field (23), many partially overlapping subsets had been described, leading to confusion in the investigation of their biological role. Three main categories of human MDSCs exist: polymorphonuclear-MDSC (PMN-MDSC, Lin$^-$CD11b$^+$CD15$^+$CD14$^-$), monocytic MDSC (M-MDSC, Lin$^-$CD11b$^+$CD14$^+$HLA-DR^{low}) and early-stage MDSC (eMDSC, Lin$^-$CD11b$^+$CD33$^+$CD14$^-$CD15$^-$HLA-DR$^-$), each containing different subsets with peculiar biochemical and molecular markers. Besides the phenotypic characterization, the gold standard for MDSC definition remains however their immunosuppressive activity (23). Since these cells play a pivotal role in the establishment of a potent immunosuppression, both at a systemic and at the tumor level, some studies have started to explore their potential as biomarkers of response to ICIs (6–8).

Besides MDSCs, the modulation of the expansion and function of monocyte subsets has also been demonstrated to have a role in different diseases (2, 24). Human monocytes can be quantified by Coulter Counter impedance technology through the absolute monocyte count (AMC) (25) or by multi-color flow cytometry. Three major populations can be discerned based on CD14 and CD16 expression: classical (CD14$^+$CD16$^-$), non-classical (CD14^{dim}CD16$^+$), and intermediate (CD14$^+$CD16$^+$) monocytes, with distinct surface markers and functions. Classical monocytes exhibit a pro-inflammatory phenotype and are mainly involved in anti-microbial responses, adhesion to the endothelium, migration, and phagocytosis Intermediate and non-classical monocytes emerge sequentially from the pool of classical monocytes: intermediate monocytes are specialized in antigen presentation and transendothelial migration, while non-classical monocytes are responsible for complement and FcR-mediated phagocytosis, transendothelial migration and anti-viral responses (24, 26).

In addition to MDSCs and monocytes, the systemic expansion of neutrophils, eosinophils and immature myeloid cells has also been shown to reflect the immunosuppressive status of TME during immunotherapy (27). Neutrophil quantification can be done with Coulter counters through the absolute neutrophil count (ANC) or by flow cytometry (CD15$^+$CD16$^+$ cells). The neutrophil-to-lymphocyte ratio (NLR), calculated as the ratio between the ANC and the absolute lymphocyte count (ALC), is also used to illustrate the expansion of the neutrophil component to the detriment of lymphocytes, unveiling the patient inflammatory status, while its derivative form, dNLR, is given by the following formula: ANC/(WBC-ALC), where WBC is the total number of white blood cells.

Besides blood markers, investigators have also analyzed the tumor in search for predictive biomarkers. With an increasing

number of ICI-treated patients and the intensified use of multiparametric analyses, new biomarkers are continuously discovered. For the approved anti-PD-1 blockers, several *tumor biomarkers* have been proposed, with variable level of clinical validation on large cohorts of patients and different cancers: PD-L1 staining (on tumor cells or both tumor and immune cells) evaluated by immunohistochemistry IHC, TMB, and the microsatellite instability (MSI) status, the infiltration of CD8 T cells, some transcriptomic signatures as TIS (Tumor Inflammation Signature) and TIDE (Tumor Immune Dysfunction and Exclusion), the presence of B cells and tertiary lymphoid structures, as reported elsewhere (9–11, 28–30). However, the predictive power of these biomarkers varies across tumors and it seems important to combine at least some of them to better distinguish potential responders from non-responders.

Even though the first markers were linked to features of tumor cells and effector T cells, the growing interest in profiling the whole TME is unveiling the potential predictive value for immunotherapy of tumor-infiltrating myeloid cells. The main myeloid populations found in tumors are tumor-associated neutrophils (TANs), dendritic cells (DCs), tumor-associated macrophages (TAMs), and monocytes (31). Depending on the TME, these myeloid populations can adopt very different phenotypes with distinct impact on the anti-tumor immune response, angiogenesis and invasion. Many reviews have analyzed the influence of tumor-infiltrating myeloid cells on the prognosis of cancer patients (32–35) and the mechanisms of negative and positive regulation of the anti-tumor immune response (3, 4, 36). Although most findings come from murine tumors, some clinical data are emerging that link the presence of myeloid cells in the TME with the outcome of approved immune checkpoint therapies, as discussed later (6–8).

ANTI-PD-1 INHIBITORS

The immune-checkpoint Programmed cell Death protein 1 (PD-1) is physiologically up-regulated following lymphocyte activation, and, through the direct interaction with its ligands PD-L1 and PD-L2, limits the activity of T lymphocytes to prevent excessive immune responses (37–39). The anti-PD-1 monoclonal antibodies (mAb) Nivolumab and Pembrolizumab block the interaction of tumor-reactive T cells expressing PD-1 with leukocytes and tumor cells expressing PD-L1 and PD-L2 (40). These antibodies are approved for the treatment of several cancer types, in monotherapy or combination with chemotherapy and anti-angiogenic drugs. Despite these antibodies have improved the clinical outcome in a wide range of tumors, the long-term benefits are restricted to a small proportion of patients, emphasizing the need for more reliable biomarkers and new drug combinations (40–45).

The evaluation of PD-L1 expression in tumors by IHC, TMB, and MSI status at baseline are the only biomarkers used in the clinical practice with ICIs but none of them alone is a strong and universal predictor of response. As an example, PD-L1 staining shows intra-patient tumor heterogeneity, evidence of response to ICIs in patients with low/negative PD-L1 expression and a lack of technical standardization (9–11, 13, 46).

Together with baseline biomarkers, on-treatment evaluation of tumors can give important insights into the mechanisms of action and resistance of ICIs, helping clinicians rapidly refine the therapy in a personalized way. Responder biopsies are characterized by an infiltration of T cells, especially $CD8^+$, the upregulation of genes related to T cell recruitment, activation, proliferation, and cytotoxicity (e.g., *CXCL9, CXCL10*, *PDCD1*, *MKI67, GZMB, IFNG,* and IFNγ-regulated genes) and an augmentation of PD-L1 expression, as a consequence of IFNγ release in the TME (47, 48). As the modulation of the TME by ICIs is still unclear, especially for non-T cells, we highlight here the studies that suggest the use of circulating and tumor myeloid cells, at baseline or during treatment, as novel predictive or pharmacodynamic biomarkers for anti-PD-1 inhibitors.

Circulating Biomarkers

Monocyte Lineage

Several reports indicate the baseline presence of the circulating M-MDSCs as potentially related to the response to anti-PD-1 blockade. As an example, advanced melanoma patients with fewer M-MDSCs among peripheral blood mononuclear cells (PBMCs) before Nivolumab treatment more likely belonged to the responder and stable disease groups, thus suggesting that long-term responses might be seen after Nivolumab even in patients that have failed prior immunotherapy (49). This concept was recently reinforced by the observation that an early accumulation of M-MDSCs expressing the immunomodulatory molecule galectin-9, associated with the concomitant expression of Tim-3 on lymphocytes, was related to primary and secondary resistance to Nivolumab in metastatic NSCLC. Compared to healthy volunteers, a statistically significant increase in $CD14^-$CD15-HLA-DR^+ dendritic cells and M-MDSCs (defined here as $CD14^+$HLA-DR^+CD33^+), together with a reduced number of granulocytes, was found in these patients. Two weeks after Nivolumab administration a rapid decrease in the M-MDSC was observed in responders and in patients with stable disease, while the number remained constant in non-responders. In addition, the authors showed that the combined expression of Tim-3 on $CD8^+$ T cells and galectin-9 on M-MDSCs impaired the secretion of IFNγ by activated $CD8^+$ T cells in the presence of anti-PD-1 *in vitro*, further suggesting that M-MDSCs can confer resistance to Nivolumab treatment (50).

M-MDSC expansion was similarly associated with poorer response in a cohort of advanced melanoma patients treated with a combination of Nivolumab and a multi-peptide vaccine. In these patients, the presence of different suppressive populations, including an M-MDSC subset (defined as $CD11b^+$HLA-$DR^{low}CD14^+$), a population of PMN-MDSCs ($Lin^-CD14^-CD11b^+CD15^+$) and regulatory T cells ($CD3^+CD4^+CD127^{low}FoxP3^+$), was assessed at baseline and during treatment. The authors showed a trend toward lower baseline Tregs and M-MDSC levels in non-relapsing patients as compared with relapsing ones, thus suggesting the negative impact of these circulating populations on the clinical outcome,

without clearly distinguishing their predictive vs. prognostic value (51).

In a cohort of metastatic urothelial carcinoma (mUC) patients treated with Pembrolizumab, successive doses of anti-PD-1 decreased the frequency of PD-1$^+$ M-MDSCs and eMDSCs, even if these changes were not statistically significant predictors of response. This decrease may indicate that MDSCs can downregulate immune checkpoints at their surface as a mechanism of resistance to ICIs, but it may also be the mirror of a positive immunotherapeutic response with reduction of immunosuppressive populations (52).

The accumulation of monocytes is also sometimes associated with a worse outcome in different tumor types, both at baseline and during anti-PD-1 therapy. A decrease in CD11c$^+$CD14$^+$CD16$^+$HLA-DR^{hi} monocytes, accompanied by a significant increase in overall survival (OS), has been observed in recurrent glioblastoma after post-surgery Pembrolizumab, but only in patients that had also received neo-adjuvant anti-PD-1 immunotherapy before surgery (53). Chasseuil et al. reported a statistically significant decrease in OS in relation to an increase in the total monocytic fraction in pre-treatment blood samples from advanced melanoma patients treated with Nivolumab, suggesting its potential as prognostic biomarker (54). In addition, in a cohort of NSCLC patients, the post-treatment AMC was higher in non-responders compared to responder patients, suggesting a predictive role of monocytes in anti-PD-1 therapy (27). Interestingly, Sekine et al. also identified the increase in the lymphocyte-to-monocyte ratio (LMR) after the start of Nivolumab treatment as a good predictor of response for NSCLC patients (55). An explanation to the negative impact of the cells of the monocytic lineage on ICI response may rely on the variety of mechanisms by which these cells can alter T cell effector functions, including nutrient depletion, generation of reactive oxygen species (ROS) and up-regulation of immune checkpoint molecules (36, 56–58).

A high monocyte frequency is however not always associated with a poorer response, as demonstrated by Krieg et al. who found a higher frequency of classical monocytes (CD14$^+$CD16$^-$CD33^{hi}HLA-DR^{hi}), measured before therapy by single-cell mass cytometry, in melanoma patients responding to anti-PD-1. A flow cytometry validation confirmed that a frequency of classical monocytes higher than 19.38% before therapy was associated with a better treatment outcome. These conflicting results might be explained by the fact that these monocytes have higher amounts of migration and activation markers, such as ICAM-1 and HLA-DR, and might thus be actively involved in the anti-tumor immune response induced by anti-PD-1 (59). Indeed, classical monocytes express high levels of chemokine receptors to migrate to inflamed tissues and secrete pro-inflammatory soluble factors, potentially shaping inflammation, and being a key player in the anti-tumor response (24).

Similarly, in a cohort of advanced NSCLC patients, an AMC higher than 700/mm^3 (median AMC for responders) at baseline was related to a shorter TTR (time to response) to either Nivolumab or Pembrolizumab. According to the authors, the positive role of monocytes could reflect a more intense macrophage-mediated tumor cell cytotoxicity that synergizes with the activity of tumor-reactive T lymphocytes. However, the impact of AMC on response to ICIs is likely highly specific to the tumor type, given that different tumors may release specific cytokines promoting the polarization of TAMs toward either an immunosuppressive or an anti-tumor phenotype (60).

Granulocyte Lineage

In a longitudinal study performed on the blood of NSCLC patients before and after Nivolumab treatment, the ratio of Tregs to PMN-MDSCs, TMR, was chosen as a predictor of response to treatment: a TMR $\geq$ 0.39 after the first infusion was associated with a higher probability of being a responder. This suggests that PMN-MDSCs, distinguished from normal neutrophils by the expression of the lectin-type oxidized LDL receptor-1 (Lox-1) in this study, could impair the efficacy of anti-PD-1 therapy, while highlighting at the same time an association between a higher frequency of Tregs and a better response to treatment. In these patients, CXCL2, CCL23, CX3CL1, and HMGB1 levels, known to be related to MDSC recruitment and proliferation, were also significantly higher in non-responders (61).

However, the role of PMN-MDSCs in tumors is still debated: in fact, they have also been proven to be associated with a better response to Nivolumab in advanced NSCLC patients. In this cohort, high baseline levels of PMN-MDSCs (Lin$^-$HLA-DR$^{low/neg}$CD33$^+$CD13$^+$CD11b$^+$CD15$^+$CD14$^-$) and low baseline CD8/PMN-MDSC ratios were associated with a better OS. As a further confirmation of this results, researchers identified an immunological asset as a possible prognostic biomarker of OS and progression-free survival (PFS) after Nivolumab treatment: in a multivariate analysis, the combination of NLR $<$ 3, baseline levels of PMN-MDSCs $\geq$ 6 cell/μl, eosinophil count $\geq$ 90 cells/mm^3 and neutrophil count $<$5,840 cells/mm^3 showed a statistically significant association with good prognosis (62).

Different studies performed on melanoma and metastatic renal-cell carcinoma (mRCC) patients under anti-PD-1 treatment showed that neutrophil-to-lymphocyte ratio values $\geq$5 were are strongly associated to shorter survival, thus assessing its potential use as a strong prognostic, and maybe predictive, biomarker (54, 63–67). Other retrospective studies in cancer patients showed that high NLR values during or after Nivolumab treatment, but not at baseline, were significantly associated with a worse outcome (27, 55, 68–71). The predictive value of NLR was recently formally assessed in a cohort of mUC patients, in which associations between candidate biomarkers and clinical benefit were investigated comparing a cohort treated with anti-PD-1/anti-PD-L1 to a cohort treated with taxanes. A NLR $<$ 5 and a high single nucleotide variant count were proposed as independent predictors of treatment response for ICIs (72).

The ANC, if above a certain threshold, also results negatively associated with treatment response either at baseline (73, 74) or on-treatment for different malignancies (27, 60, 75). In this context, Pan et al. observed that metastatic melanoma patients with high baseline ANC were more likely to undergo disease progression than patients with low values (73). Additionally, Nivolumab decreased the levels of ANC in the responder group

of another cohort of melanoma patients, mirroring the decrease in systemic inflammation levels (75). Multiple mechanisms by which neutrophils may boost cancer growth have been proposed, including the release of immunosuppressive cytokines and chemokines that affect the recruitment and phenotype of different immune cells. Neutrophils can also exert their immunosuppressive function through production of arginase 1 (ARG1) and ROS. Arginase depletes a fundamental nutrient, arginine, from the surrounding environment, leading to the inhibition of T cell proliferation and function, while ROS can suppress lymphocyte activation and, at high concentrations, induce T cell apoptosis (76).

Interestingly, within the granulocytic fraction, eosinophils seem to have a protective role and are usually associated with favorable treatment outcomes (54, 74, 77). Even if their predictive role remains unclear, a relative eosinophil count <1.5%, as well as elevated baseline levels of LDH and CRP, were independently associated with poor OS in a cohort of uveal melanoma patients undergoing anti-PD-1 monotherapy (78). A possible explanation behind the positive effect of eosinophils is their capacity to recruit cytotoxic T lymphocytes through CCL5, CXCL9, and CXCL10, to induce an anti-tumor phenotype in macrophages through TNFα and IFNγ and to normalize the tumor vasculature (79).

To conclude, several reports indicate that MDSCs, monocytes and neutrophils may reflect a compromised inflammatory status and thus be a reliable negative predictor of response to anti-PD-1 therapy. Nonetheless, some works underline how a high monocyte frequency could have a positive impact on patient outcome since it may mirror a macrophage-mediated cell cytotoxicity at tumor site. Moreover, the role of granulocytes is also debated, since eosinophils appear to have a protective role given their ability to recruit cytotoxic T lymphocytes.

Tumor Biomarkers

Monocyte/Macrophage Lineage

One of the first papers comparing the tumor transcriptome at baseline of melanoma patients undergoing anti-PD-1 therapy described an "innate anti-PD-1 resistance signature" that comprised genes involved in the mesenchymal transition (*AXL, ROR2, WNT5A, LOXL2, TWIST2, TAGLN, FAP*), immunosuppression (*IL10, VEGFA, VEGFC*) and monocyte and macrophage chemotaxis (*CCL2, CCL7, CCL8,* and *CCL13*) in non-responder patients (80). This signature was associated with resistance to anti-PD-1, but not with anti-CTLA-4, in three additional melanoma cohorts and was also described in other tumor types, where its predictive value was however not proven. In another paper describing predictive gene signatures related to T cell dysfunction and T cell exclusion (TIDE) in melanoma patients, the presence of TAM and MDSC signatures, along with cancer-associated fibroblasts, was related to reduced T cell infiltration and resistance to anti-PD-1 and anti-CTLA-4 (29). Moreover, Neubert et al. observed that IFNγ and TNFα produced by antigen-specific $CD8^+$ T cells induced the macrophage-colony stimulating factor CSF-1 in melanoma cells, possibly recruiting and activating TAMs. Circulating CSF-1 levels in melanoma patients are significantly higher than in healthy donors and positively correlated with disease progression. In the same cohort of melanoma patients treated with anti-PD-1 analyzed in (80), the authors observed a co-enrichment of $CD8^+$ T cells with CSF-1 or various TAM-specific markers in pre-treatment biopsies of non-responders, suggesting that the recruitment of $CD163^+$ M2-like macrophages by activated lymphocytes might be a mechanism of resistance to immunotherapy. Interestingly, in the presence of IFNγ and TNFα melanoma cells can also upregulate *TGFB, IL10, VEGFA,* and *VEGFC* genes, which can further modulate the immunosuppressive phenotype of TAMs (81). A recent retrospective analysis in NSCLC patients uncovered an epigenetic signature, called EPIMMUNE, predictive for PFS upon anti-PD-1 treatment. The authors observed that EPIMMUNE-negative tumors, prevalent among non-responders, were particularly infiltrated by macrophages and neutrophils, in contrast with EPIMMUNE-positive biopsies, characterized by a strong lymphoid infiltrate (82). $CD73^{hi}$ myeloid cells overexpressing several chemokine receptors and immunosuppressive factors are also highly abundant in the TME of glioblastoma, where they are negatively correlated with OS. These cells persist in glioblastoma patients after Pembrolizumab and hamper the efficacy of anti-PD-1 and anti-CTLA-4 in murine models (83).

Another interesting report of a predictive negative role of macrophages in ICI therapy regards the phenomenon of hyperprogression, an accelerated growth of tumors observed in 9–29% of the patients under ICIs that is still poorly understood. In baseline biopsies of a small cohort of NSCLC cancer, Lo Russo et al. have described that a subtype of clustered $CD163^+CD33^+PD\text{-}L1^+$ macrophages with epithelioid morphology was significantly enriched in all hyperprogressor patients compared to patients not experiencing hyperprogression (84).

Another important aspect that should be considered when analyzing the TME is the *localization* of the different cell types. On a small cohort of melanoma patients treated with anti-PD-1, non-responders displayed a significantly higher proximity of $CD68^+$ myeloid cells to $CD8^+$ T cells compared to responders in pre-treatment and on-treatment biopsies (85). Intriguingly, long-lasting contacts between macrophages and $CD8^+$ T cells in surgically resected NSCLC tumors are associated with impaired motility and reduced infiltration of lymphocytes in tumor islets; in pre-clinical models resistant to anti-PD-1, the concomitant depletion of macrophages can restore T cell motility and infiltration into tumor islets with increased tumor cell killing, suggesting that myeloid cells can modulate the TME not only through soluble mediators but also by physical contact with the surrounding cells (86).

Given the plasticity of myeloid cells and the differences in the microenvironment among tumors, the phenotype of this lineage can greatly vary. The simple abundance of $CD68^+$ cells, classically considered to represent macrophages, is thus rarely informative, while the *functional orientation* of myeloid cells by multiparametric IHC, flow cytometry or RNA sequencing allows to define a clearer relationship between the distinct subsets and the clinical outcome (32). Therefore, functionally different myeloid subpopulations can have an opposite effect on the response of patients to ICIs. In some reports, for instance, the

presence of PD-L1$^+$ macrophages seems more valuable as a predictive biomarker than the abundance of macrophages *per se*. Clinical responses are linked to high expression of PD-L1 in macrophages and dendritic cells in melanoma patients treated with a combination of anti-CTLA-4 and anti-PD-1 and in ovarian cancer patients treated with anti-PD-1, even if in the latter case the results are not statistically significant due to poor responses (87). Similarly, in the SARC028 trial, sarcoma patients who had an objective response to Pembrolizumab had a significantly higher average percentage of tumor cells and TAMs expressing PD-L1 at baseline compared to non-responders (88). Moreover, high counts of PD-L1$^+$ macrophages, but not PD-L1$^+$ tumor cells, were predictive of better OS after anti-PD-1 or anti-PD-L1 therapy in NSCLC (89).

In melanoma patients treated with anti-PD-1, anti-CTLA-4 or the combination, myeloid cells were enriched in non-responder lesions (90). However, when the myeloid compartment of the same cohort was analyzed in detail by single-cell RNA sequencing (scRNAseq), TAMs of responders were found to express CXCL10 and CXCL11 which, together with CXCL9, are predictive markers of response to anti-PD-1 and anti-PD-L1 in metastatic melanoma and mUC. The authors described distinct gene expression profiles in macrophages from responder and non-responder patients, unveiling other potential markers of response and resistance that could help understand the complex biology of the myeloid compartment in ICI therapy (91).

In another recent report, peripheral T cell and M1 macrophage signatures have shown to be enriched in NSCLC patients that displayed durable clinical benefit after anti-PD-1 treatment compared to non-responders. In particular, PFS was longer in patients with high peripheral T cell or M1 signatures, but OS was not significantly different. The same authors observed that these signatures behaved similarly in metastatic melanoma patients treated with Nivolumab [analyzed in (92)], although the differences between responders and non-responders did not reach statistical significance (93).

These examples demonstrate the importance of a more detailed analysis of the TME by multiparametric flow cytometry, multidimensional IHC, as well as scRNAseq, to better understand the role of each cell subset as biomarkers for immunotherapy.

Data regarding human myeloid cells during or after treatment are limited and conflicting. As an example, in two different studies with melanoma patients treated with Nivolumab or Pembrolizumab, responder biopsies displayed an increase in CD8$^+$ T cells in both studies, but in one case there was a reduction in the macrophage transcriptomic signature (92), while in the other there was an increase in peritumoral CD68$^+$ macrophages after treatment (48). However, in the latter cohort there was a significant increase in PD-L1 expression in macrophages of responder patients, suggesting that the function of these cells might be modified after anti-PD-1 therapy (48).

Neoadjuvant immunotherapy, currently explored for some tumor indications to ameliorate the efficacy of surgical resection, is giving important insights into the activity of ICIs and pharmacodynamic biomarkers. In a cohort of advanced melanoma patients that received neoadjuvant Pembrolizumab before resection, anti-PD-1 therapy provoked an increase in CD8 TILs and an upregulation of PD-L1 and other genes involved in T cell activation and migration. While these parameters were associated with clinical benefit, an increase in CD163$^+$ myeloid cells and a decrease in CD3$^+$ lymphocytes were observed in patients that recurred after surgery. In one of these relapsing patients, Nanostring analysis revealed the presence of T cell activation transcripts but also the presence of a myeloid signature (comprising *CD14, CCL8, CXCL14, CLEC5A,* and *CSF1R* genes), confirming the immunofluorescence data (94). Moreover, the same patient experienced p53 loss at recurrence, an event that could further increase immunosuppression, as p53 activation has been linked to MDSC reduction and anti-tumor immunity in mice (95). In another cohort, neoadjuvant Nivolumab in resectable NSCLC induced necrotic areas with large infiltrates of lymphocytes and foamy macrophages in tumors of patients with major pathological response at surgery (96, 97).

In glioblastoma patients treated with neoadjuvant anti-PD-1, increased T cell infiltration and chemokine transcripts have been described, even though there was no clear clinical benefit. In these patients no obvious modulations of myeloid cells have been observed (98), in line with what had been reported in glioblastoma patients after Pembrolizumab (83).

Granulocyte Lineage

In two small cohorts of metastatic melanoma treated with anti-PD-1, the infiltration of neutrophils, but not of macrophages, was higher in patients with progressive disease relative to those with clinical responses. The authors have shown that in murine models the infiltration of macrophages and neutrophils are, at least in part, mutually regulated and are also influenced by tumor-intrinsic factors, thus pointing at the need of a better understanding of the cross-talk between different cells subsets in the TME (99). However, the role of TANs or other tumor-infiltrating granulocytes in ICI treatment still needs to be clarified.

These results suggest that, at least in some tumor types, on-treatment myeloid cell density and phenotype in the TME might be potentially used as both predictive and pharmacodynamic biomarkers.

ANTI-PD-L1 INHIBITORS

Three drugs are currently approved to target PD-L1: Durvalumab, Avelumab and Atezolizumab. These antibodies are approved for fewer indications than anti-PD-1 blockers, i.e., mUC, Merkel Cell carcinoma, NSCLC and, in combination with chemotherapy, triple-negative breast cancer and small cell lung cancer, with less reports so far about the predictive role of myeloid cells on the clinical response.

Circulating Biomarkers

Bocanegra et al. analyzed the systemic differences in PD-L1 expression that could explain the opposite response of two patients with PD-L1-negative NSCLC tumors treated with Atezolizumab. PBMCs were divided into CD11b^{high} (monocytes, M-MDSCs and neutrophils), CD11b^{low} (DCs, PMN-MDSCs, some T cells and NK cells), and CD11b$^-$ (T and B lymphocytes

and plasmacytoid DCs) subsets. Responding patients exhibited high percentages of PD-L1$^+$ cells in CD11b$^-$ and CD11b^{high} immune cell types, but not in CD11b^{low} cells. To confirm and extend these data, other NSCLC patients under PD-1/PD-L1 blockade were analyzed, showing a significant association between a high percentage of circulating PD-L1$^+$CD11b$^+$ cells at baseline and an objective clinical response. Moreover, a tendency for responders to express high amount of PD-L1 within CD11b$^+$CD14$^+$ cells was observed, although it did not reach statistical significance. Intriguingly, patients with high percentages of circulating memory CD4$^+$ T lymphocytes and low percentages of PD-L1$^+$ immune cells did not respond to ICIs, highlighting the relevance of PD-L1 expression by myeloid cells in predicting treatment efficacy (100).

In advanced NSCLC patients treated with Atezolizumab, disease control was associated with decreased frequencies of Tregs and Lin$^-$HLA-DR$^-$CD33$^+$CD11b$^+$ MDSCs and a reduction in NLR after treatment (101).

In mUC patients, successive doses of Atezolizumab and Avelumab correlated with a significant decrease of PD-L1$^+$ M-MDSC and PD-L1$^+$ eMDSC (CD33$^+$HLA-DR$^{low/-}$CD14$^-$CD15$^-$) after the first dose. However, changes in PD-L1 expression in MDSCs either before or after therapy did not predict and neither correlate with ICI response, showing the need for further studies to find predictive biomarkers in mUC patients (52).

In a clinical trial involving patients with metastatic prostate cancer treated with the PARP inhibitor Olaparib combined with Durvalumab, a baseline Lin$^-$HLA-DR$^-$CD11b$^+$CD33$^+$ MDSC fraction lower or equal to the median of the group correlated with longer PFS. In this study, myeloid cells were also useful on-treatment markers, as patients with increased expression of CD83 on CD141$^+$ mDC after treatment had prolonged PFS (102).

A case study report of a NSCLC patient treated with Durvalumab as a maintenance therapy after chemotherapy and radiotherapy showed a 3-fold drop in the level of IL-4Rα$^+$ M-MDSC and in the expression of the *CD274* (PD-L1), *PTGS2*, *IL10,* and *IDO1* genes in PBMCs after two administrations of the anti-PD-L1 antibody, accompanied by a reduction in the suppressive potential of these cells compared to baseline. After 6 months of Durvalumab, this patient is still in clinical and radiologic disease remission (103).

As previously discussed, it should be considered that none of these studies compared anti-PD-L1 therapy to other treatments. Further investigation is thus needed to clarify the predictive and/or prognostic role of myeloid cells in this context.

Tumor Biomarkers

Compared to anti-PD-1, the amount of information regarding tumor biomarkers under anti-PD-L1 antibodies is still limited. In a clinical trial with mRCC patients, a myeloid signature comprising *IL6, CXCL1, CXCL2, CXCL3, CXCL8,* and *PTGS2* genes has recently been proposed as a resistance mechanism to Atezolizumab. Atezolizumab alone was more effective in patients with tumors enriched in cytotoxic T cells (T_{eff}^{high}) that were also Myeloid^{low}. In the T_{eff}^{high} Myeloid^{high} subgroup, the combination of Atezolizumab with the anti-VEGF Bevacizumab showed better activity than Atezolizumab alone, suggesting that the inhibition of VEGF could counteract the presence of immunosuppressive myeloid cells (104).

In addition, myeloid-associated genes (*COX2, IL8, IL1B*) in the tumor and circulating cytokines (IL-8 and IL-6) were associated with resistance and shorter OS in urothelial bladder cancer patients treated with anti-PD-L1 (Atezolizumab or Durvalumab) (105–108).

In summary, myeloid cells have been investigated as predictive markers of response to anti-PD-L1 treatment only in few studies, comprising different tumor types and a variety of myeloid subsets, hindering up to now the definition of biomarkers clearly correlated to patient response.

ANTI-CTLA-4 INHIBITORS

CTLA-4 is another immune checkpoint that mediates the physiological inhibition of activated T cells by competing with CD28 for the binding of CD80 and CD86 costimulatory molecules on antigen-presenting cells (APCs). Two monoclonal anti-CTLA-4 antibodies, Ipilimumab and Tremelimumab, are currently used in cancer to release the brake induced by CTLA-4 and build an effective immune response. Ipilimumab has been approved by the FDA for metastatic melanoma and, in combination with Nivolumab, for RCC and MSI CRC. Tremelimumab has been evaluated in the treatment of melanoma, mesothelioma, NSCLC, head and neck squamous cell carcinoma, prostate, pancreatic and hepatocellular carcinomas. Initial phase I and II studies of Tremelimumab in metastatic melanoma were promising, but a phase III trial was stopped because the antibody did not demonstrate superiority to standard chemotherapy, although responses were more durable (109).

Circulating Biomarkers

Monocyte Lineage

Many papers report the role of MDSCs as predictive markers for anti-CTLA-4 treatment, especially in Ipilimumab therapy. Among the three MDSC subsets, CD14$^+$HLA-DR$^{low/-}$ M-MDSCs are more commonly associated with resistance to this treatment. In regionally advanced melanoma patients treated with neoadjuvant Ipilimumab, circulating CD14$^+$HLA-DR$^{low/-}$ M-MDSC levels were lower at baseline but tended to increase, although not reaching statistical significance, in the relapse-free group, while frequencies in the relapsed group remained stable (110). Another work on malignant melanoma patients treated with Ipilimumab showed similar results: patients having distant metastasis in the skin or lymph nodes had lower levels of Lin$^-$CD14$^+$HLA-DR$^-$ M-MDSCs compared to patients having distant metastasis in vital organs or increased LDH. When comparing responders to Ipilimumab with non-responders, significantly lower percentages of Lin$^-$CD14$^+$HLA-DR$^{low/-}$ M-MDSCs were observed in the former group (111). Similarly, a high baseline frequency of M-MDSCs and high levels of IL-6 were associated with a reduced response to Ipilimumab in melanoma patients (112).

A study partially contradicting these results showed no significant differences between baseline levels of M-MDSCs in

patients with clinical benefit and those with progressive disease. However, after 3 and 9 weeks from Ipilimumab administration, patients with clinical benefit showed lower frequencies of this cell population, while no significant changes were observed in patients with progressive disease. Moreover, at week three after Ipilimumab M-MDSC were inversely correlated to survival (113). A low frequency of M-MDSCs was associated with long-term survival in another study on metastatic melanoma patients treated with Ipilimumab. The 2-years survival probability after Ipilimumab was 34.5% for patients with low MDSC frequency, while there were no survivors among patients with higher baseline levels.

A low AMC and a low frequency of $CD14^+$ monocytes were also strongly associated with a favorable outcome. A combination model was defined including LDH, MDSCs, Relative Lymphocyte Count, AMC, and Absolute Eosinophil Count, where each of them also remained in the model as a significantly independent biomarker (114).

In melanoma patients treated with Ipilimumab, the baseline number of monocytes and neutrophils was significantly higher in non-responder patients compared to responders. Interestingly, before treatment, non-responders displayed a tendency for an increased frequency of $CD14^+CD11b^+HLA\text{-}DR^{low/-}SSC^{low}$ M-MDSCs as compared to responders and this difference became significantly higher upon the first Ipilimumab infusion. Moreover, M-MDSCs in responders were strongly reduced after the first infusion, whereas they increased upon the second Ipilimumab cycle in non-responders. $CD15^+CD11b^+HLA\text{-}DR^{low/-}SSC^{low}$ PMN-MDSC levels were also evaluated, but no differences were detected between the two groups of patients. Interestingly, the level of intracellular nitric oxide was significantly elevated in M-MDSCs from non-responders compared to responders and higher M-MDSC percentages in non-responders significantly correlated with elevated nitric oxide production in these cells upon the first Ipilimumab infusion. Moreover, PD-L1 expression was downregulated in PMN-MDSCs of responders after the first Ipilimumab dose. Besides MDSCs, a significant increase in the eosinophil count after treatment was associated with an improved clinical response (115).

An interesting work has also demonstrated that miRs inducing MDSCs could represent predictive markers of response to ICIs in advanced melanoma patients (5). In this study, extracellular vesicles potentially derived from melanoma tumor cells were able to convert healthy donor monocytes into MDSCs (EV-MDSCs), by downregulating HLA-DR at the RNA level. Monocytes skewing to EV-MDSCs also showed changes in miR expression as compared to normal monocytes. MiR-146a, miR-146b, let-7e, miR-99b, and miR-125b were enriched in the extracellular vesicle fraction responsible for MDSC generation and were found to modulate the phenotype and function of monocytes toward MDSCs *in vitro*. In metastases from melanoma patients, high levels of miR-146a, miR-155, miR-125b, miR-100, let7e, miR-125a, miR-146b, and miR-99b were detected and correlated with CD163, CD14, CD209, CD68, ITGAM, and CD33 myeloid markers. An increased level of MDSC-miRs was detected in plasma samples from melanoma patients with advanced disease. A retrospective analysis in metastatic melanoma patients treated either with Ipilimumab, Nivolumab, or targeted therapy showed that patients with a low miR-score had a significantly longer OS, thus underlying the prognostic (and maybe predictive) value of these M-MDSC-inducing miRs (116).

Given the relevance of M-MDSCs as predictive biomarkers for response to Ipilimumab, Kitano et al. proposed a computational algorithm-driven analysis of PBMCs, demonstrating that melanoma patients with a pre-treatment M-MDSC frequency lower than 14.9% had a significantly longer OS and that M-MDSC levels inversely correlated with peripheral $CD8^+$ T cell expansion following Ipilimumab treatment (56). Beside melanoma patients, this algorithm will constitute a useful tool to evaluate M-MDSC frequencies in other tumor types.

High pre-treatment levels of M-MDSCs were also associated with reduced OS in castration-resistant prostate cancer treated with combined GVAX/Ipilimumab immunotherapy. In these patients, treatment-induced activation of conventional cDC1 and cDC3 dendritic cells was associated with prolonged OS, but also an increased risk of immune-related adverse events. In an unsupervised cluster analysis, patients with low pretreatment M-MDSCs, high pretreatment $CD4^+CTLA\text{-}4^+$ T cells and high levels of cDC1/cDC3/monocyte activation during treatment displayed prolonged survival (117).

$CD14^+IL\text{-}4R\alpha^+$ M-MDSCs were identified by Damuzzo et al. as negative predictors of response to Ipilimumab. In this study, four MDSC subsets were analyzed in the PBMCs of advanced melanoma patients, at baseline and 12 weeks after Ipilimumab: $CD14^+IL\text{-}4R\alpha^+$ M-MDSCs, $CD14^+HLA\text{-}DR^{low/-}$ M-MDSCs, $Lin^-HLA\text{-}DR^-CD33^+CD11b^+$ eMDSCs, and $CD15^+IL4\text{-}R\alpha^+$ PMN-MDSCs. A significant expansion of the two subsets of M-MDSCs and of PMN-MDSCs was observed at baseline compared to healthy controls and, upon treatment, high levels of $CD14^+IL\text{-}4R\alpha^+$M-MDSCs were independent prognostic factors of reduced OS. Moreover, longer OS was associated with low levels of IL-6, CRP, S100B, and LDH at baseline and after treatment. In a multivariate survival model, high levels of LDH and $CD14^+IL\text{-}4R\alpha^+$M-MDSCs post-treatment were identified as negative independent markers of reduced OS, thus showing that the $IL\text{-}4R\alpha^+$M-MDSC subset should be considered, together with $CD14^+HLA\text{-}DR^{low/-}$ cells, to select patients that could get most benefit from anti-CTLA-4 (118).

Interestingly, the population of non-classical monocytes has on the contrary been associated to a positive response to ICIs. In fact, advanced melanoma patients responding to Ipilimumab displayed the highest percentages and absolute counts of circulating non-classical monocytes at baseline. The authors showed that non-classical $CD14^+CD16^+$monocytes, but not classical $CD16^-$ monocytes, were able to lyse Tregs *ex-vivo* through CD16-Fc-mediated antibody-dependent cellular cytotoxicity (ADCC) mediated by Ipilimumab (119).

In metastatic melanoma patients, low levels of $CD33^+CD11b^+HLA\text{-}DR^-$ MDSCs before Ipilimumab correlated with an objective clinical response, long-term survival, increased CD3ζ chain expression in T cells and an improved clinical status. Conversely, patients with more than 55.5% circulating MDSCs had a significantly shorter OS

(120). In a neoadjuvant study with Ipilimumab in advanced melanoma, the treatment induced an expansion of activated $CD4^+$ and $CD8^+$ melanoma-specific T cell clones, an increase in circulating Treg, with greater Treg increase associated to improved PFS, and a reduction in all MDSC subsets, especially the M-MDSC fraction. A greater decrease in the circulating $HLA\text{-}DR^-CD33^+$ MDSC population was related to improved PFS in this cohort (121).

Granulocyte Lineage

Concerning PMN-MDSCs, this cell population is less frequently associated with response to Ipilimumab. Only the level of PD-L1 in PMN-MDSCs has been reported to be lower in responders as compared to non-responders in (115). In a cohort of melanoma patients treated with Ipilimumab, the level of PMN-MDSCs decreased after the first dose of Ipilimumab, but no information is given about the impact on treatment response (122). Another work on advanced melanoma patients treated with Ipilimumab showed that patients with high ANC and dNLR at baseline had an increased risk of death or disease progression (123).

Besides MDSCs, the relative eosinophil count, together with an elevated serum LDH and CRP, was significantly associated with survival in metastatic uveal melanoma patients treated with combined Ipilimumab and anti-PD-1 (78).

In conclusion, the collected data point mostly to the monocytic subsets, particularly $CD14^+HLA\text{-}DR^+$ M-MDSCs, $CD14^+IL4\text{-}R\alpha^+$M-MDSCs and non-classical monocytes, as useful markers for the selection of patients that could benefit more from Ipilimumab immunotherapy.

Tumor Biomarkers

For anti-CTLA-4 Ipilimumab, the predictive value of tumor biomarkers remains to be consolidated. The TIDE and TIS transcriptomic signatures, as well as genes linked to T cells cytotoxicity, Th1 chemokines and antigen presentation seem useful for the identification of responders among melanoma patients (29, 124). The mutational and neoantigen load (125) and a high ratio of $CD8^+$ density in the intratumoral region have also been related to clinical benefit to Ipilimumab in melanoma (126), while PD-L1 staining by IHC alone does not seem to be predictive (127).

Monocyte/Macrophage Lineage

Even if few studies exist on the predictive role of myeloid subsets, Capone et al. observed that BRAF WT melanoma patients with durable clinical benefit from Ipilimumab had a reduced transcriptomic "myeloid score." In the same cohort, the downregulation of CD73 gene, expressed by tumor-infiltrating myeloid cells as previously discussed, was also associated with response, irrespective of the BRAF status (128).

Interestingly, macrophage infiltration at baseline was even more useful than CD8 density in the distinction of responders vs. non-responders in a small cohort of melanoma patients treated with Ipilimumab. Responders displayed a higher $CD68^+/CD163^+$ ratio and higher $CD68^+CD16^+$ density at baseline than non-responders, with a concomitant reduced infiltration of $CD163^+CD16^+$ macrophages. The authors hypothesized that the enrichment of "inflammatory" $CD68^+CD16^+$ macrophages over immunosuppressive $CD163^+$ macrophages could create a more favorable TME for the anti-tumor activity of Ipilimumab. Moreover, post-treatment tumor biopsies from responders had lower Treg infiltration than lesions from non-responder patients and the authors hypothesized that this could be explained by the increased presence of FcγR "inflammatory" macrophages capable of inducing ADCC in the presence of the IgG1 Ipilimumab antibody and depleting Tregs (119). This hypothesis is also supported by Arce Vargas et al., who demonstrated how human Fcγ receptors expressed by myeloid cells can induce ADCC after binding to a chimeric murine anti-CTLA-4 with a human IgG1 Fc, *in vitro* and in human-FcγR murine models. In addition, melanoma patients with high TMB and the CD16-V158F polymorphism (conferring higher binding affinity to IgG1 antibodies) had higher response rates than all the other patients, suggesting that $Fc\gamma R^+$ myeloid cells in the tumor might contribute to the anti-tumor activity of Ipilimumab through ADCC-dependent Treg depletion (129).

The localization of different subpopulations in the TME (tumor region, stroma, invasive margin) is crucial for response, in addition to their phenotype and functional status. Madonna et al. observed that melanoma patients having baseline biopsies with few $CD8^+$ T cells combined with high numbers of $CD163^+PD\text{-}L1^+$ macrophages at the invasive margin survived significantly longer than any other group upon Ipilimumab (127).

Little information is currently available for on-treatment tumor biomarkers for anti-CTLA-4 therapy. A study of neoadjuvant Ipilimumab in advanced melanoma showed an increased infiltration of memory $CD4^+$ and $CD8^+$ T cells in the TME. At the same time, a reduction in tumor-infiltrating Tregs was associated with response or stable disease and decreased levels of tumor $HLA\text{-}DR^-CD33^+CD11b^+$ MDSCs after treatment were associated with a longer PFS. As mentioned in the previous section, in these patients an association between reduction in systemic $HLA\text{-}DR^-CD33^+CD11b^+$ MDSC and improved PFS was also observed (121).

As previously stated, the predictive vs. prognostic value of these biomarkers should be confirmed through randomized trials since the treatment with anti-CTLA-4 has not been formally compared to other treatments in most studies.

BINDING OF ICIs TO MYELOID CELLS: POTENTIAL MECHANISMS OF ACTION AND IMPACT ON BIOMARKERS

Together with their well-known regulatory functions on lymphocytes extensively reviewed elsewhere (6, 7, 22, 23, 35), circulating and tumor-infiltrating myeloid cells can express PD-L1, PD-1 and Fcγ receptors (FcγR) and can thus directly bind to ICIs and modulate their activity. On the other side, ICIs can affect the phenotype of myeloid cells either through a direct binding to these cells or through the indirect effects of IFNγ and other mediators released by activated lymphocytes. Even though murine and human myeloid subsets are identified through different markers, preclinical models are crucial for

the understanding of the mechanistic role of these cells in immunotherapy.

As for other antibody-based therapies, an important aspect to consider for ICIs is the expression of CD16, CD32, and CD64 FcγRs on the surface of myeloid cells. Depending on the isotype, the backbone of the mAb and the FcγR, the binding of the Fc part of antibodies can have different consequences. As reported before, the binding of Ipilimumab (119, 129), a potentially depleting IgG1, to the FcγR of myeloid cells can lead to the elimination of CTLA-4$^+$ Tregs through ADCC or antibody-dependent phagocytosis (ADCP). In this context, the infiltration of the TME by myeloid cells can potentially be a positive predictive biomarker for Ipilimumab therapy.

On the contrary, most anti-PD-1 and anti-PD-L1 antibodies have low or significantly reduced binding to FcγR to avoid potential ADCC and complement-dependent cytotoxicity (CDC), especially when the target molecule is expressed on effector T cells. In murine tumor models, the anti-PD-1 antibody can be transferred from CD8$^+$PD-1$^+$ T cells to PD-1$^-$ macrophages through FcγRIIb/III receptors and the same phenomenon can be reproduced *in vitro* with human cells and Nivolumab. Besides, the use of FcγRIIb/III blocking antibodies prior to anti-PD-1 improved its anti-tumor efficacy in mice (130). In a similar way, another paper showed that a human IgG4 anti-PD-1 antibody, bearing an S228P mutation as most approved immune checkpoint blockers, mediated a crosslink with FcgRI(CD64)$^+$ macrophages, resulting in the activation, rather than inhibition, of PD-1 signaling in T cells, the elimination of PD-1$^+$ CD8 cells by ADCP and increased secretion of IL-10 by macrophages. Compared to an identical anti-PD-1 antibody that lacked FcγR binding, the S228P-mutated antibody displayed a reduced anti-tumor effect *in vivo*, highlighting the potential role of FcγR-expressing myeloid cells in the negative regulation of ICIs (131). These observations need to be considered for the drug development of antibodies because, even though most mAbs are IgG4 or mutated IgG1 with no or low ADCC, ADCP, or CDC, they can still bind to different FcγR with unclear clinical consequences. Further studies are needed to elucidate whether the described mechanisms can also be observed in patients, further supporting the predictive role of specific subsets of myeloid cells in anti-PD(L)1 therapy.

In addition to this, several preclinical experiments have tried to shed light on the impact of ICIs on myeloid cells, which can express PD-1 and PD-L1. ScRNAseq of MC38 tumors in immunocompetent mice treated with anti-PD-1 revealed an expected increase in IFNγ, immune checkpoints and costimulatory molecules in CD8 lymphocytes in responders. Interestingly, this was accompanied by an enrichment in an M1-like signature including *HLA-DR, CXCL9, CXCL10, CCL5, CCL8,* and *STAT1* transcripts, while, conversely, an M2-like signature comprising *SPP1, PTGS1, MRC1, MSR1, ARG1,* and *CCR2* mRNA was observed in non-responders (132). In the T3 murine model, progressing tumors are highly enriched in CD206$^+$ macrophages, but anti-PD-1, anti-CTLA-4 or combination treatment induced the accumulation of iNOS$^+$ inflammatory macrophages. ScRNAseq and mass cytometry further confirmed the transformation of control tumors, mainly infiltrated by CCR2$^+$ monocytes and CX3CR1$^+$CD206$^+$CCL2$^+$CD49d$^+$ macrophages, into tumors enriched in iNOS$^+$PD-L1$^+$CXCL2$^+$ cells after ICI treatment (133). In a similar way, Dhupkar et al. have shown that anti-PD-1 treatment induced a significant reduction in lung metastases and a decrease in PD-L1 expression by metastatic tumor cells in human LM7 osteosarcoma-bearing mice. In this T cell-deficient model, NK and macrophages were PD-1$^+$ and their fraction was increased in the tumor after treatment; the anti-tumor effect of anti-PD-1 blockade was lost after macrophage, but not NK cell, depletion. Moreover, anti-PD-1 provoked an increase in CD86 and a reduction in CD163 staining in lung metastases compared to control mice, suggesting a shift from M1-like to M2-like macrophages (134).

PD-1$^+$ macrophages have also been described in mice and human colorectal cancer (CRC), where they display M2-like features (CD206$^+$CD64$^+$ large, foamy macrophages with uncleared phagocytic material) and are involved in tumor growth and invasion. In the CT26 model these macrophages had reduced phagocytosis compared to the PD-1$^-$ counterpart, restored by the knock-out of PD-L1 on tumor cells. In immunodeficient mice bearing a PD-L1 human CRC xenograft, anti-PD-(L)1 inhibitors were able to reduce tumor growth and this effect was abrogated by macrophage depletion (135). PD-1$^+$ macrophages with an M2-like phenotype have also been described in NSCLC biopsies and in the murine LLC model. In murine tumors, PD-1$^+$ macrophages have a distinct transcriptomic profile compared with PD-1$^-$ cells. In NSCLC tumors, PD-1$^+$ macrophages are mainly stromal CD163$^+$ macrophages and are associated with poor prognosis, suggesting once more that the phenotype and the localization of myeloid cells are likely crucial parameters to take into account for ICI biomarkers (136).

In a similar way, Hartley et al. investigated the direct effect of anti-PD-L1 antibodies on PD-L1$^+$ macrophages at the tumor site, given the prevalence of this cell subset in human tumors. The authors discovered that the treatment of murine and human macrophages with anti-PD-L1 antibodies increased their proliferation, survival and activation, as measured by the upregulation of CD86, MHC II, CD40, TNFα, and IL-12 and of several transcripts linked to myeloid inflammation. The same effects could be induced when macrophages were pre-treated with anti-FcRII/III antibodies. The authors have hypothesized that PD-L1 provides a constitutive negative signal in macrophages that can be reversed by anti-PD-L1 antibodies. The anti-PD-L1 treatment in syngeneic mouse models increased the number of TAMs and upregulated CD86 and MHC II, even in the absence of T cells. The authors also showed that the *in vivo* combination of anti-PD-1 and anti-PD-L1 antibodies, given their non-redundant effects, is more effective than either monotherapies in mice (137).

These preclinical experiments suggest that PD-1 and PD-L1 could negatively signal in macrophages, keeping them in a non-inflammatory, non-phagocytic state. If confirmed in the clinical setting, this could imply that anti-PD-(L)1 immunotherapy, together with its effect on T cells, might also cause an enrichment of M1-like macrophages either directly (though the binding to PD-1 or PD-L1) or indirectly (through cytokines release by activated lymphocytes). This phenotypic and functional switch

could also be used as a tumor pharmacodynamic biomarker for these antibodies.

COMBINATION STRATEGIES FOR IMPROVING ICI THERAPY BY TARGETING MYELOID CELLS: AN OVERVIEW OF CLINICAL DATA

Despite the great success of ICIs, the large majority of patients present a *primary* (never-responders) or *acquired* resistance after a period of response (138), but to date the reasons remain largely unclear. Combinatorial approaches with drugs that target immunosuppressive networks have become attractive to extend the benefits of immunotherapy to non-responding patients and are currently being tested in various clinical trials, as shown in **Table 1**. Through the modulation of distinct cell subsets, these combinations can be useful to overcome primary, as well as acquired, resistance to ICIs.

Given the impact of myeloid cells on immunotherapy reported in the previous paragraphs, it seems reasonable to combine ICIs with drugs that target these subsets (3, 5, 7). A huge amount of preclinical data supports this hypothesis and the relevance of these combinations is also emerging in the clinic. In the following paragraphs, we discuss the main myeloid-targeting strategies designed to enhance the antitumor activity of ICIs by either decreasing the suppressive potential of myeloid cells, through the inhibition of their recruitment, differentiation or function, or boosting the anti-tumoral capabilities of specific myeloid subsets.

Inhibitors of Colony-Stimulating Factor 1 Receptor (CSF-1Ri)

As previously discussed, TAMs and MDSCs are critical players within the immunosuppressive microenvironment. CSF-1 binds to the CSF-1R tyrosine kinase receptor on myeloid cells leading to myeloid cell proliferation, differentiation and recruitment into tumors (182). CSF-1/CSF-1R blockade promotes antitumor T cell responses and reduces tumor growth in several preclinical models in combination with immunotherapy, despite showing minor effects on tumor growth as a monotherapy (81, 86, 183, 184). Inspired by these encouraging preclinical results and by the first clinical results from CSF-1Ri monotherapy (185), several clinical trials combining ICIs with small molecules (as ARRY-382 or Pexidartinib) or mAbs (e.g., Emactuzumab or Cabiralizumab) directed against CSF-1R are currently ongoing in patients with solid tumors (**Table 1**).

Initial results from the combination of the anti-CSF-1R Cabiralizumab with Nivolumab showed a durable clinical benefit in heavily pre-treated patients with microsatellite stable pancreatic cancer. A durable depletion of circulating non-classical monocytes, a pharmacodynamic marker of Cabiralizumab and other CSF-1R targeting agents (186, 187), was observed with the Cabiralizumab monotherapy and the combination with Nivolumab (139), with a dose-dependent increase in the systemic levels of CSF-1 and IL-34 (CSF-1R ligands). Within tumors, a decrease from baseline of M2-like CSF-1R$^+$CD163$^+$ and total CD68$^+$ macrophages, together with a concomitant increase in CD8$^+$ effector T cells, was shown in patients treated with the combination. Furthermore, a significant increase in the expression of CSF-1R ligands and pro-inflammatory genes, associated with an M1-type polarization, were observed only in the tumors of responders to the combination (140). These results supported a Phase 2 study of a triple combination of Cabiralizumab plus Nivolumab with or without chemotherapy in advanced pancreatic adenocarcinoma (188). The results of ICI combinations with other CSF-1Ri are awaited to support the relevance of this promising approach.

Inhibitors of CD73 and Adenosine Pathway

Apart from molecules that interfere with the myeloid cell recruitment, another interesting therapeutic approach is to target their ability to create an immunosuppressive environment. As previously described, CD73 is a myeloid marker that is emerging as an important modulator of the response to ICIs (83, 128). CD73 hydrolyses the adenosine monophosphate (AMP) into adenosine and inorganic phosphate. The increased expression of CD73 in TME directly associates with adenosine accumulation and exerts multiple immunosuppressive actions on the anti-tumor immunity (189, 190).

Adenosine signals through cyclic AMP that inhibits T cell receptor activation (191). Preliminary data shows that patient exposure to anti-PD-1/PD-L1 therapy increased the expression of adenosine A2A receptor (A2AR) and CD73, suggesting that the adenosine pathway might be a potential mechanism of resistance to ICIs (192). As previously mentioned, CD73 inhibition may be a useful strategy to improve the clinical outcome of glioblastoma patients treated with immunotherapies (83). As a matter of fact, the human anti-CD73 mAb MEDI9447 is currently being tested in a Phase I study as monotherapy and in combination with Durvalumab (144).

In a Phase 1/1b clinical trial, an oral small molecule inhibitor of A2AR (CPI-444) has shown anti-tumor activity in monotherapy and in combination with Atezolizumab in anti-PD-1/PD-L1 resistant and PDL-1-negative patients (146). CPI-444 induced CD8$^+$ T cell infiltration into tumors and IFNγ- and Th1 signatures (192). The use of adenosine analogs or agonists on PBMCs has allowed to identify a transcriptomic "adenosine signature," dominated by myeloid cytokines and chemokines, nearly identical to the "myeloid signature" associated with poor response to Atezolizumab in RCC patients (104). CPI-444 blocks the induction of these genes *in vitro* and seems to have a better anti-tumor activity in RCC patients with a high adenosine signature compared to patients with low expression (147).

Anti-semaphorin 4D Antibodies

Semaphorin 4D (SEMA4D) is a transmembrane glycoprotein that binds to Plexin receptors, regulating the movement and differentiation of cells and displaying immunomodulatory effects in the TME (193). High levels of SEMA4D positively correlate with the presence of immunosuppressive TAMs and MDSCs, with concomitant exclusion of activated APCs and CD8$^+$ T lymphocytes from the tumor (194). In preclinical models, blockade of SEMA4D was associated with an increased penetration of inflammatory F4/80$^+$CD11c$^+$ APCs and a

TABLE 1 | Clinical Trials of combinations of ICIs with myeloid-targeting drugs.

	ICI	Drug	Target	Phase	Clinical trial	References
α-PD-1	Nivolumab	Cabiralizumab	CSF1R	Phase 1	NCT02526017	(139)
	Pembrolizumab	Cabiralizumab		Phase 2	NCT03336216	(140)
		Pexidartinib		Phase 1/2	NCT02452424	(141)
		ARRY-382		Phase 1/2	NCT02880371	(142)
		AMG 820		Phase 1/2	NCT02713529	-
α-PD-L1	Atezolizumab	Emactuzumab	CSF1R	Phase 1	NCT02323191	-
	Durvalumab	Pexidartinib		Phase 1	NCT02777710	(143)
α-PD-1	Pembrolizumab	LY3475070	CD73	Phase 1	NCT04148937	-
α-PD-L1	Atezolizumab	TJ004309	CD73	Phase 1	NCT03835949	-
	Durvalumab	MEDI9447		Phase 1	NCT02503774	(144)
α-PD-1	Spartalizumab	PBF-509	Adenosine-A2A Receptor	Phase 1/2	NCT02403193	(145)
α-PD-L1	Atezolizumab	Ciforadenant (CPI-444)	Adenosine-A2A	Phase 1	NCT02655822	(146, 147)
	Durvalumab	AZD4635	Receptor	Phase 1	NCT02740985	(148)
α-PD-1–	Nivolumab-Ipilimumab	VX15/2503	Semaphorin 4D	Phase 1	NCT03690986	(149)
α-CTLA-4		(Pepinemab)		Phase 1	NCT03373188	(149)
				Phase 1	NCT03425461	-
				Phase 1	NCT03769155	(150)
α-PD-L1	Avelumab	VX15/2503 (Pepinemab)	Semaphorin 4D	Phase 1/2	NCT03268057	(151)
α-PD-1	Nivolumab	Epacadostat	IDO-1	Phase 1	NCT03707457	-
	Pembrolizumab	Epacadostat		Phase 1/2	NCT02178722	(152)
				Phase 3	NCT02752074	(153)
				Phase 3	NCT03260894	-
				Phase 3	NCT03374488	(154)
				Phase 3	NCT03358472	(155)
				Phase 2	NCT03322540	(156)
				Phase 2	NCT03322566	(157)
				Phase 3	NCT03361865	(158)
α-PD-L1	Durvalumab	Epacadostat	IDO-1	Phase 1/2	NCT02318277	(159)
α-CTLA-4	Ipilimumab	Epacadostat	IDO-1	Phase 1/2	NCT01604889	(160)
α-PD-1	Nivolumab	IPI-549	PI3K-γ	Phase 1	NCT02637531	(161)
				Phase 2	NCT03980041	
α-PD-L1	Atezolizumab	IPI-549	PI3K-γ	Phase 2	NCT03961698	-
α-PD-1	Nivolumab	APX005M	CD40	Phase 1/2	NCT03214250	(162)
				Phase 1/2	NCT03123783	-
α-PD-1	Pembrolizumab	MIW815	STING	Phase 2	NCT03937141	-
	Spartalizumab	MK-1454		Phase 1	NCT03010176	(163)
		GSK3745417		Phase 1	NCT03843359	-
		MIW815		Phase 1	NCT03172936	(164)
α-CTLA-4	Ipilimumab	MIW815	STING	Phase 1	NCT02675439	-
α-PD-1	Pembrolizumab	ATRA	Retinoic Acid Receptor	Phase 1/2	NCT03200847	-
α-CTLA-4	Ipilimumab	ATRA	Retinoic Acid Receptor	Phase 2	NCT02403778	(165)
α-PD-1	Nivolumab	Trabectedin		Phase 2	NCT03590210	-
				Phase 2	NCT03886311	(166)
α-PD-L1	Avelumab	Trabectedin		Phase 1/2	NCT03074318	-
	Durvalumab	Trabectedin		Phase 1	NCT03085225	-
α-PD-1–	Nivolumab-Ipilimumab	Trabectedin		Phase 1/2	NCT03138161	(167)
α-CTLA-4						
α-PD-1	Pembrolizumab	Axitinib	VEGF-R	Phase 1	NCT02133742	(168)
				Phase 3	NCT02853331	(169)
α-PD-L1	Avelumab	Axitinib	VEGF-R	Phase 3	NCT02684006	(170)
α-PD-1	Nivolumab	Bevacizumab	VEGF	Phase 1	NCT03382886	-
				Phase 2	NCT03890952	-
				Phase 2	NCT03452579	(171)
α-PD-L1	Atezolizumab	Bevacizumab	VEGF	Phase 1	NCT01633970	(172)
				Phase 1	NCT02715531	(173)
				Phase 2	NCT01984242	(104)
				Phase 3	NCT02366143	(174, 175)
				Phase 3	NCT02420821	(176)
α-CTLA-4	Ipilimumab	Bevacizumab	VEGF	Phase 1	NCT00790010	(177–181)
α-PD-1	Pembrolizumab	Trebananib	Angiopoietin-2	Phase 1	NCT03239145	-

decreased density of pro-tumorigenic CD206$^+$ M2-like TAMs in the TME. Combination with anti–CTLA-4 led to tumor regression accompanied by enhanced T cell activity, increase in activated CD86$^+$ monocytes in the tumor, augmentation of pro-inflammatory IFNγ, TNFα, and IL-6 and decrease in immunosuppressive MCP-1 and IL-10 cytokines (195). Based on these preclinical results, Pepinemab, a humanized anti-SEMA4D mAb, is currently being evaluated in combination with Ipilimumab and Nivolumab in solid tumors (149–151), as reported in **Table 1**.

Inhibitors of Indoleamine 2,3-Dioxygenase 1 (IDO1)

Another important molecule involved in T cell immunosuppression in the TME is IDO1, which catalyzes the cleavage of L-tryptophan into kynurenine, leading to the inhibition of effector T cell proliferation and to the increase of Tregs (196, 197). IDO1 can be constitutively expressed by tumor cells or by macrophages, MDSCs and DCs in the tumor or the lymph nodes (198, 199) but can also be induced by inflammatory cytokines, such as IFN-γ, potentially inducing resistance to immunotherapy (200). High baseline IDO1 expression in tumors has been shown to predict response to anti-CTLA-4 in metastatic melanoma patients (201). In the B16 murine model, IDO1 inhibition combined with anti-CTLA-4 blockade resulted in increased infiltration of effector T cells, while attenuating Treg and MDSC accumulation (202). Expression of IDO1, PD-L1 and CTLA-4 in PBMCs of melanoma patients have been shown to be associated with a negative outcome, independently from disease stage (203). Based on these evidences, IDO1 inhibitors have been investigated for their potential to enhance the efficacy of ICIs.

Epacadostat is a highly selective oral inhibitor of IDO1 that induces enhanced proliferation of effector T cells and NK cells, increased activation of CD86^{high} dendritic cells and a contraction of human Tregs *in vitro* and murine Tregs *in vivo* (204, 205). Based on the encouraging results obtained in a Phase 1/2 study (152), several Phase 2 and Phase 3 trials (**Table 1**) were started to define the efficacy of the combination of Epacadostat with Pembrolizumab. However, in patients with advanced melanoma, the results of the Phase 3 study ECHO-301 were disappointing, with no improvement in PFS or OS in the combination vs. Pembrolizumab alone (153). Moreover, this study lacked biomarkers, which could have answered several key questions. In a recent review several explanations have been proposed for the negative outcome of ECHO-301, including a possible insufficient inhibition of IDO1, due to the inhibitor itself or the clinical dose, and the inadequate selection of patients; the authors however suggest to pursue the clinical development of inhibitors of IDO1, which still remains an attractive target for cancer immunotherapy (206).

Inhibitors of Phosphoinositide 3-Kinase γ (PI3Kγ)

The PI3Kγ, highly expressed in myeloid cells, has recently emerged as another key regulator of immunosuppressive macrophages (207, 208). In preclinical models, PI3Kγ selective targeting has been shown to reprogram macrophages into an immune-activating phenotype and to enhance ICIs activity (209). A Phase I study of the oral PI3Kγ inhibitor IPI-549 in combination with Nivolumab showed favorable tolerability and early signs of clinical activity in solid tumors. Upregulation of PD-L1 and CXCL9/10 and re-invigoration of exhausted PD1$^+$CD8$^+$CD45RA$^-$ T cells were observed in blood samples during treatment, suggestive of immune activation and reduced immunosuppression (161). Even if no data are available for the modulation of myeloid cells in these patients, these encouraging results show that PI3Kγ inhibition might help overcome resistance to ICIs and have led to Phase II IPI-549 combinations with Nivolumab or Atezolizumab (**Table 1**).

CD40 Agonists

As discussed previously and elsewhere (4, 5), myeloid cells can also have an anti-tumoral role through antigen-presentation and effector functions. The costimulatory protein CD40 is expressed by myeloid cells and DCs and, when activated by its ligand, promotes antigen presentation (210). A strong correlation between survival of CRC patients and CD40 expression in tumors was previously uncovered (211). In murine pancreatic tumor models, CD40 agonists were combined with anti-PD-1 and chemotherapy to trigger effective T cell immunity (212, 213). In a CRC model, a CD40 agonist led to PD-L1 increase on tumor-infiltrating monocytes and TAMs, PD-1 upregulation on T cells and a synergistic tumor growth inhibition in combination with an anti-PD-1 (214). Based on this preclinical evidence, the combination of the APX005M CD40 agonist with Nivolumab plus standard gemcitabine and nab-paclitaxel is currently being tested with promising antitumor activity in pancreatic cancer, where ICIs have been ineffective as single agents (215). In these patients, baseline biopsies revealed a low CD8$^+$ T cell and a high macrophage infiltration. Moreover, the immune-profiling of PBMCs showed a rapid activation of dendritic cells in most patients upon treatment (162).

STING Agonists

Type I interferon pathway is crucial in linking the innate and adaptive immune responses to mediate tumor rejection in mice and humans (216, 217). The activation of the STimulator of INterferon Genes (STING) pathway increases IFN-β production by tumor-resident DCs and induces the recruitment and priming of T cells against tumor antigens (218). The discovery of agonists of STING in mice [5,6-dimethyllxanthenone-4-acetic acid or DMXAA (219, 220)] and humans [MIW815/ADU-S100 and MK-1454 synthetic cyclic dinucleotides or small molecules like GSK3745417 (221, 222)] extended the possibilities of rational combinations with ICIs. Until the development of small-molecules suitable for systemic administration (223), clinical trials with the first STING agonists were focused on intratumoral delivery and thus limited to patients with accessible tumors.

In preclinical models, DMXAA, previously known for its antivascular properties (224) was shown to indirectly affect the release of TNFα and nitric oxide by TAMs (225, 226) and to induce the repolarization of M2-like into M1-like macrophages (227). DMXAA was able to promote rejection of B16 melanoma cells with an increased influx of CD8$^+$ TILs (228) and triggered the cooperation between lymphocytes and monocytes, macrophages and neutrophils in murine breast cancer (229).

However, due to distinct amino acids, DMXAA does not activate the human STING (219, 220), as confirmed by the negative results of Phase 3 trial in NSCLC patients (230). Several agonists specific for human STING have since been developed and recently entered the clinic. The combination of intratumoral MK-1454 plus Pembrolizumab resulted in encouraging efficacy and an acceptable safety profile in solid tumors or lymphomas (163). Moreover, the well-tolerated combination of intratumoral MIW815/ADU-S100 with the anti-PD-1 Spartalizumab has demonstrated antitumor activity in breast cancer and relapsed melanoma (164). MIW815/ADU-S100 is also being investigated in combination with anti-CTLA-4 in a Phase I trial. These STING agonists have demonstrated evidence of myeloid cell activation in patients through the induction of IL-6, CCL2 and type I IFN in the bloodstream and PD-L1 upregulation in tumors (231).

All-Trans Retinoic Acid (ATRA)

One of the first molecules that has shown an effect on myeloid cells is ATRA, a vitamin A derivative that binds to the retinoic acid receptor on MDSCs and immature monocytes, differentiating them into mature DCs (232). This molecule is a standard treatment for patients with acute promyelocytic leukemia (233) but it has been tested in clinical trials for other indications, such as small-cell lung cancer, where anti-tumor immune responses where accompanied by a decrease in circulating total MDSC ($Lin^{-}CD33^{+}HLA\text{-}DR^{-}$) and M-MDSCs (234). In a small clinical trial, melanoma patients treated with the combination of Ipilimumab and ATRA had significantly decreased circulating MDSCs when compared to Ipilimumab alone. Additionally, while a decrease in MDSCs was observed with the combination, the frequency of MDSCs increased over time in patients treated with Ipilimumab alone. Interestingly, compared to Ipilimumab alone, the combination induced an increased in circulating $HLA\text{-}DR^{+}$ myeloid cells over time, accompanied by a significant decrease in eosinophils. The combination treatment was also associated with improved $CD8^{+}$ T cell responses and the frequency of activated lymphocytes inversely correlated with that of circulating MDSCs in all patients (165). Even though patient enrollment in this study was halted following the approval of anti-PD-1 antibodies, the NCT03200847 clinical trial was launched with the aim of testing the combination of ATRA and Pembrolizumab, with an estimated completion date in June 2020.

Trabectedin

Another myeloid-targeting agent that could improve the efficacy of ICIs is Trabectedin, a DNA-binder of marine origin approved as a single agent for the treatment of soft tissue sarcoma and, in combination with doxorubicin, for relapsed platinum-sensitive ovarian cancer (235, 236). Trabectedin not only directly kills tumor cells by interfering with cell cycle progression, but also modulates the TME via a selective depletion of TAMs and MDSCs (237). In a murine ovarian cancer model, the combination of Trabectedin with anti-PD-1 significantly prolonged mice survival, with a concomitant decrease in MDSCs and TAMs and a significant increase of effector $CD4^{+}FoxP3^{-}$ T cells and $CD8^{+}$ T cells (238). Based on this evidence, several combination trials of Trabectedin and ICIs have been launched but the efficacy in patients is still undefined (**Table 1**).

Anti-angiogenic Molecules

Myeloid cells in tumors can also be indirectly affected by drugs that are not specifically design to target them. As an example, the vascular endothelial growth factor (VEGF), in addition to its role in angiogenesis, has profound effects on immune cell functions: it inhibits DC maturation, antigen presentation and lymphocyte infiltration, while promoting Treg and MDSC expansion in the TME (239–243). Preclinical models and phase 1 studies suggest that anti-VEGF molecules might enhance the antitumor activity of ICIs by improving T cell infiltration, upregulating MHC I expression and reversing myeloid immunosuppression (244).

Based on this rationale, several clinical trials combining ICIs and antiangiogenic agents are currently ongoing (**Table 1**). The potential synergy of Ipilimumab and the anti-VEGF Bevacizumab (Ipi-bev) has been investigated in metastatic melanoma. Compared with pre-treatment or with post-treatment samples from the Ipilimumab group, the combination enhanced the intratumoral endothelial activation, resulting in increased trafficking of $CD8^{+}$ T cells and $CD163^{+}$ dendritic macrophages across the tumor vasculature. Although not functionally characterized, macrophages displayed extensive dendritic processes, suggesting that Bevacizumab might have increased their maturation and antigen-presenting capacity (177).

In the same trial, the authors found that high circulating Angiopoietin-2 (ANGPT2) [a vessel-destabilizing molecule and critical regulator of blood vessel maturation (74)] levels at baseline and early during treatment were associated with shortened OS and reduced response. Treatment with PD-1 blockade or Ipilimumab alone increased, whereas Ipi-Bev decreased, serum ANGPT2 in a significant proportion of patients (178). ANGPT2 binds to the Tie2 receptor and can have an impact on monocytes and macrophages subsets that express it (245–247). Tumor biopsies with high tumor vascular ANGPT2 expression showed an increase in $CD68^{+}$ and $CD163^{+}$ macrophages after Ipilimumab or Ipi-bev treatment. Ipi-bev treatment, however, decreased tumor vascular ANGPT2 expression in a subset of patients, together with a decreased $CD68^{+}$ and $CD163^{+}$ macrophage infiltration, suggesting that ANGPT2 might have a role in resistance to ICI through TAM recruitment and that Bevacizumab might influence myeloid infiltration also by acting on the ANGPT2 levels. Moreover, ANGPT2 promoted PD-L1 expression on M2-polarized macrophages *in vitro*, hinting at another potential mechanism of resistance in ICI-treated patients with increased amounts of ANGPT2. In conclusion, ANGPT2 might serve as a potential predictive biomarker for ICIs and a possible target for combinations that could help reduce myeloid cell infiltration and tumor immunosuppression (178). As a consequence, the ANGPT2 inhibitor Trebananib is currently being tested in combination with Pembrolizumab (**Table 1**) (248).

Several clinical trials combining ICIs with Bevacizumab are also ongoing in mRCC (**Table 1**), in which elevated serum and tumor VEGF levels have been associated with poor survival

(249). In a study combining Bevacizumab with Atezolizumab, the authors demonstrated the ability of Bevacizumab to induce a Th1 signature with chemokines involved in lymphocyte trafficking, tumor MHC I protein expression and infiltration of tumor-specific T cell clones. As reported for Ipi-bev combination (177), the combination of Atezolizumab and Bevacizumab reduced the presence of $CD31^+$ blood vessels, especially of immature, unstable ones, with a widespread infiltration of immune cells. Notably, the on-treatment localization of $CD68^+CD163^+$, but not $CD68^+CD163^-$ macrophages, was observed adjacent to immature, but not mature, vessels. Nonetheless, the role and modulation of distinct TAM subsets during Bevacizumab treatment needs to be further explored to better understand the immune-related mechanisms of action of anti-angiogenic drugs in ICI combos (172). Moreover, the anti-tumor activity seen with the combination was associated with a further increase in $CD8^+$ T cells and unique T cell clones in the tumor, supporting the evaluation of this combination in phase 2 and 3 trials in mRCC and in other tumor types.

As discussed in the previous paragraphs, the IMmotion150 study was the first randomized trial to investigate the clinical activity of Atezolizumab with or without Bevacizumab against the standard-of-care Sunitinib in mRCC. Sunitinib efficacy was enriched in highly angiogenic tumors, while the combination of Atezolizumab and Bevacizumab improved clinical benefit compared with Sunitinib in T_{eff}^{high} tumors. Atezolizumab monotherapy was effective in tumors with pre-existing immunity and a relatively low expression of myeloid-associated genes, while the combination with Bevacizumab improved the clinical outcome in $T_{eff}^{high}Myeloid^{High}$ patients, confirming the ability of Bevacizumab to overcome myeloid-mediated resistance in these tumors (104).

CONCLUSIONS

The use of ICIs has greatly changed the survival of a substantial fraction of patients with cancer in the last years. However, the knowledge about the mechanisms of primary and acquired resistance is still limited. The exploration of biomarkers in clinical trials is essential to understand how the immune system and the TME of each patient influence the response to ICIs and thus how the therapy should be personalized.

In this review we have drawn attention to the impact of myeloid cells on ICI therapy, with a special focus on clinical data. The existing evidence supports the exploration and the formal validation of myeloid subsets in blood and tumor as both predictive and pharmacodynamic biomarkers and the use of myeloid-targeting agents as rational partners for ICI combinations. Even though most studies point to a regulatory role of cells of the monocyte/macrophage lineage, different subsets might be prevalent in different cancer types. Accordingly, multiparametric technologies (multicolor flow cytometry, mass-cytometry, multiplex immunofluorescence and bulk or scRNA sequencing) are crucial for the study of biomarkers, as they allow a more detailed characterization of the phenotype, function and localization of subsets that are more informative than the simple abundance of macro-populations detected with classical methods. At last, the encouraging data from clinical combinations of ICIs with myeloid-targeting drugs support the idea that controlling the expansion, recruitment and function of myeloid cells in tumors is crucial to extend the benefit of these immunotherapies to non-responding patients.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01590/full#supplementary-material

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REVIEW
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The New Era of Cancer Immunotherapy: Targeting Myeloid-Derived Suppressor Cells to Overcome Immune Evasion

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Suppression of antitumor immune responses is one of the main mechanisms by which tumor cells escape from destruction by the immune system. Myeloid-derived suppressor cells (MDSCs) represent the main immunosuppressive cells present in the tumor microenvironment (TME) that sustain cancer progression. MDSCs are a heterogeneous group of immature myeloid cells with a potent activity against T-cell. Studies in mice have demonstrated that MDSCs accumulate in several types of cancer where they promote invasion, angiogenesis, and metastasis formation and inhibit antitumor immunity. In addition, different clinical studies have shown that MDSCs levels in the peripheral blood of cancer patients correlates with tumor burden, stage and with poor prognosis in multiple malignancies. Thus, MDSCs are the major obstacle to many cancer immunotherapies and their targeting may be a beneficial strategy for improvement the efficiency of immunotherapeutic interventions. However, the great heterogeneity of these cells makes their identification in human cancer very challenging. Since both the phenotype and mechanisms of action of MDSCs appear to be tumor-dependent, it is important to accurately characterized the precise MDSC subsets that have clinical relevance in each tumor environment to more efficiently target them. In this review we summarize the phenotype and the suppressive mechanisms of MDSCs populations expanded within different tumor contexts. Further, we discuss about their clinical relevance for cancer diagnosis and therapy.

Keywords: immune evasion, melanoma, breast cancer, hepatocellular cacinoma, non-small cell lung cancer (NSCLC), myeloid derived suppressor cell (MDSC), prostate cancer, colorectal cancer

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INTRODUCTION

Cancer immune surveillance is an important process by which the immune system can eliminate nascent tumor cells and to control tumor evolution. Eventually, due to the genetic instability, new tumor cell variants can become resistant to immune effector cells by decreasing their immunogenicity and/or secreting and recruiting immunosuppressive factors in the tumor microenvironment (TME). During this phase of equilibrium, if the immune system is unable to eliminate these clonal variants, then tumors evolve mechanisms to escape from the immune

attack allowing malignant progression (1, 2). These mechanisms are diverse but primarily induce attenuation of anti-tumor $CD8^+$ T lymphocyte. Immunosuppressive myeloid cells, including myeloid-derived suppressor cells (MDSCs) are key mediators in assisting tumors to escape immune surveillance, contributing to tumor development and progression. MDSCs are a heterogeneous group of immature myeloid cells (IMCs) with strong immunosuppressive patterns and functions. Under normal condition, IMCs quickly differentiate into mature granulocytes, macrophages, or dendritic cells (DCs) which play essential roles in host defense against pathogens. However, in a variety of pathologic conditions such as inflammation, cancer and infection IMCs fail their normal differentiation and acquire the features of immature and dysfunctional myeloid population, which include MDSCs (2). Recently, it has been introduced the hypothesis that MDSCs could also be derived from mature myeloid cells such as monocytes and neutrophils in cancer settings (3, 4). In particular, it has been demonstrated that $CD14^+$ cells exposed to extracellular vesicles (EVs) (containing proteins, lipids, and genetic material) isolated from melanoma cells, show a suppressive activity on T cells thus referred as EV-MDSCs. Similarly, it has been reported that the treatment of healthy donor-derived monocytes with chronic lymphocytic leukemia (CLL) cells-derived exosomes induced MDSCs functional characteristics on monocytes mainly driven by miRNA-155 (5). Thus, deregulated myelopoiesis is a common occurrence in cancer and it is accompanied by a reciprocal decline in the quantity/quality of the lymphoid response (6). Myelopoiesis is a tightly controlled process. Certain transcription factors, such as CCAAT/enhancer binding protein-α (C/EBPα), and interferon regulatory factor-8 (IRF-8), are instrumental for normal myeloid cell development, differentiation and function and they can be targets of tumor-derived factors (TDFs). Thus, such TDFs may impair their expression, which ultimately affect the fate of the resulting myeloid response. Indeed, interventions that target atypical myelopoiesis by enhancing IRF-8 expression demonstrated to abrogate MDSC-mediated immunosuppression and to promote MDSCs differentiation in effector myeloid cells including DCs and mature neutrophils with anti-tumor activity (7–9). About 10 years ago, two major subsets of MDSCs have been identified based on their phenotypic and morphological features: monocytic-MDSCs (M-MDSCs) and granulocytic-MDSCs (G-MDSCs). G-MDSCs are phenotypically and morphologically similar to neutrophils, whereas M-MDSCs are similar to monocytes (10). In tumor-bearing mice these cells are characterized by the expression of CD11b and Gr-1 surface markers. The granulocyte marker Gr-1 includes the isoforms Ly6C and Ly6G, and these subsets can be more accurately identified based on their expression as $CD11b^+Ly6C^{hi}Ly6G^-$ (M-MDSCs) and as $CD11b^+Ly6C^{lo}Ly6G^+$ (G-MDSC) (11). However, several other cell surface markers are introduced such as F4/80, CD124 (IL-4Rα), CD115 (M-CSF-1R), and CD80 (B7.1), which are used for identification of MDSCs subsets and to distinguish MDSCs from neutrophils and monocytes (2, 12). In cancer, the frequency of G-MDSCs in the peripheral lymphoid organs is higher than M-MDSCs. In contrast, MDSCs in tumor sites are mainly M-MDSCs (13, 14). MDSCs are generated in the bone marrow from myeloid progenitor cells and then traffic through the circulatory system into solid tumors. The accumulation of MDSCs in TME mainly depends on two groups of signals. The first group include factors that are mainly secreted by tumor cells, such as stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF). These factors stimulate myelopoiesis and promote the expansion of MDSCs in lymphoid organs and TME by activating the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathways. In particular, the transcriptional factors/regulators STAT3, STAT5, IRF8, C/EBPβ, NOTCH play a major role in this process. The second kind of signals includes inflammatory cytokines and chemokines, produced mostly by the tumor stroma, such as IFN-γ, IL-4, IL-6, IL-1β, and CXCL1, which are responsible of inducing the suppressive activity of MDSCs via NF-κB, STAT1, and STAT6 (10, 15). Studies focusing on the role of MDSCs in cancer progression showed that the main activity of these cells is to suppress immunity by perturbing both innate and adaptive immune responses. In tumors, MDSCs have been demonstrated to inhibit cytotoxic T cells proliferation and activation leading to the failure of the anti-tumor immune response, promotion of cancer progression and chemoresistance (16). The main mechanisms implicated in MDSCs-mediated immune suppression include: (i) deprivation of T cells from essential amino acids; (ii) decreased expression of l-selectin by T cells; (iii) induction of oxidative stress; (iv) induction of immunosuppressive cells like regulatory T (T-regs) and T helper (Th) 17 cells (16, 17). Although the role of MDSCs as potent inducers of T-regs has been widely described in different types of cancer, recent findings also demonstrate that T-regs control MDSCs differentiation and function through different molecules such as transforming growth factor (TGF)-β and the programmed death ligand 1 (B7-H1) (18, 19). However, more research is needed to better dissecting the cross-talk between MDSCs and T-regs in the TME. In addition to suppression of immune surveillance, MDSCs can also directly promote tumor progression and metastasis through non-immunological functions by affecting the remodeling of the TME and tumor angiogenesis via production of VEGF, bFGF, Bv8, and matrix metalloproteinase (MMP)-9 (20). The main factors implicated in MDSC-mediated immune suppression include arginase 1 (ARG1), inducible nitric oxide synthase (iNOS), TGF-β, IL-10, cyclooxigenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO) and many others. M-MDSCs and G-MDSCs can utilize different mechanisms to suppress immune response. M-MDSCs express high levels of ARG1 and of iNOS, thus, they suppress T-cell responses, both in antigen-specific and non-specific manners, trough high production of nitric oxide (NO) in the TME. On the other hand, G-MDSCs are capable of suppressing immune responses primarily in an antigen-specific manner and they act mostly through production of high levels of reactive oxygen species (ROS) (14, 21). Several evidences suggest that on a per cell basis M-MDSC are more potent than G-MDSC (13). In contrast to murine models, the phenotype of MDSCs in humans is not as well-defined. Tipically, human tumor

infiltrating MDSCs express the markers CD33 common to cells of myeloid lineage, but lack the expression of the maturation myeloid marker HLA-DR. Analogously to the murine MDSCs, human MDSCs are broadly classified into two different subsets, monocytic and granulocytic, based on the expression of the monocyte differentiation antigen CD14 and the mature monocyte marker CD15. Thus, human M-MDSCs are mostly $CD33^{+}CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}$ whereas human G-MDSCs are $CD33^{+}CD11b^{+}HLA\text{-}DR^{-/low}CD14^{-}CD15^{+}$. However, the gating strategies used to identify MDSCs populations can vary among researcher. G-MDSCs and neutrophils share similar phenotype; however, they have different density. Recently, identified lectin-type oxidized LDL receptor 1 (LOX-1) allows for better distinction between human neutrophils and G-MDSC. Immune suppressive $LOX\text{-}1^{+}$ cells represent 4–15% of all neutrophils in blood of cancer patients and up to 40% in tumor tissues, whereas in healthy individuals, these cells represent <1% (22). Conversely, human M-MDSC can be easily separated from monocytes based on the expression of MHC class II molecules which is expressed only on monocytes ($HLA\text{-}DR^{+}$) (23). In addition to the granulocytic and monocytic subtypes, a third small population of putative MDSCs that includes cells with colony-forming activity and promyelocytic appearance was described in humans. These cells, termed immature or early-stage MDSCs (e-MDSCs), have the phenotype $CD33^{+}CD11b^{+}HLA\text{-}DR^{-}CD14^{-}CD15^{-}$ cells (11, 24). Human M-MDSCs and G-MDSCs, like murine MDSCs, have been shown to exhibit distinct functional attributes. In particular, G-MDSCs primarily use ROS as the mechanism of immune suppression whereas M-MDSCs show up-regulation of iNOS, ARG1, and an array of immunosuppressive cytokines (17). In recent years, the clinical role of MDSCs has emerged. Numerous studies have reported the expansion of MDSCs in various human cancers including breast, colon, lung, pancreatic, renal, esophageal, and melanomas (24–26). Moreover, the frequency of MDSCs have also been negatively correlated with the response to immunotherapy (27). Therefore, targeting MDSCs in cancer patients may be a viable therapeutic approach to reverse immune escape and to maximize immune based treatments.

However, an important issue in this viewpoint is the great heterogeneity of these cells, which make the identification and isolation of human MDSCs subsets very challenging. Several data found a significant diversity in the MDSCs subsets in different human cancers. Moreover, the frequency and the mechanisms of action of each MDSCs subset seems to be influenced by the cancer type (26). Thus, the precise identification of cell surface markers and the exact definition of human MDSCs in different types of malignancies can be useful to improve the efficacy of immunotherapeutic interventions and cancer treatment. In this review, we summarize the phenotype and the biological function of MDSCs populations expanded within different tumor contexts which have showed the strongest negative association with MDSCs, as well as discuss their clinical relevance for cancer diagnosis and therapy.

MAIN STRATEGIES TO THERAPEUTICALLY TARGET MDSCs IN CANCER

Inhibition of MDSCs in cancer therapy has proven to be a potentially promising and well-tolerated treatment. Increasing numbers of pre-clinical studies and clinical trials have been performed over the past years in order to evaluate the safety and the efficacy of MDSCs inhibition, alone or in combination with other therapy (radiotherapy, chemotherapy, surgery or immunotherapy) in cancers. Currently, different therapeutic strategies aimed at eliminating MDSCs and/or abrogating their pro-tumor activities are being investigated. These approaches include (1) depletion of MDSCs; (2) inhibition of MDSCs recruitment to the tumor site; (3) inhibition of MDSC's suppressive activity; (4) promoting MDSCs differentiation (**Figure 1**).

In mouse models, depletion of MDSCs has been generally accomplished by the use of antibodies that target the surface markers Gr-1 or Ly6G (28). More recently, novel approaches have been developed to more preferentially target and deplete MDSCs. For example, "peptibodies" consisting of S100A9-derived peptides conjugated to antibody Fc fragments have shown potential in eliminating MDSCs in mouse models without targeting other proinflammatory immune cells (29). In addition, induction of Fas-FasL mediated apoptosis of MDSCs have been resulted effective in suppressing tumor growth and restoring T cells immune response in different murine tumor models (30–32). Similarly, targeting the TNF-related apoptosis-induced ligand (TRAIL) receptor could be a potent and selective method for MDSCs depletion (33). Some chemotherapeutics such as gemcitabine, 5-fluorouracil, paclitaxel, and doxorubicin were shown to selectively eliminate MDSCs in the spleen, blood, and tumor beds in several mouse tumor models resulting in the enhancement of the function of immune effector cells (34–38). These findings reinforce the concept that depleting MDSCs has great therapeutic promise. In cancer patients, "conventional" therapies including surgical resection (39), radiotherapy (40) or chemotherapy with gemcitabine or 5-fluorouracil, showed a decrease of MDSCs leading to the immune recovery and tumor regression (35, 36). However, MDSC numbers and/or function have been assessed in few chemotherapy clinical trials and have shown mixed results.

Intensive investigations have been performed to reduce MDSCs trafficking to peripheral lymph nodes and tumor sites. Chemokine receptors are a key driving force for the migration of MDSCs and blocking the interactions with their ligands is a rational approach to inhibit MDSCs accumulation in the TME (41). In particular, therapeutic blockade of CCL2-CCR2 interaction by using CCL2 neutralizing antibodies or CCR2 antagonist has demonstrated promising antitumor efficacies in several preclinical cancer models (42–44). However, in a phase II clinical trial, was reported that carlumab (anti-CCL2 mAb) in patients with metastatic castration-resistant prostate cancer, induced a rapid rebound of the circulating concentration of free CCL2 to value higher than the pretreatment serum levels (45). The CCR5–CCL5 axis has also a critical role in tumor

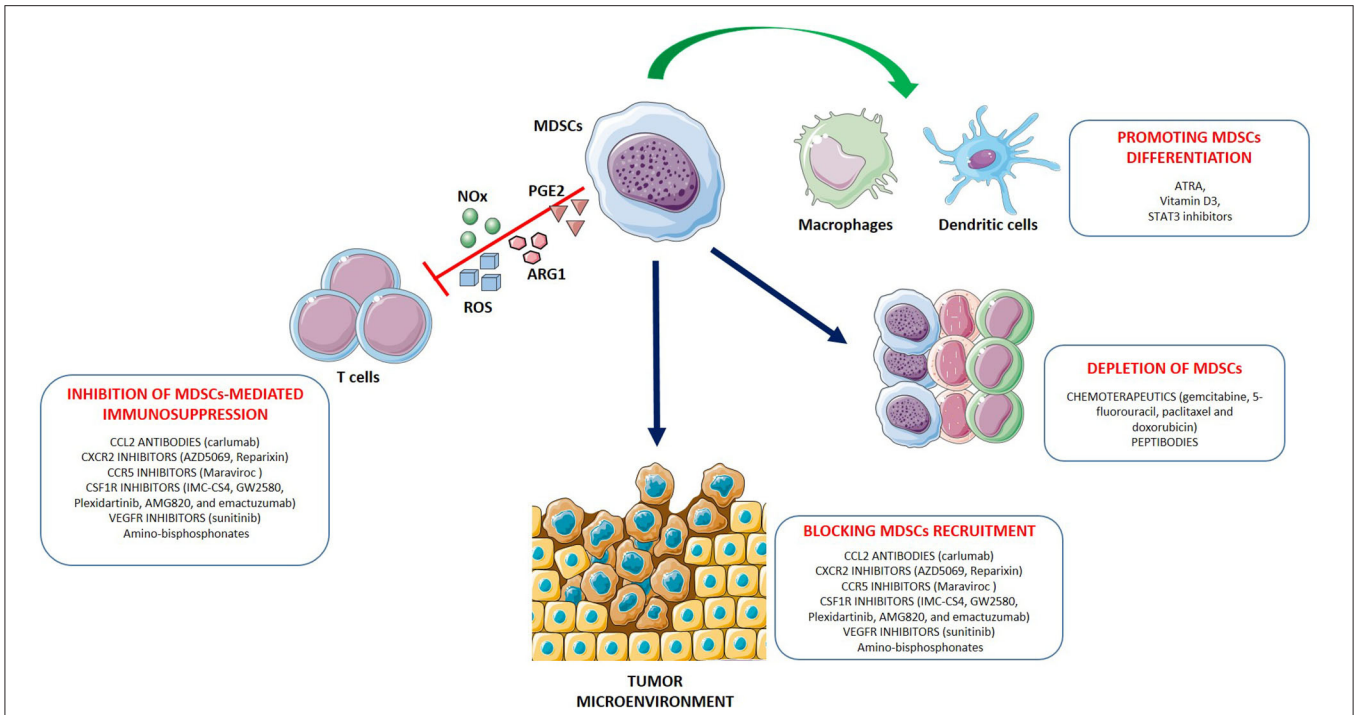

FIGURE 1 | Strategies for myeloid-derived suppressor cells (MDSC) targeting. The main approaches to target MDSCs include: (1) depleting MDSC populations; (2) preventing MDSC recruitment and migration to the TME; (3) attenuating the immunosuppressive mechanisms of MDSCs by downregulating the expression of ARG1, iNOS, COX-2 and reducing ROS generation; (4) promoting the differentiation of MDSCs into mature non-suppressive myeloid cells like macrophages and dendritic cells. Examples for each therapeutic approach are shown.

progression since it supports tumor invasion and migration of MDSCs to the tumor site (46). Indeed, by targeting the CCR5-CCL5 interaction, tumor growth and invasiveness were suppressed in colorectal, prostate, breast cancer and melanoma (47–50). Another well-characterized target to reduce MDSCs trafficking is the colony-stimulating factor 1 receptor (CSF1R) whose expression is restricted to monocytes and macrophages. Various inhibitors against CSF1R (such as IMC-CS4, GW2580, PLX3397, AMG820, and emactuzumab) have shown promising antitumor efficacies by inhibiting the survival of M-MDSCs and tumor associated macrophages (TAMs) and are being tested in combination with chemotherapy or immunotherapies in cancer patients (51). The following MDSCs inhibitors have been evaluated in clinical trials (52): Reparixin and AZD5069 (CXCR2 antagonists), respectively, in phase II for breast cancer and in phase Ib/II for advanced solid tumors and metastatic squamous cell carcinoma; Plexidartinib (CSF-1R inhibitor) in phase II for recurrent glioblastoma; Maraviroc (CCR5 antagonist) in phase I for metastatic colorectal cancer. The expansion and recruitment of MDSCs to the tumor sites is also mediated by MMP9. It has been shown that administration of amino-bisphosphonates drugs can prevent MMPs from undergoing prenylation, a post-translational modification that is essential for their function. As a result of reduced MMP9 prenylation, cleavage of the tyrosine kinase c-Kit is diminished, causing reduced mobilization of MDSCs (53). Amino-bisphosphonates have shown a good safety and tolerance and seem to exert therapeutic effects, making them promising candidates to target MDSCs (54–56). The inhibition of VEGF receptor signaling also leads to a reduction of MDSCs infiltration (57). Indeed, the tyrosine kinase inhibitor (TKI) sunitinib was reported to decrease the number of circulating MDSCs in renal cell carcinoma patients via blockade of VEGF and c-KIT signaling (58). Interestingly, sunitinib treatment resulted also in a significant reduction of STAT3 activation and ARG1 expression in M-MDSCs that was accompanied with an elevated activity and proliferation of $CD8^+$ T cells (59).

Blockade of MDSCs immunosuppressive mechanisms represents the major therapeutic approach to re-establishing T-cells activity and immunotherapy success. MDSCs can be functionally inactivated by targeting their suppressive machinery. For example, disruption of the COX-2/prostaglandin E2 (PGE2) signaling has been successful in repressing MDSC-associated suppressive factors such as ARG1 expression and ROS production, and improving T-cells frequency and immune response (60, 61). Phosphodiesterase-5 (PDE-5) inhibitors are also able to inhibit MDSCs functions by the downregulation of iNOS and ARG1 expression and activities. In preclinical mouse models, administration of PDE-5 inhibitors, such as sildenafil and tadalafil, has been demonstrated to reactivate antitumor immunity through T-cells and natural killer (NK) cells and to prolong survival of tumor-bearing mice (62–64). Recent clinical trials with PDE-5 inhibitors have also shown enhanced intra-tumor T-cells activity and improved patients' outcome in head and neck squamous cell carcinoma

(HNSCC) and metastatic melanoma (65–67). Blocking the immunosuppressive function of MDSCs can also be achieved by targeting phosphatidylinositol 3-kinase (PI3K). Knockout of PI3K was found to reduce the accumulation of G-MDSCs in tumor-bearing mice, breaking immune tolerance to cancer (68). Anti-inflammatory triterpenoids, have been demonstrate to reduce intracellular ROS production by MDSCs by upregulating the nuclear factor erythroid 2-related factor 2 (Nrf2) which plays an important role in the cellular protection against free radical damage (69). Moreover, synthetic triterpenoids, such as CCDO-IM and CCDO-Me, have shown promising anticancer results in phase I clinical trials (69, 70). Administration of ATRA, a vitamin A derivative binding to the retinoid receptor, also led to the downregulation of ROS production in MDSCs by activating the extracellular-signal regulated kinase (ERK)1/2 pathway (71). The selective class I histone deacetylase (HDAC) inhibitor entinostat has been reported to have an inhibitory effect on MDSCs immunosuppressive functions in several preclinical tumor models (72–74). Indeed, analysis of MDSCs response to entinostat revealed significantly reduced ARG1, iNOS, and COX-2 levels in both M- and G-MDSCs subsets. Interestingly, the combination of entinostat with immune checkpoint inhibitors resulted in prolonged survival and delayed tumor growth along with an increase of $CD8^+$ T effector cells in tumor-bearing mice (73, 74). Clinical trials involving entinostat are currently underway (52). Recently, the inactivation of class II HDAC (HDAC6) with ricolinostat was found to further increase the inhibitory effect of entinostat on the MDSCs suppressive activity and on tumor progression (75). STAT3 is another promising target to reduce MDSCs immunosuppressive functions. Various approaches for STAT3 inhibition, such as inhibiting the (1) SH2 domain or dimerization, (2) upstream TKIs (e.g., JAK and Src inhibitors), (3) antisense oligonucleotides, and (4) peptide mimetics of physiological negative modulators of STAT3, have been tested in pre-clinical model and in clinical trials. However, their clinical use in advanced solid tumors have revealed limited efficacy or excessive toxicities (76). Recently, the antisense oligonucleotide STAT3 inhibitor, AZD9150, has been tested in phase I/Ib clinical trials for the treatment of diffuse large B-cell lymphoma. Systemic administration of AZD9150 in patients showed a positive immunomodulatory effect, with a marked decrease in G-MDSCs in the peripheral blood, and a meaningful antitumor activity. Trials to combine this agent with checkpoint-targeting immunotherapies are in progress (77).

Finally, another therapeutic approach used for targeting MDSCs is aimed to induce MDSCs differentiation, converting them into mature non-suppressive cells. One promising therapeutic appears to be ATRA which was reported to induce the rapid differentiation of MDSCs into mature myeloid cells, such as macrophages and DCs, and to improve T-cells response in cancer patients (78, 79). The mechanism of ATRA-induced differentiation of MDSCs involves specific up-regulation of glutathione synthase and neutralization of high ROS production in these cells (80). Several studies indicate that vitamin D3 is another agent that can promote myeloid cells maturation and reduce the number of MDSCs in cancer patient. In particular, vitamin D3 administration in HNSCC patients increased levels of IL-12, IFN-γ, and improved T-cells blastogenesis (81). Transcription factors instrumental for normal myeloid cells development, differentiation and function can also be a target to reducing aberrant myelopoiesis. In particular, the interferon regulatory factor (IRF)-8 is a "master regulator" of normal myelopoiesis, indispensable for producing monocytes, DCs and neutrophils from myeloid progenitors (82). Thus, enforced expression of IRF-8, either directly or indirectly, may facilitate myeloid differentiation and improves immunotherapy efficacy (83). Further, it has been hypothesized that tumor-induced IRF8 downregulation occurred through a STAT3-dependent interaction. Indeed, STAT3 inhibition can induce MDSCs differentiation into immunogenic DCs or macrophages (84, 85).

MDSCs IN BREAST CANCER

Breast cancer (BC) is the most commonly occurring cancer and the leading cause of cancer-related deaths in females worldwide (86). Clinically, BC is a heterogeneous disease. Analyses of gene-expression profiling have identified three main groups of BC based on estrogenic receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2/neu) expression (87). This classification is critical for guiding treatments, which mainly include surgery (mastectomy or lumpectomy), radiotherapy, anthracycline-based chemotherapy or hormonal therapies with anti-HER-2 monoclonal antibodies (mAb), i.e., trastuzumab, pertuzumab, and TDM1 (88). Immunotherapy is not yet considered a routine form of treatment for BC patients. However, a recent pooled analysis of 1,954 breast tumor demonstrated that some BC, based on their different immunogenic sensitivity, can be distinguished into two discernible subtypes termed *immune benefit-enabled* and *immune benefit-disabled* which showed significant differences in distant metastasis-free survival (89). A better understanding of the factors that regulate BC immunogenicity will contribute to create more effective and personalized therapeutic strategies that target specific immunogenic subtypes. In particular, BC weak immunogenicity derive from mechanisms that diminish immune recognition and promote strong immunosuppression. Infiltration of immunosuppressive cells like T-regs, MDSCs or TAMs in the TME has been demonstrated to be the major mechanism of tumor escape from the immune system and the main cause in the reduction of the efficacy of immunotherapy (90). Indeed, circulating MDSCs in peripheral blood of BC patients have been shown to be elevated in all stages of the disease and to be positively correlated with clinical cancer stage and extensive metastatic tumor burden (91, 92). Conversely, tumors showing greater infiltration of about 50–60% of tumor-associated effector cells, such as cytotoxic T cells, memory T cells, NK cells, tend to be more immunogenic and more sensitive to chemotherapy. Thus, their presence has been associated with the suppression of metastatic recurrence resulting in a relatively good prognostic outcome (93–96). Most of the research on MDSCs in the TME has been performed in murine models, which have provided the first evidence that MDSCs are involved in the development and progression of BC. Thus, eliminating

MDSCs can result in increased immune-mediated anti-tumor responses and decreased tumor-burden (97–101). Nevertheless, also in human it has been showed a direct correlation between MDSCs levels in the peripheral blood of BC patients, disease malignancy and poor prognosis. In one of the earliest study by Diaz-Montero et al. (91), the percentage and the absolute number of circulating MDSCs were significantly increased in cancer patients compared to normal volunteers. A population of MDSCs, defined as $Lin^{-/Lo}$ HLA-$DR^{-}CD33^{+}CD11b^{+}$, was detected in fresh whole blood from 106 BC patients. In these patients, it was found that both percentage and absolute number of circulating MDSCs were associated with the clinical cancer stage. Significant differences were observed in mean MDSCs between patients with early stages I/II cancer (1.96%) stage III (2.46%) and advanced stage IV (3.77%). Overall, stage IV patients with widely metastatic disease had the highest percent (4.37%). In that report, it has been also observed that MDSCs levels in the peripheral blood corresponded to circulating tumor cells levels, which are another emerging prognostic marker. Similarly, Solito et al. (102) also identified MDSCs ($Lin^{-/Lo}$ HLA-$DR^{-}CD33^{+}CD11b^{+}$) in 25 stage IV BC patients. They showed that subjects with higher circulating MDSCs > 3.17% (median) at baseline had a poorer overall survival (OS) than patients with circulating MDSCs ≤ 3.17%, with median OS times of 5.5 and 19.32 months, respectively. Interestingly, Yu et al. identified a unique population of MDSCs in BC with the phenotype $CD45^{+}CD33^{+}CD13^{+}CD14^{-}CD15^{-}$. They found that these cells increased both in primary cancer tissues and in peripheral blood. The proportion of this cell population correlated with clinical stage and lymph node metastasis status in BC patients and exerted potent immunosuppressive activity on T cells. Further, they reported that IDO, a rate-limiting enzyme of tryptophan catabolism, was significantly upregulated in tumor-infiltrating MDSCs than in periphery, thereby suggesting a pivotal role in developing and maintaining MDSCs-mediated immunosuppressive functions in tissue (103). Recent studies also confirmed that tumor progression and invasion paralleled the development of MDSCs. For instance, Gonda et al. (104) reported that the levels of circulating MDSCs ($CD33^{+}CD11b^{+}CD14^{-}$) in the peripheral blood were increased in BC patients compared with healthy controls. Moreover, MDSCs levels were considerably higher in preoperative patients and decreased in postoperative patients or following chemotherapy, while they reached again high levels in patients with recurrent disease. They found that, in preoperative patients, MDSCs levels positively correlated with IL-6 production while they negatively correlated with IFN-γ and IL-12 production. IL-12 is known to be a modulator of immune suppression which induces Th1 cells while IL-6 promotes a Th2-dominant status. Thus, the immune suppressive function of MDSCs in BC patients may involve multiple immunological pathways, which impair the Th cell balance promoting a shift from Th1 to Th2 predominance. Additionally, Bergenfelz et al. (92), reported an expansion of circulating $CD14^{+}$HLA-$DR^{-/low}$ M-MDSCs in patients with locoregional recurrence or metastatic BC, which was correlated with increased metastasis to lymph nodes and visceral organs, suggesting that circulating M-MDSCs could be a potential biomarker for disease progression and a guide to individualize efficient immunomodulatory treatments. Also Safarzadeh et al. (105) showed that M-MDSCs (HLA-$DR^{-}CD33^{+}CD14^{+}$) represent a high percentage compared with the G-MDSCs (HLA-$DR^{-}CD33^{+}CD15^{+}$) subpopulation in BC patients. A recent study found that cells with the M-MDSCs phenotype $CD14^{+}$HLA-$DR^{-/low}$ are present at significantly higher frequencies in early-stage BC patients (40 patients with clinical stages I/II), suggesting that M-MDSCs mostly participate to the development of BC by protecting tumor cells from immune attack. In particular, one of the suppressive mechanisms proposed by the authors for M-MDSCs-mediated immunosuppression is represented by ROS (106). Conversely, Toor et al. (107) found that BC patients had significant elevated levels of granulocytic $CD33^{+}$ $CD11b^{+}$HLA-$DR^{-/low}CD15^{+}$ MDSCs in the TME vs. surrounding healthy tissue whereas no significant differences were observed in their peripheral blood compared to healthy individuals. However, a weakness of this study may be the small number of patients included (23 patients). In BC, after differentiation and recruitment, MDSCs suppress T cells via several pathways including the ARG1, ROS, RNS, and NO pathways (108). Indeed, nitration/nitrosylation of T cell receptors (TCRs) and CD8 molecules on the surface of T cells induces T cell tolerance (109). The JAK/STAT pathway is also important in regulating the various functions of MDSCs. Indeed, the transcription factor STAT-3 modulates the expression of target genes involved in various proinflammatory functions. Among them, STAT-3 promotes IDO expression. As mentioned before, IDO act as a major immune regulator inhibiting immune surveillance and promoting immune tolerance by suppressing TCR-mediated activation of T cells, as well as inducing amplification of T-regs (110, 111). Besides their canonical immunosuppressive functions, MDSCs have also direct effects on BC cells contributing to invasiveness and metastasis through the activation of the intracellular phosphatase and tensin homolog (PTEN)/Akt pathway. Upregulation of Akt in MDSCs results in increased expression of MMPs, including MMP2, MMP13, and MMP14, in BC cells which in turn promote invasion and metastasis (108). Moreover, MDSCs can act as osteoclast progenitors promoting BC metastasis to the bone. Through NO signaling and cross talk with BC cells, MDSCs can differentiate into osteoclasts in the bone microenvironment to exacerbate osteolysis in metastasizing BC which represent important issue for BC patients, causing high morbidity and mortality (98). In summary, these studies further strengthen the observations that MDSCs numbers increase in patients with BC as compared to healthy people, suggesting that targeting MDSCs may significantly improve the effect of immunotherapy protocols in patients with BC. In preclinical studies it has been demonstrates that CCR5 antagonists inhibited the metastatic potential of basal BC and reduced tumor growth (49). CSF-1R inhibition and CXCR2 antagonism has also been used in combination to reduce TAMs and G-MDSCs populations and improve anti PD-1 efficacy (51, 112). Further, the HDAC inhibitor, entinostat, in combination with the checkpoint inhibitors anti–PD-1 and anti–CTLA-4, led to a significant suppression of G-MDSCs in the TME and significantly improved

tumor-free survival in HER2/neu transgenic BC mouse model (74). Combination of entinostat with nivolumab and ipilimumab is, currently, under evaluation in a phase I trial in patients with invasive and metastatic BC (NCT02453620). Other clinical studies aimed to investigate the effect of MDSCs inhibitors in combination with immunotherapy are ongoing.

MDSCs IN COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer and the second cause of cancer deaths worldwide (86). Only 5–6% of CRC cases involve inherited genetic alterations while environmental factors, lifestyle (such as physical inactivity, smoking, alcohol consumption and obesity) and gut microbiota are responsible of ∼90% of CRC occurrence (113). The current approaches to treat metastatic CRC (mCRC) involve multimodal therapy based on chemotherapy (including the combination of cytotoxic drugs) or targeted agents (such as bevacizumab, cetuximab, and panitumumab) (114). In the last few years, immunotherapy, which typically rely on the activation of T cells in the TME, has been considered for mCRC patients (115). Checkpoint inhibitors such as antibodies directed against cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death protein (PD-1)/PD-1 ligand (PD-L1) resulted ineffective to produce durable clinical responses due to tumor-mediated immune evasion and resistance, caused by the presence, into the TME, of immunosuppressive cells like MDSCs (116). In CRC, MDSCs are widely considered the link between chronic inflammation and cancer. Indeed, patients with inflammatory bowel disease, such as ulcerative colitis, show an increased risk of developing CRC (117). Evidences from studies in mouse models of colitis-associated cancer (CAC) indicate that chronic inflammation can drive tumor initiation and progression by enhancing MDSCs accumulation and immune suppression (118, 119). Accumulating data also support a role for the microbiota in CRC carcinogenesis (120). Recent studies have shown that symbiotic bacteria like *Fusobacterium nucleatum* and *Helicobacter hepaticus* can exacerbate the development of cancer by inducing MDSCs expansion in the inflamed colon of mice (121, 122). Although both MDSCs subtypes have been found increased in several colon cancer mouse models, the expansion of G-MDSCs resulted much greater compared to M-MDSCs (118, 119, 122, 123). In CRC patients, at first, MDSCs were identified generally as $CD33^+HLA\text{-}DR^-$ (124, 125). Both circulating and tumor-infiltrating MDSCs have been found significantly expanded in patients with various stage of CRC compared with healthy donors. Interestingly, their frequencies were shown to increase with tumor stage and with the presence of nodal and/or distant metastasis, indicating a correlation with clinical cancer stage. These MDSCs displayed characteristics of immature myeloid cells expressing no level of the lineage markers CD3, CD14, CD19, and CD56. Notably, they showed up-regulation of CD18/CD11b expression, which is critical for cell adhesion and migration, suggesting the involvement of MDSCs in CRC tumor development (124). Further, Zhang et al. (125) demonstrated that $Lin^{-/low}HLA\text{-}DR^-CD33^+CD11b^+$ MDSCs had immunosuppressive effect on T cells and expressed high level of the ectonucleotidase molecule CD39, which plays a key role in mediating the suppressive activity of MDSCs on T cells, by converting immunostimulatory ATP into immunosuppressive adenosine. A better phenotypical characterization of MDSCs in CRC patients was originally reported by OuYang et al. (126). They observed an increased proportion of $CD33^+CD11b^+HLA\text{-}DR^-$ MDSCs in peripheral blood and tumor tissues which correlated with advanced disease stages and tumor lymph node metastases. In particular, this population consisted for the major part of a M-MDSCs subset ($CD33^+CD11b^+HLA\text{-}DR^-CD14^+CD15^-$) and an atypical G-MDSCs subset, with a moderate expression of the granulocyte-monocyte progenitor cell markers CD117 and a weak expression of the granulocytic marker CD15. These MDSCs populations were found to suppress both $CD8^+$ and $CD4^+$ T cells proliferation through the oxidative metabolism, including the generation of NO and ROS, as demonstrated by the high expression levels of the immune mediators ARG1, iNOS, and NOX2. Conversely, Toor et al. (127) identified $CD33^+CD11b^+HLA\text{-}DR^{-/low}CD15^+$ G-MDSCs as key players among others in CRC progression. They found a significant expansion of G-MDSCs in both circulation and in tumor tissues of 21 CRC patients with different tumor stages. In particular, circulating G-MDSCs were significantly elevated in CRC patients with regional and distant metastases and exerted their immunosuppressive functions trough the activation of ARG1. Several factors have been implicated in the regulation of the accumulation and the suppressive functions of MDSCs in CRC. IL-17 appears one of the main driving chemoattractant forces, especially for G-MDSCs, within the TME (128). In murine tumor models, IL-17 promotes MDSCs tumor infiltration, in a CXCL5/CXCR2-dependent manner, and enhances the immunosuppressive activity of MDSCs (129). Chun et al. (119) postulated that CCL2 acts as a neoplastic regulator of MDSCs, contributing to their intratumoral accumulation and to G-MDSC-mediated suppression of $CD4^+$ and $CD8^+$ T cells via STAT3-mediated pathway. Indeed, increased CCL2 in patients with early-stage colon cancer (colitis-associated CRC, adenocarcinomas, and adenomas) influences carcinogenesis inducing MDSCs. Thus, CCL2 neutralization may afford therapeutic opportunities to decreased MDSC accumulation and function. Recent data indicate that Yes-associated protein 1 (YAP1) and PTEN can mediate CRC tumorigenesis through the induction of MDSCs in the TME. In fact, Yang et al., describe that up-regulation of YAP1 in the tumor promoted MDSCs expansion through suppressing PTEN expression and subsequently inducing the secretion of GM-CSF (130). Further, inhibition of Kit has been demonstrated to enhance the antitumor activity of immune checkpoint inhibitors (anti–CTLA-4 and anti–PD-1) by selectively reducing the immunosuppressive M-MDSCs population in Colon26 mouse tumor model (131). The humanized anti-Kit mAb KTN0158 has also been evaluated in clinical trials for patients with Kit positive advanced solid tumors (NCT02642016). Notably, the inhibition of STAT3 signaling pathway with nifuroxazide inhibited lung and abdomen metastasis in mice and reduced the number of MDSCs in the blood, spleens and

tumors, accompanied by the increased infiltration of $CD8^+$ T cells (132). Targeting TRAIL-R2 with the agonist antibody DS-8273a was applied in a phase I clinical trial in patients with advanced cancers, including CRC patients, in combination with nivolumab and caused selective depletion of MDSCs without affecting mature myeloid or lymphoid cells (133). Thus, a better identification of the molecular mechanism driving MDSCs expansion in CRC may guide the future development of new therapeutic strategies for CRC patients based on targeting MDSCs.

MDSCs IN MELANOMA

Melanoma is the most aggressive and fatal form of skin cancer with a high mortality rate. Primary melanoma is usually curable with surgery when diagnosticated in early stages (134). Nonetheless, melanoma is characterized by a lively progression that is correlated to rapid metastasis development to regional lymph nodes and distant organs as well as therapy resistance by reducing the patients median survival to <1 year (135). In fact, despite the recent introduction of encouraging immunotherapies such as ipilimumab and pembrolizumab, that target CTLA-4 and PD-1 respectively, the majority of patients experience resistance and tumor progression (136). This critical condition is partially due to the immunosuppressive mechanisms established within the TME mediated by immunoregulatory cells including T-regs and MDSCs that contributes to immune evasion (137). In particular, multiple reports have highlighted the role of MDSCs as one of the most important restrictions preventing efficient melanoma treatment (116). Several reports indicated an increased frequency of both M-MDSCs and G-MDSCs in melanoma patients (138–141). For instance, Jordan et al. demonstrated that the frequency of both M-MDSCs ($Lin^-CD11b^+HLA\text{-}DR^{-/low}CD33^+CD14^+$) and G-MDSCs ($Lin^-CD11b^+HLA\text{-}DR^{-/low}CD33^+CD14^-$) subsets was significantly increased in the peripheral blood of stage IV melanoma patients and was associated with disease progression and decreased OS (142). Similarly, Filipazzi et al. reported an expansion of $CD14^+CD11b^+HLA\text{-}DR^{-/low}$ M-MDSCs in fresh whole blood from 70 advanced melanoma patients suggesting an inverse correlation with immune responses to cancer vaccine (138). Additionally, Weide et al. also reported that circulating $CD14^+CD11b^+HLA\text{-}DR^{low}$ M-MDSCs were inversely correlated to both OS and the presence of functional antigen-specific T cells in patients with advanced melanoma (140). Conversely, more recently Stanojevic et al., demonstrated that $HLA\text{-}DR^{-/low}CD11b^+CD33^{low}Lin^-CD14^-CD15^+$ G-MDSCs population was significantly higher in different clinical melanoma stages according to both TNM and AJCC classification (143). Thus, MDSCs abrogation and inhibition, could be the next biggest aims for melanoma treatment (144). In fact, in the last few years, various preclinical studies have been focused in measuring and targeting MDSCs in melanoma patients, resulting in tumor growth inhibition and survival prolongation (145). Nevertheless, there are different ongoing clinical trials focused on evaluating the effect of new molecules that target MDSCs in melanoma patients such as ATRA), SX-682 or omaveloxolone in combination with classical immune checkpoint inhibitors (116, 144). ATRA, that has previously demonstrated to induce differentiation of MDSCs into macrophages and DCs in mice, (80) has been applied in a phase II clinical trial in combination with ipilimumab in melanoma patients. The study demonstrated that this combination improved the clinical outcome by increasing tumor antigen-specific T cell responses and reducing MDSCs frequency as compared to ipilimumab alone (146). SX-682 is a selective and potent antagonist of CXCR1/2 chemokine receptors that are expressed on both melanoma cells and MDSCs supporting tumor growth, immunosuppression and angiogenesis in response to CXCL1, CXCL2, or CXCL8 (147–149). Omaveloxolone (also referred as RTA408), is a semisynthetic oleanane triterpenoid that represses ROS production and NO signaling in MDSCs showing promising preclinical antitumor activity (150). Both SX-682 and RTA408 have been applied in two different clinical trials in combination, respectively with pembrolizumab (NCT03161431) and ipilimumab or nivolumab (NCT02259231) (116, 144, 151). Interestingly, MDSCs enrichment in melanoma patients has been frequently associated to heightened amounts of inflammatory mediators such as IFN-γ, IL-1β, IL-4, IL-13, TNF-α, toll-like receptor (TLR) ligands, and PGE2 that support MDSCs accumulation and activation (152, 153). PGE2 is one of the best-characterized prostaglandins synthesized by COX-2. Recently, we and others reported that COX-2 has a crucial role in melanoma development and progression by affecting patients progression free survival (PFS) (154–156). In particular, PGE2 production by MDSCs has been associated to ARG1 overexpression, STAT3 and STAT1 phosphorylation and IL-10, ROS, and NO production that are correlated to MDSCs suppressive activity (157–160). Thus, PGE2–dependent activation of MDSCs result to be a potent additional mechanism of tumor immune escape which is driven by COX-2 (161). Indeed, COX-2 pharmacologic inhibition reverts MDSCs suppressive phenotype by reducing the production of ROS and NO, the expression of ARG1 and restoring the differentiation of bone marrow cells (162, 163). Nevertheless, a better understanding is necessary to figure out which mechanisms PGE2 exploits for triggering MDSCs immunosuppressive effects in malignant melanoma. Recently, a new class of compound defined as hydrogen sulfide donors, has been shown to inhibit both the expansion and the suppressive functions of MDSCs in melanoma-bearing mice (164). Interesting results have also been achieved in the field of microRNAs (miRNAs) (165). miRNAs are relevant multifunctional post-transcriptional modulators of gene expression which have been reported to play a key-role in various human cancers including melanoma (166–171). Different evidences established an emerging role for miRNAs in the expansion and functional activation of MDSCs during tumor development (165). For instance, miR-155 has been shown to promote tumor growth by triggering MDSCs ripening, endurance and function through SOCS1 inhibition (172). More recently, Huber et al., discovered a set of miRNAs that are associated with the phenotypic and functional features of MDSCs in melanoma

patients (173). Most importantly, they reported that higher expression of these miRNAs is correlated to shorter PFS in patients receiving ipilimumab and nivolumab (173). Finally, miRNAs identification as MDSC regulators, could be an additional and promising strategy to fight and monitor systemic immunosuppression that occur in melanoma patients, mainly driven by MDSCs.

MDSCs IN PROSTATE CANCER

Prostate cancer is the most commonly diagnosed cancer in males in the world and is responsible for about 20% of cancer-related deaths (174). Prostate cancer diagnosis is divided in low, intermediate and high risk according to Gleason patterns, prostate specific antigen (PSA) levels and clinical stage (175). Surgical or chemical androgen deprivation therapy (ADT) is the first-line treatment once the disease spreads outside the prostate in order to reduce circulating testosterone levels (176, 177). Nevertheless, an important percentage of patients experience resistance and tumor progresses to a more aggressive form referred as castration-resistant prostate cancer (CRPC) after 18–36 months (178, 179). This advanced form of prostate cancer is usually treated with classical chemotherapy regimens including docetaxel and cabazitaxel (179). Moreover, there are also novel hormone therapies available for CRPC such as abiraterone and enzalutamide (180, 181). In 2010, the U.S. Food and Drug Administration (FDA) approved PROVENGE (sipuleucel-T), the first immunotherapy agent for the treatment of patients with asymptomatic or minimally symptomatic metastatic CRPC. Sipuleucel-T stimulates T-cell immune response against prostate cancer cells by targeting prostatic acid phosphatase (PAP), an antigen that is highly expressed in most prostate cancer cells (182). Despite these recent advances, treatments only provide scanty survival benefits and most patients develop disease relapse (183). Investigating on the mechanisms that may drive prostate cancer progression, different data reported that it is surrounded by a complex TME (184, 185). In particular, MDSCs are the most renowned immune cells subset that has been reported to infiltrate the prostate TME (186–188). In fact, by evaluating the frequency of MDSCs in the blood of prostate cancer patients the $CD14^{+}HLA\text{-}DR^{low}$ monocytic subset result to be augmented compared with sex- and age-matched healthy donors, whereas it is decreased after ADT (39, 189). Conversely, Chi et al., reported that circulating $CD33^{+}CD11b^{+}HLA\text{-}DR^{-}CD14^{-}$ granulocytic MDSCs represented the major subtype of MDSCs in patients with prostate cancer and their level were significantly elevated compared with both healthy donors and patients with benign prostatic hyperplasia (BPH) (190). Interestingly, Idorn et al., showed that the levels of $CD14^{+}HLA\text{-}DR^{low/-}$ M-MDSCs were increased in both untreated and docetaxel-treated CRPC patients and that they were correlated with a shorter median OS, suggesting that MDSCs support prostate cancer progression (191). Additionally, they also reported a significant positive correlation between MDSCs and T-regs frequency in peripheral blood of CRPC patients denoting a cross-talk between these two immunomodulatory cells (191). This intricate scenario is orchestrated by different mediators such as cytokines, chemokines and growth factors that contribute to the accumulation of MDSCs in prostate tumors (192). In particular, elevated levels of IL-6 pro-inflammatory cytokine, have been reported to promote cancer cell growth and significantly correlate with MDSCs expansion (193–195). In fact, it has been showed, in mice, that high serum levels of IL-6 were positively associated to MDSCs recruitment (195). This data has been further reinforced by using IL-6 KO mice in which the inhibition of tumor-produced IL-6 significantly reduced MDSCs recruitment (195). Similarly, Chi et al. reported that MDSCs frequency was correlate with serum levels of IL-6 and IL-8 in prostate cancer patients (190). This IL-6-mediated immunosuppressive effect involves different signaling pathways including PI3K/PTEN/AKT pathway which in turn triggers MDSCs recruitment (196, 197). Interestingly, more recently, Calcinotto et al., reported that IL-23 cytokine is another important MDSC-secreted factor that drives CRPC progression in both human and mice by sustaining the growth and the endurance of prostate cancer cells as well as the transcription of androgen dependent genes such as Nkx3-1, Pbsn, and Fkbp5 (186, 198). Moreover, co-administration of anti IL-23 antibody with enzalutamide, reverted resistance to castration in tumor-bearing mice by reducing tumor volume and proliferation (186). These findings demonstrated that MDSCs are the major players involved in prostate cancer progression and resistance. Thus, immunotherapies focused on the inhibition of either MDSCs recruitment or the inhibition of other mediators that sustain MDSCs immunosuppressive effect (e.g., IL-6 and IL-23) can be a promising therapeutic strategy for prostate cancer patients. Several clinical trials targeting MDSCs in prostate cancer are ongoing (197). One promising agent is tasquinimod, an oral second-generation quinoline-3-carboxamide derivative (199). Tasquinimod inhibits S100A9 protein that interacts with the receptor for advanced glycation end products (RAGE) and TLR4, triggering the inflammatory response (200). S100A9 is also involved in MDSCs recruitment in solid tumors sustaining tumor growth and metastasis development (201). A phase II clinical trial demonstrated that tasquinimod improved both PFS and OS in prostate cancer patients compared to placebo (202, 203). Nonetheless, in a phase III randomized controlled trial, tasquinimod significantly improved PFS but did not improve OS (204). However, larger controlled clinical trials are needed to confirm and validate tasquinimod as a standard agent for the treatment of CRPC.

MDSCs IN HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. Cirrhosis and liver inflammation are frequently associated with HCC, and inflammation is considered one of the main factors driving hepatocarcinogenesis (205). HCC is a highly chemotherapy-resistant tumor and the applicability of most cytotoxic drugs

is severely limited by the underlying liver cirrhosis. Currently, sorafenib and lenvatinib, oral multi-TKIs with antiangiogenic activity, are the most widely used systemic therapeutic agents which have showed increase in median survival in patients with unresectable HCC, respectively of 12.3 and 13.6 months (206, 207). Recently, other oral multi-TKIs, regorafenib and cabozantinib, have been added as second line systemic therapeutic options in patients with disease progression on sorafenib (208, 209). In the last few years, the interest in immunotherapies for HCC has been growing giving great opportunities for treating HCC with newer and more sophisticated agents (210). In particular, encouraging results has been obtained with the anti PD-1 mAbs nivolumab and pembrolizumab, which exhibited an objective tumor response of about 20% in HCC patients who had been previously treated with sorafenib (207, 211). Optimizing this response is challenging, especially because of the immune environment on which HCC arises. Although ∼25% of HCC show high or moderate levels of lymphocyte infiltration (TILs), within the TME (212), they often prove insufficient to control tumor growth because the expansion of immunosuppressor cells like MDSCs and Tregs (213). Indeed, there is a general consensus that various dysfunctions of the immune system contribute to HCC development and progression (214, 215). In the chronic inflammatory milieu present in the liver of HCC patients, myeloid cells infiltrating the tumor can acquire suppressive capability and contribute to immune escape of HCC cells. In the last decade, the clinical importance of MDSCs in HCC patients has been investigated. Several authors have reported elevated level of total MDSCs with the phenotype HLA-DR$^{-/low}$CD11b^{+}CD33^{+} in HCC patients compared with healthy controls (216–218). In other studies, MDSCs were identified as CD14^{+}HLA-DR$^{-/low}$, which are considered to be M-MDSCs. These M-MDSCs were found to be significantly elevated in the peripheral blood or tumor of HCC patients compared with chronic hepatitis patients and healthy controls. Moreover, the frequency of circulating MDSCs, both total and M-MDSCs, was significantly correlated with reduced OS and tumor progression (213, 219, 220). Later, Hetta et al. observed that HCV-HCC patients with advanced stage had higher percentage of total MDSCs and M-MDSCs in the peripheral blood compared with those with early-stage HCC and healthy control. The frequency of M-MDSCs subsets was positively correlated with liver related laboratory parameters, especially AFP and ALT, which reflects a hepatic insult whereas, was inversely related to the frequency of CD4^{+}, CD8^{+} T, and CD19^{+} B cells. Moreover, patients with chronic liver disease had a significantly higher percentage of MDSCs suggesting that an increased level of MDSCs may contribute to the progression from chronic hepatitis to HCC (221). In a recent publication, an extensive study on 183 HCC patients showed the prognostic value of CD14^{+}HLA-DR$^{-/low}$ M-MDSCs for predicting early recurrence (within 2 years) in patients undergoing curative resection. In particular, the authors observed a significant positive correlation between the frequency of MDSCs and the systemic immune-inflammation index (SII), which is a powerful prognostic indicator of poor outcome in HCC patients after resection. Thus, HCC patients with high MDSCs level and high SII level had significantly shorter time to recurrence (TTR) and OS than those with low MDSC level and low SII level (219). However, due the limitations of this study, such as relatively small cohort size, short follow-up time, and data from a single study center, the prognostic significance of MDSCs requires further validation. Clinical studies of MDSCs in HCC have mainly focused on analyzing M-MDSCs. Recently, Nan et al. employed a novel marker, LOX-1, to analyze G-MDSCs in HCC patients and determined that LOX-1^{+}CD15^{+} cells were significantly increased both in the peripheral blood and in tumor tissue of patients compared with healthy controls and were positively related to OS. Moreover, LOX-1^{+}CD15^{+} MDSCs suppressed T-cell proliferation through the ROS and ARG1 pathway and reduced interferon IFN-γ production (222). Mechanistically, also M-MDSCs isolated from the peripheral blood of HCC patients have been proven to be immunosuppressive by inducing CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells and inhibiting autologous NK cells, as well as they shown to have high ARG1 activity (213, 223). Nonetheless, Shen and colleagues, described an immature subset of Lin^{-} HLA-DR^{-}CD33^{+} MDSCs in the peripheral blood of patients with primary HCC and their frequency was found to be positively correlated with tumor stage and splenomegaly. In the same way, the immature MDSCs were able to inhibit tumor-specific T-cell responses and IFN-γ secretion through a suppressive mechanism involving ARG1 and iNOS enzymes (224). Regarding the mechanism of MDSCs expansion, it was found that the serum levels of suppressive cytokines like IL-10 and IL-13 as well as of tumor-promoting factors like G-CSF, VEGF and MMP-13 were significantly increased in patients with high frequency of MDSCs (220, 224). Indeed, these cytokines, that trigger JAK-STAT signaling pathways are considered to be the main regulators of the activation of MDSCs, which leads to stimulation of myelopoiesis and inhibition of myeloid-cells differentiation (225).

Most published studies on human MDSCs in HCC patients have been done using blood samples. Thus, in order to better understand the complex immunobiology of MDSC in HCC, different murine HCC models have been employed: carcinogen-induced, spontaneous and transplantable HCC. Although all tumor bearing mice demonstrated elevated MDSCs level (identified as CD11b^{+}Gr-1^{+} cells), subtle differences in frequency, location and function of MDSCs were found among the murine models (226). Pre-clinical models of HCC have been also used to evaluate the ability of sorafenib to modulate MDSCs. Several studies have reported that sorafenib could enhance the antitumor immunity by reducing MDSCs in tumor-bearing mice (226, 227). On the other hand, targeting MDSCs with anti-Ly6G or anti-IL-6 antibody significantly reduced the frequency of Ly6G^{+} MDSCs in orthotopic liver tumors improving the therapeutic effect of sorafenib (228). However, Chen et al. (229) demonstrated that sorafenib increased the intratumoral infiltration of Gr-1^{+} MDSCs through the SDF1α/CXCR4 pathway while reduced the accumulation of Gr-1^{+} myeloid cells in the surrounding fibrotic liver tissue. Differences in these studies might depend on the mouse liver cancer model, the sorafenib dose or the gating strategy used.

Further, recent studies have investigated the role of MDSCs in the efficacy of checkpoint inhibitors in mouse HCC models. Chiu et al. found that targeting the enzyme, ectonucleoside triphosphate diphosphohydrolase 2 (ENTPD2), which support the maintenance of MDSCs, enhanced the efficacy of PD-1/CTLA-4 blockade (230). Likewise, depletion of the cell cycle-related kinase (CCRK) reduced tumor-infiltrating MDSCs and increased intratumor $CD8^+$ T cells, thus enhancing the efficacy of PD-L1 inhibitor to eradicate HCC (217). In addition, an *in vitro* study demonstrated that combination of sorafenib with an anti-CTLA-4 mAb restored the proliferation of $CD8^+$ lymphocytes co-cultured with MDSCs (231). Radiotherapy is commonly used as alternative approaches for HCC patients who may experience serious adverse effects to chemotherapeutics. Interestingly, a decrease in percentages of $CD14^+HLA\text{-}DR^{low/-}$ MDSCs was observed in patients who received curative radiofrequency ablation (220). Recently, it has been reported that hypofractionated irradiation with high dose per fraction reduced the level of circulating MDSCs in two HCC tumor-bearing mouse models and decreased the expression of MDSC-related stimulatory cytokines: IL-6, G-CSF and RANTES (232). Collectively, these preclinical studies not only confirmed the roles of MDSCs in tumor formation and progression but also indicated the importance to reduce MDSCs in order to improve the efficacy of therapeutic strategies in HCC. However, these results remain to be confirmed in cancer patients. In this regard, a recent phase I/Ib study (NCT01839604) tested the effect of danvatirsen (AZD9150), a STAT3 oligonucleotide inhibitor, in 39 patients with advanced/metastatic HCC. At the end of the study the results reported that only one patient had a partial response. A phase I/IIa clinical trial is evaluating the outcome of HCC patients, progressing under sorafenib, following the treatment with regorafenib, a multi-TIKs that targets angiogenic (VEGFR1–3, TIE2), stromal (PDGFR-β, FGFR), and oncogenic receptor tyrosine kinases (KIT, RET, and RAF) in combination with nivolumab (NCT04170556).

MDSCs IN LUNG CANCER

Lung cancer is one of the most commonly diagnosed malignancies that is strongly correlated with cigarette smoking and is a leading cause of cancer-related death (233). Lung cancer is generally divided into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Both SCLC and NSCLC are treated with similar chemotherapeutic agents often in combination such as cyclophosphamide, doxorubicin, and vincristine (CAV) or cyclophosphamide, doxorubicin and etoposide (CDE) (234–236). In addition, different targeted antibodies and immunomodulators are currently used for the treatment of lung cancer (237, 238). However, a high percentage of patients do not respond or develop resistance to treatment promoting cancer progression (239, 240). MDSCs represent, together with Tregs as well as TAMs, the major immunosuppressive cells that make up the TME in lung cancer patients (241). For lung cancer, the main body of literature reports increases of monocytic $CD33^+CD11b^+CD14^+$ MDSCs or granulocytic-like $CD33^+CD11b^+CD14^-$ MDSCs (242–245). For instance, Feng et al., defined MDSCs as $CD11b^+CD14^+$ expressing high levels of the proinflammatory molecule S100A8/A9 whose expression was highly correlated with the ability to suppress T-cells proliferation (244). Recently, de Goeje et al., showed for the first time that the immunoglobulin-like transcript 3 (ILT3) represent a novel immunosuppressive molecule expressed by defined MDSCs subsets in lung cancer patients. In particular, ILT3 high expression on a specific subset of G-MDSCs, defined as $CD11b^+CD14^-HLA\text{-}DR^-CD33^+CD15^+ILT3^{high}$, was correlated with reduced survival into NSCLC patients (246). Interestingly, increased frequency of both M-MDSCs ($HLA\text{-}DR^{-/low}CD11b^+CD14^+CD15^-$) and G-MDSCs ($HLA\text{-}DR^{-/low}$ $CD11b^+CD14^-$ $CD15^+$) has been found not only in the peripheral blood of patients but also in the tumor lesions. Indeed, a strong elevation of both tumor-infiltrating MDSCs subsets compared with the circulating subsets has been showed, confirming that the tumor site is characterized by the strongest immunosuppression. In particular, the frequency of tumor infiltrating and circulating G-MDSCs correlated with tumor progression (247). Among the different mediators that have been reported to regulate MDSCs suppressive functions, gp91phox, which is correlated to NADPH oxidase enzyme (248), results to be upregulated in MDSCs of lung cancer patients (242). The activity of NADPH oxidase enzyme translates into an increase in ROS production which mediates tumor immunosuppression and might thus represent a potential target for therapeutic intervention. Other important mediators involved in cancer immunosuppression are IDO and the adenosine (ADO)-producing enzymes CD39 and CD73 (249–253). It has been reported that ADO-producing enzymes are expressed in MDSCs isolated from the peripheral blood of NSCLC patients and favor their immunosuppressive function. Further analysis identified a novel MDSCs subpopulation enriched in CD39 and CD73 in tumor lesions of NSCLC patients defined as $Lin^-CD14^-CD11b^+CD39^+CD73^+$ and $Lin^-CD14^+CD11b^+CD39^+CD73^+$ that were found to be positively correlated to disease progression and were reduced after chemotherapy cycles suggesting them as predictive tools for chemotherapy response (254). Moreover, the ratio between Treg cells and G-MDSCs may also have an impact on the response to nivolumab treatment, since patients with a high frequency of circulating Tregs and low frequency of G-MDSCs show improved PFS in NSCLC patients (255). However, more research is needed to better understand the correlation between MDSCs and Tregs in this type of cancer. Given these evidences about the association between MDSCs and anticancer therapies, strategies focusing on the functional targeting of MDSCs are fast approaching clinical realization. For example, depletion of MDSCs increases the frequency and activity of NK and T cell effectors in the tumor and enhance therapeutic vaccination responses (256). Furthermore, it has been also demonstrated that dopamine receptor D2 (DR2) agonists and histamine type-2 receptor antagonists, such as carbegoline and cimetidine respectively, inhibit the progression of lung cancer in both human and mouse models by affecting

at least in part MDSCs proliferation and function (30, 257). Interestingly, different natural compounds, such as resveratrol and curcumin, have been defined as novel synergistic agents for tumor immunotherapy. It has been demonstrated that resveratrol reduces *in vivo* lung cancer development and progression by inducing MDSCs apoptosis and reducing the recruitment of G-MDSCs (258). Likewise, curcumin reduced the frequency of MDSCs in the tumor and the spleen of tumor-bearing mice that was correlated to the reduction of IL-6 which is known to influence the function of MDSCs (259, 260). Giving the promising data regarding the targeting of MDSCs in mouse lung cancer, several clinical trials are now ongoing in NSCLC patients (NCT02922764; NCT03846310; NCT03801304; NCT04262388).

TABLE 1 | Phenotype and immunosuppressive features of MDSCs subsets in cancer patients.

MDSCs type	Phenotype	Immunosuppressive features	Tumor	Site	References
T-MDSCs	$Lin^{-/Lo}$ $HLA\text{-}DR^{-}$ $CD33^{+}CD11b^{+}$	-	BC	PBMCs	(91, 102)
T-MDSCs	$Lin^{-/Lo}$ $HLA\text{-}DR^{-}$ $CD33^{+}CD11b^{+}$	CD39	CRC	PBMCs	(125)
T-MDSCs	$Lin^{-/Lo}$ $HLA\text{-}DR^{-}$ $CD33^{+}$	ARG1, iNOS, MMP-13, VEGF	HCC	PBMCs	(224)
T-MDSCs	$CD45^{+}CD11b^{+}$ $CD33^{+}$	-	CRC	TT	(125)
T-MDSCs	$HLA\text{-}DR^{-}$ $CD33^{+}$	-	CRC	PBMCs/TT	(124)
T-MDSCs	$CD33^{+}CD45^{+}CD13^{+}CD14^{-}$ $CD15^{-}$	IDO, IL-4R	BC	PBMCs/TT	(103)
T-MDSCs	$CD33^{+}CD11b^{+}CD14^{-}$	↑IL-6 ↓IL-12, INF-γ	BC	PBMCs	(104)
T-MDSCs	$HLA\text{-}DR^{-}$ $CD33^{+}CD11b^{+}$	↓INF-γ	HCC	PBMCs/TT	(217, 218)
M-MDSCs	$HLA\text{-}DR^{-/low}CD14^{+}$	HMGB1, ARG1, S100P, MMP-9, MMP-25 ROS	BC	PBMCs	(92, 105, 106)
M-MDSCs	$HLA\text{-}DR^{-/low}CD14^{+}$	-	PC	PBMCs	(39, 191)
M-MDSCs	$HLA\text{-}DR^{-/low}CD14^{+}$	↓INF-γ ↑IL-10, IL-13, VEGF	HCC	PBMCs/TT	(213, 219, 220)
M-MDSCs	$HLA\text{-}DR^{-/low}CD14^{+}$	Nkp30 blocking	HCC	PBMCs/TT	(223)
M-MDSCs	$HLA\text{-}DR^{-/low}CD14^{+}$	gp91phox	NSCLC	PBMCs	(242)
M-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}$ $CD14^{+}CD15^{-}$	ARG1, CD39, iNOS, CXCR4	CRC	PBMCs/TT	(126)
M-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{+}$	TGF-β	MEL	PBMCs	(140–142)
M-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{+}$ $CD15^{-}$	-	HCC	PBMCs/TT	(216)
M-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}CD14^{+}$	-	HCC	PBMCs/TT	(221)
M-MDSCs	$CD11b^{+}CD14^{+}S100A9^{+}$	ARG1, iNOS, IL-4Rα, IL-10	NSCLC	PBMCs	(224)
M-MDSCs	$CD16^{low}CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}$ $CD14^{+}CD15^{+}$	ARG1, ROS	NSCLC	PBMCs	(245)
M-MDSCs	$CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{+}$ $CD15^{-}$	CCR5, PDL-1	NSCLC	TT	(247)
M-MDSCs	$Lin^{-}CD11b^{+}$ $CD14^{+}$ $CD73^{+}$ $CD39^{+}$	IL-4R, HIF-1α, IL-10, COX-2	NSCLC	PBMCs/TT	(254)
G-MDSCs	$HLA\text{-}DR^{-/low}CD15^{+}$	-	BC	PBMCs	(105)
G-MDSCs	$CD15^{+}$	ARG1	BC	TT	(107)
G-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}$ $CD17^{+}CD15^{+}$	↑ROS; PDL-1	CRC	PBMCs/TT	(126)
G-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD15^{+}$	ARG1	CRC	PBMCs/TT	(127)
G-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}$ $CD14^{-}$	-	MEL	PBMCs	(142)
G-MDSCs	$CD33^{low}CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{-}CD15^{+}$	-	MEL	PBMCs	(143)
G-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}$ $CD14^{-}$	↑IL-6, IL-8	PC	PBMCs	(190)
G-MDSCs	$CD33^{+}CD11b^{+}CD15^{+}$	IL-23	PC	TT	(186)
G-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{-}CD15^{+}$	-	HCC	PBMCs/TT	(216)
G-MDSCs	$LOX\text{-}1^{+}CD15^{+}$	ROS, ARG1	HCC	PBMCs/TT	(222)
G-MDSCs	$CD33^{+}CD11b^{+}$ $CD14^{-}CD15^{+}$	ARG1, iNOS, IL-4R, INF-γR	NSCLC	PBMCs	(243)
G-MDSCs	$CD16^{low}CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}$ $CD14^{-}CD15^{+}$	ARG1, ROS	NSCLC	PBMCs	(245)
G-MDSCs	$CD33^{+}CD11b^{+}$ $HLA\text{-}DR^{-}$ $CD14^{-}CD15^{+}ILT3^{high}$	-	NSCLC	PBMCs	(246)
G-MDSCs	$CD11b^{+}$ $HLA\text{-}DR^{-/low}$ $CD14^{-}$ $CD15^{+}$	CCR5, PDL-1	NSCLC	TT	(247)
G-MDSCs	$Lin^{-}CD11b^{+}$ $CD14^{-}$ $CD73^{+}$ $CD39^{+}$	IL-4R, HIF-1α, IL-10, COX-2	NSCLC	PBMCs/TT	(254)

BC, breast cancer; CRC, colorectal cancer; HCC, hepatocellular carcinoma; MEL, melanoma; NSCLC, non-small cell lung cancer; PBMCs, peripheral blood mononuclear cells; PC, prostate cancer; TT, tumor tissue; M-MDSCs, monocytic-MDSCs; G-MDSCs, granulocytic-MDSCs; T-MDSCs, total-MDSCs.

CONCLUSION

To overcome tumor immune evasion is the new challenge of our era. Cancer immunotherapy has experienced remarkable advances in recent years, and significant improvements have been achieved in the treatment of several solid cancer types (e.g., melanoma, non-small cell lung cancer, bladder cancer). However, for most patients a favorable initial response to treatment changes afterwards, thereby leading to cancer relapse and recurrence. A key factor underlying the limited response to immunotherapies is the existence of multiple mechanisms mediating tumor immune suppression (261). In this context, MDSCs have been recognized to have a crucial role. Recent studies demonstrated the value of MDSCs in predicting the response to cancer immunotherapies. In particular, a close association of MDSCs level with patient response to the checkpoint inhibitors anti-CTLA4 (262, 263) and anti-PD-1 (264) has been observed. Moreover, a growing number of studies have demonstrated a significant correlation between circulating MDSCs frequency in cancer patients with tumor stage, metastatic spreading, and course of the disease. Indeed, a recent meta-analyses including 40 studies and 2,721 patients with solid cancer support the existence of an association between higher MDSCs levels and worse OS as well as shorter disease-free survival/progression-free survival/recurrence-free survival. The negative prognostic value of MDSCs was observed for all MDSCs subtypes, most tumor types, and all tumor stages suggesting a potential novel and promising use of MDSCs as prognostic biomarkers and/or therapeutic target (265). Initial studies monitored MDSCs in cancer patients, analyzed total MDSCs population (G-and M-MDSC together). The diversity of cell surface markers used to identify the main subsets of tumor-derived MDSCs in human is very high, which is in part due to the differences in the factors that are involved in the development and activation of MDSCs. The complexity of the human MDSCs phenotype is summarized in **Table 1**, with the main MDSCs phenotypes expanded in cancer patients and the common immunosuppressive mechanisms. The M-MDSCs subset defined as HLA-DR$^{-/low}$CD14^{+}, resulted to be predominant in melanoma, breast cancer and hepatocellular carcinoma. Conversely, in colorectal cancer G-MDSCs defined as HLA-DR$^{-/low}$ CD15^{+} were the most abundant in both circulation and in tumor tissues. In prostate cancer and in lung cancer both G-MDSCs and M-MDSCs subsets were significantly elevated in patients and positively correlated to disease progression. However, despite most of the suppressive mechanisms and phenotype differences reported seemed shared among MDSCs subsets and tumor types, it is necessary to further dissect their role in order to define whether these

TABLE 2 | Summary of clinical trials targeting MDSCs in cancer patients.

Drug	Target	Combination partner	Tumor	ClinicalTrials.gov identifier
ENTINOSTAT	class I HDAC	Nivolumab	BC	NCT02453620
IPI-549	PI3K	Nivolumab	NSCLC, MEL, BC	NCT02637531
IPI-549	PI3K	Tecentriq and Abraxane	BC	NCT03961698
REPARIXIN	CXCR2	Paclitaxel	BC	NCT02370238
AB928	A_{2a}R and A_{2b}R	IPI-549, PLD, NP	BC	NCT03719326
DS-8273a	TRAIL-R2	Nivolumab	CRC	NCT02076451
PEXIDARTINIB	CSF1R	Durvalumab	CRC	NCT02777710
MARAVIROC	CCR5	-	CRC	NCT01349036
DANVATIRSEN (AZD9150)	STAT3	-	HCC	NCT01839604
REGORAFENIB	multi-TKIs	Nivolumab	HCC	NCT04170556
ATRA	Retinoic acid receptor	Ipilimumab	MEL	NCT02403778
SX682	CXCR1/2	Pembrolizumab	MEL	NCT03161431
RTA408	Nrf-2	Ipilimumab/Nivolumab	MEL	NCT02259231
Tasquinimod	S100A9	-	PC	NCT01234311
AZD5069	CXCR2	Enzalutamide	PC	NCT03177187
Granocyte	G-CSF	Cabazitaxel plus Prednisone	PC	NCT02961257
RGX-104	LXR	Nivolumab/Ipilimumab/ Docetaxel/Pembrolizumab	NSCLC	NCT02922764
AB928	A_{2a}R and A_{2b}R	Carboplatin/Pemetrexed Pembrolizumab	NSCLC	NCT03846310
vinorelbine	Cytotoxic	Atezolizumab	NSCLC	NCT03801304
Oleclumab	CD73	Durvalumab	NSCLC	NCT04262388
PD-0360324	CSF1	Avelumab	NSCLC, MEL, BC	NCT02554812
ARRY-382	CSF1R	Pembrolizumab	NSCLC, MEL,	NCT02880371

AR, adenosine receptor; BC, breast cancer; CCR5, C-C chemokine receptor type 5; CXCR1/2, C-X-C motif chemokine receptor 1/2; CRC, colorectal cancer; CSF1, colony-stimulating factor 1; CSF1R, colony-stimulating factor 1 receptor; G-CSF, granulocyte colony-stimulating factor; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; LXR, liver X receptor; MEL, melanoma; NP, nanoparticle albumin-bound paclitaxel; Nrf-2, nuclear factor erythroid 2-related factor 2; NSCLC, non-small cell lung cancer; PC, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PLD, pegylated liposomal doxorubicin; STAT3, signal transducer and activator of transcription-3; TKIs, tyrosine kinase inhibitors; TRAIL-R2, TNF-related apoptosis-induced ligand receptor 2.

differences are real or related to some bias from analysis of some markers/mechanisms. Numerous preclinical studies carried out in mouse tumor models, have showed that targeting MDSCs improved the effect of anti-cancer therapies (266–268). Although tumor mouse models could be useful for a better understanding of the mechanisms of induction, expansion, trafficking, and function of MDSCs in tumor, and for a rapid screening of anti-MDSCs agents *in vivo*, the translation in human is not so straightforward. First, the identification of human MDSCs phenotype is still challenging, owing the great heterogeneity of MDSCs in different cancers. Second, most human studies focus only on circulating MDSCs while little is known about tumor infiltrating MDSCs. Thus, a better and univocal characterization of the predominant subsets of MDSCs in several types of cancer as well as their further evaluation at the tumor site represent a compelling requirement in order to develop new effective strategies for targeting MDSCs. It is well-known that different subsets of MDSCs could use different mechanisms to suppress T-cells function. Therefore, the identification of the specific immunosuppressive mechanism is also essential to find the proper agent to block it and, consequently, to inhibit their function. Reduction of MDSCs expansion and recruitment to peripheral lymph nodes and tumor sites, inhibition of MDSC's suppressive activity and promotion of their differentiation into mature non-suppressive cells are the current therapeutic approaches that are being investigated to target MDSCs (**Figure 1**). So far, only few agents approved by FDA have been reported to have direct effects on MDSCs accumulation, maturation, and function (e.g., ATRA, Vitamin D, Suitinib, Gemcitabine, Bevacizumab, Tadalafil). However, a wide number of therapies and combination therapies are currently being tested in human clinical trials (**Table 2**) demonstrating an improvement of the patients' clinical outcome (146, 202, 203). In sight of this, further studies are needed to identify or confirm key mechanisms and upstream signals involved in MDSCs generation, expansion and immunosuppressive function in different malignancies. Advances in this field should facilitate rational design of new strategies to target MDSCs in cancer in order to enhance clinical responses to current immunotherapies and improve OS in patients.

AUTHOR CONTRIBUTIONS

PD and GE did the bibliographic research and wrote the manuscript. AI critically revised the article for intellectual content. All authors contributed to the article and approved the submitted version.

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Myeloid-Derived Suppressor Cells in Colorectal Cancer

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Colorectal cancer (CRC) remains one of the most common malignancies diagnosed worldwide. The pathogenesis of CRC is complex and involves, among others, accumulation of genetic predispositions and epigenetic factors, dietary habits, alterations in gut microbiota, and lack of physical activity. A growing body of evidence suggests that immune cells play different roles in CRC, comprising both pro- and anti-tumorigenic functions. Immunosuppression observed during cancer development and progression is a result of the orchestration of many cell types, including myeloid-derived suppressor cells (MDSCs). MDSCs, along with other cells, stimulate tumor growth, angiogenesis, and formation of metastases. This article focuses on MDSCs in relation to their role in the initiation and progression of CRC. Possible forms of immunotherapies targeting MDSCs in CRC are also discussed.

Keywords: colorectal cancer (CRC), myeloid-derived suppressor cells (MDSCs), inducible NO synthase (iNOS), arginase-1 (ARG1), T regulatory cells (Tregs)

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INTRODUCTION

Colorectal Cancer (CRC): Epidemiology and Immunity

According to the World Cancer Research Foundation, colorectal cancer (CRC) (referring to malignancy of colon, rectum, or anus) is the third most common malignancy worldwide. In 2018, more than 1.8 million new cases of CRC were diagnosed (1). About 20–25% of CRC cases are caused by genetic predispositions, including monogenic mutations in mismatched repairing genes associated with, e.g., DNA repair, the cell cycle, and apoptosis (2). Alongside inherited genetic mutations, epigenetic changes also play a significant role in CRC development (3). The remaining 75–80% of cases develop spontaneously and are related to environmental factors such as lack of physical activity, dietary habits, and smoking or alcohol abuse (4). Currently, alterations in the composition of the gut microbiome and its metabolites (playing a role in damaging local tolerance) are also considered as risk factors for CRC (5). An increased risk of CRC is often associated with chronic inflammation of the mucous membrane, which may lead to cell dysplasia, as was proven for patients with inflammatory bowel disease (IBD) (6).

The role of inflammation in CRC development was further supported by data showing that non-steroidal anti-inflammatory drugs (NSAIDs) may decrease the risk of both CRC and colon polyps, which are considered as a premalignant stage (7, 8). The tumor-infiltrating leukocytes (TILs), especially lymphocytes, contribute to the immunoscore classification, where the density of $CD3^+$ and $CD8^+$ T-cell infiltrate is used as a predictor of anti-tumor response and the prognostic marker in CRC (9, 10). However, further studies have shown that most of the immune cells may actually have a dual activity—anti- and pro-tumor, depending on the signals received from the tumor microenvironment. Interestingly, the so-called myeloid-derived suppressor cells (MDSCs) can switch the polarization of other cells to the status with pro-tumorigenic activity (11).

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)

Already in the early 1900s, it was shown that cancer development is often accompanied by extra-medullary hematopoiesis (EMH) and neutrophilia (12). These "fresh" leukocytes were further characterized by suppressive activity and were called immature myeloid cells (ImC) or myeloid suppressor cells (MSC) (13). Eventually, in 2007, their name was changed to MDSCs (13). These cells represent a heterogeneous population of granulocytes and monocytes that rapidly expand during infection, inflammation, and cancer (14, 15). MDSCs, together with the tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs), and regulatory dendritic cells, compose the population of myeloid regulatory cells (MRC), strongly cooperating with each other during cancer development, and progression (16). Based on mouse data, the MDSC population has been divided into two subgroups: of monocyte (Mo-MDSCs), defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$, and polymorphonuclear (PMN-MDSCs), $CD11b^{+}Ly6G^{+}Ly6C^{low}$, origin (11, 17, 18). Reflecting MDSC populations already defined in mice, human MDSCs have been described as Lin^{-} HLA-DR^{-}/low $CD11b^{+}$ $CD14^{-}$ $CD15^{+}$ $CD33^{+}$ for PMN-MDSCs and Lin^{-} HLA-DR^{-}/low $CD11b^{+}$ $CD14^{+}$ $CD15^{-}$ $CD33^{+}$ for Mo-MDSCs. Very recently, a population of early-stage MDSCs (e-MDSCs) was detected and defined as Lin^{-} HLA-DR^{-}/low $CD11b^{+}$ $CD14^{-}$ $CD15^{-}$ $CD33^{+}$ (17, 19). As their name suggests, these cells possess immunosuppressive function and help cancer to escape the surveillance of the immune system and support further tumor development (17). Most studies point out that the suppressive role of MDSCs in cancer is associated with the activation of their two enzymes, namely inducible NO synthase (iNOS) and arginase-1 (ARG1) (20–22). These enzymes are responsible for metabolism of L-arginine, which is essential for the proliferation and proper functioning of T cells (23). Moreover, NO and ROS produced in these reactions are involved in the inactivation of the T-cell receptor (TCR), causing a decrease in the expression of CD3ζ chain and inducing T-cell apoptosis (18, 19, 22).

Expansion and Activation of MDSCs in CRC

It is widely accepted that the level of circulating MDSCs increases in the late stage of cancer, correlating with disease progression and formation of metastases (15, 24–26). However, recently, Ma et al. showed that the MDSC level in circulation also increases in premalignant states, such as colon polyposis (27).

The development of MDSCs is caused by various mediators released under chronic inflammatory conditions, including the release of chemokines (11, 15, 28, 29). One of them that is particularly relevant is CCL2, which contributes to tumor growth, progression, and metastasis development in many tumors, including breast, ovarian, prostate, and CRC (30–33). Previous studies in mice showed that CRC growth could be supported by myeloid cells recruited by the CCL2-CCR2 signaling pathway (33). CCL2 caused accumulation of MDSCs and enhanced their immunosuppressive function during colorectal carcinogenesis (34). It was also shown that the level of CCL2 increased simultaneously with the progression of CRC (humans), while the deletion of CCL2 led to the reduction of the MDSC level (mouse model) (34). Further, RNS produced by MDSCs may nitrite chemokines, e.g., CCL2 to N-CCL2, which do not attract CD8+ T cells (like unmodified CCL2 does) but instead recruit myeloid cells, e.g., monocytes (35). On the other hand, several studies documented that CXCL1 is elevated in human CRC (36–38). Further data indicated that CXCR2-positive MDSCs are recruited through CXCR2 ligands, e.g., CXCL1 and CXCl2 are essential for chronic colonic inflammation and colitis-associated tumorigenesis (39).

In addition to chemokines, an important role in the regulation of MDSC activity is attributed to other inflammatory mediators such as histamine and prostaglandins. It has been documented that histamine induces MDSC proliferation and promotes ARG1 and iNOS expression in Mo-MDSCs. At the same time, histamine inhibits the expression of ARG1 and iNOS in PMN-MDSCs, promoting the production of IL-13 and IL-4 (40). Thus, histamine may activate Mo-MDSCs and PMN-MDSCs in different ways (40, 41). Prostaglandin E2 (PGE2), on the other hand, is a strong proinflammatory mediator produced by COX-2 (42) and may activate MDSCs through STAT3 phosphorylation (43, 44). In CRC, persistent STAT3 activation is associated with tumor growth (45, 46) and activation of MDSCs (47, 48). These observations are consistent with the results showing effectiveness of COX-2 inhibitors in the reduction of the MDSC level through blocking COX-2 and subsequent inhibition of the STAT3 pathway (43, 44, 49, 50). Another arachidonic acid metabolite, leukotriene B4 (LTB4), a product of 5-lipoxygenase (5LO), acts as a chemoattractant for MDSCs, leading to their accumulation. Deficiency of 5LO is associated not only with a lowered circulation level of MDSCs but also with decreased activity of ARG1 and iNOS (51).

The tumor microenvironment stimulates MDSCs also by other factors induced by local hypoxia and low pH (52, 53). One of them is hypoxia-inducible factor (HIF). Over-expression of HIF-1α and also HIF-2α is associated with poor prognosis in the majority of cancers, including CRC (54). HIF-1α is associated with increased activity of ARG1 and iNOS in MDSCs, leading to stronger inhibition of T-cell functions (55). Moreover, HIF-1α can also enhance the suppressive nature of MDSCs by inducing expression of programmed death-ligand 1 (PD-L1) (56), a ligand for PD-1, leading to inhibition of IL-2 production and decreased proliferation of cytotoxic T cells (56, 57). Additionally, HIF-1α, by binding to a conserved hypoxia response element in the *V-domain of Ig suppressor of T-cell activation* (VISTA*)* promoter, upregulates VISTA expression on MDSCs, thereby inducing their suppressive activity in the tumor microenvironment (58).

Many studies have shown that not only soluble mediators but also extracellular vesicles, e.g., exosomes secreted by tumor cells, may directly induce MDSC development and modulate their activity (59). This was demonstrated for many malignancies, including melanoma, breast, lung, and CRC (60). The role of cancer exosomes in CRC is complex, based on the type of cargo material transferred from cancer cells to the cells of the immune system, including MDSCs. This may occur through the delivery

of tumor proteins, e.g., FasL (61) and Hsp72 (62), mRNA (63), and non-coding microRNAs (miRNA) (64). The role of miRNA in CRC, in particular, has been documented recently, with an elevated level of miRNA-21 in patients' sera correlating with poor prognosis (65, 66).

MDSC Action in CRC

The suppressive function of MDSCs in CRC is mainly associated with their ability to inhibit T-cell proliferation and to stimulate Treg development (15). One of the important factors involved in interactions between T cells and MDSCs is L-arginine, an amino acid that is essential for T-cell proliferation and proper functioning. MDSCs highly express ARG1, which uses L-arginine, causing its depletion from the microenvironment (21, 22), which in turn affects T-cell functionality. Lack of L-arginine blocks T-cell proliferation and decreases expression of CD3ζ chain and IFNγ production (67–69). Studies on CRC have shown that MDSCs impair T-cell activation through O_2^- production and iNOS activity (70, 71), which can be reversed by MDSC depletion or the use of iNOS and O_2^- inhibitors (72). The mechanism of ARG1- and iNOS-dependent T-cell suppression has been explained by studies showing that, under conditions where the L-arginine level is reduced due to ARG1 activity, L-arginine is preferentially used by iNOS for O_2^- and NO production, while under normal conditions, where the L-arginine level is high, only NO is produced (73). After mutual reaction of NO with O_2^-, a strongly reactive oxidizing agent, peroxynitrite ($ONOO^-$), is formed. It can cause nitration of proteins (74, 75) as well as the induction of T-cell apoptosis through the TCR/CD3 complex tyrosine phosphorylation pathway (22, 76, 77). Recent results have also shown that MDSC level correlates with reduction in the adaptive immune response to tumor antigens, e.g., MUC-1, both by lowering the production of specific antibodies and activation of tumor-specific T cells (27).

The interactions between MDSCs and Tregs in cancer are well-documented. Mainly, the activation of Tregs by MDSCs is caused by cytokines, including IL-10 and TGF-β, where the latter is also associated with MDSC induction (78). However, the relationship between MDSCs and Tregs in CRC is questionable. Some authors indicate that MDSCs in CRC do not induce Tregs development *in vitro* (70). On the other hand, mouse MDSCs were able to induce Tregs *in vitro* and *in vivo* through the IL-10- and IFN-γ-dependent pathway (79).

In addition to the role of MDSCs in immunosuppression that is observed during tumor progression, they may also directly stimulate tumor growth and metastases, inducing, in cooperation with VEGF, angiogenesis. Furthermore, MDSCs may introduce high levels of MMP9 and pro-MMP9 into the extracellular milieu, regulating VEGF bioavailability for colorectal cancer cells (80, 81). At the initial stage of cancer, MDSCs, through TGF-β, can also induce the epithelial to mesenchymal cell transition (EMT) process, which is essential for metastases at the late stage. These cells participate in extracellular matrix degradation in order to prepare distant tissue for receiving metastatic cells (82, 83). The latest findings reveal that PMN-MDSCs also enhance CRC growth by exosomes and exosomal protein S100A9 in the tumor microenvironment, especially under hypoxic conditions (84).

Both populations of MDSCs can effectively inhibit T-cell activity but using different mechanisms (85, 86). Some authors suggest that Mo-MDSCs are more suppressive than PMN-MDSCs (87), while others show the opposite result (88, 89). PMN-MDSCs are mainly responsible for ROS production, while Mo-MDSCs have high expression of iNOS, producing large amounts of NO, which has a longer activity than ROS. Thus, PMN-MDSCs, in contrast to Mo-MDSCs, need direct cell-to-cell contact to suppress T cells (85, 90). In this context, it has been documented that PMN-MDSCs preferentially settle the peripheral lymphoid organs, while Mo-MDSCs mainly persist in the tumor bed (85). In addition, MDSCs can also downregulate innate immune response, e.g., affecting the activity of NK cells (91). The crosstalk between MDSCs and cells in the CRC microenvironment is summarized in **Figure 1**. According to some authors, in human CRC, a major proportion of the MDSCs in peripheral blood are PMN-MDSCs (86). However, there are also studies showing an increased level of both populations (92–95). Additionally, an e-MDSC population was also detected in CRC patients (27, 96).

DETECTION OF MDSCs IN CRC

The composition of phenotype markers used for MDSC detection and characterization in CRC quite often differs between studies. The phenotype markers and functional characteristics of MDSCs from various studies on human CRC are presented in **Table 1**. While the majority of the authors agree that the general phenotype of MDSCs is $CD11b^+$ $HLA\text{-}DR^-$ Lin^- $CD33^+$ or functional markers, e.g., $iNOS^+$ and $ARG1^+$, there is no consensus with respect to more specific markers such as CD14, CD15, PD-L1, or CD124 (IL-4αR). The recent recommendations of the COST-Mye-EUNITER consortium provide the minimal phenotype characteristics necessary to identify cells as MDSCs: $CD14^-CD11b^+CD15^+$ (or $CD66b^+$) for PMN-MDSCs; $CD11b^+CD14^+HLA\text{-}DR^{low/-}$ $CD15^-$ for Mo-MDSCs, and Lin^-(CD3/14/15/19/56)/$HLA\text{-}DR^-/CD33^+$ for e-MDSCs (17).

TARGETING MDSCs IN CRC

Despite the availability of chemo- and immunotherapy, surgery is still the primary method of CRC treatment. However, in a mouse model, it was shown that surgical removal of tumor mass recruits MDSCs to the peritoneal cavity and promotes tumor progression due to the surgical trauma, downregulating the CXCL4 expression. CXCL4 inhibits tumor growth and angiogenesis, which might be due to its inhibitive impact on the recruitment of MDSCs (97). In this context, it seems that MDSC-targeted therapy is urgently required for this type of cancer.

There are numerous studies concerning different small-molecule compounds that are able to inhibit the suppressive activity of MDSCs. In this section, however, the compounds with potential for CRC treatment are mainly being discussed.

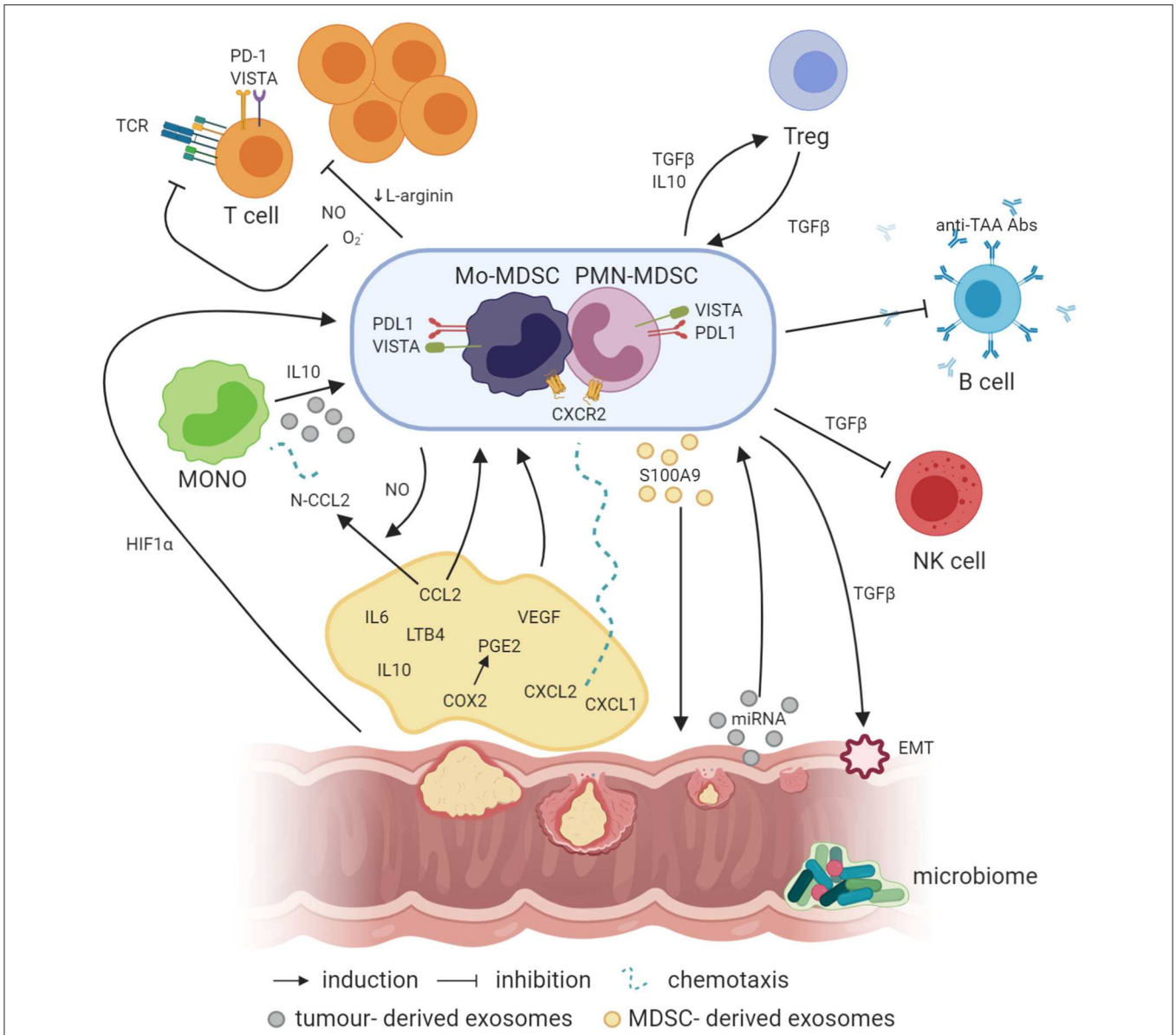

FIGURE 1 | Crosstalk between MDSCs and other cells in the cancer microenvironment (created with BioRender.com). Factors like PGE2, IL-6, IL-10, and LTB4 are involved in the induction of MDSCs, where IL-10 can also be involved in the generation of Mo-MDSCs from circulating blood monocytes. In addition, NO produced by iNOS is required for the production of N-CCL2 from CCL2, acting as a chemoattractant for monocytes. In a similar manner, CXCL1 and CXCL2 binding to CXCR2 may recruit MDSCs to the tumor bed. Simultaneously, exosomes containing exosomal S100A9 protein are released by PMN-MDSCs, supporting the tumor growth. On the other hand, EVs generated by the tumor transfer biologically active tumor-related factors, e.g., proteins and miRNAs, which may also be involved in the induction of MDSCs from infiltrating monocytes. Moreover, hypoxia *per se* and hypoxia-related factors, including HIF1a, are also responsible for the induction of the expression of suppressive molecules such as VISTA or PD-L1 on the surface of MDSCs, which act through VISTA receptor and PD-1 on the T cells, respectively. TGFβ produced by MDSCs has a number of suppressive actions, e.g., MDSCs, through TGFβ, can induce the epithelial to mesenchymal cell transition (EMT) process, which is essential for metastasis formation, or inhibit NK cells. Moreover, TGFβ has a great influence, together with IL-10, on the induction of Tregs, while Tregs, producing TGFβ, induce in return MDSCs as a result of a positive feedback loop. In addition, MDSCs may also inhibit the production of antibodies and T cells directed against tumor-associated antigens (TAA), such as MUC1. Additionally, NO, O2-, and a reduced concentration of L-arginine, which are associated with MDSC activity in the tumor microenvironment, inhibit T-cell proliferation. Moreover, NO by itself can modify TCR structure and induce T-cell apoptosis.

One such is AT38, an inhibitor of RNS, which was used in a mouse model of CRC where it proved to effectively reduce nitration of chemokines, including CCL2. Administration of AT38 also decreased the level of iNOS and ARG1 (35). Another example is nitroaspirine, which, in a mouse model, increased the number of tumor antigen-specific T cells and reduced both ARG1 and iNOS activity in MDSCs (98). Triterpenoids were also shown to reduce the suppressive functions of MDSCs through

TABLE 1 | The phenotype markers and functional characteristics of MDSCs as published in various studies on human CRC.

Orgin/Tumor stage	Phenotype	Suppressive activity	References
Circulating/I-IV	Lin^- $HLA\text{-}DR^-$ $CD11b^+$ $CD33^+$ $CD13^+$ $CD115^{low}$ $CD117^{low}$ $CD124^{low}$ $CD14^-$ $CD15^-$ $CD66b^-$ $CD34^-$ $CD39^+$ $CD73^-$ $PD\text{-}L1^{low}$ $PD\text{-}L2^-$ $PD\text{-}1^-$	MDSCs correlate with tumor metastasis. Inhibition of CFSE-labeled autologous CD3+ T cell proliferation at 2:1 ratios with MDSCs in the absence or presence of CD3/CD28 antibody stimulation for 3 days.	(26)
CD33+ from PBMC were co-cultured with SW480/SW620 cells to induce tumor MDSCs	CD33+ CD11b+ $HLA\text{-}DR^-$, $CD14^+$ $CXCR4^+$ $CD39^+$ ARG-1^+ $iNOS^+$ ROS^+ $PD\text{-}L1^+$ $CD73^-$ $CD117^{+/-}$ $CD34^{+/-}$ $CD66b^{+/-}$ $CD15^{weak}$	Tumor-induced MDSCs promoted SW480 and SW620 cell growth in a co-culture system *in vitro*. Tumor-induced MDSCs suppressed the proliferation of PBMCs labeled with CFSE more strongly than $CD33^+$ cells cultured in medium alone.	(70)
Circulating/tumor tissue	$CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ $CD14^+$ $CXCR4^{+/-}$ $CD39^{+/-}$ ARG-1^+ $iNOS^+$ $PD\text{-}L1^+$ ROS^+ $CD73^-$ $CD117^{+/-}$ $CD34^{+/-}$ $CD66b^{+/-}$ $CD15^{weak}$ MDSCs from tumor tissue have higher PD-L1 expression	Advanced disease stage was associated with an elevated level of circulating MDSCs; also, tumor resection reduces the level of circulating MDSCs and Tregs measured 7 days after surgery.	
Circulating/IV	CD14+ $HLA\text{-}DR^{-/low}$ $S100A9^{high}$ $iNOS^+$	–	(71)
Circulating/tumor tissue/III IV	$CD124^+CD14^+$ $CD124^+CD15^+$ tumor tissue $CD15^+$ $CD14^+$	Mixed lymphocyte reactions in which gamma-irradiated PBMC, $CD14^+$, $CD14^-$, and PMN from CRC patients were added as stimulator to responder PBMC derived from healthy donors. These experiments showed two main subpopulations with suppressive activity present among $CD14^+$ monocytes in one and among PMN in the other.	(93)
Colorectal tumor/III	PMN-MDSCs $CD45^+$ Lin^- $HLA\text{-}DR^-$ $CD11b^+$ $CD33^+$ $CD66b^+$ Mo-MDSCs $CD45^+$ Lin^- $HLA\text{-}DR^-$ $CD11b^+$ $CD33^+$ $CD14^+$	PMN-MDSCs isolated from tumor inhibited the proliferation of activated autologous CFSE-labeled T cells and IFN-γ production in medium containing CD3 and CD28.	(94)
Circulating	$CD33^+$ $HLA\text{-}DR^-$ $CD11b^+$ $CD15^+$ $CD33^+HLA\text{-}DR^-CD11b^+CD15^-$ $CD33^+HLA\text{-}DR^{-/low}CD14^+$	Upregulated plasma levels of IL-6 and IL-10, where IL-6 correlates with 15^+ MDSCs and IL-10 with 15^- MDSCs. Also, $CD15^+$ and $CD15^-$ MDSCs correlated with reduced IFN-α responsiveness in $CD4^+$ T cells.	(95)
Circulating/Metastasis	PMN-MDSCs $CD33^+$ $HLA\text{-}DR^{-/low}$ $CD15^+$ $CD124^+$ $PD\text{-}L1^+$ $CD73^+$ $CD39^+$ Mo-MDSCs $CD33^+$ $HLA\text{-}DR^{-/low-}$ $CD14^+$ $PD\text{-}L1^+$ $CD73^+$ $CD39^+$	Accumulation of PMN-MDSCs was associated with poor prognosis; also, PMN-MDSCs have higher levels of PD-L1, CD39, and CD73 expression and a stronger immunosuppressive function than Mo-MDSCs. Reduced TNF-α production and Ki67 proliferation marker of $CD3^+$ T cells, especially by PMN-MDSCs.	(89)
Circulating/I-IV	$CD33^+$ $CD11b^+$ $HLA\text{-}DR^{-/low}$ $CD15^-CD14^+$ ARG-1^+ $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ $CD15^+$ $CD14^-$ARG-1^{++}	–	(96)
Tumor tissue/I-IV	$CD33^+$ $CD11b^+$ $HLA\text{-}DR^{-/low}$ $CD15^-CD14^+$ ARG-1^+ $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ $CD15^+$ $CD14^-$ARG-1^+ $CD33^+$ $CD11b^+$ $HLA\text{-}DR^-$ $CD15^-$ $CD14^-$	–	
Circulating	PMN-MDSCs $CD14^-CD33^+HLA\text{-}DR^-CD66b^+$	Human MDSCs increase fatty acid uptake and expression of FAO-related enzymes, and, in mice, inhibition of FAO blocked the tolerogenic function and immunosuppressive mechanisms of MDSCs. Inhibition of CFSE-labeled $CD3^+$ T-cell proliferation after co-culturing with MDSCs from mice in the presence of anti-CD3.	(86)
Circulating	Mo-MDSCs $CD14^+HLA\text{-}DR^{-/lo}$ PMN-MDSCs $CD33^+$ $CD11b^+$ $CD14^-$ $CD15^+$ SSC ^{hi}	Mo-MDSC population was significantly expanded in CRC patients; the immunosuppressive capacity of these cells was evaluated in a T-cell suppression assay using a 3-way allogenic mixed leukocyte reaction (MLR).	(92)
Circulating/cancer and adenoma	Total MDSCs: $CD11b^+HLA\text{-}DR^{-/low}$ $CD33^+$ PMN-MDSCs: $CD11b^+HLA\text{-}DR^{-/low}$ $CD33^+$ $CD15^+$ $CD14^-$ Mo-MDSCs: $CD11b^+HLA\text{-}DR^{-/low}$ $CD33^+$ $CD15^-$ $CD14^+$ e-MDSCs: $CD11b^+HLA\text{-}DR^{-/low}$ $CD33^+$ $CD14^-$ $CD15^-$	PMN-MDSCs are the main immunosuppressive population, as depletion of $CD15^+$ cells spares Mo-MDSCs and eliminates most of the suppression of T-cell proliferation and interferon production. MDSC levels negatively correlated with anti-MUC1 IgG levels.	(27)

downregulation of ROS and inhibition of STAT3. However, they did not exert any effects on ARG1 activity, on NO production, or on the frequency of MDSCs (99). In the human CRC, amiloride, normally used to reduce high blood pressure, can also inhibit tumor exosome formation, which has been shown to induce suppressive functions in MDSCs (62). It was also reported that H_2 blockers, e.g., cimetidine, appear to induce apoptosis of MDSCs through a Fas-FasL-dependent pathway (100).

Another therapeutic approach involves the reduction of MDSC expansion by using COX2 or PGE2 inhibitors, as PGE2 production could be associated with MDSC expansion in cancer (43). Such inhibitors, e.g., indomethacin, celocoxib, melocoxib, and acethylosalicylo acid (ASA), were able to reduce tumor growth in various tumor models, including CRC (101–103). This treatment could also modulate MDSC functions by inhibiting ARG1 expression and ROS and NO production (104, 105). ASA also reduced the level of chemokines, including CCL2, a potent chemoattractant for MDSCs (106). Another way to block MDSC accumulation is the inhibition of stem cell factor (SCF), which causes MDSC recruitment when produced in the tumor environment (107).

Another option for targeting MDSCs is inducing their differentiation. For example, curcumin used in a mouse model of CRC was able to decrease the level of PMN-MDSCs and to induce differentiation of Mo-MDSCs into cells with M1-like phenotype (108).

Another strategy for potential MDSC-targeted therapy was suggested by Condamine et al. who pointed to a shorter lifespan for MDSCs compared with neutrophils and monocytes (109). This was associated with their increased apoptosis rate in the periphery, related to high expression of *TNF-related apoptosis–induced ligand* receptors (TRAIL-Rs) due to the stress in endoplasmic reticulum (ER) occurring under pathophysiological conditions like cancer. Thus, targeting TRAIL-Rs by selective agonists can be considered as a future therapy for reducing MDSC activity and number (109).

Immunotherapy designed to target the checkpoint inhibitors of the PD-1–PD-L1 pathway is currently one of the most promising possibilities for reducing MDSC activity. Currently, four monoclonal antibodies are already approved by the FDA for the inhibition of this pathway: anti-PD-1 nivolumab and pembrolizumab, and anti-PD-L1 atezolizumab and avelumab. These inhibitors and several other checkpoint modulators are under clinical investigation for CRC treatment (110). In the clinical studies, nivolumab and pembrolizumab showed good response rates of 26 and 57%, respectively (111). Better results were obtained in the case of nivolumab combined with ipilimumab (anti-CTLA-4) (111–113). However, in the context of MDSCs, more satisfactory results were obtained where the PD-L1 inhibitor was used (56). Recently, several chemotherapeutic agents, e.g., gemcitabine, 5-fluorouracil, and doxorubicin, which are used in conventional cancer chemotherapy have been found to reduce MDSC numbers through the induction of apoptosis in tumor tissues as well as in the peripheral lymphoid organs (114–116), and combining these agents with immunotherapy improved survival of tumor-bearing hosts. In keeping with this, Limagne et al. in their study, provided a clinical rationale for combining chemotherapy with anti-PD-1/PD-L1 antibodies for more effective reduction of the immunosuppression caused by PMN-MDSCs in metastatic CRC (89). In this context, FOLFOX (5-fluorouracil + oxaliplatin) chemotherapy was shown to act synergistically with anti-PD-1 (117).

In the context of immunotherapy, it is worth mentioning the heterogenic genetic composition of CRC, which has important therapeutic implications. The effectiveness of immunotherapy, particularly immune checkpoint inhibition therapy, such as CTLA-4 and PD-1, has been confirmed in mismatch-repair-deficient (dMMR) and microsatellite instability-high (MSI-H) (dMMR-MSI-H) tumors, while it was ineffective in mismatch-repair-proficient (pMMR) and microsatellite instability-low (MSI-L) (pMMR-MSI-L) tumors (118). This resistance for immunotherapy of MMR-MSI-L tumors results from the inability of immune cells to recognize MSI-L mutated tumor cells and thereby reduced T-cell infiltration (119). However, it was noticed that pMMR-MSI-L tumors are more extensively infiltrated by Tregs and MDSCs than dMMR-MSI-H, which may also explain the poor immune response (120). Thus, to use of MDSC-targeted therapy seems to be a beneficial opportunity to assist the effectiveness of surgery in patients with pMMR-MSI-L cancer.

CONCLUSIONS

Tumor develops a variety of mechanisms to escape from immune system surveillance, including the generation of MDSCs. There is substantial evidence that MDSCs are involved in CRC development and progression. MDSCs can be detected both in the peripheral blood and tumor tissue; however, it is not known if both or one of them are relevant for predicting the prognosis for patients in the clinic. Therefore, more in-depth investigation of the mechanisms of MDSC actions in the tumor bed is still needed. Finally, more advanced pharmacological data on specific treatments targeting MDSCs are required. This could significantly improve the effectiveness of the treatment of CRC patients, and also those with pMMR-MSI-L tumors, who respond poorly to current forms of immunotherapy.

AUTHOR CONTRIBUTIONS

IS wrote the paper. JB critically revised the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Analyzing One Cell at a TIME: Analysis of Myeloid Cell Contributions in the Tumor Immune Microenvironment

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Tumor-mediated regulation of the host immune system involves an intricate signaling network that results in the tumor's inherent survival benefit. Myeloid cells are central in orchestrating the mechanisms by which tumors escape immune detection and continue their proliferative programming. Myeloid cell activation has historically been classified using a dichotomous system of classical (M1-like) and alternative (M2-like) states, defining general pro- and anti-inflammatory functions, respectively. Explosions in bioinformatics analyses have rapidly expanded the definitions of myeloid cell pro- and anti-inflammatory states with different combinations of tissue- and disease-specific phenotypic and functional markers. These new definitions have allowed researchers to target specific subsets of disease-propagating myeloid cells in order to modify or arrest the natural progression of the associated disease, especially in the context of tumor-immune interactions. Here, we discuss the myeloid cell contribution to solid tumor initiation and maintenance, and strategies to reprogram their phenotypic and functional fate, thereby disabling the network that benefits tumor survival.

Keywords: MDSC, TAM, TIME, sc-RNAseq, reprogram

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INTRODUCTION

In recent decades the traditional view of tumor development and metastasis has evolved to include new and emerging cell types, extrinsic to the tumor itself. Over time it has become apparent that tumors are composed of many cell types from different origins, all with varying functions. By defining the tumor as a distinct organ, cell populations can be broadly separated into two categories: parenchymal tumor cells and stromal tumor-associated cells. Tumor-associated cells can originate either from the tissue in which the malignancy arises, or they can migrate from the periphery and infiltrate the tumor after it forms. The tumor itself and the tumor-associated cells together comprise what is termed the tumor microenvironment (1). When the tumor microenvironment being discussed relates to the influx and function of the immune system, it is termed the tumor immune microenvironment (TIME) (2). Therapies targeting different components of the tumor microenvironment, such as neovascularization, cellular proliferation, growth factors, extracellular matrix proteins, and more, have all been utilized to regulate tumor growth, each with various levels of success (3). More recently, targeting the immune component of the malignancy, deemed immunotherapy, has shown great promise and curative potential in several tumors (4).

Fundamentally, the goal of immunotherapy is to modulate the mechanisms that tumors use to suppress the immune response. The ability of a tumor to evade immune mediated killing is one of the hallmarks of cancer development, highlighting the importance of the immune response in preventing cancer formation (1). Classically, the immune system is divided into two branches: adaptive and innate. The innate division determines how to respond to danger by sensing the environment with an array of pattern recognition receptors and cytokine receptors that allow them to sense tissue damage, pathogens, and inflammation. The defining feature of the adaptive branch is its ability to respond in an antigen specific manner and memory responses (5). The importance of the innate immune system in regulating malignancies has come into sharper focus with the discovery of immunomodulatory myeloid cells residing within and around tumors. These myeloid cells are known to play a central role in suppressing adaptive immunity and are comprised of diverse clusters that fulfill various roles in promoting the viability of the developing malignancy. Two central groups of suppressive myeloid cells are the tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) (6). Initially called natural suppressor cells, these cells were shown to inhibit cytotoxic lymphocyte activity and support tumor growth (7). A body of work has shown that tumor development frequently causes defects in the differentiation and activity of myeloid cells, ultimately leading to a functional state that favors the tumor progression. Given the massive heterogeneity of infiltrating leukocytes found in tumors, and the striking difference in the TIME seen between different tumor types, there is a need to better understand the mechanisms contributing to this overall immune suppressive environment at the single cell and high-dimensional level. Advances in single-cell RNA sequencing (scRNAseq) and mass cytometry have enabled these types of studies and comparisons and are giving rise to new generations of data that may provide greater understanding of the mechanisms leading to immune suppression, TAM and MDSC polarization, and immune evasion.

Studies testing the potential of modulating the TIME via altering cellular recruitment, differentiation, proliferation, and survival are currently underway. These are reviewed elsewhere (8–11). Here, we discuss tumor associated suppressive myeloid cells, analyze recent findings obtained through high resolution dissection of their phenotypes, and highlight potential reprogramming strategies to orient cells toward anti-tumor functionality.

THE PLAYERS: TAMs AND MDSCs

In the 1960's, it was first observed that tumor bearing mice developed a leukemoid reaction with expanded myeloid cell populations in both the circulation and in the tumor. This correlated with enhanced tumor growth and these cells were subsequently shown to suppress cytotoxic T cell activity (7). Over time additional research has demonstrated that these myeloid cells exist as two separate populations: TAMs and MDSCs (12). Studies seeking to understand the factors that led to the differentiation of these populations demonstrated that tumor-associated macrophages develop from both tissue resident and circulating monocyte populations (13). New myeloid cells recruited from the bone marrow exhibit different programming from embryonically derived tissue resident macrophages (TRMs) (14), and commonly represent the definition of "tumor-associated macrophage" populations (15, 16), albeit not without debate, depending on tumor model (17–19).

Myeloid-Derived Suppressor Cells

Correctly identifying MDSCs *in vivo* remains challenging despite decades of intense study. MDSCs are commonly identified in tumor bearing mice by the Gr-1 surface marker, and recently, CD84 has arrived into the spotlight as another potential marker in murine models. There is potential for application of CD84 to differentiate MDSCs from conventional myeloid cells in human studies, but this has yet to be validated (20). Despite shortcomings in MDSC phenotypic definitions, several surface markers are employed in the literature with varying degrees of success and have been discussed elsewhere (12, 21). Thus, the gold standard and only reliable method to correctly identify MDSCs is to evaluate their ability to suppress CD3-mediated T cell activation and function *in vitro* (22–24).

MDSC recruitment and maintenance within the tumor tissue is thought to be more complex than that for TAMs, in part because of the hypothesized signaling required to maintain MDSCs in an immature state. This is thought to be accomplished by a combination of multiple growth factors and polyunsaturated fatty acids (25). Supplementary inflammatory signals generated by the tumor traps these immature cells in a pathogenic suppressive state (25, 26). A combination of TLR4/IFNγ/GM-CSF signaling and activation of intracellular STAT3 is needed to control the development and function of MDSCs (27–30). MDSCs are typically replenished by bone marrow precursors

Abbreviations: TIME, tumor-immune microenvironment; TAM, tumor-associated macrophage; MDSC, myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell; scRNAseq, single-cell RNA sequencing; TRM, tissue resident macrophages; TLR, toll-like receptor; IFN, interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor; STAT, signal transducer and activator of transcription; C-EBP, CCAAT/enhancer-binding protein; IRF, interferon regulatory factor; ROR, retinoic acid-related orphan receptor; CCL, C-C motif chemokine ligand; M-CSF/CSF1, colony stimulating factor 1; VEGF, vascular endothelial growth factor; IL, interleukin; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; CTLA, cytotoxic lymphocyte antigen; iNOS, inducible nitric oxide synthase; PPAR, peroxisome proliferator activated receptor; SOCS, suppressor of cytokine signaling; ARG1, arginase 1; IDO, indoleamine-pyrrole 2,3-dioxygenase; Bcl-xL, B-cell Lymphoma-extra large; ROS, reactive oxygen species; GCN2, general control non-derepressible 2; CREB, CAMP response element-binding protein; ATF, activating transcription factor; TGF, transforming growth factor; SPARC, secreted protein acidic and rich in cysteine, osteonectin; CCR, C-C motif chemokine receptor; MARCO, macrophage receptor with collagenous structure; NRP2, neuropilin 2; APOE, apolipoprotein E; IFITM1, interferon-induced transmembrane protein; TSPO, translocator protein; NSCLC, non-small-cell lung cancer; LILRB, leukocyte immunoglobulin like receptor B; PIRB, paired immunoglobulin-like receptor B; IL1RN, interleukin 1 receptor antagonist; NFKBIA, nuclear factor κ B inhibitor α, I κ B α; VISTA, V-domain Ig suppressor of T cell activation; CNS, central nervous system; GBM, glioblastoma multiforme; HIF, hypoxia-induced factor; PDAC, pancreatic ductal adenocarcinoma; TME, tumor microenvironment; PMN-MDSC, polymorphonuclear myeloid-derived suppressor cell; α-, anti-.

and the spleen functions as their reservoir (31), but it is unclear as to how extramedullary hematopoiesis contributes to their replenishment.

A growing body of evidence supports that MDSCs retain some ability to polarize to a cell displaying more characteristics of typical monocytes (32, 33). Genetic and pharmacologic methods can promote maturation or polarization in MDSCs, with multiple groups reporting that M-MDSCs can be functionally characterized into not only suppressive states, but also into reactive states (32, 34, 35). Transcriptional programs initiated by c-EBPβ, STAT3, PU.1, IRF8, and RORC1, among others, regulate the suppressive activities of MDSCs (36). Blocking these programs to force MDSCs into an activating, rather than suppressive role, is a potential therapeutic strategy, with several mechanisms to do so (37). MDSCs represent just one of the suppressive populations in the TIME; quantifying the phenotypes and functional states of the environment at the single cell level will offer more clues for therapeutic applications.

Tumor-Associated Macrophage

Tumor-associated macrophages comprise the macrophage populations located in and around a solid tumor (38). Originating from both tissue resident macrophages and circulating monocytes, TAMs are also known to perform a prominent role in modulating immune responses to tumors (39). TAMs can arise from peripheral monocytes in response to a combination of CCL2 and CSF1 produced by the tumor (27–30, 40, 41). Once monocytes reach the tumor site, they follow a maturation course that leads to their TAM finale (42, 43), under the influence of tumor factors, local cytokine milieu, and integrin signaling (44). Other than replenishment of TAM populations, the role of undifferentiated monocytes within the TIME has not been clearly defined at the single-cell level. Additional important signaling pathways resulting in macrophage recruitment and subsequent TAM differentiation include VEGF, IL-4, CCL2, CCL18, and CCL9 (45). TAMs further mobilize additional TAMs to the tumor niche by signaling to the bone marrow via CCL8 (14) to replenish and maintain their populations, although an undefined mechanism for the transition of TRMs to TAMs has been observed (15). Through a combination of TLR and cytokine signaling, infiltrating MDSCs can also differentiate into TAMs and function as a source of TAM replenishment (46–49).

TAMs are identified and distinguished from MDSCs by the presence of characteristic surface markers that are shared with mature macrophages (22). Frequently described as M2-like macrophages, TAMs have distinct phenotypic and transcriptional characteristics that can be used to distinguish them from conventional M2-macrophages. Additionally, TAMs demonstrate marked immunosuppressive functionality not seen in the M2 macrophage population (50).

TRMs have an interesting role in tumorigenesis. Because they develop with the tissue, they are present long before any noticeable malignancy, but are thought to contribute to the early stages of tumor development (2). The contribution of various myeloid cell ontologies to tumor development and immunosuppression is highly debated (51), although myeloid cells recruited from the periphery seemingly have a more important role in propagating the growth and invasiveness of malignancies (17, 52). However, this might be a tumor-specific phenomenon, as evidence from breast cancer patients and murine models shows proliferating resident macrophages in the tumor contributing to the bulk of the myeloid compartment (19). Interestingly, there is some evidence that both populations may also play distinct roles in supporting tumor growth, and their origins bias their transcriptional networks (53). Therefore, it is possible that the developing tumor modulates both the tissue resident and infiltrating myeloid cell populations concurrently.

SUPPRESSIVE MECHANISMS

The mechanisms of immunosuppression employed by TAMs and MDSCs are targeted toward inhibiting the activity of the adaptive immune system, namely T-cells, and NK cells. Suppressive myeloid cells do so by either direct cell-cell interaction with target cells, or through secreted factors. The mechanisms to suppress anti-tumor immune responses *in vivo* and have been extensively reviewed elsewhere (45, 50, 54–56). Briefly, they utilize four distinct functions to suppress T-cell mediated immunity: (1) signaling via the stereotypical inhibitory receptors PD-1 and CTLA-4 mediate leukocyte apoptosis and anergy (57–61); (2) depriving the local environment of nutrients necessary for T-cell activation and function (62–67); (3) generation of nitric oxygen and reactive nitrogen species, by iNOS expression, that induce T-cell exhaustion (12, 23, 62, 68, 69); (4) production of reactive oxygen species (12, 70). These mechanisms ultimately lead to a decrease in the effect and numbers of anti-tumor T-cells while enhancing the populations of tumor supporting regulatory T-cells (23, 24, 71, 72).

Suppressive Programming

Stereotypically, STAT and PPAR signaling pathways are independently responsible for programming that drives suppressive functionality of myeloid cells (73, 74), but there are studies that describe their joint interaction in programming as well (75). STAT3 signaling in myeloid cells can be initiated by tumor derived factors, including IL-10 and lactate. Activation of STAT3 typically results in activation of SOCS to block intracellular inflammation cascades and initiate an "M2-like" state, complete with functional and phenotypical markers, such as ARG1 and CD206. More importantly, STAT3 activation also results in the production of factors that benefit tumor viability and invasiveness, such as VEGF, matrix metalloproteases, and IDO (76–78). With respect to MDSCs, STAT3 has been identified as a crucial factor for both their development and function. STAT3 is capable of modulating gene expression of anti-apoptotic proteins Bcl-xL, c-Myc, Cyclin D1, and others to promote cell survival. STAT3 also engages programs that prevent monocytic lineages from terminal differentiation to maintain an immature phenotype, a hallmark of M-MDSCs (27). Supporting the central role STAT3 plays in MDSC function, inhibition or deletion of STAT3 abrogates the function and development of MDSCs *in vivo* (79, 80).

STAT6 signaling also promotes a suppressive program in myeloid cells. IL-4 and IL-13 induce a cascade of phosphorylation events that eventually lead to phosphorylation and homodimerization of STAT6, translocation to the nucleus, and binding to the promoters for various "M2-like" genes, such as ARG1 and CD206. As is the case for STAT3, STAT6 can also bind to IFNγ-induced activation sites and repress the transcription of associated genes (81). One of the transcriptional targets of STAT6 is PPARγ, which augments the effect of the suppressive programming set in place by STAT6 (75, 82). Moreover, PPARγ also increases oxidative pathways that result in increased ROS production (83), among other suppressive pathways (84).

GCN2, an intracellular nutrient sensor, also regulates macrophage function and promotes the pro-tumorigenic phenotype of both TAMs and MDSCs by enhancing translation of the CREB-2/ATF4 transcriptional factor responsible for promoting their differentiation (64). Fundamentally the changes induced by these altered differentiation pathways results in a pro-tumorigenic response rather than mediating tumor elimination.

TUMOR-ASSOCIATED MYELOID CELL SUPPORT OF TUMOR GROWTH & PROGRESSION

In addition to their role in aiding tumor immune evasion, TAMs and MDSCs also help orchestrate tumor progression. MDSCs remodel the extracellular matrix and promote blood flow to increase nutrient delivery via the production of various metalloproteases, cathepsins, and pro-angiogenic factors (24, 69). M-CSF can promote recruitment of peripheral myeloid cells to the tumor site and differentiate them into directors of angiogenesis (85). This distinct proangiogenic TAM subset, identified by surface TIE2 expression, secretes classic proangiogenic factors, such as VEGF proteins and SEMA4D (86, 87). These factors simultaneously retain anti-inflammatory functionality via autocrine and paracrine signaling through TIE2 (88). The combination of neovascularization and immune suppression can promote early dissemination of malignant cells (89), potentially through the breakdown of cadherin junctions between vascular endothelial cells (90). In some cases, the mobilization of $TIE2^+$ macrophages is initialized as a response to chemotherapy, highlighting the complex systemic reaction to therapy.

Myeloid cell support of tumoral fitness isn't limited to the primary site of malignancy, as subsets of patrolling monocytes have been found to increase angiogenesis to distal metastatic sites (19). MDSCs can serve a similar role and "fertilize the soil" in pre-metastatic sites for malignant cells to settle. Through undefined mechanisms, MDSCs can be recruited to a premetastatic niche before TAMs and establish a nutrient-rich, vascularized, and immunosuppressive environment for tumors to seed (91, 92). Along the same lines, a subset of $CCR2^+$ myeloid cells has also been associated with primary tumor recurrence (19), or re-fertilizing the soil for any remaining local or circulating tumor cells to grow.

TAM/MDSC IDENTIFICATION ACROSS TUMOR TYPES

Identification of cells implicated in facilitating cancer growth is imperative for several reasons. Despite established knowledge that TAM/MDSC infiltration is associated with worse prognosis (93), it is clear that not all myeloid cells in the tumor microenvironment directly benefit the growing malignancy. Finding a defined population specifically associated with tumor aggressiveness or invasiveness can serve as a prognostic marker. Furthermore, chemotherapy is not a "silver-bullet" to diminish or deplete malignant cells. It results in changes to the local and distant environment that are not easy to predict without studying the effects *in vivo* or *ex vivo* (40). Beneficial off-target effects are possible, such as concurrently depleting myeloid cells from the tumor microenvironment (94). Some therapies, however, can exacerbate the suppressive actions of TAMs, MDSCs, and other local myeloid cells, reducing their *in vivo* efficacy (95–97). It is also unclear as to which myeloid cell subsets are most affected by the therapy. Defining the myeloid cell subsets that are resistant, or even retaliatory, to a particular therapy is crucial for response prediction. Lastly, defining the myeloid suppressive phenotype that is most associated with malignancy and most associated with therapy resistance brings therapeutic efforts one step closer to targeting a specific cell cluster that contributes to several requirements of the hallmarks of cancer (1, 98, 99).

Historically, identification of stromal contribution was achieved with immunohistochemistry and staining for a limited set of markers on serial sections. This practice, however, can be quite wasteful of precious biological specimens and data due to the limited number of concurrent stains that can be performed. As the definitions of all of the players in the tumor microenvironment are expanding exponentially, an expanded panel of markers must be employed to adequately study the TIME. Tissue analysis at single-cell resolution is allowing for discoveries of distinct myeloid cell phenotypes and connecting their gene and protein expression patterns to immunosuppressive and tumor-promoting mechanisms (98, 100). The myeloid compartment has vast heterogeneity in itself, even within monocyte/macrophage subsets (43). Commonly identified subsets are TAMs, monocytes, TRMs, and MDSCs. TAMs and MDSCs are the most interesting populations, as they seem to have the highest correlation to tumor progression and are typically present in the greatest quantities, compared to other immune cells (69). Within these populations are even more complex subsets. Technologies such as scRNAseq (101) and mass cytometry (102, 103) have created new definitions for these populations that highlight heterogeneity previously unappreciated by conventional flow cytometry, allowing for discoveries of rare cell populations. These technologies have also effectively outdated the standard classification scheme of M1- vs. M2-like phenotypes for macrophages. Standard M1/M2-like phenotypic markers should not be applied with absolute exclusivity, as many of the stereotypic genes that represent classical or alternative activation states can be co-expressed and even correlated with each other (43). Therefore, it is crucial

to perform deeper statistical analyses to identify these smaller subsets that are more closely associated with the initiation, progression, and maintenance of the malignant niche, in addition to patient outcomes.

In defining the PD-1/PD-L1 (104) interaction and CTLA-4/CD80/86 (105), the search for novel immune checkpoints broadened into identifying new mechanisms that keep the adaptive immune cell out of the tumor environment and immunologically ignorant (2). More recently, myeloid cells in and around the tumor microenvironment have been recognized, as their utility for prognostication becomes more delineated. Generally speaking, TAMs, and MDSCs perform the same task of nurturing tumor growth among all cancers (106). The subset of culprit cells and the mechanisms by which they cloak or support the cancer can range. The surface markers of TAMs and MDSCs are not easily defined. Some markers of alternative activation are shared among TAMs and MDSCs, such as $CD163^+$, $CD68^+$ (40), or $CD206^+$ (107), the same cells can also express markers of classical activation, such as CD169 and CD163 (107). Additionally, TAMs and MDSCs of different malignancies have different phenotypes, indicating differences in mechanisms of suppression, albeit with minimal conservation. Below, we highlight breast, lung, and central nervous system malignancies to address the myeloid cell heterogeneity, as these are the tumor models that have sufficient studies defining single-cell immune populations. For quick reference, immunosuppressive mechanisms discussed throughout the text are summarized in **Figure 1** and **Table 1**. We have also summarized outstanding myeloid cell populations discussed in the text in **Table 2**.

Breast Malignancy

Without stratifying by breast cancer subtypes or stages, the myeloid landscape presented by different studies shows similarities. Notably, individual TAMs co-express both M1-like and M2-like associated genes along the same positive correlation trajectory (43, 107). Azizi et al. (43) identified TAM populations from human samples that expressed both classical and alternative activation markers, such as *CCL3* and *MARCO*, respectively, in addition to enrichment of signaling networks that are associated with each of the activation states. Highlighting a potential role for further recruitment of additional TAMs to the malignant site, one TAM cluster in the study by Azizi et al. (43) had distinctly enhanced expression of *STAT3*, *B7H3*, *CSF1R*, and *CCL3*. This same cluster also had upregulated *SIGLEC1*, which can serve as an independent predictor of poor prognosis [(14, 43), Supp.]. A separate TAM cluster in the same study was enriched in *PPARG* and *NRP2*, indicating distinct functional properties as a potential suppressor of T-cell activity through NRP2 (43, 119). Azizi et al. (43) further validated the individuality of the clusters and rejected the null hypothesis of unimodality across components that explain their variation.

Using scRNAseq information, Wagner et al. (107) detailed TAMs and MDSCs present in human breast cancer. A unique population of $PD\text{-}L1^+$ TAMs and a population of MDSCs with high expression of CD38 is also identifiable among breast cancer samples (107). Notably, CD38 has been found to aid the proliferation and migration of tumor cells and is also independently associated with the establishment of an immunosuppressive environment, even when expressed on M-MDSCs isolated from peripheral blood (120–122). As a note of caution, studying peripheral blood immune cells as biomarkers for diseases comes with its own challenges, as PBMC phenotypes don't necessarily agree with tumor-infiltrated immune cells (106). The complexity and heterogeneity of intra-tumoral myeloid cell populations is not well-represented by peripheral myeloid cells, possibly due to the effect of local tumor-associated signaling, therefore care must be taken when associating peripheral cells to the local disease. However, locally expressed CD38 can bypass disinhibition from PD-1/PD-L1 targeted therapy (123).

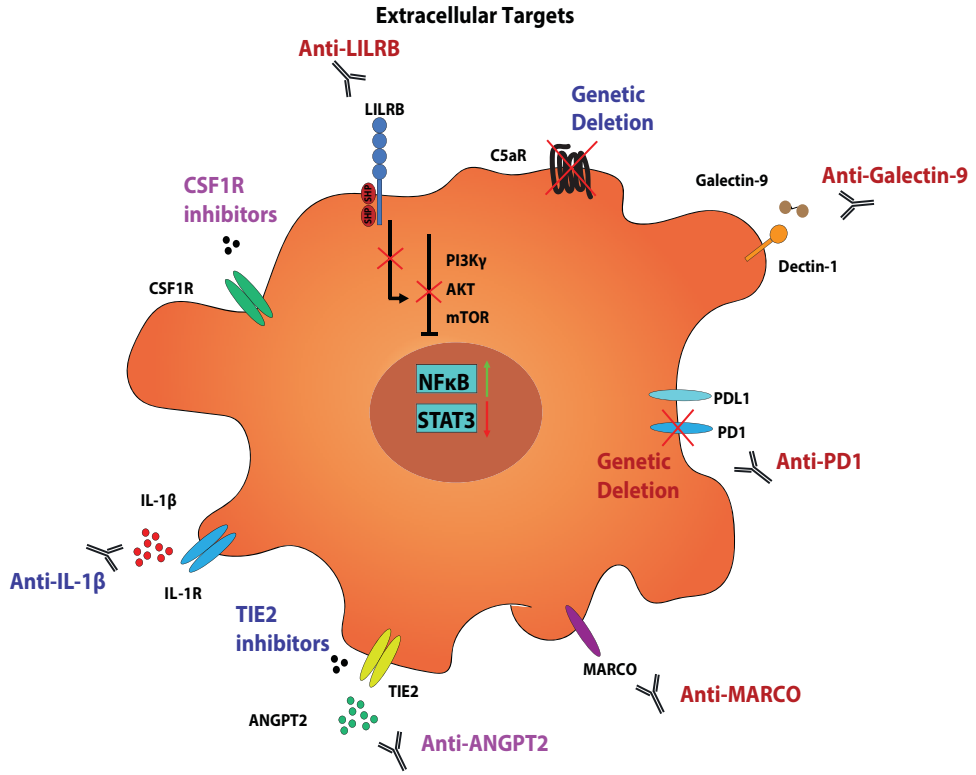
The TAM population in breast cancer studies seems to be the most mature cell population, defined by a signature defined by several factors, such as *TREM2*, *APOE*, and *MARCO* (43). All can be used as phenotypic markers of mature myeloid populations, such as macrophages, but TREM2 can serve as a functional marker of an anti-apoptotic state (124). Similar populations of TAMs are described in other cancers later (14, 112). Several studies showed the presence of undifferentiated monocyte populations within the breast TIME. Azizi et al. (43) described several monocyte populations with no enrichment of immune gene sets in addition to several other populations that are on track to dendritic cell differentiation. Likewise, Wagner et al. (107) described a border of monocytes to wall off the TAMs within the tumor core.

In murine models of breast cancer by Alshetaiwi et al. (20) MDSCs can be distinguished with scRNAseq. However, their identification presents a sizable challenge, as they do generally do not form distinct clusters by standard informatics analyses. With deeper analysis, they are distinguishable from other myeloid cell populations by their own transcriptional signature (20). Most notable in their transcriptional signature is the dramatic upregulation of *IFITM1* and *SOCS3*, marking their suppressive programming, in addition to *TSPO* (translocator protein) when compared to other myeloid cell clusters, highlighting their functional role in the TIME. TSPO is a mitochondrial membrane protein that, when activated, results in a respiratory burst and generates reactive oxygen species from myeloid cells, subsequently causing inhibition of T-cell activity (125). Unfortunately, no studies to date have evaluated the phenotypes of individual MDSC clusters to differentiate their functional roles in the TIME, although it is hypothesized that distinct clusters do exist (126).

Taken together, phenotypically distinct populations of TAMs/MDSCs have different functional responsibilities within the TIME in breast cancer. Notably, the majority of these suppressive cells are more mature TAMs, rather than MDSCs. Yet to be determined is the ontogeny of TAMs, i.e., whether they are the product of MDSC maturation or monocyte differentiation.

Lung Malignancy

Normal lung tissue is rich in immune cells responsible for eliminating foreign bodies and infections, therefore it is important to segregate TAM/MDSC populations from the



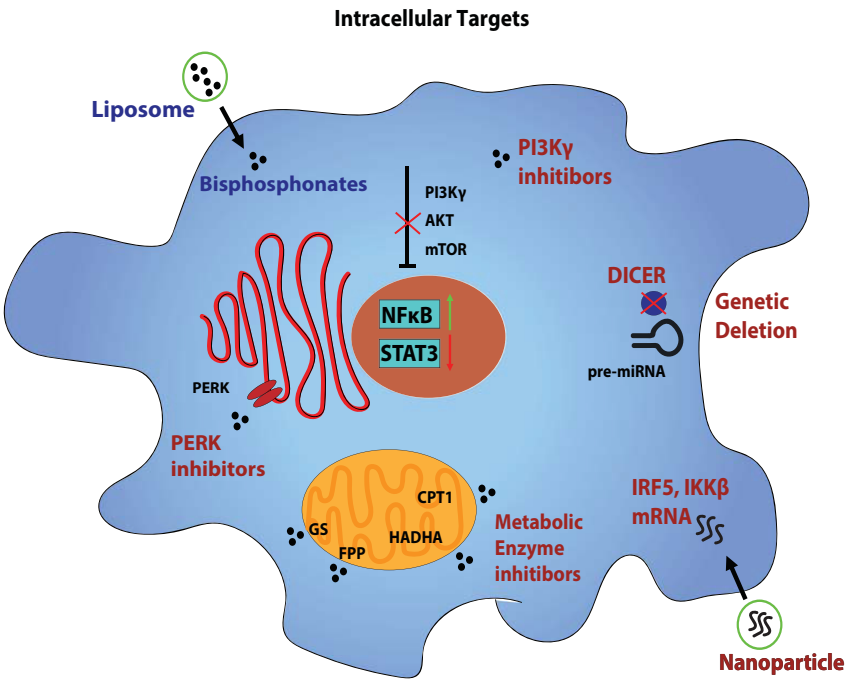


FIGURE 1 | Myeloid cell populations within the TIME and strategies to target them. Tumor-infiltrating myeloid cells, such as MDSCs and TAMs, perform a variety of functions in order to keep the TIME a hospitable niche for tumor growth and progression. Mechanisms include direct secretion of growth factors to support the viability of the tumor, in addition to maintaining an immunosuppressive environment to prevent recognition by cytotoxic immune cells. These functional states are controlled by

(Continued)

FIGURE 1 | the growing tumor itself through various combinations of ligand-receptor interactions, and can be propagated by the tumor-associated myeloid cells. Several markers, both surface and intracellular, can be used to not only identify the individual populations of tumor-associated myeloid cells, but also as therapeutic targets. Therapies aimed at these targets generally serve to either deplete the individual clusters of cells from the TIME, or to reprogram them from pro- to anti-tumor states. Presented are conserved targets on MDSCs and TAMs across tumor types, although they exist in different combinations amongst various tumor-associated clusters. The simplified cell diagram on the top presents various surface targets to reprogram (red), deplete (blue), or a combination of both (purple), tumor-associated myeloid populations, and the simplified diagram on the bottom presents intracellular targets. While only a single cell diagram is portrayed, these strategies represent individualized therapies in targeting specific tumor-associated myeloid cell populations. While some receptors may overlap between populations, we hypothesize that a multifactorial approach is imperative to abolish myeloid cell support of tumor growth.

TABLE 1 | Immunosuppressive mechanisms employed by MDSCs and TAMs, as well as stereotypic programming that regulate the mechanisms.

	Effect	Tumor type	References
Immunosuppressive mechanism			
PD-1/PD-L1	T-cell exhaustion/suppression Myeloid cell suppressive programming	Glioma, Breast, Lung (non-small cell)	(45, 55, 58–61) (108, 109)
CTLA-4/CD80/86	T-cell exhaustion	Breast, Lung	(45, 58, 110, 111)
B7-H3 Receptor/B7-H3	T-cell exhaustion/suppression	Breast, Lung	(206, 207)
ARG	Environmental nutrient depletion	Breast, Lung	(45, 62, 63)
IDO	Environmental nutrient depletion	Breast, Lung	(55)
NOS	T-cell suppression	Breast, Brain, Lung	(12, 23, 45, 55, 62, 68, 69)
ROS	T-cell suppression	Breast, Brain, Lung	(12, 55)
Immunosuppressive program			
STAT	Inhibition of intracellular inflammation cascade in suppressive myeloid cells Anti-apoptosis in suppressive myeloid cells	Breast, Brain (GBM), Lung	(75–78, 81, 82) (27, 79, 80)
PPARγ	Inhibition of intracellular inflammation cascade in suppressive myeloid cells Metabolic reprogramming in suppressive myeloid cells	Lung, Breast	(74, 75) (83)

normal lung myeloid populations for correct analysis. In adenocarcinoma, TAMs may have expression networks that make them more readily identifiable from normal myeloid cells, but deeper analyses like scRNAseq is required in order to differentiate their signatures and identify distinct populations (112, 113). TAMs in a later stage of macrophage differentiation within lung adenocarcinoma are distinguishable from resident myeloid cells via concurrent expression of *TREM2*, *MARCO*, and *APOE*, as mentioned earlier. As in other tumors, the TAMs from early lung adenocarcinoma express M1- and M2-like markers, including *HLA-DR* and *CD163*, respectively. Importantly, subsets of TAM populations in non-squamous cell lung cancer (NSCLC) show an enrichment of *PPARG* expression that can initiate anti-inflammatory transcriptional networks that propagate immune ignorance (127, 128), differentiating them from both normal lung macrophages and peripheral myeloid cells (106, 112, 115, 129). Zilionis et al. (106) also describe a population of tumor-infiltrating monocytes that express anti-inflammatory-like markers, such as *LILRB2*, a potent activator of the STAT6 signaling network. As this population has comparably low *CD14* expression, we speculate that this population of monocytes could represent newly-trafficked cells [(106), Supp.] that display immunosuppressive functionality early in the TAM differentiation process. This supports the notion that tumoral recruitment of suppressive cells happens early and at a systemic level. Our group has shown that the murine homolog to LILRB2, PIRB, can regulate the entire network of suppressive functionality of myeloid cells, making the expression of LILRB2 an interesting therapeutic target (130). Additionally, we have shown that targeted therapy against LILRB2 on tumor-infiltrating myeloid cells can reverse their suppressive fate initiated by the malignancy and diminish lung cancer tumor burden in murine models (131).

Tumor associated myeloid cells in lung cancer have the ability to further recruit new myeloid cells, as seen in other cancer types. Lambrechts et al. (113) describe the heterogeneity of immune cells within NSCLC, and describe a particular myeloid cell compartment that is enriched in several genes that recruit more immune cells to its location, such as *CCL2*, *CCL3*, and *CCL8*, in addition to *IDO1*, *IL1RN* (132). The same cluster exhibits high expression of *IL4I1*, *NFKBIA*, *VISTA,* and *LILRB4*. Like LILRB2, LILRB4-mediated ITIM signaling has a strong effect on the anti-inflammatory phenotype of myeloid cells (133), and we hypothesize that LILRB4 could act as a central regulator of the immunosuppressive cascade network in this myeloid cluster, as Deng et al. (134) showed a significant decrease of NFKBIA (IκB) at the protein level, following genetic ablation of LILRB4 in myeloid cells (135). The additional correlation to VISTA within the same cluster is of particular importance, as VISTA is proving to be an attractive target to prevent inhibition of T-cell cytotoxicity (136). In concert, this network would presumably directly program newly recruited myeloid cells to a suppressive

state and add to the immunosuppressive border surrounding the growing malignancy.

Clusters of suppressive myeloid cells can incorporate other cell types to augment their effect. A population of macrophages has been shown to induce T-regulatory cells to further fortify the immune barrier to cancer recognition (115). This macrophage cluster expresses markers of T-cell recruitment, such as *CXCL9*, *CXCL10*, and *CXCL11*, but the cluster is also enriched for anti-inflammatory-like genes, such as *STAT3*, *CCR2*, and *LILRB2* [(115), Supp.]. Most importantly, the same cluster is extraordinarily enriched for *PDL1*, *IL4I1*, and *IDO1*—genes heavily implicated in suppression of cytotoxic T-cell activity and induction of T-regulatory cell programming (137–142). According to Maynard et al. (115), this cell population is expanded in patients that show progression of malignancy on therapy, highlighting a crucial mechanism for therapy failure that corroborates previous work (143). This demonstrates another role of myeloid cells in tumoral viability—creating a hospitable environment for recurrence. While the entire population of myeloid cells is frequently targeted for cancer therapeutics (144), it's clear that more efficient strategies are needed. From the study by Lambrechts et al. [(113), Supp.], there does not appear to be any one particular myeloid cluster that has outstanding expression of PD-L1, PD-1, or B7-H3, underscoring the relevance of the other strategies employed by TAMs to keep the adaptive immunity at bay.

In summary, the lung cancer studies show off the power of deep analysis of the tumor microenvironment. Even in the case of the TAM compartment, which is frequently depicted as a single cell type, there is substantial heterogeneity in cell types that seemingly assume different roles to protect and contribute to the tumor growth. This also underlines a key aspect of immunotherapy targeted against the tumor microenvironment: it is unlikely a single therapeutic would have the capability to transform or reprogram all involved cells—in this case, TAMs/MDSCs. While targets such as PD-1/PD-L1 or CTLA4/CD80 are important, these mechanisms address just one mechanism of TAM-mediated suppression, and a downstream effector, which could explain the limited clinical benefit.

Central Nervous System Malignancy

Central nervous system (CNS) malignancies account for a small percentage of all diagnosed cancers (145), but they are frequently associated with abysmal prognoses. The resident immune system of the CNS, namely the microglia, are established contributors to CNS malignancies (146), but there are several other phagocytic myeloid cell populations in the CNS that are also, if not more so, implicated in a poor prognosis of the most aggressive form of CNS malignancy, glioblastoma multiforme (GBM). Perivascular, meningeal, and choroid plexus macrophages of the CNS have generally been overlooked as contributors to GBM (147, 148), but the involvement of bone marrow-derived myeloid cells has recently been established, and even positively correlated, to poor outcomes in GBM models (116, 149). As seen in the previous cancer studies, GBM TAMs co-express M1- and M2-associated markers, again making simple surface phenotyping of cells rather difficult, and creating the need for mechanism and pathway analysis (116). Invading peripheral myeloid cells show a greater suppressive potential than do microglia, marked by increased expression of *IL10* and *TGFB2*—potent inducers of T-regulatory cells (12, 43)—compared to the resident immune cells (116, 117). Likewise, peripheral myeloid cells were also enriched in genes involved in the citric acid cycle and *TSPO* compared to the resident microglia, resembling TAMs that we speculate to directly inhibit T-cell functionality mentioned previously in the Breast Cancer section [(116), Supp.].

Unfortunately, current scRNA-seq studies of the TIME in GBM use consensus clustering only to distinguish the roles of microglia and peripheral macrophages. This method limits the resolution and only allows for the evaluation of two myeloid cell clusters. Despite this, Muller et al. (116) describe myeloid cell heterogeneity that is the result of their spatial relationship with the malignancy, suggesting that suppressive myeloid cells perform different roles according to their physical location. Likewise, Darmanis et al. (114) show that macrophages make up the majority of myeloid cells within the tumor core and microglia make up the myeloid population of the surrounding stroma. The macrophages in the core seemingly contribute more to the overall viability of the tumor via their expression of *VEGFA* and *HIF1A*, while the juxtatumoral microglia serve as the main masqueraders of the malignancy with increased expression of *PDL1*, *B7H3*, *CD80*, and *CD86* (114). The myeloid cells within the tumor core are also the main source of *LILRB2* expression, offering a selective target for reprogramming a significant cell population for maintaining tumoral viability. Also interesting is that the majority of *LILRB2*-expressing myeloid cells do not co-express *MARCO*, a pattern recognition receptor enriched on TAMs (150); we speculate that these cells could be MDSCs (114). Most GBM-associated myeloid cell populations are involved in recruiting additional immunosuppressive myeloid cells, marked by exorbitant expression of *CCL3* and *TGFB2* in numerous GBM specimens (116). Combined expression of *CCL3* and *TGFB2* in a variety of bulk tumor samples from tissues of different origin is strongly associated with the local presence of MDSCs, despite the difficulty in their identification (151). More importantly, high expression of the combination is associated with a worse overall median survival in high grade glioma, referenced in multiple data repositories (152).

While there are limited studies that recognize the presence of MDSCs, and specifically analyze heterogeneity of MDSCs, in models of CNS malignancy, it is imperative that we discuss them in this context. MDSCs have been detected in the tumor microenvironment and play a significant role in tumor progression (153). They do not exist in healthy CNS tissue outside of the context of malignancy (149, 153). Alban et al. (118) use MDSC infiltration in GBM as prognostic markers and indicate a hazard ratio of 4.7 (1.69–13.4) when comparing overall survival of patients with high MDSC GBM infiltration to low infiltration. Under the assumption that all M-MDSC populations that infiltrate GBMs are programmed into the same functional state, their role is to secrete IL-10 and TGF-β, just like their macrophage counterparts. The presence of these cytokines is correlated to overall stage of the malignancy [(118, 149), Supp., (154)], indicating that there is most likely a dose effect as a

greater amount of MDSCs in the local environment is correlated to staging as well.

In addition to the local involvement of suppressive myeloid cells, the peripheral differential cell count offers insight to prognosis of GBM patients (118, 149). MDSCs in the periphery are heavily implicated in higher grade, more aggressive CNS malignancies. Peripheral MDSCs have a strong positive correlation with worse prognoses in GBM patients, and the converse is true as well. Alban et al. (118) showed that, after surgical resection of GBMs, patients with increasing fractions of MDSC populations had inferior survival time, compared to those of decreasing MDSC fractions. A cohort of newly diagnosed patients in the study received standard-of-care adjuvant therapy (155), but the expansion of M-MDSCs were variable, indicating a potential difference in activation of myeloid cells following chemo- or radiotherapy (156, 157). Additionally, a number of studies show increased peripheral MDSC counts in subsets of patients who received dexamethasone perioperatively, indicating a potential confounder, or contributor, in correlating overall survival with MDSC levels (118, 148, 149).

GBM is well-known to be an extraordinarily heterogeneous malignancy, making it very difficult to target with "off-the-shelf" therapy. However, it is striking to see that even across the heterogeneity of malignancies from different patients, the myeloid cell clustering, and signaling networks seem to remain conserved (114). Manipulating the programming of both bone marrow-derived myeloid cells and resident microglia is important in regulating the entire network of immune suppression and pro-tumor functionality. While microglia appear to be attractive targets for the popular therapies targeting PD-L1 or B7 family of proteins, involvement of the peripheral immune system within the tumor microenvironment is more closely associated to prognoses and should also be considered for immunomodulation. Whether the infiltrating TAMs, the malignancy itself, or a combination of both is causing the suppressive programing of the microglia remains to be determined. **Table 1** details the pathways and receptors that mediate immunosuppression along with the specific effect and tumors impacted by the signaling pathway.

METHODS TO PREVENT MYELOID CELL CONTRIBUTION TO CANCER GROWTH

Currently, there are two main strategies for manipulating tumor associated myeloid cells: depletion and reprogramming. Depletion involves broad, systemic targeting of myeloid cells, although newer, more specific approaches are aimed at depleting only the myeloid cells that are specifically involved with the malignancy (40). The therapeutic strategies are summarized in **Table 3**, along with recent clinical trial information.

Depletion

Strategies to deplete myeloid cells from the TME include mechanisms to prevent myeloid cell trafficking to the malignancy or initiate apoptosis. Tumoral recruitment and expansion of bone marrow-derived myeloid cells occurs through a CCR2-CCL2–dependent signal and, along with increasing serum levels of CCL2, is independently associated with worse prognosis. Disruption of CCR2 signaling prevents the recruitment and development of suppressive myeloid cells, while suppressing tumor metastasis and prolongs survival across several cancer models (16, 176, 177). Importantly, disrupting CCR2 signaling also reduces TAM/MDSC recruitment to premetastatic niches (16).

Antagonizing the CSF1–CSF1R axis is an interesting approach as it disrupts several mechanisms for therapeutic effect. Blocking the axis can disrupt localization of suppressive TAMs to the site of malignancy (178) as well as reprogram TAMs for anti-tumor activity (162), in addition to preventing the conversion of M-MDSCs to TAMs (12). JNJ-28312141, a CSF1R inhibitor, depleted F4/80$^+$ TAMs in a subcutaneous H460 human lung tumor xenograft model and increased plasma CSF1, a potential biomarker in CSF1R inhibition (179). Biologics have also been studied in this regard—RG7155, a monoclonal CSF1R antibody, greatly reduced F4/80$^+$ TAMs in animal models of colon cancer. RG7155 showed promise in human applications as well, as it induced apoptosis of CSF1R$^+$CD163$^+$ macrophages in patients with diffuse type giant cell tumor tissue (Dt-GCT) (178). However, as CSFR1 blockage with pexidartinib has proven to be ineffective in patients, targeting the CSF1–CSF1R signaling axis might have limited applications (180). Combination therapy of CSF1R blockade with immune checkpoint blockade is currently ongoing in a solid malignancy clinical trial (Trial # NCT02713529).

Targeting CD38 is proving to be a good strategy for antibody-mediated depletion in some cancer models. CD38$^+$ MDSC populations are expanded in cancer patients and can even serve as an escape mechanism after PD-1/PD-L1 therapy. Daratumumab, a CD38 antagonist antibody, can deplete immunosuppressive myeloid cells from circulation, as well as serve as an independent therapy for CD38$^+$ myelomas. CD38 antibody therapy initiates apoptosis via antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity. Other suppressive cell types, such as T-regs, are also sensitive to anti-CD38 treatment (122, 123, 181).

Liposomal delivery of dichloromethylene biphosphonates is another effective method to deplete tumor associated myeloid cells, as it deposits its payload directly into the intracellular space. Liposomes are enclosed multifunctional structures that consist of one or more phospholipid bilayers surrounding a hydrophilic core. This organization allows for hydrophobic therapies to associate with the lipid bilayer, and hydrophilic therapies, including genetic material such as RNA, DNA or siRNA, to be carried in the core. Clodronate and other bisphosphonates are a class of drugs typically used for the treatment of osteolytic bone disease and osteoporosis by inhibiting bone resorption, as they specifically target the phagocytic cells involved (182). By encapsulating clodronate in liposomes, clodronate can be delivered to the tumor site where it is phagocytosed by macrophages, ultimately initiating apoptosis.

TABLE 2 | Specific clusters of myeloid cells highlighted in the text are summarized here.

Outstanding clusters	Hypothesized role	Cell type	Tumor studied	Species	Reference
STAT3, B7H3, CSF1R, CCL3, SIGLEC1	Additional myeloid recruitment	TAM	Breast cancer	Human	(43)
PPARG, NRP2	T cell suppression	TAM	Breast cancer, lung cancer	Human, murine	(43, 106, 112)
PD-L1	T cell suppression	TAM	Breast cancer	Human	(107)
CD38	Tumor proliferation and migration	M-MDSC	Breast cancer	Human	(107)
TREM2, APOE, MARCO	Mature TAM markers; global immunosuppression; anti-apoptosis	TAM	Breast cancer, lung cancer	Human	(43, 112, 113)
IFITM1, SOCS3, TSPO	Global immunosuppression; ROS production and T cell suppression	MDSC	Breast cancer	Murine	(20)
LILRB2	Global immunosuppression	Monocyte-early TAM/MDSC	Lung cancer, GBM	Human	(106, 114)
CCL2, CCL3, CCL8, IDO1, IL1RN, IL4I1, NFKBIA, VISTA, LILRB4	Additional myeloid recruitment; global immunosuppression	Monocyte-early TAM	Lung cancer	Human	(113)
CXCL9, CXCL10, CXCL11, STAT3, CCR2, LILRB2, PDL1, IL4I1, IDO1	Global immunosuppression; T cell recruitment & suppression; chemotherapy resistance	TAM	Lung cancer	Human	(115)
IL10, TGFB2	Global immunosuppression; tumor progression	MDSC & infiltrating macrophage	GBM	Human, rat	(116–118)
TSPO	ROS production and T cell suppression	Infiltrating macrophage	GBM	Human	(116)
VEGFA, HIF1A	Tumor progression	Infiltrating macrophage	GBM	Human	(114)
PDL1, B7H3, CD86	T cell suppression	Microglia	GBM	Human	(114)
CCL3, TGFB2	Additional myeloid recruitment	MDSC	GBM	Human	(116)

Several clusters overlapped between various malignancies.

However, these effects have only been shown *in vitro* and animal models (183–185).

An interesting, albeit controversial, aspect of MDSCs in the TIME is the effect of chemotherapies on MDSC quantities and suppressive programming. 5-fluorouracil (5-FU) and gemcitabine were able to induce apoptosis and deplete MDSCs in both spleens and tumors in 4T1 murine breast cancer model. Moreover, both 5-FU and gemcitabine can activate caspase-1 and induce IL-1β production via the NLRP3 inflammasome pathway (186, 187). Evidence points to conflicting effects of IL-1β with respect to the TIME. While some studies show a beneficial effect of increased IL-1β in the TIME (64), others show that blockade of IL-1β signaling can prevent immunosuppressive cell recruitment (163). Other secondary effects of chemotherapy on the immune system are discussed in depth elsewhere (40, 54).

Reprogramming

The tumor microenvironment can polarize TAMs to an immunosuppressive M2-like functional state, leading to enhanced tumor growth, progression, and metastasis. Besides depleting TAMs and MDSCs, myeloid cells can be reprogrammed toward a pro-inflammatory state by direct intervention via small molecules and antibodies targeting key receptors. Two reprogramming strategies can be used—blocking a receptor that normally transduces an inhibitory intracellular signal, or using an exogenous ligand to activate a receptor that stimulates pro-inflammatory intracellular cascades (188). Despite its success in diminishing tumor burden, pro-inflammatory agonist therapy is frequently associated with systemic toxicity (189, 190), therefore, we will discuss the former strategy.

Surface Targets

In some cases where disruption of the CSF1-CSF1R signaling axis is unsuccessful in depleting TAMs, antagonism of CSF1R signaling can reprogram TAMs away from an M2-like state. Using glioma xenograft models, Pyonteck et al. (162) describe how CSF1R antagonism did not decrease TAM numbers nor did it alter their CSF1R expression pattern. However, inhibition of AKT phosphorylation and M2-related gene expression, such as *ARG1* and *CD206*, indicated that CSF1R antagonism initiated a functional shift to a pro-inflammatory state to block glioma progression (162).

In a murine pancreatic ductal adenocarcinoma (PDAC) model, crosstalk between B-cells and FcRγ^+ TAMs resulted in an M2-like phenotype through Bruton's tyrosine kinase (BTK) activation in a PI3Kγ-dependent manner. Using the BTK inhibitor ibrutinib, PI3Kγ inhibition in PDAC tumor-bearing mice reprogrammed TAMs toward an M1-like state and

TABLE 3 | Therapies used to reprogram tumor associated macrophages and MDSCs.

Target	Therapy/Treatment	Clinical Trials	References
BTK/ PI3Kγ	Small molecule BTK inhibitor: Ibrutinib Small molecule PI3K inhibitor: IPI-549	NCT03379428 NCT02403271 NCT02321540 NCT02950038 NCT02403271 NCT03535350 NCT03961698 NCT03719326	(158) (159)
LILRB	Anti-LILRB2 antibody	N/A	(131)
C5a/C5aR	C5aR genetic deletion	N/A	(160)
Dectin-1 (CLEC7A)/Gal-9	Anti-Gal-9 antibody	N/A	(161)
CSF-1/CSF-1R	Small molecule CSF-1R inhibitor: BLZ945	NCT02829723	(162)
IL-1β	Anti-IL-1β antibody	NCT02900664 NCT03742349 NCT03447769 NCT03968419 NCT03631199 NCT03626545 NCT03064854	(163)
HIF1α/β	HIF1 genetic deletion	N/A NCT01036113	(164)
ANGPT2/TIE2	Small molecule TIE2 inhibitor: Rebastinib Anti-ANGPT2 antibody: Nesvacumab	NCT02824575 NCT03717415 NCT03601897 NCT01688960	(164) (165)
PERK (UPR)	Inhibitor of unfolded protein response: Tauroursodeoxycholic acid (TUDCA)	N/A	(166)
Glutamine Synthetase (GS)	Methionine Sulfoxamine	N/A	(167)
CPT1 (FAO enzyme) HADHA (FAO enzyme)	Small molecule CPT1 inhibitor: Etoximir/Perhexiline Small molecule HADHA inhibitor: Ranolazine	N/A	(168) (169) (170)
FPP	Small molecule FPP inhibitor: Zoledronic Acid	NCT02347163 NCT00295867 NCT00320710 NCT03664687	(171) (172) (173)
MARCO	Anti-MARCO antibody	N/A	(150)
IRF5 IKKβ	Nanoparticle encapsulated mRNAs	N/A	(174)
DICER	DICER genetic deletion	NCT01353300 NCT00565903	(175)
PD-1/PD-L1	PD-1 genetic deletion Anti-PD-1 antibody	NCT04173325 NCT03414684 NCT03925246	(108) (109)

increased $CD8^+$ T-cell cytotoxicity to slow PDAC tumor growth (158). PI3K is a critical switch to promote suppressive activity in macrophages, as PI3K signaling via AKT and mTOR inhibits NFκB to promote M2-like functionality in TAMs. Conversely, inhibiting PI3K prevents C/EBPβ activation and disinhibits NFκB to induce a pro-inflammatory phenotype. Combining PI3K blockade with anti-PD-1 therapy can promote tumoral T-cell infiltration to slow tumor growth and enhance survival in tumor-bearing mice (159).

Signaling pathways that activate NFκB to initiate pro-inflammatory functionality represent valuable therapeutic strategies. Our group found that PIRB/LILRB signaling pathways can function as crucial regulators of NFκB activity. Ablation of PIRB in MDSCs forced a transition to an M1-like phenotype, resulting in decreased suppressive function, T-reg activation, tumor growth, and metastasis (130). $PIRB^{-/-}$ monocytes expressed stereotypic markers of inflammatory functionality, such as increased iNOS, TNFα, with decreased IL-10 and ARG1 when compared to WT monocytes. $PIRB^{-/-}$ MDSCs also demonstrated increased ERK, MAPK, and NFκB activation upon LPS stimulation, and enhanced IFNγ-related inflammatory responses. LILRB2—the human ortholog to murine PIRB—blockade via monoclonal antibodies favored the activation of NF-κB and STAT1 and the inhibition of STAT6 activation by IL-4. *In vitro*, we observed decreased levels of CD14, CD163, CD16, and DC-SIGN in A549-derived macrophages cultured

in the presence of αLILRB2 antagonist antibodies. Humanized MISTRG (M-CSF^{hi}, IL-3/GM-CSF^{hi}, and TPO^{hi}) mice treated with αLILRB2 antibodies to reprogram human macrophages to a M1-like classically activated phenotype. Our group has also generated BAC-transgenic mice expressing LILRB2 for various studies. Recently we showed that αLILRB2 antibody therapy had a synergistic effect when combined with αPD-1 therapy to diminish tumor burden in a lung cancer model with BAC-transgenic LILRB2 mice, while simultaneously suppressing MDSC and T-reg infiltration into the tumor site (131).

High Dectin-1 and the novel Dectin-1 agonist Galectin-9 expression were found in the TME of PDAC bearing mice. Dectin-1 is a c-type Lectin expressed mainly on macrophages and other myeloid-monocytic lineage cells. It is postulated that Dectin-1 ligation in TAMs leads to immunosuppression, thereby promoting PDAC growth. Dectin-1 does not have direct pro-tumorigenic effect on transformed PDAC but its deletion in tumor infiltrating macrophages induced immunogenic reprogramming. Similar to the outcome of Dectin-1 deletion, Galectin 9 neutralization enhanced intra-tumoral T-cell activation in PDAC (161).

MARCO (macrophage receptor with collagenous structure) is a scavenger receptor found on M2 immunosuppressive TAMs. We discussed the presence of this receptor in TAMs across multiple tumor types. Conditioned medium from cultured B16 melanoma cells and IL-10 stimulated culture resulted MARCO expression on M0 bone marrow derived macrophages (BMDM). Treatment with anti-MARCO antibodies decreased tumor sizes, increased M1-like, and decreased M2-like TAM populations in the TIME in 4T1 breast cancer and B16 melanoma mouse models. The TIME displayed decreased immature macrophages, increased CD4/T-reg cell ratio, and an upregulation of M1-like genes such as *TNF*, *IL-1β*, *NOS2*, and a downregulation of *IL-10* suggesting polarization of TAMs to a more inflammatory phenotype (150).

Last but not least, the PD-1/PD-L1 axis is one the best studied and most clinically successful checkpoint inhibitors. In cancer, the PD-1/PD-L1 axis is best known for T cell regulation. Previously, macrophages were known to express PD-1 during pathogenic infections (191, 192). Since then, it was discovered that TAMs can also express high levels of PD-1, with increasing levels over time in murine models and higher expression in increasing human cancer disease stage. PD-1/PD-L1 blockade *in vivo* increased PD-1^{+} macrophage phagocytosis activity and reduced tumor growth in murine colon carcinoma models (108). A more recent study showed that PD-1 ablation or blockade with monoclonal antibodies prevented the accumulation of granulocyte/macrophage progenitors under cancer driven emergency myelopoiesis. Interestingly, PD-1 deficient myeloid progenitors also had increased cholesterol synthesis which is required for the differentiation of inflammatory macrophages. Additionally, PD-1 ablation on myeloid cells decreased tumor growth more effectively than T-cell specific PD-1 ablation in a murine fibrosarcoma and melanoma models (109). Cumulatively, PD-1/PD-L1 blockade or ablation on myeloid cells promotes phagocytosis in macrophages, reprogramming of myeloid progenitors and even furthers myeloid differentiation via metabolic pathways. None of the aforementioned single-cell studies show exceptional levels of PD-1 on myeloid cells, but that does not exclude it from being a potential target for diminishing immunosuppressive phenotypes of myeloid cells.

Soluble Targets

C5a is a protein fragment released from cleavage of complement C5 that may be involved with PMN-MDSC recruitment. In one study, C5a was found to enhance tumor growth and inhibit CD8 T-cell mediated cytotoxicity by recruiting PMN-MDSC (CD11b^{+}Gr1^{+}) to the tumor microenvironment. C5a also enhanced PMN-MDSC's suppressive capacity by increasing the production of reactive oxygen (ROS) and nitrogen species (RNS) which inhibits CD8+ T cell response (193). Ablation of C5aR reduced the ratio of PMN-MDSC to M-MDSC in tumor bearing mice compared to wild type mice. C5aR blockade is a potential strategy to modulate the tumor microenvironment by preventing the recruitment of immunosuppressive PMN-MDSC (160).

IL-1β, a proinflammatory cytokine, is a potential target for macrophage reprogramming because it impacts CSF1/CSF1R signaling. In early tumor progression models using 4T1 cells in Balb/c mice, IL-1β acts as a master cytokine, exhibiting both pro- and anti-tumoral functionality (163). IL-1β recruited CCR2^{+} inflammatory monocytes to the tumor site through the induction of CCL2 but also promoted the differentiation of these monocytes into immunosuppressive macrophages by inducing CSF1. IL-1β deficient mice displayed significant reduction in inflammatory monocytes recruitment and macrophage differentiation. Combination therapy of αIL-1β and αPD-1 completely abrogated breast tumor progression (163).

Microenvironment

Besides cell surface receptors, cytokines and chemokines, the oxygen level in the tumor also affects the microenvironment. Tissue hypoxia develops as tumor cells proliferate until oxygen demand overwhelms the supply. To restore oxygen to the microenvironment, malignant cells initiate a hypoxic response to drive a more aggressive phenotype, promoting angiogenesis, cell proliferation, self-renewal, and other pro-tumoral programs. Two master regulators of hypoxia in cells are HIF1α and HIF2α. TAMs within this hypoxic environment are more strongly associated with M2-like functionality (194), and HIF2α ablation in TAMs resulted in a more favorable outcome in models of hepatocellular carcinoma and colitis associated colon carcinoma (164).

Just as oxygen levels affect the TIME, tumor vascularization also plays a role. The angiopoietin (ANGPT2)/TIE2 kinase signaling axis is essential to angiogenesis. TIE2 can be found on a subset of pro-angiogenic macrophages (TIE2^{+} macrophages) and promote tumor angiogenesis and tumor metastasis. Rebastinib, a TIE2 kinase inhibitor, suppressed the infiltration of TIE2^{+} macrophages to the tumor site in the PyMT mouse model of breast cancer (195). Another study showed that vascular endothelial production of ANGPT2 recruited TIE2^{+} macrophages to the tumor and the inhibition of ANGPT2 binding suppressed TAM recruitment (165).

Recent studies have attempted to explain why tumor-associated immunosuppressive myeloid cells cannot simply be binned in an M1/M2-like dichotomy, and Mohamed et al. (166) describe ER stress as a potential mechanism. Undefined tumoral signaling causes an upregulation in the unfolded protein response of MDSCs, leading to an activation of the intermediate media PERK and NRF2 drive the immunoregulatory phenotype. PERK ablation led to a reprogramming of MDSC functionality, specifically, to initiate a type I interferon anti-tumor response. More importantly, similar anti-tumor effects can be achieved with the exogenous administration of PERK inhibitors (166).

Metabolism

Recent studies have shown that immunometabolism plays a very important role in the regulation of macrophage function in the tumor microenvironment. The metabolic profile of TAMs determines their status as pro- or anti-tumoral effector cells. M1-like macrophage metabolism is generally characterized with increased glycolysis, fatty acid synthesis, and a truncated TCA cycle whereas M2-like macrophage metabolism is skewed toward fatty acid oxidation (FAO) and the TCA cycle (196–200). For example, tumor-derived lactate induces an M2-like state in macrophages, measured by the induction of the M2-related genes *VEGF, RELMA, MGL1*, and *MGL2*. Lactate can also promote the expression of ARG1 and stabilize HIF1α–key functional elements of a suppressive macrophage (201). Preventing the metabolic profile initiated by lactate using a small molecule inhibitor may reduce the presence of immunosuppressive myeloid cells (77). Similarly, methionine sulfoximine, a potent inhibitor of glutamine synthetase, skewed M2-polarized macrophages toward an M1-like state characterized by reduced intracellular glutamine and increased succinate to promote glycolysis (167).

However, promoting glycolysis in macrophages of the TIME is a risky endeavor as cancer cells also preferentially use glycolysis as an energy source, according to the Warburg effect. Therefore, targeting a metabolic pathway that inhibits tumor progression while simultaneously promoting an anti-tumor immune response would be an attractive strategy. FAO is one potential pathway, as it is the defining metabolic program of M2-like macrophages. FAO inhibition can impair the proliferation of leukemia cells (168) and reduced cellular ATP and viability in glioma (169). In multiple tumor models, tumor infiltrating MDSC were found to have increased fatty acid uptake and activated FAO (170). Etomoxir, a pharmacologic inhibitor of FAO, decreased the overall metabolic activity of MDSCs, their ability to prevent T-cell proliferation, and production of critical cytokines that maintain the induction and differentiation of MDSCs. Tumor-bearing mice treated with etomoxir and a related inhibitor, ranolazine, showed delayed tumor growth attributable to increased T-cell mediated cytotoxicity (170).

As previously mentioned, liposomal delivery of bisphosphonates can be used to deplete macrophages via apoptosis. Zoledronic acid (ZA) is a bisphosphonate containing a double nitrogen group. It inhibits the active site of the enzyme farnesyl pyrophosphate synthase in the mevalonate pathway, which is critical for isoprenoid and cholesterol synthesis (171). ZA also has a direct proapoptotic effect on tumor cells and reduces their metastatic potential (172). TAMs were significantly reduced in a TUBO cell murine mammary tumor model. Peritoneal macrophages and TAMs in ZA-treated mice displayed enhanced M1-like markers, shown by nuclear translocation of NFκB, NOS expression, and NO production (173).

Genetic Modification

Gene therapy is a unique strategy to polarize TAMs. Zhang et al. (174) describe using *in vitro*-transcribed mRNA encoding IRF5 and its activating kinase IKKβ encapsulated in nanoparticles to reprogram TAMs in models of ovarian cancer, melanoma, and GBM. The nanoparticles were engineered with D-mannose on the surface to efficiently and specifically target the mannose receptor $CD206^{+}$ on TAMs. Upon mRNA uptake, TAMs adopted a tumor-clearing, pro-inflammatory profile (174).

Similarly, endogenous RNA processing mechanisms can be exploited to reprogram TAMs. MicroRNAs (miRNA) are a class of small non-coding RNAs that negatively regulate RNA transcription and transcript levels through a sequence dependent mechanism. Normally, DICER, an RNAse-III enzyme, processes hairpin-shaped precursor miRNAs into mature miRNAs (202). Baer et al. (175) describe conditional deletion of DICER in TAMs to prevent maturation of miRNAs that otherwise inhibit M1-like functionality, rewiring the cells toward a pro-inflammatory state characterized by the activation of IFNγ and STAT1 signaling. Moreover, DICER-deficient TAMs promoted the recruitment of cytotoxic T cells that completely eradicated tumors in mouse models when combined with PD-1 checkpoint blockade (175). A summary of these methods, specific targets, and ongoing clinical trials to target them is provided in **Table 3**.

CONCLUSION, QUESTIONS, LIMITATIONS

Emerging techniques such as scRNAseq and mass cytometry have allowed for enhanced analyses of previously uncharacterized cell subsets in the tumor immune microenvironment, offering new avenues for discovering potential novel therapeutic targets and pathways that support tumor progression. Although these have not translated into the clinic yet, there is optimism that greater understanding of the tumor immune microenvironment and associated immunomodulatory mechanisms will allow for targeted therapeutic strategies to improve patient survival. While the tumor-associated myeloid cell population collectively functions to support the growing malignancy, subsets of the population are driven by assorted environmental cues that induce different functional programs. Different subsets of tumor-associated myeloid cells can directly contribute to the viability of the tumor, prohibit recognition of the tumor by the adaptive immune system, and drive chemotherapy or immunotherapy resistance. The questions left to be answered are: what combinations of signals cause the heterogeneity within the microenvironment and do they originate from the parenchyma, stroma, or both? What effect does chemotherapy or immunomodulation have on the various populations? Is there a specific population that is correlated with local or distal recurrence? For any of these cases, is one subset enough to drive any of these phenomena, or is the collection of these subsets

necessary? Is there a combination of therapies that would be most effective in eradicating these detrimental subsets?

Defining previously uncharacterized subsets of immune cells by single-cell analyses is crucial to the understanding of tumor biology, but *in situ* cell relationships also require attention. Loss of tissue architecture is a major limitation to suspension-cell-based assays, such as scRNAseq and suspension mass cytometry, thereby discounting important spatial information that comes from delineating cell-cell interactions. Several of the studies referenced above underscored heterogeneity of myeloid cell phenotypes based on their physical orientation to the tumor—within the tumor or surrounding the periphery of the tumor (18, 107, 116, 203). The location in which cells are found also dictates their functional role in the development of the malignancy, as juxtatumoral immune cells most likely serve as a suppressive barrier to cloak the malignancy, while intratumoral immune cells directly contribute to the viability of the growing tumor (114). Techniques that incorporate spatial information also offer the ability to determine direct cell-cell interaction using Cell Neighborhood Analysis (204) and predict the roles of immune cells (205). These functional states can serve as additional prognostication metrics, as several studies to date have already defined the presence of bulk TAMs and MDSCs in tumor parenchyma vs. stroma in terms of patient outcomes (19, 40, 100, 206, 207). Further work in associating the added dimension of space to the tumor immune microenvironment is required to fully understand the complex interplay between myeloid cells and malignancies.

AUTHOR CONTRIBUTIONS

VD, GJ, SM, S-HC, and P-YP contributed to the intellectual content of the manuscript and contributed to the drafting of the manuscript. All authors read and approved of the final manuscript.

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The Development and Homing of Myeloid-Derived Suppressor Cells: From a Two-Stage Model to a Multistep Narrative

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Myeloid-derived suppressor cells (MDSC) represent a heterogeneous population of immature myeloid cells. Under normal conditions, they differentiate into macrophages, dendritic cells, and granulocytes. Under pathological conditions, such as chronic inflammation, or cancer, they tend to maintain their immature state as immature myeloid cells that, within the tumor microenvironment, become suppressor cells and assist tumor escape from immune eradication. MDSC are comprised of two major subsets: monocytic MDSC (M-MDSC) and polymorphonuclear MDSC (PMN-MDSC). Monocytic myeloid cells give rise to monocytic cells, whereas PMN-MDSC share similarities with neutrophils. Based on their biological activities, a two-stage model that includes the mobilization of the periphery as myeloid cells and their activation within the tumor microenvironment converting them into suppressor cells was previously suggested by D. Gabrilovich. From the migratory viewpoint, we are suggesting a more complex setup. It starts with crosstalk between the tumor site and the hematopoietic stem and progenitor cells (HSPCs) at the bone marrow (BM) and secondary lymphatic organs, resulting in rapid myelopoiesis followed by mobilization to the blood. Although myelopoiesis is coordinated by several cytokines and transcription factors, mobilization is selectively directed by chemokine receptors and may differ between M-MDSC and PMN-MDSC. These myeloid cells may then undergo further expansion at these secondary lymphatic organs and then home to the tumor site. Finally, selective homing of T cell subsets has been associated with retention at the target organs directed by adhesion molecules or chemokine receptors. The possible relevance to myeloid cells is still speculative but is discussed.

Keywords: CCR5, CCR2 chemokines, cancer, myeloid derived suppressor cells, chemokine

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INTRODUCTION

The tumor microenvironment (TME) is the environment around the tumor that includes sounding blood vessels; immune cells; fibroblasts; soluble mediators, such as cytokines, chemokines, and growth factors; and extracellular matrix (ECM). Among the immune cells that enable tumor escape from immune eradication are myeloid-derived suppressor cells (MDSC). These are a heterogeneous

population of cells that consists of myeloid progenitor cells and immature myeloid cells (IMCs). Under nonpathological conditions, these IMCs differentiate into monocytic cells that later become macrophages, dendritic cells (DC), and mature granulocytes. However, under stress and during chronic inflammation, particularly cancer, they tend to response to "emergency signals" (1, 2), and as a result, their maturation into fully differentiated cells is inhibited while retaining their suppressive activity (3–7). Their mechanism of action includes secretion of Arginase 1 (encoded by ARG1) and inducible nitric oxide synthase (iNOS, also known as NOS2) as well as an increase in their production of nitric oxide (NO) and reactive oxygen species (ROS) [for a recent review, see (8)]. MDSC also express immune checkpoint inhibitors, among them PD-L1 and also PD-1 (9). Along with this, very recently it has been reported that targeted deletion of PD-1 from MDSC induces highly effective antitumor immunity (10). Altogether, these render MDSC immune suppressive, in particular of effector T cells, which enables tumor escape from immune eradication (3–7). It is, thus, believed that these cells play a major role in enabling tumors to escape their elimination or blockade, which could be beneficial for cancer immunotherapy (11).

Myeloid cells, as other bone marrow (BM)-derived cells, are generated from hematopoietic stem and progenitor cells (HSPCs) in a process termed myelopoiesis and then are mobilized from the BM to the blood. HSPCs also migrate from the BM to secondary lymph nodes and spleen (12). At these organs, the presence of myeloid cells has also been recorded [reviewed in (13)]. Recently, it has been reported that under "emergency" conditions occurring during stress, inflammation, and cancer diseases, the retinoic acid–related orphan receptor (RORC1/ROR/γ) orchestrates emergency myelopoiesis by suppressing negative (Socs3 and Bcl3) and promoting positive (C/EBPb) regulators of granulopoiesis as well as the key transcriptional mediators of myeloid progenitor commitment and differentiation to the monocytic/macrophage lineage (IRF8 and PU.1) (2). This may suggest that, under emergency conditions, myelopoiesis and rapid extension of myeloid cells may also take place at secondary lymphatic organs and spleen and, by so doing, allow massive accumulation of these cells at tumor sites (2) [(see also reviews in ((1) and (14)) (**Figure 1**)].

Chemokines are a subgroup of chemotactic cytokines that are well associated with chemo-attraction of various leukocytes, either from the BM to the blood (mobilization); from the blood to sites of inflammation, autoimmune sites, tumor sites, etc.; and from tissues and blood to the lymph nodes (21–23). The current review focuses on elaborating a sequential multistep model for characterizing their myelopoiesis, mobilization, recruitment, retention, and biological function. In this model, the migratory properties of myeloid cells from BM (and perhaps also from secondary lymphatic organs) to the blood (mobilization), is likely to be directed by specific chemokine receptors (**Figure 1**). The model that we are suggesting does not contradict the two-stage model of Gabrilovich (11), but adds several steps that are associated with the migratory properties of these cells. For example, the first step in Dr. Gabrilovich's model corresponds to activation of myelopoiesis, mobilization to the blood, and migration of myeloid cells to the tumor sites as suggested in our multistep model as different steps.

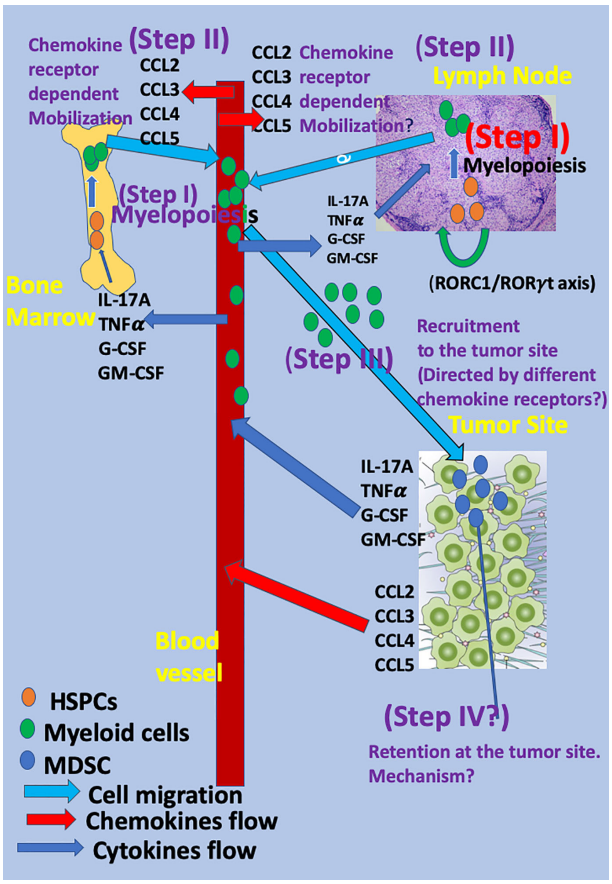

FIGURE 1 | *The mobilization and migration of myeloid cells to the tumor site as a multistep event* The mobilization and migration of myeloid cells to the tumor site is a multistep event in which cytokines, chemokines, and transcription factors released from the tumor site reach the blood and, thereafter, the BM and LNs and direct the different steps in myeloid cell differentiation and migration. The first step (Step I) is rapid myelopoiesis of myeloid cells at the BM and secondary lymphatic organs (LNs and spleen) and is directed by several cytokines, among them interleukin-17A (IL-17A), G-CSF, GM-CSF, TNFα, and others. Recently, the key role of the retinoic acid–related orphan receptor (RORC1/ROR/γ) in directing myelopoiesis in LNs has been observed (2). The subsequent step (Step II) includes the mobilization of myeloid cells to the blood and is directed by specific chemokine receptors: CCR2 for monocytic myeloid cells (15) and CCR5 for the polymorphonuclear myeloid cells (16) *via* CCR2 key ligand CCL2 and the CCR5 key ligands: CCL3, CCL4, and CCL5 (Step II). Homing to the tumor site is likely to be directed by many chemokines and chemokine receptors and is likely to have low specificity (Step III). Step IV includes the retention of these cells at the tumor site and, thus far, has been mostly studied for T cells (17–20). For myeloid cells, it is still speculative.

MDSC SUBTYPES

MDSC are comprised of two major subsets: monocytic MDSC (M-MDSC), and polymorphonuclear MDSC (PMN-MDSC). In

human, M-MDSC are defined as CD11b+ CD14+ CD15−HLA-DRlow/− cells. Due to the low or absent HLA-DR expression, M-MDSC can be distinguished from monocytes. Human PMN-MDSC are characterized as CD11b+ CD14−CD15+ HLA-DR− or CD11b+CD14−CD66b+ (24).

In mice, M-MDSC are defined as CD11b+Ly6G−Ly6C^{high} and share phenotypical and morphological characteristics with monocytes. PMN-MDSC are described as CD11b+Ly6G^{high} Ly6C^{low} cells and resemble neutrophils (24).

M-MDSC and tumor-associated macrophages (TAMs) share many features (25). Thus, it is believed that, at the tumor site, M-MDSC may become TAMs. The question of whether PMN-MDSC may also become mature granulocytes is still an open question. There are two lines of evidence that support this hypothesis: 1. Tumor-associated neutrophils and G-MDSC represent similar functional states of cells originating from the same cell type and induced within a tumor host. 2. Neutrophils isolated from a normal tumor-free host substantially differ from tumor-associated neutrophils or G-MDSC obtained from a tumor-bearing host [reviewed in (26)].

Both types of MDSC express many chemokine receptors, among them CCR5 and CCR2 (27). Within the TME, a vast majority of MDSC are PM-MDSC (about 80%) even though they have a shorter lifetime (11). Both also operate *via* similar mechanisms of immunosuppression with a few differences: Arginase-1 and prostaglandin E2 (PGE2) are preferentially produced by PMN-MDSC, whereas NO is by M-MDSC [for a recent review, see (3)]. It is also believed that M-MDSC are more prominent than PMN-MDSC as they are thought to rapidly differentiate to TAMs at the tumor site (28–35), whereas PMN-MDSC play a major role in inducing peripheral T cell tolerance (3, 11).

THE TWO-STAGE MODEL OF MYELOID CELLS MOBILIZATION AND FUNCTION

Myelopoiesis during acute infection, stress, or trauma results in rapid terminal differentiation of myeloid cells. By contrast, in cancer and chronic inflammation, myelopoiesis is associated with defective myeloid cell differentiation, which results in the accumulation and persistence of immature myeloid cells at cancer sites or chronic inflammatory sites. These cells then function as suppressor cells and are, therefore, referred to as MDSC (4, 6–8). Based on the above, D. Gabrilovich et al. suggest a two-stage model that is based on the biological function of myeloid cells during cancer and chronic inflammation (11). It includes the myelopoiesis of these cells in BM, their mobilization to the blood and secondary lymphatic organs as myeloid cells (stage I), and later their transition and maintenance as MDSC (stage II), which mostly takes place at the tumor site (11) or, respectively, sites of chronic inflammation (36).

In both type of diseases, the rapid myelopoiesis of myeloid cells at the BM is likely to be directed by several cytokines and transcription factors, among them interleukin-17A (IL-17A) ROR1C that induces IL-17A, G-CSF, GM-CSF, TNFα and others (2, 4, 6, 14, 37, 38), whereas maintenance of the suppressive function is driven by several components that affect the activities of MDSC at the tumor site, including interaction with other cells, particularly T cells cytokines, chemokines, and transcription factors, and the effect of microRNA released from exosomes (39–41).

The second stage includes two distinct yet partially overlapping types of signals. The first is associated with the expansion of the immature myeloid cells and inhibition of their terminal differentiation, and the second is their pathologic activation as suppressor cells (42). The first group of signals is mostly driven by tumor-derived growth factors as well as STAT3, IRF8, C/EBPb, Notch, adenosine receptors A2b signaling, and NLRP3 (43) and of microRNA released from exosomes (39–41). The second type of signal is mediated by factors produced mostly by the tumor stroma (proinflammatory cytokines, HMGB1) and includes the NF-kB pathway, STAT1, STAT6, prostaglandin E2 (PGE2), and cyclooxygenase 2 (COX2) as reviewed in (42).

It should be noted that the mechanisms controlling the suppressive activities may vary between PMN-MDSC and M-MDSC. The first are short-lived (44), and their activity may require close cell-to-cell contact with T cells (45), whereas M-MDSC are long-lived and are likely to give rise to TAMs that, under the TME milieu, suppress antitumor immune reactivity by different mechanisms (46).

Despite the clear definition between myeloid cells in the periphery and MDSC at the tumor site (11), it has been reported in cancer MDSC in spleen, and secondary lymphatic organs function as suppressor cells and execute far-reaching immune suppression by reducing expression of the L-selectin lymph node (LN) homing receptor on naive T and B cells, and impair T cell activation also by inhibiting the homing of naïve CD4+ and CD8+ T cells to LNs (47).

THE RECRUITMENT OF MDSC AT TUMOR SITES AS A MULTISTEP EVENT DIRECTED IN PART BY CHEMOKINE–CHEMOKINE RECEPTOR PATHWAYS

The generation of myeloid cells and their recruitment to the tumor site could be viewed as a multistep event, in which the cross-talk between the tumor site and myeloid cells play a major role. We are suggesting a four-step event that characterizes the homing of these cells (step I–IV) and an additional two steps that aim to focus on two complementary signaling events within the TME that enable the transformation of myeloid cells into suppressor cells and maintains their immature state as such (**Figure 1**) as follows:

Step I

The first step is myelopoiesis. It could occur in the BM and also possibly in the LNs and spleen as HSPCs also migrate from BM to LNs, spleen, and peripheral tissues (12) and undergo myelopoiesis there (2). Several key cytokines take a major role in this step, including IL-17A, granulocyte-colony stimulating

factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNFα. All these cytokines are largely produced at tumor sites, and their blood levels increase during cancer diseases (48–56). Concurrent myelopoiesis at the BM and secondary lymphatic organs may allow intensive accumulation of myeloid cells at the tumor site (1, 2, 14).

Step II

The subsequent step (step II) is the mobilization of myeloid cells that rapidly proliferated along myelopoiesis from the BM and possibly secondary lymphatic organs to the blood. It is not yet clear whether the mechanism by which these cells are mobilized from the BM to the blood differs from the one by which they are mobilized from the lymph nodes to the blood. Accumulating evidence votes for a pivotal role of chemokine–chemokine receptor interactions at this step (15, 16, 57). Several key chemokines are largely produced at tumor sites, and their blood level increases during cancer diseases, among them the CCR2 ligand CCL2 (58), and CCR5 ligands, in particular CCL5 (59, 60). These soluble mediators are likely to participate in the inter-talk between the tumor and leukocytes, either within the tumor site or at the periphery. The CCR2–CCL2 axis is highly relevant for monocytic and monocytic myeloid cells (15, 57), particularly in inflammation (15) and cancer (28–31, 33, 34). In 2003, Geissmann, Jung, and Littman reported different migratory properties for CX3CR1^{low}CCR2+Gr1+ and CX3CR1^{high}CCR2-Gr1+ cells, showing that those that are CCR2+ preferentially home to inflammatory sites, whereas the others go to normal tissues (57). This links CCR2 to selective homing of monocytic myeloid cells to inflammatory sites (57). Three years later, Sebrina et al. demonstrated the pivotal role of CCR2 in directing the mobilization of Ly6C^{high} monocytes from BM to the blood (15). This study shows that CCR2KO mice display fewer circulating Ly6C^{high} monocytes and, after infection with listeria monocytogenes, accumulate activated monocytes in BM (15). This study also shows that the later chemotaxis of these cells to the inflammatory site is not necessarily CCR2-dependent and also occurs if using monocytic cells from CCR2 KO mice (15). Later studies further explore the pivotal role of CCR2 in directing the recruitment of CCR2+ monocytic cells to the tumor site to support its development and suppress antitumor immunity (28–31, 33, 34). More recently, Chang et al. observed in murine glioma that CCL2 produced by microglia recruited CCR2+Ly6C+ monocytic MDSCs (M-MDSCs) to the tumor site, which is absent in CCR2KO mice (61). Among the different ligands that bind CCR2, CCL2 has been thought to be the dominant chemokine. An additional chemokine that is likely to hold similar properties is CCL12 (62).

Less is known about the mobilization of polymorphonuclear myeloid cells from the BM (and perhaps lymphatic organs) to the blood. Our group found interest in exploring the role of CCR5 and its ligands in cancer. Individuals with a functional mutation in CCR5 (deletion of the N-terminal 32 nucleotides) display a high state of HIV resistance (63). Later, it was found that they also show low prevalence of cancer diseases, particularly cancer of the prostate (64). This motivated us to explore the underlying mechanism by which the absence of CCR5 confers cancer resistance. In so doing, we have used CCR5KO mice and an autologous model of prostate cancer in immunocompetent mice (16). In this study, we observed that 1. CCR5 ligands directly support tumor growth *via* CCR5, and thus, blockade of CCR5 ligands in a chimeric system in which CCR5KO mice bearing CCR5+ tumor cells, targeting CCR5 ligands restrains tumor growth (16). 2. CCR5 drives the accumulation of MDSC at the tumor site; thus, in CCR5KO mice, the relative number of GR1+ CD11b+ myeloid cells at the tumor site is very low, and tumor development is arrested. Reconstitution of these mice with GR1+ CD11b+ myeloid cells from WT mice (CCR5+) reconstituted tumor growth (16). Further investigation shows that, along with tumor development the level of CCR5 ligands that are largely expressed with the TME, increases in the blood. This leads to a rapid increase in the expression of CCR5 on myeloid cells at the BM to a rapid mobilization of CD11b+Ly6G^{high}Ly6C^{low} myeloid cells that become PMN-MDSC at the tumor site (16). It has yet to be studied if limited accumulation of PMN-MDSC at the tumor site in CCR5KO mice exclusively results from reduction in their mobilization from the BM to the blood, and/or from secondary lymphatic organs to the blood, or also due to possible roles of CCR5 in directing the accumulation of these cells at the tumor site. In this study, we also observed that the CCR5–CCR5 ligands interaction also potentiates the suppressive activities of PMN-MDSC by increasing the expression of Arginase 1 and possibly other mediators that suppress effector T cell function (16). A recent manuscript used the technology of deleting the genomic locus incorporating the iCCRs of different chemokine receptors that have been associated with myelomonocytic cell population migration, including CCR1, CCR2, CCR3, and CCR5 to show that tissue-resident myelomonocytic cell populations are established even in their absence, whereas during inflammation, CCR2 holds a key role in their targeted recruitment (65). Dyer et al. have not explored their setup in a cancer disease model.

Step III

The third step (step III) includes the accumulation of MDSC at the tumor site and their retention there. In our opinion, this step is more complex and less understood than most leukocyte subtypes. The major obstacle is that myeloid cells express many different chemokine receptors and may, thus, respond to many different chemokines that are largely expressed at tumor sites. Then, what causes chemokine receptor specificity? Indeed, many studies show a significant role of different chemokines in myeloid cell recruitment to tumor sites (**Table 1**). Among them, 1. the CCL15-CCR1 signaling pathway (68, 69), 2. the CX3CL1 - CCL26 pathway for recruiting M-MDSC (70), 3. the CXCL5/CXCL2/CXCL1 chemokines were suggested to recruit PMN-MDSCs to tumor tissue *via* CXCR2 in murine spontaneous melanoma model (71), 4. CXCL13-CXCR5 signaling mediates the migration of MDSCs to tumor tissue (72). Moreover, in different cancer diseases, poor or good prognosis was associated with high or low levels of these chemokines (**Table 2**) [for a recent review, see (73)]. How could these observations take place with the predominate role of the CCR5-axis for directing PMN-MDSC recruitment and the CCR2-axis for M-MDSC selective recruitment at tumor sites? We are suggesting, within the three-step model described above, the highly selective step that

TABLE 1 | The role of chemokines, cytokines, and other mediators in directing the different steps in myeloid cell migration and function.

Step	Mediators	References
Step I: Myelopoiesis	IL-17A, G-CSF, GM-CSF, TNFα, RORC1,	(1, 2, 43, 66, 67)
Step II Mobilization to the blood (and possibly also homing to the tumor site):	CCR2 ligands (mostly CCL2) for monocytic cells, and CCR5 ligands, preferentially CCL5 for PMN-MDSC	(15, 16, 57)
Step III: Homing to the tumor site	CCL15-CCR1 signaling pathway, CX3CL1 - CCL26 pathway, the CXCR2-CXCL5/CXCL2/CXCL1 pathway, the CXCL13-CXCR5 pathway	(68–72). Also recently reviewed in (73)
Step IV: Retention at the tumor site	Firm data only for T cells. Yet to be identified for myeloid cells.	For T cells: (17–20, 74–76),
expansion of the immature myeloid cells and inhibition of their terminal differentiation at the tumor site	STAT3, IRF8, C/EBPb, Notch, adenosine receptors A2b signaling, and NLRP3 and of microRNA released from exosomes	(39–41, 43)
Transformation of the immature myeloid cells into suppressor cells	proinflammatory cytokines HMGB1, STAT1 STAT6, prostaglandin E2 (PGE2) cyclooxygenase 2 (COX2)	reviewed in (42)

TABLE 2 | Key chemokines associated with myeloid cell homing and cancer prognosis.

Chemokine	Key Target receptor	Step	Association with prognosis in the following cancer diseases	Reference
CCL2	CCR2	II III?	Pancreatic cancer, Bladder cancer, Breast cancer, Lung Adenocarcinoma, Renal cell carcinoma, Ovarian cancer, Cervical carcinoma	(77–83)
CCL5	CCR5	II III?	Breast cancer, Glioblastoma, Colorectal cancer, Osteosarcoma, Gastric cancer, Hepatocellular carcinoma	(59, 60, 84–91)
CCL15	CCR1	III	Head and Neck Squamous Cell Carcinoma (HNSCC), Colorectal cancer, Gastric cancer, Hepatocellular carcinoma, Lung cancer	(69, 92–97)
CCL26	CX3CL1	III	Colorectal cancer	(98)
CXCL5/CXCL2/CXCL1	CXCR2	III	Pancreatic ductal adenocarcinoma, Glioblastoma, Non-small cell lung cancer, Gastric Cancer, Prostate cancer, Colorectal cancer, Bladder cancer	(99–107)
CXCL13	CXCR5	III	Clear Cell Renal Cell Carcinoma (ccRCC), Gastric cancer, HBV-related hepatocellular carcinoma, Breast cancer, Lymphoma	(108–114),

determines receptor specificity is the mobilization from the BM and perhaps from the secondary LNs to the blood and that this step serves as a bottleneck for selectivity in myeloid cell migration.

Step IV

Retention at the tumor site: This step is still speculative and has been mostly studied for T cells thus far. It suggests that, tentatively, myeloid cells could be recruited to tumor sites by many different chemokine receptors, but their retention there is more specific and may involve a limited number of chemokine receptors and/or adhesion molecules (74). This option has been explored thus far only for T cell migration. Key examples are the retention of CD103+ memory T cells to tissues where they become tissue resident memory T cells due to the interaction of CD103 (an αE integrin) that binds a β7 counter integrin (17–20), the L-selectin serving as a homing receptor for naïve T cells (75), and the role of the α4β1 integrin in the retention of CD4+ T cells in the inflamed brain (76). The relevance of this concept to other leukocyte subtypes is yet to be studied.

POST-TRANSLATIONAL MODIFICATION (PTM) OF CHEMOKINES AND SELECTIVE MIGRATION OF PMN-MDSC

An important mechanism of fine-tuning chemokine activity is PTM of chemokines and their receptors. One of the mechanisms that may show high relevance to CCR5-dependent selective migration of PMN-MDSC is PTM by CD26 [for a recent relevant review, see (115)]. CD26 is a cell-bound enzyme ubiquitously expressed on blood cells, especially on activated T cells, fibroblasts, and epithelial cells. Two of the three CCR5 ligands, CCL4, and CCL5 are truncated by CD26, which may selectively reduce CCL4/CCL5 activity on T cells but to a lesser degree extend PMN-MDSC.

CLINICAL IMPLICATIONS IN CANCER DISEASES: COULD REDUNDANCY IN CHEMOKINES BE OVERCOME *VIA* MONOTHERAPY?

Thus far, many clinical trials in humans in which single chemokines or their receptors were targeted for therapy of inflammatory autoimmunity or cancer failed. Two major potential reasons could be taken into account: redundancy, that is, different chemokines with similar properties bind the same chemokine receptor, and overcompensation by production of increased levels of targeted chemokine. Two possible approaches to partially overcome this obstacle is by designing a compound that would target all ligands of a single receptor or prefer a receptor blockade over targeting a single chemokine. We have taken the first approach and generated a chimeric CCR5 soluble receptor study (116) that could effectively inhibit cancer

of the prostate in C57Bl/6 mice (16). Then, together with Viktor Umansky and his team, this study was further extended to a transgenic model of melanoma showing that indeed the CCR5-CCR5 ligand axis directs the accumulation of PMN-MDSC at the tumor site and that CCR5-Ig also effectively inhibited the development and progression of this disease (117). Others used blocking mAbs to CCR5 or even one of its three ligands, CCL5, to inhibit metastasis and improve the survival of tumor-bearing mice (118, 119) and also enhance anti-PD1 efficacy in gastric cancer (120). As for humans, Halama et al. recently showed success in blocking colorectal cancer using a CCR5 small molecule inhibitor that was previously developed for therapy of HIV (121). If successful, we think that extension of this therapeutic approach as a monotherapy or part of a combined therapy could be further considered.

A FUTURE VIEW OF THE CLASSICAL TWO-STAGE MODEL IN LIGHT OF MODERN TECHNOLOGIES

The traditional classification of myeloid cells in the periphery and MDSC at the tumor site have recently been revised using several modern technologies, aiming at categorizing single cells based on their gene signature (single-cell RNAseq) and expression of cell surface receptors and some intracellular proteins (mass cytometry, CyTOF). The basic hypothesis is that, beyond the variety between human and mouse in the classification of these cells (24), in each species, the gene signature and cell surface protein expression may vary depending on the organ from which cells are isolated (BM, spleen, blood, LNs, tumor site) and may also differ between tumor types (122–126). These studies are still in early development but may pave a new direction in scientific research and its translational implications.

CAN THE MULTISTEP MODEL EXPLAIN THE PARADOX OF REDUNDANCY IN CHEMOKINE–CHEMOKINE RECEPTOR INTERACTIONS AND SELECTIVE MIGRATION?

MDSC express many chemokine receptors and may, therefore, potentially migrate in response to each of them (**Table 1**). The multistep model suggests that, among the four different steps (myelopoiesis, mobilization to the blood, recruitment, and retention) the step of mobilization to the blood is likely to be the more highly selective stage. In this event, CCL2 signals *via* CCR2 to allow the effective mobilization of monocytic cells, including monocytic myeloid cells (15, 57), whereas CCR5 *via* its ligands, mostly CCL5, is likely to direct the mobilization of PMN myeloid cells (16). The last has mostly been studied in our laboratory and has to be further confirmed by others. It is conceivable that the CCR2 and CCR5 axes are also involved, together with other axes in step III of homing to the tumor site (**Table 1**). This may explain why CCR2 and perhaps CCR5 are indeed key drivers in the migratory cascade of myeloid cells.

Among these four steps, not much is known for the last one (retention) for myeloid cells. For T cells, its selectivity and specificity are mostly directed by selective adhesion receptors (18–20, 75, 76). We do not exclude the possibility that a key adhesion molecule or a key chemokine receptor may direct this stage, making it a highly selective step as well.

CONCLUSIONS

Based on their biological function, the development of MDSC includes two major stages: the first starts with myelopoiesis in the BM and lymphatic organs and the second upon their entry to the tumor site where they acquire suppressive capabilities and retain their amateur state of development. Nevertheless, based on their migratory properties, their generation and migration to the tumor site could be described as a more detailed multistep event in which their mobilization to the blood seems to be chemokine-receptor dependent and also determines the selectivity of their migration. We have uncovered a key role of the CCR5 axis in directing the mobilization of PMN-MDSC and suggest CCR5 blocking as a potential way for monotherapy or part of combined therapy for cancer diseases.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Analysis of Spatial Organization of Suppressive Myeloid Cells and Effector T Cells in Colorectal Cancer—A Potential Tool for Discovering Prognostic Biomarkers in Clinical Research

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The development and progression of solid tumors such as colorectal cancer (CRC) are known to be affected by the immune system and cell types such as T cells, natural killer (NK) cells, and natural killer T (NKT) cells are emerging as interesting targets for immunotherapy and clinical biomarker research. In addition, $CD3^+$ and $CD8^+$ T cell distribution in tumors has shown positive prognostic value in stage I–III CRC. Recent developments in digital computational pathology support not only classical cell density based tumor characterization, but also a more comprehensive analysis of the spatial cell organization in the tumor immune microenvironment (TiME). Leveraging that methodology in the current study, we tried to address the question of how the distribution of myeloid derived suppressor cells in TiME of primary CRC affects the function and location of cytotoxic T cells. We applied multicolored immunohistochemistry to identify monocytic ($CD11b^+CD14^+$) and granulocytic ($CD11b^+CD15^+$) myeloid cell populations together with proliferating and non-proliferating cytotoxic T cells ($CD8^+Ki67^{+/-}$). Through automated object detection and image registration using HALO software (IndicaLabs), we applied dedicated spatial statistics to measure the extent of overlap between the areas occupied by myeloid and T cells. With this approach, we observed distinct spatial organizational patterns of immune cells in tumors obtained from 74 treatment-naive CRC patients. Detailed analysis of inter-cell distances and myeloid-T cell spatial overlap combined with integrated gene expression data allowed to stratify patients irrespective of their mismatch repair (MMR) status or consensus molecular subgroups (CMS) classification. In addition,

generation of cell distance-derived gene signatures and their mapping to the TCGA data set revealed associations between spatial immune cell distribution in TiME and certain subsets of $CD8^+$ and $CD4^+$ T cells. The presented study sheds a new light on myeloid and T cell interactions in TiME in CRC patients. Our results show that CRC tumors present distinct distribution patterns of not only T effector cells but also tumor resident myeloid cells, thus stressing the necessity of more comprehensive characterization of TiME in order to better predict cancer prognosis. This research emphasizes the importance of a multimodal approach by combining computational pathology with its detailed spatial statistics and gene expression profiling. Finally, our study presents a novel approach to cancer patients' characterization that can potentially be used to develop new immunotherapy strategies, not based on classical biomarkers related to CRC biology.

Keywords: computational pathology, spatial statistics, tumor immune microenvironment, suppressive myeloid cells, T cells, colorectal cancer

INTRODUCTION

Currently used classification of colorectal cancer (CRC) tumors is based on classical pathological features such as tumor architecture, infiltration of bowel wall, and involvement of local lymph nodes assessed in the HE stained slides. Despite being clinically relevant, pathology staging shows its limitations especially in the era of cancer immunotherapy (CIT). With pembrolizumab being registered for Microsatellite Instable (MSI) tumors irrespective of the cancer type (1, 2), the role of non-classical parameters like Mismatch Repair (MMR) status, tumor infiltrating lymphocyte (TIL) density, or tumor mutation burden (TMB) in predicting patients outcome increased dramatically (3, 4). However, since many of the CIT regiments target directly T effector cells (5, 6), ongoing research tries to address primarily T cell biology partially overlooking the importance of other immune cell types in shaping the tumor immune microenvironment (TiME). One of them, namely tumor myeloid derived suppressor cells (MDSC), has been postulated to play an important role in generating a suppressive environment negatively affecting the function of T effector cells (7, 8). In the present study, we address the question of how the distribution of certain types of MDSC in TiME of primary CRC impact the location and function of cytotoxic T cells. By leveraging computational pathology and spatial statistics (9), we identified distinct spatial organizational patterns of immune cells in the CRC TiME. Our research suggests that the proximity of monocytic and granulocytic myeloid and cytotoxic T cells may reflect their functional interactions. The spatial analysis indicates that the location of monocytic cells correlates with the presence of TCF7 memory stem-like lymphocytes and tumor specific T cells, whereas spatial distribution of granulocytic cells associates with the activity of $CD4^+$ lymphocytes. In addition, our approach allowed to stratify CRC patients into 4 categories according to the level of overlap between myeloid and T cells irrespective of the MMR and CMS status. Categories with high levels of spatial overlap generally revealed down-regulation of cytotoxic T cell related pathways.

MATERIALS AND METHODS

CRC Samples

Human primary CRC tumor specimens of 74 treatment-naïve patients were acquired from Avaden Biosciences and Indivumed. The samples were collected after obtaining patients informed consent and approval from the respective Institutional Review Boards or equivalent agencies. For all patients, additional clinical information was provided, including gender, age, tumor stage and grade, tumor-node-metastasis (TNM) classification, tissue of excision detail, MMR status, CMS classification and TMB Score (**Table 1**). Fresh specimens were prepared as formalin fixed and paraffin embedded tissue (FFPET) blocks prior to shipment and further used for either chromogenic immunohistochemistry (IHC) staining or RNA extraction. The applied tissue processing workflow is presented in the Supplementary Material (**Supplementary Figure S3**), which includes all steps described in the following paragraphs.

IHC Staining Protocols

Sections of 2.5 μm thickness were stained with following single- and double colored chromogenic immune assays: CD11b/CD14, CD11b/CD15, CD8/Ki67, ARG1, and FOXP3. Staining procedures were performed, using Ventana Discovery Ultra, Discovery XT, or Benchmark XT automated stainers (Ventana Medical Systems, Tucson, AZ) with NEXES version 10.6 software. For all IHC assays, sections were first dewaxed, antigens were retrieved with Tris-EDTA based Cell Conditioning 1 and peroxidase inhibitor was applied to decrease endogenous peroxidase activity. For the myeloid duplex assays, CD11b/CD14 and CD11b/CD15, the primary antibody CD11b (Abcam, EPR1344, 1:400) was applied for 32 min at 37°C and then detected with UltraMap anti-rabbit HRP secondary antibody and subsequent Discovery Purple detection kit (Ventana Medical Systems). After heat denaturation, second primary antibody, either CD14 (Ventana Medical Systems, EPR3635, RTU) or CD15 (Ventana Medical Systems, MMA, RTU), was applied for 32 min at 37°C and detected with either UltraMap anti-rabbit AP or

TABLE 1 | Clinical Data of CRC patients used in this study.

Parameters	No. of patients	percent (%)
Gender		
Male	36	49
Female	38	51
Age		
< 50	3	4
50–70	14	19
≥ 70	57	77
Tumor Excision		
Right-sided	13	18
Left-sided	16	22
Rectum	17	23
NA	28	39
Tumor Grade		
Grade 1	8	11
Grade 2	42	57
Grade 3	24	32
Tumor Stage		
Stage I	2	3
Stage II	10	14
Stage III	15	20
Stage IV	47	64
pTNM Status		
pT1	0	0
pT2	5	7
pT3	49	66
pT4	20	27
pN0	19	26
pN1	23	31
pN2	32	43
pMX	26	35
pM0	1	1
pM1	47	64
MMR Status		
MSI	17	23
MSS	57	77

UltraMap anti-mouse AP secondary antibody and subsequent Discovery Yellow detection kit (Ventana Medical Systems). Sections stained with CD8/Ki67 assay were first incubated with primary antibody CD8 (Spring Biosciences, SP239, 1:12.5) for 32 min at 37°C. Bound CD8 antibody was detected with UltraMap anti-rabbit AP secondary antibody and Discovery Yellow detection kit (Ventana Medical Systems). The second primary antibody Ki67 (Ventana Medical Systems, 30-9, RTU) was added after heat denaturation for 8 min at 37°C, then detected with Hapten linked Multimer anti-rabbit HQ and anti-HQ HRP secondary antibody, followed by Discovery Purple detection kit (Ventana Medical Systems). For ARG1 assay, sections were first treated with primary antibody ARG1 [Abcam, EPR6672(B), 1:500] for 60 min at 37°C and bound antibody was detected with OmniMap anti-rabbit HRP secondary antibody and ChromoMap DAB detection kit (Ventana Medical Systems). As last, sections stained with FOXP3 assay were incubated with primary antibody FOXP3 (Abcam, 236A-E7, 1:100) for 60 min at 37°C and positive staining was detected with OptiView DAB detection kit (Ventana MedicalSystems). The nuclear counterstaining was implied for all assays by adding both Hematoxylin II and Bluing Reagent for 8 min each. Finally, slides were dehydrated and coverslipped with a permanent mounting medium.

Digital Image Analysis

Immunostained slides were histologically evaluated by an expert pathologist and then digitally scanned at 20X magnification with the high throughput iScan HT (Ventana Medical Systems). Whole-slide images were analyzed with the HALO Software (IndicaLabs) tool. On each image, tumor and normal colon regions were manually annotated and substantial areas of necrosis or tissue artefacts were excluded. The invasive margin was automatically applied, with a 500 μm width, encompassing both tumor and normal colon regions at 250 μm each. Images of the slides stained with CD8/Ki67 were registered to the images of consecutively cut slides of CD11b/CD14 and CD11b/CD15 to transfer annotations and for further spatial analysis. Annotations of ARG1 and FOXP3 images were processed separately. Next, images were used for training the algorithms to detect monocytic $CD11b^+CD14^+$ and granulocytic $CD11b^+CD15^+$ myeloid cells, $ARG1^+$ immunosuppressive myeloid cells, proliferating and non-proliferating $CD8^+Ki67^{+/-}$ cytotoxic and regulatory $FOXP3^+$ T cells (**Figure 2A**). Total cell counts, annotation areas and cell object XY coordinates were extracted for tumor, invasive margin and normal colon regions of interest (ROI). Spatial maps combining $Ki67^+$ tumor cells, $CD8^+Ki67^{+/-}$ T cells and either $CD11b^+CD14^+$ or $CD11b^+CD15^+$ myeloid cells, were used to visualize distribution patterns of immune cells in tumor annotated areas and to further perform spatial overlap analysis (see chapter 2.4.2). To provide additional information about cell co-localization at higher resolution, distances between myeloid cells and T cells were measured. Briefly, each detected $CD8^+$ cell was assigned to the nearest respective myeloid cell, the distances between formed cell pairs were extracted and a global average distance (GAD) per CRC sample was extracted.

Spatial Analysis

GAD_{norm} parameter

To avoid potential bias from the amount of myeloid cells, the GAD was normalized against the myeloid cell density and the expected mean distance for a random distribution pattern of myeloid cells (10):

$$GAD_{norm} = \frac{GAD}{\frac{0.5}{\sqrt{\frac{n}{A}}}} \quad (1)$$

Here (1), n corresponds to the total number of myeloid cells ($CD11b^+CD14^+$ or $CD11b^+CD15^+$) and A to the annotated tumor area. To differentiate between ($CD11b^+CD14^+$ and ($CD11b^+CD15^+$ derived GAD_{norm} parameters we called them: GAD_CD14 and GAD_CD15, respectively. These parameters were then used for further gene correlation analysis (see chapter 2.6).

Spatial Overlap Analysis

For better understanding the spatial relation between myeloid and T cells in the TiME, we calculated the spatial overlap between ($CD11b^+CD14^+$ or $CD11b^+CD15^+$) myeloid cells and $CD8^+Ki67^{+/-}$ T cells. First, spatial maps of annotated tumor regions, including tumor cell, myeloid cell and T cell XY coordinates, were overlaid with a hexagonal grid displayed

with a diagonal length of 250 μm (**Figure 4A**). For each single tile, we computed cell densities of both myeloid and T cells, which were then compared with corresponding median tumor density of the whole CRC cohort. Tiles with immune cell densities measured above the median tumor density were labelled with "hot" for respective $CD8^+$, $CD11b^+CD14^+$, and $CD11b^+CD15^+$ (2). So, we defined a tile h with index i to be "hot" for a cell type j if:

$$h_{ij} = \begin{cases} 1 & d_{ij} > D_j \\ 0 & otherwise \end{cases} \quad (2)$$

with $I = 1,\ldots, N$, whereas d_{ij} representing the density of a cell type j in tile i and D_j the median cell density.

This process resulted in a tiled spatial distribution map, representing single T cell or myeloid cell "hot" tiles and additional overlapping "hot" tiles with both high T cell and myeloid cell density. In order to determine the amount of $CD8^+$ "hot" areas that were also occupied by myeloid cells (3), we counted overlap tiles that were both $CD8^+$ and $CD11b^+CD14^+$ or $CD11b^+CD15^+$ "hot" and normalized this value by the total number of $CD8^+$ "hot" tiles per sample. The Myeloid-T cell Overlap (MTO) parameter for two cell types *j,k* was calculated as in the following equation:

$$MTO_{j,k} = \frac{\Sigma_{i=1}^{N} \quad h_{ij} \star h_{ik}}{\Sigma_{i=1}^{N} \quad h_{ij}} \quad (3)$$

Here (3), j accounted for cytotoxic $CD8^+$ T cells and k either for $CD11b^+CD14^+$ or $CD11b^+CD15^+$ myeloid cells. To differ between MTO calculated for $CD11b^+CD14^+$ myeloid cells and MTO calculated for $CD11b^+CD15^+$ myeloid cells, we named the respective parameters MTO_CD14 and MTO_CD15. The MTO parameters were further plotted against tumor $CD8^+$ T cell density in order to better characterize the CRC cases according to the different levels of T cell infiltration Using their median values of both parameters ($CD8^+$ T cell density/MTO level), we stratified the CRC patients into four categories: low/low (category 1), low/high (category 2), high/high (category 3), and high/low(category 4).

RNA Extraction and Sequencing

The AllPrep DNA/RNA FFPE Kit (Qiagen Cat No./ID: 80234) was used to purify genomic DNA and total RNA from 10 μm thick FFPET curls, according to the manufacturer's instructions. RNA samples were then assessed for quality and quantity using the Qubit instrument and the Agilent Bioanalyzer to determine the degradation of the RNA samples (DV200 value). To further generate the sequencing library, the hybridization-based Illumina TruSeq RNA Access method was performed according to the manufacturer's instructions, with first preparation of the total RNA library and second library enrichment for coding RNA. Finally, normalized libraries were sequenced using the Illumina sequencing-by-synthesis platform, with a sequencing protocol of 50 bp paired-end sequencing and total read depth of 25M reads per sample.

Gene Expression and Correlative Analysis

Correlation Analysis With Distance Parameter GAD_{norm}

A single sample signature scoring method, BioQC (11) was adapted to compute signature scores for patient samples in both our CRC cohort and TCGA. We performed a centering and rescaling transformation on the rank-biserial correlation output by BioQC. First, rank-biserial correlation values were multiplied by 10, and then median-centered for each signature. This is to enable qualitative comparison to gene expression values (log2 RPKM) and comparison across samples (a score above 0 indicates the sample is enriched in the signature compared to at least half of the population in the CRC cohort or TCGA dataset).

Spearmans rank correlation coefficient was then used to quantify the strength and direction of the association between a signature or gene and either the 1) measured GAD_{norm} parameter in the CRC cohort, or 2) signature representing the GADrscore CD14 or GAD_CD15 in the TCGA dataset, respectively. To identify the signature in point 2), we used the following cutoff for GAD_CD14 based correlation ($|R| > 0.4$): 64 genes in total (32 positively correlated genes, 32 negatively correlated genes) and for GAD_CD15 based correlation ($|R| > 0.3$): 271 genes in total (180 positively correlated genes, 91 negatively correlated genes) (**Supplementary Table S2**).

The permutation test was performed to evaluate the correlation between a signature or gene of interest and the signature representing the global average distance (GAD_CD14 or GAD_CD15) ("distance signature" for short). The steps in the permutation test for a signature or gene of interest (SGOI) were as follows: first we computed the correlation coefficients of the distance signature to the SGOI in each cancer cohort. Next, we generated 10,000 random signatures of similar size to the distance signature and we computed the correlation coefficients for each random signature to the SGOI in each cancer cohort. We counted how many times a random signature had absolute correlation coefficient that exceeds the absolute correlation from the distance signature. Finally, we divided this number by the total number of random signatures in order to get the p-value for the null hypothesis that the correlation of the SGOI to the distance signature could have occurred by random chance alone.

Differential Gene Expression and Pathway Analysis With Spatial Overlap Categories

redFor this analysis we used the DESeq2 (12) analysis pipeline to investigate group differences in gene expression derived from RNAseq summarized gene count data. More specifically, we compared differences in gene expression between spatial overlap category 1 and 2 and separately between category 3 and 4 in order to understand genetic differences associated with high vs low overlap when $CD8^+$ T cell density is high vs low. We made these comparisons for each of the myeloid cell subtypes of interest (i.e. ($CD11b^+CD14^+$ and $CD11b^+CD15^+$). For all comparisons, the high vs low group label was assigned using a median cutoff. We further investigated whether genes associated with differences in Myeloid-T cell Overlap were associated with

dysregulation of specific pathways using the LRpath analysis pipeline (13) using the RNA-enrich option to account for biases associated with gene count.

Statistical Methods

All statistical analysis was carried out using R (14). Statistical significance for difference between IHC extracted features were assessed with Mann-Whitney U Test. For correlation analysis between cell densities in tumor ROI, the Pearson Correlation Coefficients were calculated. The R package spatstat was used for spatial analysis (15) and GGplot2 was used for visualization (16).

RESULTS

T Cell and Myeloid Cell Subpopulations Are Distinctly Distributed Across Tumor Stromal and Tumor Epithelial Compartments

The observation of digital images representing sections stained with myeloid and T cell markers, shows specific immune cell distribution patterns in the TiME. In the tumor ROI, we focused on stromal and epithelial tumor compartments and assessed their infiltration by monocytic $CD11b^+CD14^+$, granulocytic $CD11b^+CD15^+$, and immunosuppressive $ARG1^+$ myeloid cells and also $CD8^+Ki67^{+/-}$ cytotoxic and $FOXP3^+$ regulatory T cells. Cytotoxic T cell infiltration is observed in most cases only in the stromal compartment. Minority of cases, mostly MSI type, show additional $CD8^+$ T cell infiltration into the epithelial tumor compartment (**Figure 1A**). On the contrary, myeloid cells and regulatory T cells exclusively occupy the stromal compartment, as their distribution pattern in the tumor ROI is mirroring the distribution of the stroma itself (**Figure 1B**). In the invasive margin ROI, both myeloid and T cells tend to accumulate, with myeloid infiltration being relatively higher. $CD11b^+$ and $ARG1^+$ myeloid cells are co-localizing close to the tumor epithelial border, forming an envelope covering the invasive front of the tumor ROI (**Figure 1C**).

Myeloid Cell Populations Show the Highest Density in the Invasive Margin ROI and Have Significantly Higher Density in Tumor ROI of MSI Cases

Single immune cells were detected with trained algorithms in annotated tumor, invasive margin and normal colon ROI and cell densities of $CD11b^+CD14^+$, $CD11b^+CD15^+$ and $ARG1^+$ myeloid cells, and $CD8^+Ki67^{+/-}$, $CD8^+Ki67^{+/-}$ and $FOXP3^+$ T cells were computed respectively (**Figures 2A, B**). Generally, the invasive margin ROI shows the highest immune cell infiltration among all three analyzed ROIs with median cell densities: 142.82 and 227.42 cells/mm^2 for monocytic and granulocytic myeloid cells, respectively, 192.01 and 244.08 cells/mm^2 for cytotoxic and regulatory T cells, respectively. Interestingly, the measured cell densities are significantly higher than in the tumor ROI, which is dominated by immunosuppressive myeloid and regulatory T cells with median densities: 35.32 and 122.12 cells/mm^2 formonocytic and granulocytic myeloid cells, respectively, 76.64 and 129.89 cells/mm^2 for cytotoxic and regulatory T cells, respectively. On the contrary, in the normal colon ROI both T cell subpopulations represent higher median cell densities than myeloid cells: 102.29 and 116.19 cells/mm^2 for monocytic and granulocytic myeloid cells, respectively, 233.32 and 214.11 cells/mm^2 for cytotoxic and regulatory T cells, respectively. Despite the fact that cytotoxic $CD8^+Ki67^{+/-}$ T cells show the lowest density in tumor ROI, the proportion of proliferating $CD8^+Ki67^{+/-}$ T cells to total $CD8^+Ki67^{+/-}$ T cells is higher than in invasive margin and normal colon ROIs (**Supplementary Figure S4**).

Comparing the myeloid cell subpopulations, the monocytic cells exhibit the lowest median density values, in both tumor and invasive margin ROI. The immune cell population expressing the suppressive $ARG1^+$ marker, follows the granulocytic myeloid cell expression levels in all ROIs. This is also reflected by strong positive correlation (Pearson 0.92) between $ARG1^+$ and $CD11b^+CD15^+$ myeloid cell densities in tumor ROI (**Figure 2D**). The monocytic myeloid cell density, on the contrary, shows only a moderate positive correlation to $ARG1^+$ cell density (Pearson 0.62). In addition, redproliferating $CD8^+Ki67^{+/-}$,non-proliferating $CD8^+Ki67^{+/-}$ and total $CD8^+Ki67^{+/-}$ cytotoxic T cells show a moderate positive correlation with monocytic cell density (Pearson 0.50, 0.48, and 0.50, respectively) and a weak positive correlation with granulocytic myeloid cell density (Pearson 0.36, 0.35, and 0.36, respectively). T regulatory cell density is very weakly positively correlated with the densities of other studied immune cell types. The same correlation analysis was performed in the invasive margin ROI (**Supplementary Figure S5**), only showing weak correlations between monocytic myeloid cells and proliferating, non-proliferating and total cytotoxic T cells (Pearson 0.18, 0.09, and 0.12, respectively) and weak correlations between granulocytic myeloid cells and corresponding cytotoxic T cells (Pearson 0.10, 0.14, and 0.13, respectively).

When comparing cell densities according to the patients MMR status, we observe a generally higher immune cell density in MSI cases in all ROIs, for both myeloid and T cells (**Figure 2C**). However, myeloid and T regulatory cells show significant difference only in tumor ROI, whereas proliferating and non-proliferating cytotoxic $CD8^+$ T cells are significantly higher in MSI throughout all tissue ROIs.

In addition, when cell densities of each immune cell type in all three ROIs were analyzed through hierarchical clustering, CRC patients with higher myeloid content (cluster 2) tend to cluster with both $CD8^+$ T cell high and low density groups (**Figure 2E**). Interestingly, this group of patients represents both MSI (N = 12) and MSS (N = 18) cases, whereas the cluster with low $CD8^+$ infiltration and lower myeloid content (cluster 1) only includes MSS patients (N = 20). The CMS classes, however, are inconsistently distributed throughout cluster 1 and 2, only CMS1 follows the pattern of MSI patients with one exceptional CMS1 case in cluster 1. The T regulatory cell densities were equally distributed throughout both patient clusters.

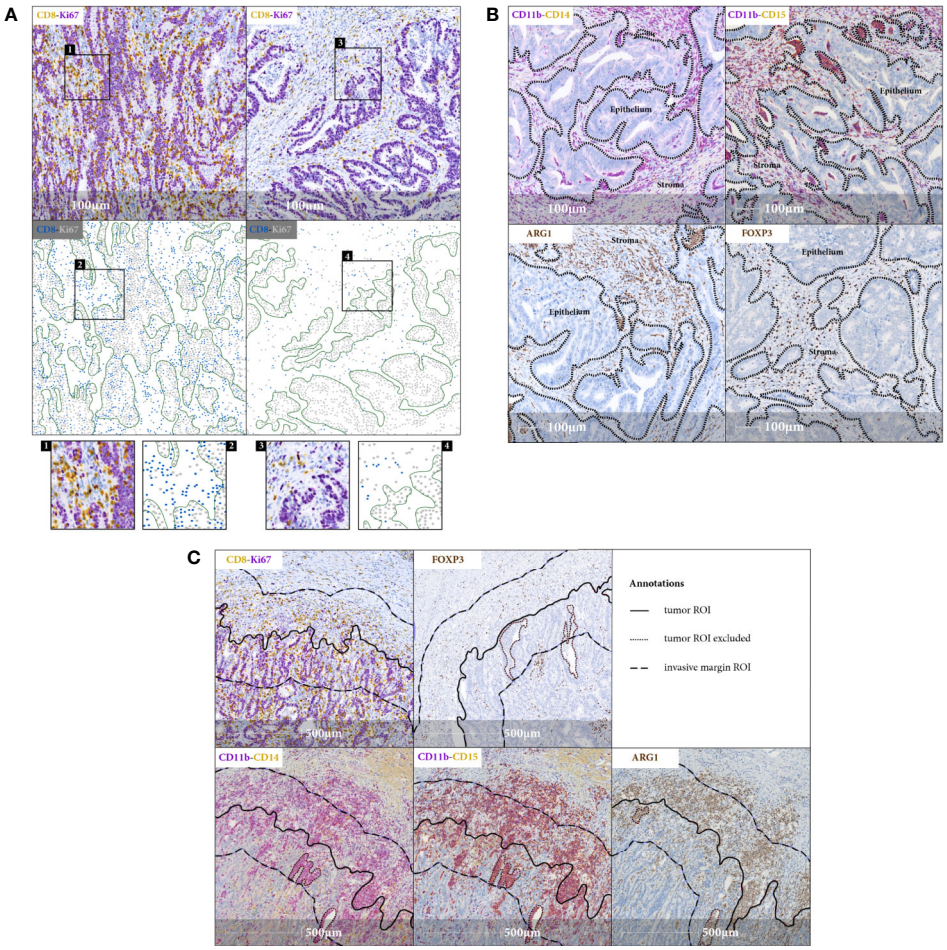

FIGURE 1 | Immune cell distribution in the TiME. **(A)** Representative IHC images (20x magnification) and corresponding spatial maps for $CD8^+$ cytotoxic T cell distribution in tumor stromal (right panel) and tumor epithelial (left panel) compartments indicating variety of lymphocytic infiltration patterns observed in CRC samples. Involvement of tumor epithelium by $CD8^+$ T cells depicted in the left panel representing an exemplary MSI case. $CD8^+Ki67^+$ staining serves as a surrogate marker for proliferating cancer cells. Green lines in spatial maps are manually annotated for FOVs and mark the tumor epithelial border for better visualization. Detailed view in the inlets. **(B)** FOVs, showing tumor stromal immune infiltration of $CD11b^+CD14^+$, $CD11b^+CD15^+$ and $ARG1^+$ myeloid cells and $FOXP3^+$ regulatory T cells, with black dashed lines indicating the epithelial borders. There is a striking accumulation of myeloid cells predominantly in tumor stromal compartment **(C)** IHC images (20x magnification), representing myeloid and T cell distribution in invasive margin ROI. Note the aggregation of myeloid cells along the tumor invasive front.

The Average Distance Between Monocytic Myeloid Cells and Cytotoxic T Cells Is Higher in MSI Cases

In order to characterize further distribution of myeloid and T cells and their spatial relation, we used the normalized distance parameter GAD_{norm} of $CD8^+$ T cells to $CD11b^+CD14^+$ and $CD11b^+CD15^+$ (GAD_CD14 and GAD_CD15), respectively.

In the comparison between MSI and MSS cases, only GAD_CD14 shows significantly higher values in MSI tumors ($p = 0.02$) (**Figure 3A**). Interestingly, this observation was confirmed when we mapped gene signatures derived from both GAD parameters to the TCGA gene expression data of CRC patients (N = 497) with known MMR status.

Additional analysis of TCGA data set revealed significant negative correlation between GAD_CD14 derived signature and the gene signature representing genes differentiating $CD8^+$ TCF7-high (CD8_G, stem-cell-like) vs. $CD8^+$ TCF7-low T-cells (CD8_B, exhausted-like or dysfunctional) (Feldman Signature) (17) (**Figure 3B**). Conversely, we found GAD_CD14 derived signature being positively correlated with the expression of *ITGAE* (CD103) and *TNFRSF9* (CD137) genes, which represent activated tumor-specific T-cells (18). GAD_CD15 derived signature shows negative correlation with the GSE6566 signature representing genes differentiating between strongly DC stimulated $CD4^+$ T cells (memory cells) vs. weakly DC stimulated $CD4^+$ T cells (effector cells) (19).

Myeloid–T Cell Overlap Allows Grouping Patients According to the Spatial Relation of Immune Suppressive and Effector Cells

Since the GAD parameter reflects only general proximity of myeloid and T cells in the TiME, we applied spatial overlap

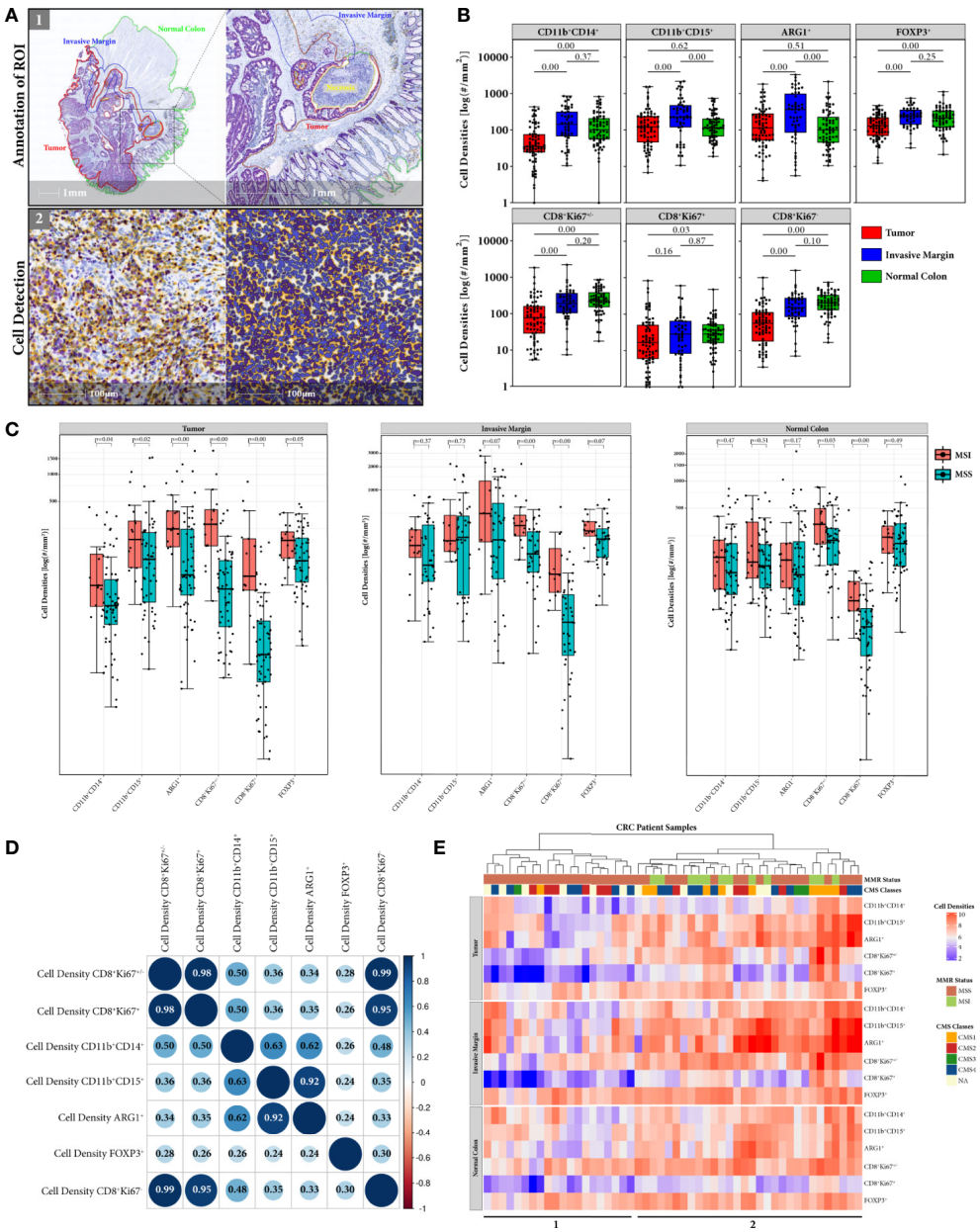

FIGURE 2 | Immune cell densities computed in tumor, invasive margin and normal colon ROIs. **(A)** HALO Workflow for processing immune cell detection in all annotated respective ROIs. **(B)** Global distribution of myeloid and T cell densities for tumor (N = 74), invasive margin (N = 51), and normal colon (N = 69) ROI. Distribution of cell densities across the compartments suggests that despite the accumulation of immune cells in tumor invasive front the tumor bed is dominated by myeloid and T regulatory immunosuppressive populations. **(C)** Comparison of myeloid and T cell densities according to CRC patients MMR status (MSI = red, MSS = blue) in all 3 ROIs. Significant difference between MSS and MSI cases are observed for immunosuppresive cells only in tumor ROI **(D)** Correlation matrix of myeloid and T cells densities measured in annotated tumor ROI. Sizes of the circles correspond to the strength of the Pearson correlation coefficient (inside the circles) and colors correspond to the direction of the correlation (blue for positive, red for negative). Note strong correlation between ARG1$^+$ and CD11b$^+$CD15$^+$ cell densities indicating the immunosuppressive nature of granulocytic myeloid cells. Weak to moderate correlation between densities of cytotoxic T cells and myeloid cell subpopulations suggests existence of other contributing factors like the spatial distribution. **(E)** Heatmap representation of hierarchical clustering of CRC patients (columns) according to their measured immune cell densities (rows) in tissue annotated regions. Only patient specimens having all 3 ROIs (N = 50) were used for the cluster analysis. Color coded bars corresponding to the MMR status and CMS classification of the cases were added on the top of the heatmap to illustrate the distribution of MMR and CMS categories across clustered samples. Cluster 1 represents generally lower myeloid cell content and consists exclusively of MSS cases, while cluster 2 shows generally higher immune cell infiltration in both MSS and MSI cases.

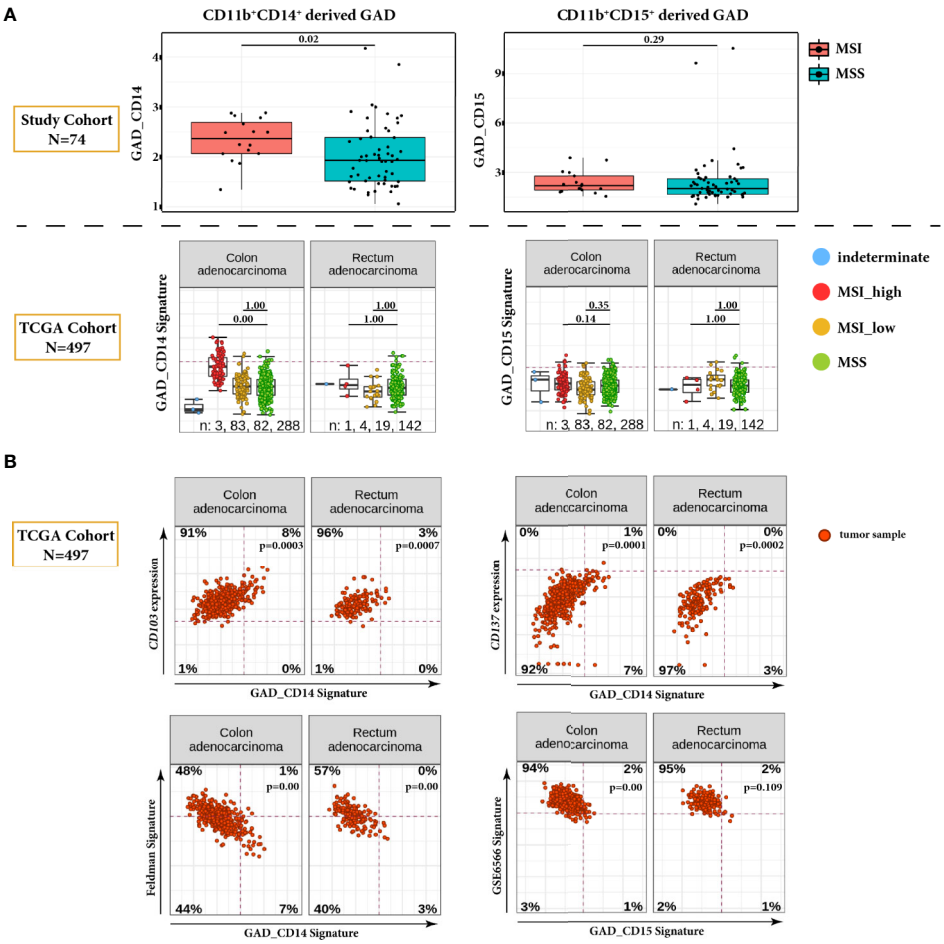

FIGURE 3 | Global Average Distance (GAD) analysis in tumor ROI. **(A)** Comparison of GAD_CD14 and GAD_CD15 parameters between MSI and MSS cases in our study CRC cohort (N = 74) (upper panel). Corresponding GAD derived gene signatures are mapped on the TCGA dataset encompassing 497 CRC tumors, and are presented for MSI_high, MSI_low, MMS, and indeterminate cases (lower panel). For the TCGA dataset, statistical significance is calculated for the differences between MSI_high and MSS categories and between MSI_low and MSS categories. Global average distance between myeloid cells and cytotoxic T cells is significantly higher in MSI cases only for $CD11b^{+}CD14^{+}$ cells, which is validated in the TCGA CRC dataset. **(B)** Correlation plots of GAD derived gene signatures and selected genes or gene signatures from TCGA dataset. Positively correlated genes *CD103* and *CD137* and negatively correlated Feldman gene signature are plotted against GAD_CD14. Negatively correlated GSE6566 gene signature is plotted against GAD_CD15. Genes and gene signatures related to anti-tumor specificity, T cell stemness and DC stimulation show association with the Global Average Distance.

analysis to detect differences in the local myeloid cell and T cell distribution in the tumor ROI (**Figure 4A**). This resulted in the MTO parameter, with both MTO_CD14 and MTO_CD15 parameters showing a very diverse distribution throughout the whole cohort reflecting the heterogeneous pattern of single and overlap tiles. The median values of MTOrscore CD14 and MTO_CD15 are comparable as they represent similar median overlap with cytotoxic T cells (0.35 and 0.33, p = 0.4, respectively). Further comparison between MSI and MSS cases shows that the median MTO is higher in MSI cases for both monocytic or granulocytic myeloid cell subtypes, however the difference is not statistically significant (**Figure 4B**).

Additional stratification of CRC patients (**Figures 4C, D**) resulted in four categories, each being characterized by two independent variables: amount of cytotoxic T cells in the tumor ROI and their spatial distribution in relation to myeloid cells. For MTO_CD14 stratification, 17, 20, 17, and 19 patients were assigned to categories 1 to 4, respectively. Most of the MSI tumors (15 out of 16, 94%) are in categories 3 and 4 (8 and 7 cases, respectively) with only 1 MSI case being assigned to category 2. For MTO_CD15 stratification, 21, 16, 21, and 16 patients were assigned to the respective categories. Similarly to MTO_CD14 stratification, the majority of MSI cases (16 out of 17 total, 94%) are found in categories 3 and 4 (11 and 5, respectively) with 1 MSI case in category 2. MSS cases are present in all four categories, with categories 1 and 2 almost being exclusive for MSS cases and categories 3 and 4 showing a mix of MSS with MSI cases. The comparison of MTO_CD14 and MTO_CD15 stratification shows that 31% (N = 23) of the CRC patients are differently distributed between MTO low and high irrespective of the $CD8^{+}$ T cell density.

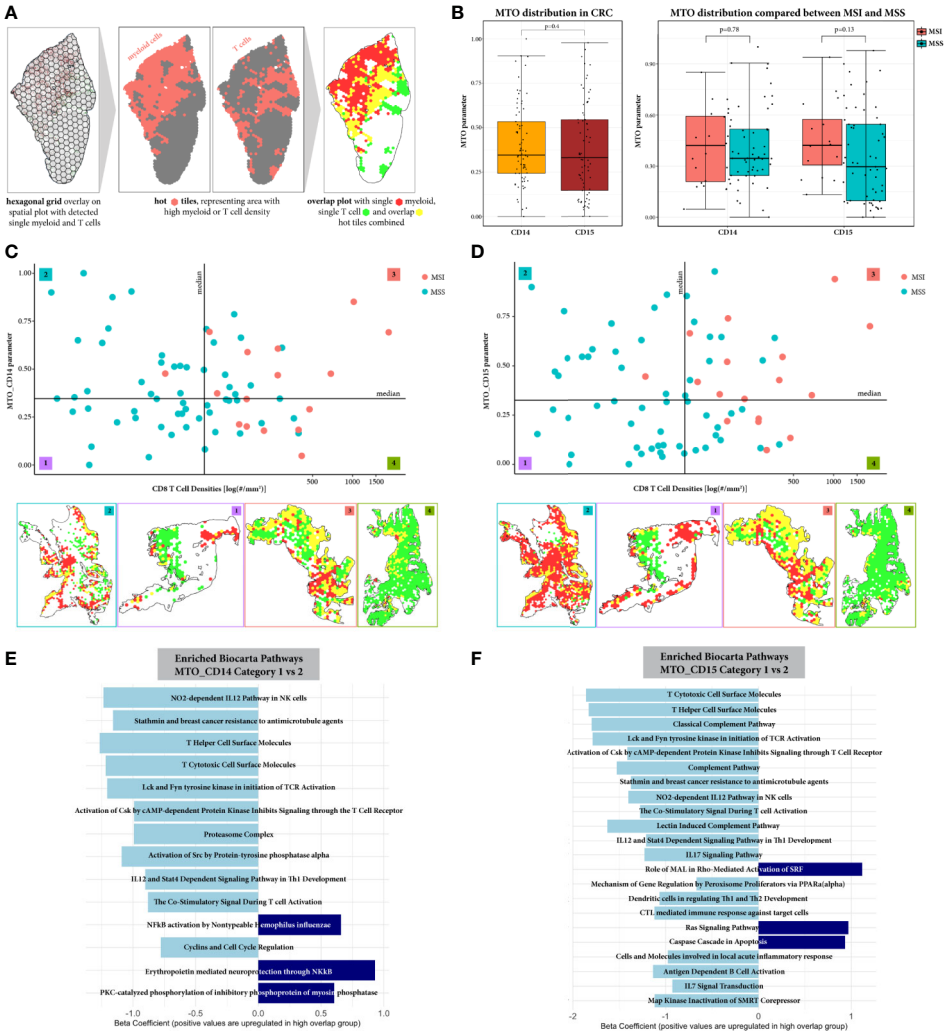

FIGURE 4 | Spatial overlap analysis in tumor ROI. **(A)** Workflow of spatial overlap analysis generating overlap maps on representative example. The tumor annotated sample was overlaid with a hexagonal grid. The density of $CD8^+$ T cells and myeloid cells (monocytic or granulocytic, respectively) was identified for every grid tile. A tile was considered as hot for a certain cell type, if its cell density was bigger than the median cell density within the whole cohort. This allowed the visualization of areas showing spatial overlap between T cells and myeloid cells. **(B)** The MTO parameter distribution is represented for both myeloid cell types $CD11b^+CD14^+$ and $CD11b^+CD15^+$, showing an overall similar distribution within the CRC cohort and when comparing between MSI and MSS cases. **(C, D)** Stratification of the samples according to $CD8^+$T cell density and Myeloid-T cell overlap (MTO). Exemplary overlap plots are depicted for each category: low/low (1), high/low (2), high/high (3) and low/high (4), for MTO_CD14 and MTO_CD15 respectively. Both, MSI and MSS cases are distributed in categories with low and high spatial overlap between myeloid and T cells, suggesting that the MTO analysis adds value to the characterization of CRC patient samples. **(E, F)** Pathway Enrichment Analysis represented for comparison between category 1 and 2, respectively. Significantly down-regulated pathways are colored in light blue, significantly up-regulated pathways are colored in dark blue. The comparison between category 4 vs 3 computed no significant differences and is therefore not shown. The spatial proximity of myeloid cells to T cells can be connected with a decreased T cell effector function, when CRC samples show low $CD8^+$ T cell density. With a high $CD8^+$ T cell density, T cells seem to overcome the immunosuppressive TiME.

We further investigated differences between CRC samples categorized for low and high spatial overlap of myeloid and T cells using differential gene expression and subsequent pathway enrichment analysis. When comparing CRC tumors with lower $CD8^+$ T cell density (categories 1 and 2), samples in category 2 characterized by high MTO show down-regulation of pathways mainly related to T cell differentiation, T cell activation and pro-inflammatory cytokine and chemokine release (**Figures 4E, F**). The MTO_CD15 samples generally reveal more significantly down-regulated pathways than MTO_CD14 samples in category 2, including additional cytokine IL17 and IL7 signaling, complement activation and DC regulation of T helper cells 1 and 2 development pathways. Up-regulated pathways, on the contrary, found in MTO_CD14 category 2 include NF-kB related pathways, while MTO_CD15 category 2 show significance for Ras signaling and caspase cascade in apoptosis. In contrast to that, CRC tumors highly infiltrated by $CD8^+$ T cells (categories 3 and 4) exhibit no significant difference in their pathway

enrichment, when comparing between samples categorized in low (category 3) and high (category 4) myeloid and T cell overlap (data not shown).

DISCUSSION

Currently, patient stratification models focus mostly on the amount of tumor infiltrating CD8+ T cells in CRC tumors. However, our data suggests that both the amount of CD8+ T cells and the spatial relationship between myeloid and T cells should be taken into account in CRC tumor immune-based classifications. As there are only a few good examples of prognostic biomarkers in clinical use for stratifying CRC patients, this observation can be of high relevance. In the present study, we focused on analyzing the myeloid cell compartment in CRC primary tumor samples and its spatial relation to $CD8^+$ T effector cells. We observed that tumor invasive margin is the tissue ROI in which most of the immune cell types accumulate. Interestingly, when MSI and MSS cases were compared, $CD8^+$ T cell densities were significantly higher in MSI cases in all 3 ROIs, i.e. tumor, invasive margin, and normal colon, whereas myeloid cells showed significantly higher accumulation only in tumor ROI. In addition, cytotoxic T cells tend to heavily infiltrate into the tumor epithelial compartment in contrast to myeloid cells which occupy almost exclusively the tumor stromal compartment (**Figures 1**, **2**). Interestingly, the normal colon ROI shows very similar immune cell infiltration patterns compared to tumor and invasive margin ROIs. It can be explained with the fact that the normal tissue represents a very heterogeneic architecture, including mucosa, submucosa, tertiary lymphoid structures (TLS), and muscle or adipose tissue. In addition, part of the invasive margin region reaches into the normal tissue. All these structures show a different immune infiltration, especially the mucosa and TLS show high densities of mainly cytotoxic and regulatory T cells, but also some myeloid cell subtypes. Previous findings of *Galon et al.* confirmed a prognostic value of $CD3^+$ and $CD8^+$ T cell distribution in tumor and invasive front ROIs in CRC patients' stage I–III (20). In addition, detailed analysis of consensus molecular subtypes (CMS) showed high $CD8^+$ T cell infiltration in CMS groups 1 (MSI-like) and 4 (mesenchymal) with the latter characterized by high myeloid content and the worst prognosis compared to other 3 CMS groups (4). Due to the lack of clinical follow up data in our study cohort, we could not correlate the myeloid cell content with patients' prognosis. However, hierarchical clustering (**Figure 2E**) according to immune cell densities in all 3 ROIs resulted in 2 main groups—with higher and lower myeloid content. Of these two, only "myeloid low" group exclusively contains MSS cases, whereas "myeloid high" represents both MSI and MSS phenotypes and has no clear molecular characteristics with respect to the CMS classification. In general, our observations based on comparison of myeloid and T cell densities show that myeloid cell concentration in TiME of CRC is not strongly dependent on the molecular phenotypes. Additionally, there is only weak to moderate positive correlation between $CD8^+$ T cells and monocytic and granulocytic densities in tumor ROI, suggesting that not the amount of immune cells but rather their distribution plays a more important role in shaping the TiME. Actually, *Si et al.* found that tumor associated neutrophils (TANs) in head and neck cancer execute their immunosuppressive role when they are in close proximity with T cells (21). Activated T cell densities, however, present both weak to moderate positive or negative correlations with neutrophils depending on TANs immunosuppressive or T cell stimulatory functions, respectively. Our IHC methodology does not allow to identify different subsets of TANs or TAMs. Despite that limitation, we could observe high and moderate association between $CD11b^+CD15^+$ and $CD11b^+CD14^+$ cell densities and $ARG1^+$ cell densities, respectively (**Figure 2D**). Therefore, we assume that the density correlations observed in our CRC cohort represent mostly correlations with myeloid immunosuppressive subsets.

When we looked closer into the distribution of myeloid and T cells in TiME by measuring the Global Average Distance (GAD), we found that MSI cases have significantly higher GAD between $CD8^+$ T cells and monocytic $CD11b^+CD14^+$ myeloid cells. This is not true, however, for granulocytic $CD11b^+CD15^+$ myeloid cells. These findings were confirmed with subsequent mapping of the gene signatures derived from GAD_CD14 and GAD_CD15 to the TCGA dataset, consisting of 497 CRC samples (**Figure 3A**). Our observation can be potentially explained with the higher tendency of cytotoxic T cells to infiltrate and reside in the tumor epithelium in MSI cases (22), resulting in the bigger spatial separation of monocytic myeloid and $CD8^+$ T cells. In addition, the GAD_CD14 derived signature shows negative correlation with TCF7 related signature and positive association with expression of *CD137* and *CD103* genes (**Figure 3B**). It indicates that tumors with lower distance between monocytic myeloid and $CD8^+$ T cells may have more TCF7 memory stem-like T cells. As described by Held et al., $TCF7^+$ cells represent $CD8^+$ T cell population residing predominantly in tumor stroma (23). Therefore, our findings suggest that the close proximity between monocytic myeloid and cytotoxic T cells reflects tumor stromal co-localization of $CD11b^+CD14^+$ myeloid cells with $CD8^+TCF7^+$ stroma-residual stem cell-like T cells. On the contrary, tumors with higher monocytic myeloid to T cell distance seem to have more intra-epithelial activated tumor specific $CD8^+$ T cells ($CD103^+$ and $CD137^+$) that are spatially separated from stroma-residual myeloid suppressive cells. For $CD11b^+CD15^+$ granulocytic myeloid cells, the distance to $CD8^+$ T effector cells does not correlate with MMR status probably due to the fact that this particular spatial parameter reflects different aspect of TiME not related to the microsatellite stability. In fact, tumor associated neutrophils are very heterogeneous cell population with several pro- and anti-tumor functions (24). They can engage with different tumor resident immune cell types performing either stimulatory or inhibitory functions (25). Our findings suggest that tumors with lower distance between granulocytic myeloid cells and cytotoxic T cells may have higher $CD4^+$ memory T cell content. This observation may indicate substituting immunostimulatory role of $CD4^+$ memory T cells in the situation when $CD8^+$ T cell activity is downregulated by granulocytic immunosuppressive myeloid cells.

Since GAD does not give a detailed insight into the distribution patterns of myeloid and T cells in TiME, we introduced the spatial overlap analysis using the Myeloid T cell overlap (MTO) parameter. Using both the amount of myeloid-T

cell overlap and the $CD8^+$ T cell density, we assigned CRC patient samples into 1 of 4 categories (low/low, high/low, high/high, or low/high) (**Figures 4C, D**). We observed CRC tumors showing high spatial overlap, with low (category 2, high/low) and high $CD8^+$ T cell density (category 3, high/high), reflecting co-localization of the majority of infiltrating T cells with myeloid cells. In comparison, categories containing CRC tumors that show low MTO and either low (category 1, low/low) or high $CD8^+$ T cell density (category 4, low/high) represent tumors with low level of co-localization. Interestingly, the MSI cases, which show predominantly high $CD8^+$ T cell density, intermingle with MSS cases and are distributed between categories 3 and 4. It potentially indicates that the spatial organization of CRC TiME does not depend on the tumor MMR status but is rather a result of local interactions between myeloid and T cell populations. This is probably the reason why 31% of CRC cases in our cohort are assigned to different overlap-derived categories when MTO_CD14 and MTO_CD15 are compared.

To better reflect the T cell activity in cases showing high amount of spatial overlap areas between immunosuppressive myeloid cells and cytotoxic T cells in tumor ROI, MTO categories 1 (low/low) and 2 (high/low) and categories 3 (high/high) and 4 (low/high) were compared by using differential gene expression and subsequent pathway enrichment analysis (**Figures 4E, F**). Only categories with low $CD8^+$ T cell density appear to show significant differences in the regulation of cytotoxic T cell activity. For both MTO_CD14 and MTO_CD15 the category 2 characteristic for high spatial overlap depicts a general down-regulation of T cell related pathways. First, the analysis suggests an impaired IL12 mediated T cell differentiation into T helper 1 (Th1) and Th2 cells when immunosuppressive myeloid cells occupy T cell infiltrated areas in the tumor ROI. This is followed by a reduced cytotoxic T cell activity, marked by lower expression of cell surface molecules, a dysfunctional activation initiation of the T cell receptor (TCR) and by down-regulated cytokine signaling. These findings are in line in with the described in literature mechanisms of T cell suppression by activated neutrophils requiring direct contact between them and T cells (26). While the effects on cytotoxic T cell function is very similar, functional differences between monocytic and granulocytic myeloid cells are mainly detected in pathways up-regulated for NF-kB signaling ($CD11b^+CD14^+$) and Ras signaling and apoptosis ($CD11b^+CD15^+$). It may indicate differences in cell specific functions, e.g. changes in monocytic MDSCs pro-inflammatory function and anti-tumor activity of neutrophils (27, 28). On the contrary, the comparison between MTO categories characteristic for high $CD8^+$ T cell density revealed no significant differences in the functional status of cytotoxic T cells. These results suggest that with increased infiltration by $CD8^+$ T cells the local immunosuppressive effect of interacting myeloid cells is overcome or is not dominating any longer in TiME.

Our study has certain limitations. The CRC cohort we analyze is relatively small (N = 74) and misses clinical follow-up information. We partially address it by validating our results on the TCGA database through mapping of the distance derived signatures. In addition, we used CMS classification as a surrogate of the clinical outcome. Due to the limitation of IHC methodology and bulk gene expression analysis using whole tissue sections we could not study presence and location of other types of cells (e.g. cancer cells, fibroblasts, certain immune cell subsets) that may have potential impact on the distribution of T cells. One of the solutions to that problem could be application of multiplex immunofluorescence and spatial genomics methods which would allow detailed analysis of several tumor compartments and more complex immune cell phenotypes. Instead, we used image registration capabilities of HALO software to analyze several immune cell types in the same coordinate system.

In summary, this study presents a multimodal approach addressing the distribution of myeloid and T cells in the TiME of CRC tumors. We combine digital image-based analysis, including cell density, cell-to-cell distance and spatial overlap, with gene expression profiling to link the tumor spatial features with the biological function of tumor infiltrating immune cells. Importantly, our data shows that myeloid cells, in general, play a crucial role in building the TiME of CRC tumors. In our cohort, we observe high variability of tumor infiltration pattern by monocytic and granulocytic myeloid cells and their spatial relation to cytotoxic T cells. Our findings suggest that the location and the function of $CD8^+$ T effector cells is influenced by the tumor stroma-residual myeloid cells. In particular, GAD_CD14 derived gene signature indicates that the location of monocytic cells correlates with the distribution of TCF7 memory stem-like lymphocytes and tumor specific T cells. Additionally, the spatial overlap analysis shows the suppressive functional effect of both monocytic and granulocytic myeloid cells on cytotoxic T cells, when co-localizing in immune dense areas in the tumor ROI. Given that current patients stratification models are focusing mostly on the amount of tumor infiltrating $CD8^+$ T cells, results of our study provide strong rationale for including spatial relation between myeloid and T cells into CRC tumor immune-based classifications. Our system for characterization of CRC samples, based on both spatial relationship and T cell density, is a promising tool for investigation as a potential prognostic biomarker for CRC and warrants additional investigation. Further validation is needed to correlate this tool with clinical outcome in the hope of supporting patients' enrichment strategies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152395).

ETHICS STATEMENT

Human primary CRC tumor specimens of 74 treatment-na¨ıve patients were acquired from Avaden Biosciences and Indivumed. The samples were collected after obtaining patients informed consent and approval from the respective Institutional Review Boards or equivalent agencies.

AUTHOR CONTRIBUTIONS

NZ, MC, FG, and KK contributed conception and design of the study. NZ organized tissue samples and data base, performed all IHC stainings, tissue annotations, and digital image analysis. KK provided quality control for annotations of digital images of histopathological slides. HF performed spatial statistics, CH-O performed gene expression and correlative analysis with distance parameter. DPH performed differential gene expression and pathway analysis. NZ and KK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.550250/full#supplementary-material

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REVIEW
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Tumor-Associated Neutrophils and Macrophages—Heterogenous but Not Chaotic

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Tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) have been extensively studied. Their pleotropic roles were observed in multiple steps of tumor progression and metastasis, and sometimes appeared to be inconsistent across different studies. In this review, we collectively discussed many lines of evidence supporting the mutual influence between cancer cells and TAMs/TANs. We focused on how direct interactions among these cells dictate co-evolution involving not only clonal competition of cancer cells, but also landscape shift of the entire tumor microenvironment (TME). This co-evolution may take distinct paths and contribute to the heterogeneity of cancer cells and immune cells across different tumors. A more in-depth understanding of the cancer-TAM/TAN co-evolution will shed light on the development of TME that mediates metastasis and therapeutic resistance.

Keywords: tumor-associated neutrophils, tumor-associated macrophages, metastasis, tumor microenvironment, tumor evolution

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INTRODUCTION

Tumors are heterogeneous at multiple levels. Genomic and transcriptomic profiles classifies many cancers into different intrinsic subtypes (1–5). Individual tumors consist of not only neoplastic cells but also a variety of stromal cells and extracellular matrix components that together constitute tumor microenvironment (TME) that determines tumorigenesis and tumor progression. Even for cancer cells within the same tumor, yet another layer of heterogeneity exists among different cells due to clonal evolution or variable status of differentiation. These different levels of heterogeneity represent a major obstacle against effective therapies that can be applied to most patients.

Neutrophils and macrophages are the most abundant immune cells that infiltrate tumors (6–8). The crosstalk between tumor cells and the infiltrated neutrophils and macrophages can contribute to drive tumor growth and metastasis. Recent research suggests that frequencies of tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) can vary across different breast cancers, thereby forming a previously unappreciated level of heterogeneity across patients, but extrinsic to cancer cells. This heterogeneity appears to be somewhat inheritable but may become altered when tumors are subjected to therapeutic interventions. It is compelling to hypothesize that cancer cells co-evolve with TAMs and TANs: whereas TAMs or TANs confer selective advantages to cancer cells with specific properties, different cancer cell clones also preferentially recruit certain

myeloid cell populations, thereby forming a loose symbiosis-like relation that is highly context-specific.

Both TAMs and TANs have immunosuppressive functions and are known to modulate anti-tumor immunity, which are covered by outstanding reviews in this issue or elsewhere (7–10). In this review, we focus on evidence showing that TAMs and TANs directly participate in tumor initiation, proliferation, and metastasis. We will highlight the heterogeneity of breast cancers and how this heterogeneity can reciprocally shape the surrounding tumor microenvironments. Finally, we will discuss our lack of knowledge in direct cancer-myeloid interactions that are selective based on different cancer-intrinsic properties and myeloid subpopulations. Although the principle and hypothesis may not be cancer type–specific, we will use breast cancer as a representative in the final discussion to integrate our knowledge and exemplify future directions.

TUMOR-ASSOCIATED NEUTROPHILS

Neutrophils are the first line of defense of our immune system, abundantly circulating in peripheral blood. When foreign pathogens invade human bodies, neutrophils are quickly recruited to the site of inflammation to exert antimicrobial moieties (11, 12). Neutrophils make up a considerable proportion of the immune cells infiltrated in primary tumors including lung, breast, gastric and others and are associated with poor overall survival and recurrence-free survival (6, 13). Meta-analysis has shown that a high density of intratumoral neutrophils are independently associated with unfavorable survival, whereas the peritumoral and stromal neutrophils were not (14). Traditionally believed as short-lived, neutrophils have been shown to have longer lifespans in tumor bearing settings, likely due to support from tumor secreted cytokines (15, 16). Tumor associated neutrophils (TANs) actively participate in various steps of tumor progression and have been reported to have both antitumor and pro-tumor roles. Direct cytotoxicity of TANs has been found to inhibit tumor progression and metastasis (17–19), however a larger number of pro-tumor functions have been uncovered. These include angiogenic switch, promotion of migration and invasion, as well as exertion of immunosuppression (7). Like TAMs, TANs of different roles on tumors were classified as N1 (antitumor) or N2 (protumor). A study by Fridlender et al. showed that blockade of TGF-beta increased recruitment of anti-tumor pro-inflammatory neutrophils. These neutrophils exhibited nuclei that were hypersegmented compared to neutrophils present under TGF-β suffice conditions. These two different kinds of neutrophils are termed as TAN N1 or N2 (20). It remains to be elucidated whether the N1 and N2 statuses result from polarization or different degrees of maturation. Regardless, this and related studies demonstrated that neutrophils are not a homogenous entity and should be studied in a context dependent manner. In the following paragraphs, we will focus on the roles of TANs in specific aspects of tumor progression (**Table 1**).

THE IMPACT OF TANS IN HUMAN CANCERS

The correlation between neutrophils and cancer prognosis remains to be precisely characterized (**Table 2**). In peripheral blood, high neutrophil to lymphocyte ratio (NLR) is associated with worse prognosis in patients with a variety of cancers, including breast cancer (13, 45). In many studies, the infiltration of TANs in cancer follows a similar trend and is associated with poor clinical outcomes (6, 13). In terms of microenvironmental characteristics, TANs were found to be inversely correlated with T cell infiltration and positively correlated with angiogenesis, consistent with pro-tumor roles (57, 58). However, there were also studies showing TANs as good prognostic factors in colorectal cancer, squamous cell carcinoma and invasive ductal breast carcinoma. The antitumor effects of neutrophils may be mediated through direct killing or coordinating with adaptive lymphocytes. These seemingly controversial results, sometime even within the same cancer type, might be derived based on different markers used. For example, frequency of high $CD66^+$ neutrophil is positive correlated with CRC malignancy, while myeloperoxidase (MPO^+) neutrophils exhibited the opposite trend as good prognosis factor (51, 52). These discrepancies highlight the urgent need for precise characterization of the heterogeneous "neutrophil" populations. The current marker system is clearly insufficient. The functionally distinct subpopulations need to be identified and separated, in both experimental and clinical studies.

TANS IN TUMOR INITIATION

Inflammation-induced damage promotes tumorigenesis independent of cancer-intrinsic genetic mutations. Studies showed that neutrophils are more frequently recruited to tumor-prone tissue through chemotaxis (21, 59). Using three genetically engineered spontaneous tumor mouse models to mimic the tumorigenesis in human, Jamieson et al. found that CXCR2 ligands were upregulated in all three models, including intestinal adenoma ($Apc^{Min/+}$), the invasive intestinal adenocarcinoma ($Ah{-}CreER;\ Apc^{F/+};\ Pten^{F/F}$) and the spontaneous oral papilloma ($K14{-}CreER;Kras^{G12D/+}$) model. CXCR2 inhibition or deficiency suppressed tumor formation in $Apc^{Min/+}$ model and $Ah{-}CreER;\ Apc^{F/+};\ Pten^{F/F}$ model, respectively. Administration of carcinogens failed to induce papilloma or adenoma in CXCR2 deficient mice, in which neutrophils trafficking was significantly impaired. Depletion of $Ly6G^+$ cells using anti-Ly6G antibodies showed a similar inhibitory effect of tumorigenesis in both chemical-induced and spontaneous models. Although the detailed mechanism was not discussed, myeperoxidase (MPO) was detected on neutrophils, which might link the reactive oxidative stress induced by neutrophils to tumorigenesis (21). The genotoxic substances released by neutrophils can initiate a carcinogenic response by inflicting DNA damage on epithelial cells. Neutrophils was reported to stimulate ROS and telomere DNA

TABLE 1 | The role of tumor-associated neutrophils in cancer.

Function	Identification Markers	Tumor model	Experimental system	Reference
Tumor initiation	$CD11b^+$ $Ly6G^+$; MPO+	$Apc^{Min/+}$, *Ah–CreER;* $Apc^{F/+}$; $Pten^{F/F}$, *K14–CreER;*$Kras^{G12D/+}$ spontaneous models, carcinogen induced model	Mouse, *in vivo*	(21)
	$CD11b^+$ $Ly6G^+$	Diethynitrosamine induced hepatocellular carcinoma, $nfkb1^{-/-}$	Mouse, *in vivo*	(22)
	Mpx	Gfap-$Kras^{G12V}$ astrocytes	Zebra fish larvae	(23)
Tumor proliferation	NE	TE-1, TE-7, TE-8, TE-12, TE-13	Human, *in vitro*	(24)
	NE	*loxP*-Stop-*loxP* K-ras^{G12D}	Mouse, *in vivo*	(25)
	$CD11b^+$ $Ly6G^+$ $Ly6C^+$	PC3	Human, *in vivo*; human, *in vitro*	(26)
	Cytoplasic granule morphology $MMP9^+$	$MMP\text{-}9^{-/-}$ HPV16 model	Mouse, *in vivo*	(27)
Proliferation Metastasis	MPO	D2.0R	Mouse, *in vivo*	(28)
Angiogenesis	Chick heterophils, gradient centrifugation	Chick embryos with 3D collagen grafts	Chick, *in vivo*; Human, *in vivo*	(29)
	$Ly6G^+$	Chick embryos with 3D collagen grafts; PC3, L929, B16, LLC	Chick, *in vivo*; Mouse, *in vivo*, Human, *in vivo*	(30)
	$CD11b^+$ $Gr1^+$	EL4, LLC, B16F1, T1B6	Mouse *in vivo*	(31)
	$CD11b^+$ $Gr1^+$	RIP-Tag2 model; HM7	Mouse, *in vivo*; human, *in vivo*	(32, 33)
Invasion and migration	Gradient centrifugation	AsPC-1, HepG2, MDA-MB-468	Human, *in vitro*	(34–36)
EMT	Gradient centrifugation, NASDCL, elastase	PDAC biopsies, T3M4, HuH7	Human, patient samples; Human, *in vitro*	(37)
	$CD66b^+$	MKN45, MKN74	Human, *in vitro*	(38)
	$CD66b^+$	Lung carcinoma samples	Human, patient samples	(39)
	$CD11b^+CD66b^+$	MCF-7	Human, *in vitro*	(40)
CTC proliferation	$Ly6G^+$, Wright-Giemsa staining	BR16-GFP	Human, *in vivo*	(41)
Extravasation	$CD11b^+$	C8161.CI9, 1205Lu; A375, MDA-MB-231	Human, *in vitro*	(42, 43),
Metastasis	$Ly6G^+$	4T1	Mouse, *in vivo*	(44)

TABLE 2 | The clinical relevance of TANs in human cancers.

Type of Cancer	Marker	Correlation	Reference
Breast cancer	Peripheral blood neutrophil to lymphocyte ratio (NLR)	Short- or long-term mortality	(45)
Renal cell carcinoma	$CD66b^+$	Short RFS	(46)
Melanoma	$CD66b^+$ and $CD123^+$ DCs	Poor prognosis	(47)
Head and neck cancer	Polymorphonuclear granulocytes	Poor survival	(48)
Hepatocellular carcinoma	$CD66b^+$	Early recurrence and decreased PFS/OS	(49)
Colorectal cancer	$CD66b^+$	Better prognosis	(50)
Colorectal cancer	$CD66b^+$	Poor prognosis	(51)
Colorectal cancer	MPO^+	Better prognosis	(52)
Gastric adenocarcinoma	$CD15^+$	Independent and unfavorable factor in prognosis	(53)
Human gliomas	$CD15^+$ and MPO^+	High tumor grade	(54)
Pancreatic adenocarcinoma	Polymorphonuclear granulocytes	More malignant subtype	(55)
Pancreatic adenocarcinoma	$CD66b^+$	Associated with shorter survival along with pan-macrophages and M2 macrophages	(56)

damage in hepatocytes. Upon depletion of hepatic neutrophils by anti-Ly6G (1A8) antibody, diethynitrosamine (DEN) induced hepatocellular carcinoma was attenuated. Anti-oxidant treatment led to protection against progression of DEN induced hepatocellular carcinoma (22). Another study reported that neutrophils were recruited to $Kras^{G12V}$-expressing astrocytes in an optical transparent larva zebrafish model of glioblastoma by CXCR1. The proliferation of these tumor-initiating astrocytes was also blunted when neutrophil chemotaxis signaling CXCR1/2 was inhibited (23). Thus, it appears that TANs can enhance tumor initiation either through exerting genotoxicity by inducing ROS or potentiating the tumor initiating cells.

TANS IN TUMOR PROLIFERATION

Several molecules (NE, MMP9, Bv8) expressed on neutrophils can mediate their positive roles in tumor proliferation. As a serine protease, the proteolysis ability of neutrophil elastase (NE) is able

to release growth factors from cancer cells. In an *in vitro* esophageal cell line model, NE treatment led to a rapid release of TGF-α, PDGF and VEGF along with EGFR phosphorylation. Increased cell proliferation and invasion was also observed in all five cell lines tested (24). In a *loxP*-Stop-*loxP* K-ras^{G12D} (LSL-K-ras) model of mouse lung adenocarcinoma, Houghton et al. showed that neutrophil elastase is endocytosed by tumor cells where it degrades IRS-1 and skews the PI3K toward tumor proliferation (25). Hammes et al. demonstrated NE is produced by infiltrating immune cells using live imaging of nude mice bearing PC3 tumors. Inhibition of NE could suppress PC3 xenograft growth. Mechanistically, NE activates MAPK and its downstream signaling in PC3 cells (26). Inhibition of NE by Elafin also shows tumor suppressing activity by inducing Retinoblastoma pathway dependent cell cycle arrest and elevated apoptotic cell death (60). Coussens et al. showed MMP9 was mainly expressed by neutrophils, macrophages and mast cells. MMP9 knockout mice exhibited reduced keratinocyte hyperproliferation and bone marrow transplantation of MMP9 expressing cells can restore the tumor growth in these mice (61). The NE and MMP9 loaded on the neutrophil extracellular traps (NETs) were also found to awaken dormant cancer cells to proliferate through sequential cleavage of laminin in the extracellular matrix of the dormant cancer cells to activate integrin and YAP signaling (28). Taken together, the protease-enriched secretome of TANs appear to be able to activate several growth factor pathways at different levels to enhance proliferation.

TANS IN ANGIOGENESIS

MMP9 produced by neutrophils in the tumor microenvironment was also found to be strongly associated with the tumor angiogenesis. Using a quantitative non-tumor *in vivo* model to induce angiogenesis in 3D collagen rafts, Quigley et al. revealed that the angiogenesis is facilitated by the MMPs of the infiltrated inflammatory cells including heterophils. And the potent angiogenic characteristic was related to the active form of MMP-9 that was free of tissue inhibitor of metalloproteinases (TIMP) (29). They also showed in a later study that tumor infiltrated neutrophils are a major source of MMP-9 and is highly linked to tumor angiogenesis in a PC3 orthotopic prostate cancer xenografts in NOD/SCID mice (30). Using a RIP-Tag2 model of pancreatic islet carcinoma, Bergers et al. revealed the specific angiogenesis role of MMP-9 by releasing VEGF from normal and hyperplastic pancreatic islets (62). The absence of MMP-9 function reduced the angiogenic switching and the growth of tumor cells. An increased intratumor infiltration of neutrophils was correlated with glioma grade as well as the resistance to anti-VEGF therapy (31, 63). Structurally similar to VEGF, G-CSF induced Bv8 secretion by bone-marrow-derived cells was implicated in tumor angiogenesis by neutrophils. Shojaei et al. elucidated the role of Bv8 in RIP-Tag angiogenic switching, where systemic depletion of Bv8 by anti-Bv8 antibody at early stage significantly reduced angiogenic islets number as well as the homing of CD11b$^+$ Gr1$^+$ cells to the emerging neoplastic lesions (32, 33). Anti-CSF or anti-Bv8 confers additional effect on anti-VEGF therapy. Therefore, both MMP-9 and Bv8 are responsible for the angiogenic effects of TANs.

TANS IN METASTASIS

The roles of TANs in metastasis are pleiotropic and highly context dependent. The 13762NF rat mammary adenocarcinoma clones with varying metastatic potentials showed a dose-dependent increase of invasion in a reconstituted basal membrane invasion culture system when co-cultured with neutrophils (a.k.a., polymorphnuclear leukocytes or PMN) from tumor bearing rats. These clones also exhibited increased lung metastases *in vivo* when co-injected with the tumor elicited neutrophils compared with those PMNs from normal rats (64). The same group also discovered later that bone marrow cellularity and myeloid erythroid ratios positively correlated with the metastatic potentials of the tumors these rats bared (65). Jung et al. showed an increased neutrophil extracellular traps formation in blood sample after co-cultured with AsPC-1 cells. Using *in vitro* Boyden chamber model, NETs increased migration and invasion of AsPC-1 cells than intact neutrophils alone, which can be inhibited by histone binding agents, some DNA-degrading enzyme as well as Toll-like receptor neutralizing antibodies (34). The interactions between cancer cells and neutrophils are not unidirectional. Reciprocally, the survival of neutrophils can be enhanced by tumor supernatant from hepatocellular, cervical, colorectal and gastric carcinoma cell lines. This effect can be mimicked by Hyaluronan fragments. Blocking the interactions between HA and TLR4 on neutrophils could mitigate this pro-survival of neutrophils as well as the migration of cancer cells (35). Strell et al. found that MDA-MB-468 cells that secreted IL-8 and GRO-α increased the migratory activity of neutrophils and recruitment to tumor cells to enable cell-cell interaction, which led to the binding of β_2-integrins expressed by neutrophils and its receptor ICAM-1 on MDA-MB-468 cells. The focal adhesion molecules including FAK were then phosphorylated by SRC kinase and the p38 MAPK was activated by Rho kinase. Eventually, the migration of tumor cells was increased (36). These studies highlighted the importance of the crosstalk between neutrophils and cancer cells during tumor progression, and demonstrated effects of neutrophils on pathways related to migration and invasion.

TANs can trigger epithelial-mesenchymal transition (EMT). Neutrophil elastase cleavage of E-Cadherin induced EMT in pancreatic and liver cancer cell line *in vitro*. Co-culture with either neutrophils or NE could induce rapid cell dyshesion and E-Cadherin degradation as early as 3 h after co-culture. In parallel, the upregulation of TWIST, translocation of β-catenin into the nucleus, nuclear expression of ZEB1, and the downregulation of keratin was also observed. Using PDAC biopsy samples, Steffen et al. showed the positive correlation of PMN infiltration with the EMT status using ZEB1 or nuclear β-catenin expression (37). Li et al. found that neutrophils were enriched in gastric cancer tissues in patients, especially in the tumor invasive edge. Coculturing of tumor associated

neutrophils with gastric cells *in vitro* significantly decreased E-cadherin expression along with the upregulation of vimentin and ZEB1. The migration and invasion of the gastric cancer cells were also increased. This effect was related to the IL-17a secreted by neutrophils. Blocking IL-17a with neutralizing antibody inhibited the TAN-stimulated activities in gastric cancer cells (38). Hu et al. showed a negative association of intratumoral $CD66^+$ PMNs expression with the E-cadherin expression. Neutrophils induced EMT was observed *in vitro* accompanied by enhanced migration of tumor cells, where TGF-β/Smad signaling was initiated and in part related to this process (39). A study by Wang et al. demonstrated that it was the neutrophils isolated from breast tumors but not from peripheral blood can significantly promote migration and invasion of a panel of breast cancer cell lines *in vitro*. MCF7 cells cultured with 30% conditioned medium from tumor infiltrating neutrophils showed mesenchymal morphology along with the downregulation of E-cadherin as well as the upregulation of Twist expression. These effects were abrogated by blocking TIMP-1 of neutrophils. Reciprocally, MCF7 cells that underwent EMT could stimulate the neutrophil expression of TIMP-1 through CD90 in a contact dependent manner (40).

Neutrophil derived enzymes also promote tumor intravasation besides angiogenesis. Using a chick embryo spontaneous intravasation assay, Bekes et al. demonstrated an essential role of proMMP9 protease in modulating certain variants of PC3 or HT-1080 cell intravasation *in vivo* (66). The neutrophils expressing MMP9 were recruited to primary tumors of highly disseminating variants to enhance their intravasation and angiogenesis. Blocking neutrophil influx by anti-IL-8 antibodies diminished both intravasation and angiogenesis.

After intravasation, it is inevitable for circulating tumor cells to encounter leukocytes. Szczerba et al. found a rare but consistent CTC-WBC clusters in peripheral blood samples from both breast cancer patients and tumor bearing mice. Most of these clusters are CTC-neutrophil clusters, which correlates with significantly worse progression-free survival in patients. Compared to CTC alone, CTCs from clusters were observed to be more proliferative with a marked enrichment in positive regulators of cell cycle and DNA replication (41).

Extravasation is a key step for disseminated cancer cells to seed in the distant organs. Neutrophils were seen to facilitate this process. Attracted by IL-8 secreted by melanoma, neutrophils interacted with the melanoma cells through β_2-integrin ICAM-1 and promoted docking along vascular endothelium. Blocking IL-8 secretion from these melanoma cells significantly decreased extravasation (42). Chen et al. employed an *in vitro* multiplexed microfluidic model of human microvasculature to observe in real-time the physiologically relevant transportation of circulating cells in a high spatial resolution. Co-injection of melanoma cells with LPS stimulated human PMNs resulted in the quick formation of tumor cell-PMN heterotypic aggregates along the endothelial under flow by both mechanical trapping and neutrophil-endothelial adhesions. By secreting IL-8, PMNs were chemotactically confined by tumor derived CXCL-1, which enhanced the extravasation of adjacent melanoma or breast cancer cells through a modulation of the endothelial barrier by IL-8. Using a neutralizing antibody against IL-8 could abrogate both PMN sequestering and the extravasation of tumor cells. Similarly, the inflamed PMNs exhibited confined migration and enhanced tumor cell extravasation in zebrafish embryos (43). The adhesion between neutrophils and disseminated tumor cells also plays a role when tumor cells arrived the organ of metastases. Clusters of neutrophil and H-59 Lewis lung carcinoma cells were seen in the liver sinusoid. This interaction was mediated by Mac-1 and ICAM-1 (67). Using two clones with different metastasis potentials from same tumor, Park et al. showed that 4T1 cells the clone with high metastasis potential recruited more neutrophils to primary tumor compared to 4T07 which have less metastasis potential. More neutrophil extracellular matrix was also found in lungs of mice injected with 4T1 cells through tail vein. Enzymatic digestion of NETs as well as anti-G-CSF antibody blocked migration and invasion *in vitro* using three different cancer cells. An intraperitoneal injection of DNase I-coated nanoparticles could prevent lung metastases in mice which received an intravenous injection of 4T1 cells (44). Neutrophils were also found to participate in the awakening of dormant cancer cells. A study from the same group showed that under inflamed conditions, NETs could awaken dormant D2.0R cells and increase metastases in mice. Neutrophil related proteases NE and MMP9 loaded on NETs' DNA scaffolding can sequentially cleave the extracellular matrix protein laminin, which reveals an epitope to trigger proliferation of dormant cancer cells through integrin activation and FAK/ERK/YAP signaling. A blocking antibody against remodeled laminin could prevent or reduce inflammation induced dormant cancer cells awakening (28).

TUMOR-ASSOCIATED MACROPHAGE (TAMS)

Differentiated from mononuclear phagocyte lineage, macrophages are a tissue-resident cell type that play a vital role in regulating immune response to maintain tissue homeostasis and organ development. Macrophages are found as key components of the infiltrating leukocytes in various types of tumors, which are considered as wounds that never heal. TAMs have been reported to actively participate in almost every step of tumor progression including tumor angiogenesis, invasion, migration, colonization at secondary organs as well as immune suppression (**Table 3**). The association between their frequency and expression patterns and poor clinical outcomes has been reported in most of the studies focusing on the clinical implications of TAMs. Bingle et al. showed in a meta-analysis that increased macrophage infiltration frequency in primary tumors was associated with poor prognosis in most of the breast cancer cases (90). Studies from Beck and Campbell linked proliferating macrophages and their related signaling like colony-stimulating factor 1 with high grade, malignant subtype as well as poor clinical outcome (91, 92). However, multivariate model analysis by Mahmoud et al. showed that overall macrophage

TABLE 3 | The role of tumor-associated macrophages in cancer.

Function	Identification Markers	Tumor model	Experimental system	Reference
Tumor initiation	$F4/80^+$	$Mdr2^{-/-}$ spontaneous model	Mouse, *in vivo*	(68)
	$CD11b^+$ $F4/80^+$	Stat3-IKO spontaneous model	Mouse, *in vivo*	(69)
Angiogenesis	$CD68^+$	Breast carcinoma samples	Human, patient samples	(70)
	$F4/80^+$	MMTV-PyMT/LysMCre+/$VEGF^{f/f}$ spontaneous model	Mouse, *in vivo*	(71)
		Breast tumor samples	Human, patient samples	(72)
	$CD11b^+$ $F4/80^+$	E0771, LLC	Mouse, *in vivo*	(73)
	$CD68^+$	K14-HPV16 spontaneous model	Mouse, *in vivo*	(74)
	$F4/80^+$ $Tie2^+$	PyMT	Mouse, *in vivo*	(75)
Migration and invasion	$CD11b^+/Gr1^{mid/low}$	MC38, LLC	Mouse, *in vitro*	(76)
	$F4/80^+$	MMTV-PyMT	Mouse, *in vivo*	(77)
	$CD68^+$ $CD163^+$	THP-1, patient samples	Human, patient samples, human, *in vitro*	(78)
	$CD11b^+Gr1^-F4/80^+$	MMTV-PyMT	Mouse, *in vivo*	(79)
	$CD68^+$ $CCL18^+$	MDA-MB-231	Human, *in vitro*	(80)
	$CD68^+$;	SKBR3, MDA-MB-231; SW48	Human, *in vitro*	(81, 82)
	$CD68^+$ $CD163^+$, $CD206^+$		Human, *in vitro*	
Intravasation	BAC1.2F5 macrophage cell line	MDA-MB-231	Human, *in vitro*	(83)
Intravasation	$MRC1^+/CD11b^+/F4/80^+/CD11c^-$	MMTV-PyMT	Mouse, *in vivo*	(84)
Extravasation, metastasis	$CD11b^+$ $F4/80^+$	Met-1	Mouse, *ex vivo*; Mouse, *in vivo*	(85, 86)
Metastasis	$CD11b^+$ $F4/80^+$	E0771-LG, Met-1,	Mouse, *in vivo*	(87)
EMT, metastasis	$CD68^+$, $CD206^+$, HLA-DR	MCF-7,	Human, *in vitro*; Humanized mouse model, *in vivo*	(88)
Anti-metastasis	$Ly6C^+$	$MT/ret^{+/-}$ spontaneous model	Mouse, *in vivo*	(89)

number ($CD68^+$) was not an independent prognostic marker, which shed light on the heterogeneity and plasticity of TAMs (93).

In an oversimplified model, macrophages polarize to two opposite states. M1 macrophages are known as classically activated macrophages, which are activated by Th1 cytokines like interferon-gamma, or together with bacterial components. These M1 macrophages exert anti-microbial properties by secreting cytotoxic molecules (e.g. reactive oxygen species and nitrogen intermediates) and pro-inflammatory cytokines (e.g., IL-6, IL-12, IL-23, TNF). As alternatively activated macrophages, M2 macrophages are activated by Th2 cytokines (e.g. IL-4, IL-10, and IL-13), which typically attenuate inflammation, promote wound healing, angiogenesis and tissue remodeling (94, 95). Polarization towards M1 or M2 requires the activation of ERK, NF-κB, and STAT1 signaling or STAT3 and STAT6 pathway, respectively. In fact, these two polarization states serve as the boundaries for a spectrum of activation states which reflects the complex tissue microenvironment that can induce simultaneous activation of different signaling pathways.

There are two sources for tumor associated macrophages. One source is from circulating $Ly6C^+$ $CCR2^+$ monocytes that enter tissues through the adherence of activated integrins (96). The other source is from tissue resident macrophages that originated from $CXC3CR1^+$ Kit^+ erythromyeloid progenitors from yolk sac or murine fetal liver independent of bone marrow (97, 98). Tumor associated macrophages tend to exhibit an M2-polarized state with impaired antigen presentation and tumoricidal capacity and high expression of angiogenic factors, tissue remodeling metalloproteases, and cathepsins. The polarization of TAM is not only regulated by intrinsic signaling, but also shaped by the complex immune and stromal cells in tumor microenvironment as well as the cancer cells. This complex interaction makes the polarization of TAM change over the dynamic evolution of microenvironment milieu. High production of inflammatory molecules from M1 macrophages may support neoplastic transformation in the early stage of tumorigenesis. However when a tumor was established, M2 macrophages can suppress immune surveillance and remodel tissue matrix to promote tumor progression (99). Besides the temporal change in the polarization status, macrophages phenotypes differs even within different areas of the same tumor. Two distinct tumor microenvironments were found in the same orthotopic mammary tumor. Perivascular TAMs showed stronger migration compared to those in avascular regions. Large number of perivascular macrophages at mouse mammary tumor margins could interact with cancer cells and migrate together (100). Macrophages within the tumor mass express less M2 markers compared with macrophages in the peri-tumor areas (101, 102). The temporal and spatial heterogeneity of TAM implies its high plasticity that can be utilized for therapeutic purposes by re-polarization strategies.

THE IMPACT OF TAMS IN HUMAN CANCERS

Like TANs, the clinical impact of TAMs has not been completely elucidated (**Table 4**). Most clinical studies have linked the density and molecular signatures of TAMs with poor clinical outcomes (113–115). A meta-analysis of literatures by Zhang et al. found that the density of TAMs was associated with poor overall survival (OS) in patients with gastric, urogenital and head and neck cancers with some exceptions in patients with colorectal cancer (113). More recently, deconvolution algorithms were developed to deduce frequencies of different immune cells in bulk tumors, which provided another way to examine potential impact of immune microenvironment

TABLE 4 | The clinical relevance of TAMs in human cancers.

Type of Cancer	Marker	Correlation	Reference
Breast cancer	$CD68^+$, $CD11c^+$, or $CD163^+$	$CD163^+$ correlated with reduced OS and DFS; $CD11c^+$ in stroma correlated with higher OS and DFS	(103)
Invasive breast cancer	$CD68^+$	High tumor grade, negative estrogen receptor	(104)
Bladder cancer	$CD68^+$	Invasive subtype, reduced 5-year survival	(105)
Hodgkin's lymphoma	$CD68^+$	Shortened patient survival	(106)
Hepatocellular Carcinoma	CSF-1R	Increased intrahepatic metastasis, tumor recurrence, reduced patient survival	(107)
Advanced thyroid cancer	$CD68^+$	Advanced histological grade, tumor invasiveness and mortality	(108)
Non-small cell lung cancer	$CD68^+$ in tumor islet and stroma	Increased survival	(109)
Follicular lymphoma	$CD68^+$	Reduced OS	(110)
Colon cancer stage II	$CD68^+$ and $CD206^+$	CD206/CD68 ratio associated with poor DFS and OS	(111)
Head and neck squamous cell carcinoma	Meta-analysis of TAMs and M2 macrophages	Both correlated with poor clinicopathologic markers	(112)

(6, 116, 117). According to a few algorithms, subpopulations of TAMs (e.g., M1 vs M2) can be distinguished, and M2 falls into the poor-prognosis category among other immune cells (118). However, single-cell RNA-seq data in human patients suggested that M1- and M2-like features may co-vary at a single cell level, and therefore, the separation between the anti- and pro-tumor TAMs is indistinctive (119). Furthermore, some generic macrophage signatures are highly correlative with T cell and B cell signatures, which are in turn associated with good prognosis (120). Thus, similar to situation of TANs, simple analysis to characterize clinical impact of TAMs as an entirety is confounded by the heterogeneity, plasticity and context-dependency of TAM functions. The simple M1-M2 bi-polarization model, which is derived *in vitro*, is insufficient to fully recapitulate these characteristics *in vivo* (121). Instead, more granular classification and functional characterization may be required before the exact clinical impact of TAMs can be determined in specific clinical contexts.

TUMOR-ASSOCIATED MACROPHAGES IN TUMOR INITIATION

It has been well noted that inflammatory conditions are positively correlated with carcinogenesis (95, 122). Since macrophages are one of the major participants in regulating the inflammation network, its role in tumor initiation has been widely reported. Cytokines IL-23 and IL-17 derived from $CD11b^+$ $F4/80^+$ are responsible for colorectal cancer initiation and growth (123). Selective ablation of IL-6 in monocytes and Kupffer cells resulted in inhibition of STAT3 signaling and delayed the tumorigenesis in a Mdr2-defecient spontaneous hepatocellular carcinoma model (68). Depletion of Stat3 in CSF1R expressing cells in mice resulted in drastic inflammatory response of the intestine and malignant tumor formation (69). These studies indicate that TAMs play an essential role in tumor initiation.

TUMOR-ASSOCIATED MACROPHAGES IN ANGIOGENESIS

Angiogenesis is crucial to maintain the fast growth of a tumor, especially after it reaches a certain size. Among many supporting factors contributing to angiogenesis in tumors, macrophages play an indispensable role. TAMs produce epidermal growth factor (EGF), fibroblast growth factor (FGF) (124), VEGF (125), transforming growth factor-α and -β (126, 127), Il-1β (128), IL-6, IL-8 (129), platelet-activating factor (130), platelet-derived growth factor (PDGF), thrombospondin-1 (131), MMPs, and other molecules that promote and stabilize the intratumoral blood vessels formation (114). The number of infiltrated macrophages correlates with the vessel density in invasive breast carcinoma (70). Overexpression of CSF-1 and its receptor correlates with poor prognosis in human breast carcinoma (132). CSF-1 was also found to direct macrophage recruitment before malignant initiation and produce VEGF to promote angiogenesis (133). Ablation of VEGFA in myeloid cells could inhibit the angiogenic switch (71). Macrophages can be recruited to hypoxic region of tumor by CCL-2, where upregulated HIF1α/HIF2α orchestrates the transcription of many angiogenesis related genes including VEGF, CXCR4, CCL2, and endothelins which reciprocally enhanced the recruitment of macrophage (72, 134). Genetic deletion of REDD1 under hypoxia can enhance glycolysis in TAMs, which raises the competition of glucose between TAMs and endothelial cells. This prevents the formation of an abnormal vascular network and reduces metastasis (73). Besides producing VEGF, macrophage can also free VEGF by degrading extracellular compartments through MMP9 expressed. Targeting MMP9 of tumor infiltrating macrophages by a bisphosphonate, zoledronic acid, inhibited the angiogenesis in a cervical carcinoma model (74). $Tie2^+$ macrophage is one well-characterized subset in primary tumor stroma that regulates the angiogenic switch (135). Forget et al. showed that CSF-1 could increase the $Tie2^+$ expressing macrophages and angiogenesis in PyMT mammary tumor bearing mice. They also uncovered that $Tie2^+$ expressing macrophages could also augment chemotactic response to endothelial cells expressed angiopoietin-2 (75).

TAMS IN METASTASIS

Tumor associated macrophages can direct tumor migration and invasion through regulating genes related to metastasis. $CD11b^+$/$Gr1^{mid/low}$ tumor infiltrating monocytes/macrophages can induce

the expression of S100A8 and S100A9 in MC38 and Lewis lung carcinoma cells. Ablation of their expression significantly diminished the migration and invasion *in vitro* culture as well as reduced liver metastasis and invasion to adjacent tissues without affecting the subcutaneous tumor growth (76). Sometimes the regulation is not uni-direction but rather a paracrine loop. CSF-1 synthesized by tumor cells and EGF derived from macrophages paracrine loop in MMTV-PyMT model were reported by Wyckoff et al. to cause tumor cells to migrate into surrounding connective tissue. The migration effect of both cell types was abrogated by blocking either CSF-1 or EGF signaling (77). $CD163^+$ TAMs derived IL-6 regulated EMT to enhance CRC cells migration and invasion. IL-6 activated JAK2/STAT3 pathway to upregulate FoxQ1 expression, which in turn increased the production of CCL2 to promote macrophage recruitment. This reciprocal loop can be blocked by inhibition of CCL2 or IL6 with reduced macrophage migration and metastasis of CTC (78). TAMs in breast patient samples were activated to an M2-like phenotype. DeNardo et al. reported that $CD4^+$ T lymphocytes skew the phenotype and effector function of $CD11b^+$ $Gr1^-$ $F4/80^+$ tumor associated macrophages to promote the invasion and metastasis in MMTV-PyMT mammary carcinoma model by stimulating the EGF signaling (79). The TAMs secreted CCL18 to promote mesenchymal breast cancer cells invasion and migration through their receptor PITPNM3 mediated extracellular matrix adherence (80). Another way TAMs promote tumor migration and invasion is through secreting exosomes, which promotes metastasis related signaling (81). Lan et al. showed that miR-21-5p and miR-155-5p encapsulated in the exosomes derived from M2 macrophages downregulate the expression of BRG1 by binding to its coding sequence to enhance the migration invasion and lung metastasis of colorectal cancer (82).

Although the underlying mechanisms of intravasation are still poorly understood, TAMs were reported to participate in this key step of metastasis. Direct contact enabled macrophages to induce invadopodium formation of breast cancer cells through activating RhoA signaling. This invadopodium facilitated transendothelial migration of MDA-MB-231 cells and patient derived triple negative breast cancer cells TN1 *in vitro* (83). Using intravital real-time imaging, macrophage-mediated vascular permeability and the dissemination of tumor cells into the blood stream was visualized *in vivo*. This permeability and intravasation, was transient and localized where macrophages were present, and was regulated by VEGFA signaling from $Tie2^+$ macrophages (84).

Having escaped from the primary site, disseminated tumor cells must survive harsh conditions when infiltrating to and colonizing distant organs. Macrophages are a vital player in preparing the metastasis soils, aiding extravasation, maintaining survival, and stimulating growth of the disseminated tumor cells (86, 136, 137). Kaplan et al. discovered that VEGFR1 expressing bone marrow-derived hematopoietic progenitor cells home to pre-metastatic sites before arrival of disseminated tumor cells through the interaction of VLA-4 and its ligand fibronectin in the resident fibroblasts. Blockade of VEGFR1 or depletion of VEGFR1+ cells from bone marrow could abrogate the formation of pre-metastatic niche and prevent metastasis of Lewis Lung carcinoma (136). $CYP4A^+$ TAMs infiltration was positively correlated with formation and metastasis. Inhibition of CYP4A showed decreased $VEGFR1^+$ myeloid cell recruitment and pro-metastatic protein expression in lung pre-metastatic niche, accompanied by skewing from M2 to M1 polarization in the 4T1 spontaneous metastasis breast cancer model and the B16F10 melanoma model (138). Deletion of S1P receptor 1 (S1pr1) in $CD11b^+$ $CD206^+$ TAMs reduced the NLRP3 expression and IL-1b production, and thus prevented pulmonary metastasis and tumor lymphangiogenesis in breast tumors (139). Qian et al. showed that tumor cells in contact with macrophages had a higher rate of extravasation. Depletion of macrophages using L-clodronate significantly reduce the extravasation of tumor cells (85). Gr1+ monocyte-derived VEGF promoted the extravasation of breast tumor cells. These monocytes also recruited to pulmonary metastases driven by CCL2 to promote the seeding of PyMT breast cancer cells (86). Kitamura et al. found that CCL2-CCR2 signaling promoted the secretion of CCL3 from metastasis associated macrophages (MAM), which increased the retention of MAM to promote lung metastasis in breast tumor models (87). Su et al. elucidated that mesenchymal breast cancer cells activated macrophages in the vicinity to skew towards a TAM-like phenotype through GM-CSF. The activated TAMs secreted CCL18 could reciprocally induce cancer cell EMT both *in vitro* and *in vivo*. Blockade of either GM-CSF or CCL18 can break this positive feedback loop, and thus reduced metastasis (88). Another study featuring the antitumor effect of TAMs revealed in a mouse model of spontaneous melanoma expressing human RET oncogene that reactive oxygen species was an essential mechanism underlying the tumor proliferation inhibition of $CD11b^+$ $Ly6C^+$ monocytes. Regulatory $CD4^+$ T cell derived IL-10 facilitated tumor progression through inhibiting the recruitment or differentiation of inflammatory monocytes in skin (89). Taken together, numerous lines of evidence support the pivotal roles of TAMs in metastasis, and the underlying molecular mechanisms appear to be diverse and complicated. Therefore, it will be crucial to identify targetable molecules that are key in each specific biological context.

CLINICAL RELEVANCE OF TAMS AND TANS

Multiple strategies are being pursued to target TAMs. One category of clinical trials is to target the CCR2-CCL2 axis, the major chemokine axis responsible for monocyte recruitment. Several clinical trials targeted CCL2 transiently (NCT00992186, NCT01204996, NCT00537368) with Carlumab, and showed acceptable tolerance and preliminary antitumor response in some solid tumors. In combination with chemotherapeutic agent Folfirinox, a CCR2 inhibitor PF-04136309 exhibited benefit in patients with pancreatic cancer (NCT01413022). Depletion of macrophages is another strategy used by many clinical trials. The colony stimulation factor CSF1R signaling is important in regulating macrophage proliferation and survival as

well as macrophage recruitment and polarization. Various CSF1R inhibitors were developed and used alone or in combination with other agents for different type of cancers (140). The caveat of macrophage depletion is toxicity, especially for liver cells (141), which highlights the need for more precise targeting of TAMs instead of normal macrophages. Since many studies showed that TAMs resemble the alternative activated M2 phenotypes that favor tumor progression, another strategy is to reprogram M2 to pro-inflammatory M1 macrophage. For instance, CD40 monoclonal antibody was reported to increase the pro-inflammatory factors (M1-promoting) and regulate innate and adaptive immune response (142). As a human immunoglobulin (IgG2) anti-CD40 monoclonal antibody, CP-870893 can specifically target the non-ligand binding site of CD40 and enhance the secretion of IL-12, IL-23, and IL-8. In combination with gemcitabine, CD40 was associated with antitumor activity in PDA patients (143). SEA-CD40 is an agonistic non-fucosylated humanized IgG1 CD40 antibody with enhanced FcγRIIIa binding. It showed superior effect over other CD40 antibodies. The phase I clinical trial in patients with relapsed or refractory metastatic solid tumors are ongoing (NCT02376699). Inhibition of PI3Kγ has been shown to induce proinflammatory gene expression in TAMs without affecting their accumulation in tumors. Suppression of tumors has been shown in some preclinical studies (144). In combination with nivolumab, the PI3Kγ inhibitor is undergoing Phase 1b clinical trial for solid tumors (NCT02637531) with the repolarization of macrophages will be assessed. Ibrutinib, with its inhibition on BTK downstream of PI3Kγ, can induce proinflammatory polarization of macrophages as well as $CD8^+$ T cells infiltration. It is in clinical trials in combination with several chemotherapeutic agents to treat pancreatic adenocarcinoma relapsed or refractory solid tumors (NCT02599324, NCT02436668, NCT02303271). Because TLRs polarize macrophages towards more proinflammatory phenotype, their agonists can be used to induce immune response against tumors. Several TLR agonists (TLR4, 7/8, 9) are in clinical trials in combination with different immune checkpoint blockade (140). Another unneglectable strategy is to unleash the phagocytosis of macrophages that was compromised in tumors. CD47 is a receptor for thrombospondin on human myeloid and endothelial cells. It protects the host cells from destruction by macrophages through binding to SIRP1a on macrophages. Targeting CD47 by antibody or other agents can stimulate phagocytosis of tumor cells in many mice models. Hu5F9-G4, a human monoclonal antibody that targets CD47 is under clinical trial against solid tumors (NCT02216409, NCT02953782). Another new agent TTI-621, a SIRPa-Fc fusion protein, is being tested for solid tumors in Phase I clinical trials (NCT02890368).

Despite the increasing recognition of importance of TANs, clinical trials that specifically focus on neutrophils are only in their fetal stage. Several drugs currently tested may have potential impact on TANs. For instance, some neutrophil elastase inhibitors, PDE5 inhibitors and COX2 inhibitors, were reported to inhibit the pro-tumor activity of neutrophils (NCT01170845, NCT02544880, NCT00752115). In addition, TGF-β was reported to skew neutrophils to a more protumor phenotype (20), and TGF-β inhibitors may stimulate neutrophil to antitumor phenotype (13). Other drugs are being tested to reduce TAN recruitment or induce TAN apoptosis. Several chemotaxis inhibitors, such as those targeting CXCR2 and CCR5, are under investigation to hinder the recruitment of neutrophils to the TME (NCT02370238, NCT02001974, NCT03274804, NCT01736813). Trail receptor expressed by neutrophils can be agonized to induce their apoptosis (NCT01088347, NCT00508625, NCT00092924). CD47-SIRPα inhibitors and CD40 monoclonal antibody that regulate TAMs could also limit the migration of neutrophils to tumor or deplete neutrophils (NCT02216409, NCT03717103, NCT02367196, NCT01103635). The clinical outcome of these above agents will provide invaluable insights into the roles of TANs in human tumors.

THE RELATIONSHIP BETWEEN TANS, TAMS, AND MYELOID-DERIVED SUPPRESSOR CELLS (MDSCS)

By definition, MDSCs are immunosuppressive and can blunt T cell cytotoxicity to create a favorable microenvironment for tumor growth. Blocking the immunosuppression of MDSCs will benefit antitumor response and improve the efficacy of the immunotherapies. Two different subgroups of MDSCs were identified in both mice and human: polymorphonuclear MDSCs (PMN-MDSCs) and monocyte MDSCs (M-MDSCs). The PMN-MDSCs resemble neutrophils in morphology and phenotypes and are defined as $CD11b^+$ $Ly6G^+$ $Ly6C^{low}$ in mice and $CD11b^+$ or $CD3^+$, $CD15^+$ or $CD66b^+$, and $CD14^-$ in human. The M-MDSCs resemble monocytes and are identified as $CD11b^+Ly6G^-Ly6C^{high}$ in mice and $CD11b^+$ or $CD33^+$, $CD14^+$, and HLA-DR^{low} in human (145). They use different mechanisms for immunosuppression with M-MDSCs more potent than PMN-MDSCs per cell but PMN-MDSCs typically outnumbering M-MDSCs. The major immunosuppressive molecules involved in their activities are ARG1, NO, ROS, prostaglandin E2, which are similar to those used by M2 macrophages or N2 neutrophils to promote tumor progression (145, 146). Thus, the major question is if and how MDSCs differ from TANs and TAMs.

While TAMs and TANs usually refer to macrophages and neutrophils infiltrating tumors, MDSCs are systemically accumulated in tumor-bearing hosts. They are derived from the bone marrow under the remote influence of tumors, and can be found in peripheral blood and spleen, in addition to the tumor microenvironment.

PMN-MDSCs are recognized using the same set of markers for neutrophils both in mice and human, although in some circumstances PMN-MDSCs can express unique markers distinct from normal neutrophils (147). As TANs are a heterogenous population that may have anti-tumor or pro-tumor functions, PMN-MDSCs are more likely the pro-tumor subset of TANs (145). It is worth noting that PMN-MDSCs and

neutrophils can be distinguished in human peripheral blood since the former are enriched in low density Ficoll gradient fraction while the latter are in the high density fraction (145, 148).

The markers used to identify M-MDSCs in mice are different with TAMs in that M-MDSCs has high expression of Ly6C while TAMs are recognized as high expression of F4/80, intermedium to low expression of Ly6C, and undetectable expression of S100A9. Unlike normal monocytes, M-MDSCs do not express or have low expression of HLA-DR (149).

MDSCs also exhibited considerable plasticity in TME. M-MDSCs had the potential to differentiate into PMN-MDSCs as reported by Youn et al, where the pathway for monocyte differentiation was dysregulated to preferentially generate G-MDSCs (150). MDSCs can also generate M2 TAMs and N2 TANs. Kuma et al. reported that STAT3 regulated the differentiation of MDSCs into immunosuppressive TAMs in hypoxic conditions (151). TGF-β secreted by MDSCs and other tumor stromal cells can deviate neutrophils into N2 TANs, which in turn recruit Treg cells through CCL17 secretion (20). The plasticity of MDSCs is also reflected by their ability to trans-differentiate into myeloid cells in different lineages. In a study by Corzo et al, MDSCs from spleen could differentiate into both macrophages and dendritic cells (DCs) while MDSCs from tumor only differentiated into macrophages. MDSCs from these two sites also differed in their T cells suppression ability. The spleen MDSCs suppressed only $CD8^+$ T cells while the tumor MDSCs suppress both antigen specific and antigen non-specific T cells (152). Thus, increased plasticity and potency for differentiation may be a general feature of MDSCs as compared to TAMs and TANs.

It is still premature to draw a concrete conclusion on the relationship between MDSCs and TANs and TAMs. However, profiling these cells at genomic and proteomic levels will facilitate solving the myth of MDSCs (153–155). Clear description of the context and markers used to study these populations is the best practice for current researches in the field of oncoimmunology (148, 156).

INTERACTIONS BETWEEN TANS, TAMS, AND TUMOR-INFILTRATING LYMPHOCYTES

TAMs and TANs extensively interact with tumor infiltrating lymphocytes, and have pleotropic effects. Several examples are provided below with a common theme that both TAMs and TANs use multiple overlapping pathways to crosstalk with T cells, including engagement of immune checkpoints and secretion of cytokines.

ROS and arginase I released by TANs inhibited T cell activation and proliferation (7, 20, 157). Arginase I produced by TANs blunt T cell response in human renal cancer carcinoma and non-small cell lung cancer (158, 159). TANs also induced apoptosis of non-activated $CD8^+$ T cells through NO and TNF-α (160). Immune checkpoints can be activated on T cells by their ligands expressed on TANs. High level of PD-L1 was expressed on TANs in patients with gastric cancer induced by tumor secreted G-CSF. These activated PD-$L1^+$ neutrophils suppressed the T cells function *in vitro* and is correlated with disease progression and patient mortality (161). PD-$L1^+$ neutrophils were also found in peritumor site of patient samples of hepatocellular carcinoma and was associated with poor disease free patient survival (162). Other than the immunosuppressive effect, Ponzetta et al. reported that neutrophils drove the polarization of a subset of unconventional $CD4^-$ $CD8^-$ αβ T cells in a IFN-γ dependent way to resist 3-methycholantrene induced murine sarcomas in mice (163).

TAMs exert immunosuppressive effect through several mechanisms (8). Arginase I and iNOS expression by TAMs partially regulated their T cell suppressive activity (164). Genetic depletion or pharmaceutical inhibition of TAMs and CSF-1 restored the cytotoxic $CD8^+$ T cell functions with tumor regression in mouse mammary and cervical models (165). Similar to TANs, T cell immune checkpoint ligands were also found to be expressed on TAMs. Circulating monocytes and TAMs in patients with glioblastoma expressed increased level of B7-H1. *Ex vivo* stimulation monocytes with conditional medium resulted in increased production of IL-10 which upregulated B7-H1 expression. These stimulated monocytes induced T cells apoptosis in co-culture (166). Tumor associated macrophages were found to be a primary source for PD-L1 in mouse and human cholangiocarcinoma, where inhibition TAMs and G-MDSCs improved immune checkpoint blockade efficacy (167). Regulatory T cells were also used by TAMs to suppress T cell immunity. Natural regulatory cells (nTreg) were recruited by TAMs to suppress the effector function of $CD4^+$ and $CD8^+$ T cells (168, 169).

INTERACTIONS BETWEEN TAMS AND TANS

Arising from a common progenitor lineage, the multifaceted roles of TANs and TAMs are implicated in almost every steps of tumor growth and metastasis. However, there are still few studies on the interactions between TANs and TAMs in the tumor microenvironment settings (170, 171). Recently, emerging studies began to integrate both populations to gain a better understanding of their interactions in the varying tumor microenvironment. Kumar et al. demonstrated in a series of mouse tumor models a significant increase of infiltrated PMN-MDSCs ($CD11b^+$ $Ly6C^{lo}$ $Ly6G^+$) in their attempt to deplete TAMs by pharmaco-inhibition or antibody neutralization of CSF1R. The infiltrated PMN-MDSCs recruited by carcinoma associated fibroblasts failed the expected therapeutic effect of CSF1R inhibition (172). Concomitantly, Janiszewska et al. found that minor subclones of breast tumor cooperated to drive breast tumor metastasis through inducing local and systematic stimulation of pro-metastatic neutrophils ($CD11b^+$ $Ly6C^{lo}$ $Ly6G^+$). Neutrophils were significantly higher in blood, primary tumors and lungs induced by an IL11-expressing minor subclone of MDA-MB-468. Although the

percentage of macrophages in primary tumors was not shown, it did decrease in blood and lungs (173). Using a panel of eight mouse triple negative breast cancer models, our recently published paper revealed that tumors did not recruit TANs or TAMs equally. Even as the same subtype of breast cancer, they could still be immuno-subtyped into neutrophil-enriched subtype (NES, $CD11b^+$ $Ly6C^{mid}$ $Ly6G^+$) or macrophage-enriched subtype (MES, $CD11b^+$ $Ly6G^-$ $Ly6C^-$ $F4/80^+$) according to their preference to recruit TANs and TAMs. A mutual exclusion between TANs and TAMs was observed. When one was depleted the other would be up-regulated (174). The mechanism underlying this mutual exclusion awaits further investigation. Yet, it shed light on a possible co-evolution between tumor associated myeloid cells and tumors.

BREAST CANCER AND ITS MICROENVIRONMENT

Breast cancer is the most common malignancy among women. Breast cancer is heterogeneous with distinct molecular and histological features that can be ascribed into luminal A-like (ER^+, PR^+, $HER2^-$), luminal B-like $HER2^-$, luminal B-like $HER2^+$, HER2-enriched (non-luminal) and triple-negative (ER-, PR-, HER2-) in current clinical practice (175). The heterogeneity of the subtypes influences treatment decision as well as the therapeutic outcomes. For instance, six detailed subclasses with distinct sensitivity to therapeutic drugs have been characterized (176). The heterogeneity exists not only across full spectrum of breast tumors as inter-tumoral but also between different regions of the tumor. Plus, molecular signatures evolve along the pressure from the microenvironment during progression as well as from the therapeutic intervention (177, 178).

Stromal cells, on the other hand, also bare heterogeneity between different tumors or within the same tumor. Tumor intrinsic signaling is one of the major factors that determines the heterogeneity of microenvironment. Studies showed that inflammatory response is downstream intrinsic oncogenic pathways (179, 180). And local production of chemokines and cytokines from cancer cells regulate the tumor infiltrating immune constituents of the microenvironment. A dichotomy of immune microenvironment was reported in different lung cancer subtypes. Macrophages are predominantly present in Kras adenocarcinoma models while neutrophils were mainly recruited to the squamous cell carcinoma region by Lkb1 and Pten inactivation but not the adjacent adenocarcinoma region (181). Our group discovered that mTOR signaling in cancer cells determines the MDSC accumulation through regulating G-CSF production. This MDSC accumulation preferentially occur in tumor models exhibiting elevated mTOR activities (182). More recently, we further demonstrated a dichotomous myeloid cell profiles across eight murine triple negative breast cancers; some of the tumors are enriched with TAMs with few TANs and some others are enriched with TANs with a minority of TAMs. We named these tumors as macrophage-enriched and neutrophil-enriched subtypes (MES and NES) respectively. This dichotomy may be driven by two forces: 1) the intrinsic properties of cancer cells, such as the mTOR activities and EMT (changing the EMT status of the tumor cells could alter the type of myeloid being recruited as shown in our work), and 2) the mutually negative impact between TAMs and TANs. Interestingly, when MES tumors that are initially sensitive to therapies acquire resistance, a shift toward NES was observed, indicating the plasticity of myeloid compartment during therapeutic interventions (174).

CONCLUDING REMARKS AND OPEN QUESTIONS

The progression of tumorigenesis and metastasis resembles the evolution of ecosystems. On one hand, tumor cells are under constant selective pressure skewing towards increased survival and proliferation (183, 184). On the other hand, tumors continuously reprogram TME systematic environment to create an abnormal ecosystem. Extensive molecular evolution of tumor-associated stroma during cancer progression has been shown by gene expression analysis (185, 186). A possible co-evolution pattern of TANs, TAMs and tumors is shown in **Figure 1**.

TANs and TAMs participate in many steps of tumor progression and metastasis. As a major part of the innate immune system, they have drawn tremendous interest to their roles in almost every step of tumor progression and metastasis. Despite this knowledge, several questions remain outstanding.

First, do the frequencies and functional roles of TAMs and TANs vary across individual tumors? As discussed in previous sections, both TAMs and TANs can play opposite roles in different contexts. Variable polarization status may create a continuous spectrum between anti- and pro-tumor functionalities. The exact positioning of TAMs and TANs in this spectrum will likely be influenced by cancer cells and other immune cells. In our previous studies, mTOR and EMT pathways were found to contribute to enrichment of TANs and TAMs in different models, respectively. Moreover, depletion of TANs led to increase of monocyte infiltration whereas depletion of TAMs resulted in influx of TANs. Thus, the frequencies of these myeloid cells in a particular tumor are jointly determined by tumor-intrinsic factors and their mutual (negative) impacts. In terms of functions, genetic depletion of macrophages from different MES models had opposite or highly distinct effects on tumor growth and therapeutic responses to checkpoint blockade therapies. Thus, additional factors seem to dictate TAM polarization independent of recruitment. In general, models or biological contexts have not been sufficiently considered as an important variable in understanding the roles of TAMs and TANs, which severely prevent the integration of our knowledge.

Second, how does intertumoral TAM and TAN variations correlate with known subtypes of tumors? A general classification of "hot" versus "cold" tumors has been used to describe tumors with or without immune cell infiltration (especially T cells). However, not all hot tumors are similar –

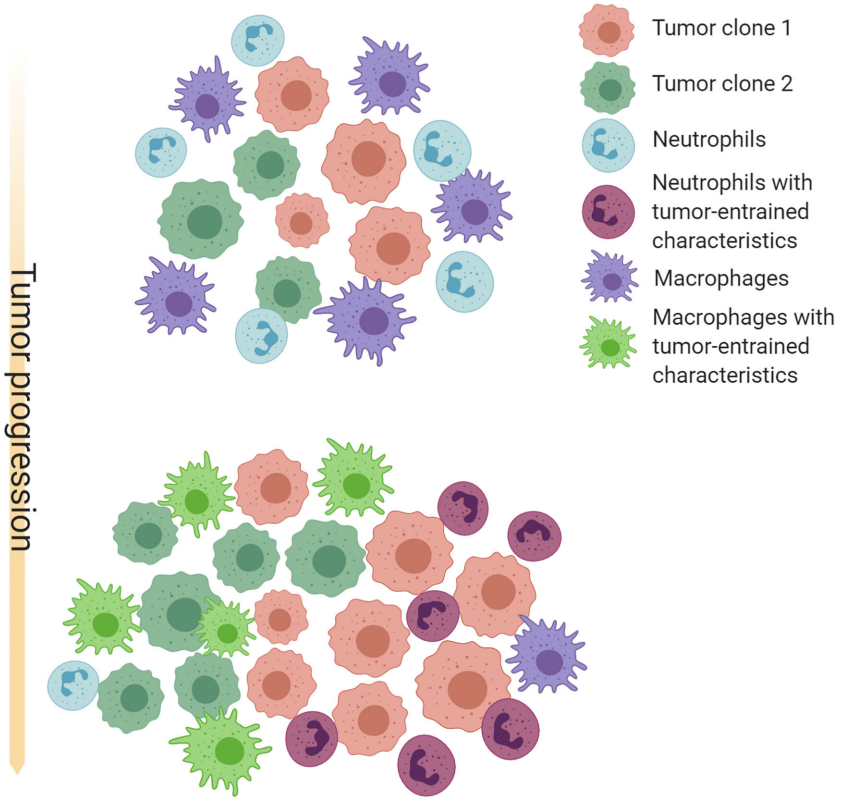

FIGURE 1 | Schematic illustration of possible co-evolution among tumor cells, TANs and TAMs. Cancer cells with different genetic or epigenetic traits may selectively recruit neutrophils or macrophages, and provide an initial milieu to influence differentiation, polarization, and survival of these myeloid cells. TAMs and TANs in turn confer selective benefit to some clones by paracrine and direct cell-cell interactions. TAMs and TANs may also compensate each other and compete for the same microenvironment niches. These interactions may often result in a feed-forward loop that favor an equilibrium of TAM-enriched or TAN-enriched microenvironment, as well as specific cancer cell-intrinsic characteristics. Thus, co-evolution with TAMs and TANs may be an important force driving intra- and inter-tumor heterogeneity. Created with Biorender.com.

the exact composition of the immune TME including TAMs and TANs should be considered independent of lymphocytes, as they may use totally distinct immunosuppressive mechanisms. Triple negative breast cancers have been shown to be heterogeneous and can be further divided into 4–6 different subtypes based on cancer-intrinsic gene expression (176, 187). However, the characteristics of some subtypes are clearly related to activation and suppression of immune system. Furthermore, the correlation between EMT and macrophages has been uncovered in a number of studies (88, 174, 188, 189), indicating a link between metaplastic histology or "claudin-low" phenotype (190) and macrophage-enriched TME. Taken together, these lines of evidence support correlations between tumor-intrinsic heterogeneity and TME heterogeneity.

Finally, how do tumor cells and immune cells co-evolve as an integrated ecosystem? The concept of immunoediting has greatly facilitated our understanding of interactions between tumor cells and the immune system (191). The selective pressure exerted by anti-tumor immunity impacts clonal evolution and ultimately leads to escape of immunosurveillance. Moreover, cancer cells also gain additional selective advantages by turning immune cells into conspirators in tumor progression (137). Thus, the crosstalk between cancer and immune cells is bidirectional, forming the foundation of co-evolution. It should be noted that this co-evolution may take a distinctive path in each individual tumor, resulting in a unique ecosystem. TAM and TAN may together provide examples illustrating this process. For instance, mesenchymal-like tumor cells are more likely to recruit TAMs, which in turn reinforce mesenchymal properties. Both mesenchymal stem cells and TAMs may repel or compete against infiltration of TANs, thereby forming a macrophage-enriched TME (174). The mTOR pathway, on the other hand, stimulates systemic and local accumulation of neutrophils, which might outcompete macrophages and drive tumor evolution toward another direction (174, 182). More in-depth and mechanistic studies are required to test these hypotheses. Furthermore, the clinical implications also need to be explored to facilitate better immunotherapies.

In conclusion, the precise influence of TAMs, TANs and other immune cells on tumor progression and metastasis needs

to be collectively analyzed together with tumor-intrinsic properties to reveal molecular mechanisms underlying the coevolution in context-dependent manners.

AUTHOR CONTRIBUTIONS

LW and XZ conceived and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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