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Therapeutic targeting of MDSC in the tumor and immune microenvironment

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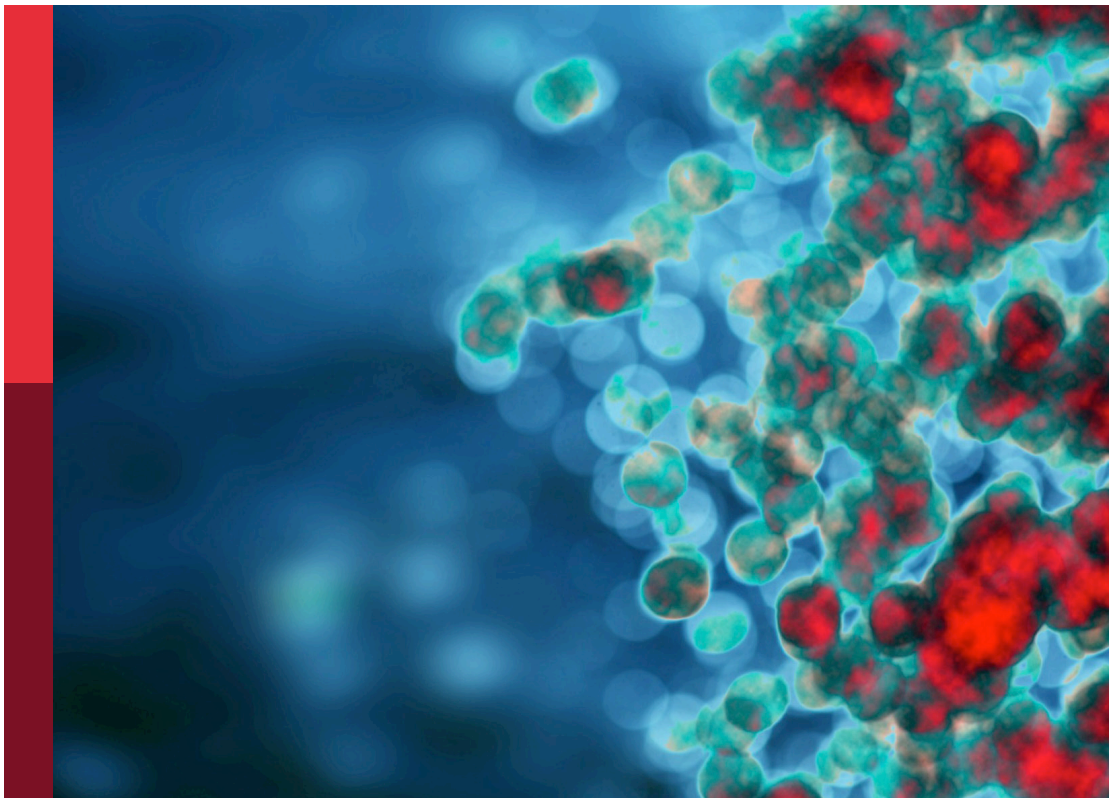

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Therapeutic targeting of MDSC in the tumor and immune microenvironment

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REVIEW
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Myeloid-Derived Suppressor Cells in Tumors: From Mechanisms to Antigen Specificity and Microenvironmental Regulation

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Among the various immunological and non-immunological tumor-promoting activities of myeloid-derived suppressor cells (MDSCs), their immunosuppressive capacity remains a key hallmark. Effort in the past decade has provided us with a clearer view of the suppressive nature of MDSCs. More suppressive pathways have been identified, and their recognized targets have been expanded from T cells and natural killer (NK) cells to other immune cells. These novel mechanisms and targets afford MDSCs versatility in suppressing both innate and adaptive immunity. On the other hand, a better understanding of the regulation of their development and function has been unveiled. This intricate regulatory network, consisting of tumor cells, stromal cells, soluble mediators, and hostile physical conditions, reveals bi-directional crosstalk between MDSCs and the tumor microenvironment. In this article, we will review available information on how MDSCs exert their immunosuppressive function and how they are regulated in the tumor milieu. As MDSCs are a well-established obstacle to anti-tumor immunity, new insights in the potential synergistic combination of MDSC-targeted therapy and immunotherapy will be discussed.

Keywords: myeloid-derived suppressor cells, immune suppression, tumor microenvironment, immunotherapy, endoplasmic reticulum stress

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INTRODUCTION

Myeloid cells are a group of highly diverse cells that are essential for the normal functioning of innate and adaptive immunity. Mononuclear myeloid cells include monocytes, macrophages, and dendritic cells (DCs), and granulocytic myeloid cells include neutrophils, eosinophils, basophils, and mast cells. In steady state, myelopoiesis is under tight control and remains predominantly quiescent. A wide range of pathological stimuli, such as infectious microorganisms, tissue damage, and malignantly transformed cells, induce emergency myelopoiesis that largely leads to robust expansion of activated monocytes and neutrophils to eliminate potential threats. If these conditions terminate in time, the homeostasis of myeloid cells will be restored, leaving no negative consequence to the host; conversely, the persistent presence of low-strength stimuli leads to the

accumulation of immature myeloid cells characterized by powerful immunosuppressive capacity, which may serve as a protective mechanism to prevent excessive tissue damage caused by unresolved immune response (1).

Studies since the 1970s have highlighted a group of systematically expanded and pathologically activated immature myeloid cells in tumor-bearing hosts. Based on their myeloid origin and immunosuppressive potency, these cells were termed myeloid-derived suppressor cells (MDSCs) in 2007 (2). In addition to cancer, MDSCs are implicated in other diseases, such as chronic inflammation or infection, autoimmune disorder, trauma, and graft-versus-host disease (2). MDSCs are a heterogeneous population consisting of myeloid progenitor cells and immature myeloid cells, characterized by the lack of surface markers associated with fully differentiated myeloid cells and by their morphological resemblance to granulocytic and monocytic cells (3).

MDSCs are generally divided into two main subsets: polymorphonuclear MDSCs (PMN-MDSCs, also known as granulocytic MDSCs) and monocytic MDSCs (M-MDSCs), which morphologically and phenotypically resemble neutrophils and monocytes, respectively. In tumor-bearing mice, MDSCs are generally defined as positive for myeloid lineage differentiation markers CD11b and Gr-1, with PMN-MDSCs being $Ly6G^{+}Ly6C^{low}$ and M-MDSCs being $Ly6G^{-}Ly6C^{high}$ (4). On the other hand, their counterparts in cancer patients are less definite, since studies on human MDSCs have been hampered by cellular diversity and a lack of unequivocal markers. Nonetheless, human PMN-MDSCs are now commonly defined as $CD11b^{+}CD14^{-}CD15^{+}$ or $CD11b^{+}CD14^{-}CD66b^{+}$ and M-MDSCs as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}CD15^{-}$ (4). Another population of immature MDSCs has recently been identified. These LIN^{-} (including CD3, CD14, CD15, CD19, and CD56) $HLA\text{-}DR^{-}CD33^{+}$ cells contain mixed groups of MDSCs comprising more immature progenitors and have been defined as "early-stage MDSCs (e-MDSCs)" (4). However, the murine equivalent of these e-MDSCs has not yet been defined.

Activated MDSCs actively participate in multiple aspects of tumor progression, including immune evasion, angiogenesis, pre-metastatic niche formation, and epithelial-mesenchymal transition (EMT) (5–7). Among these tumor-promoting activities, suppression of immune cells is the defining feature of MDSCs. Since the aforementioned surface markers are not exclusive to MDSCs and some are shared by other myeloid cells, phenotyping together with suppressive function assessment has been proven to be the optimal strategy for identifying bona fide MDSCs (4). Studies in the past decade have provided us with a clearer view of the immunosuppressive nature of MDSCs. In this work, we intend to thoroughly review the ever-expanding list of suppressive machineries and cell targets of MDSCs (**Figure 1**). The nature of MDSC-mediated immune suppression will be discussed in detail, highlighting the antigen specificity of suppression and the regulatory role of the tumor microenvironment.

SUPPRESSIVE MECHANISMS AND CELL TARGETS OF MDSCs

Nitric Oxide, Reactive Oxygen Species, and Peroxynitrite

It is well-established that MDSCs are capable of inhibiting T-cell function. MDSCs express a high level of inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO) (8–11). It is reported that NO suppresses T-cell proliferation, probably directly by inhibiting the Jak/STAT5 pathway or indirectly by inhibiting the antigen presentation from DCs (11, 12). Meanwhile, NO induces apoptosis of T cells (13). On the other hand, MDSCs produce a high amount of reactive oxygen species (ROS) via NADPH oxidase (NOX2) (8, 14). The inhibitory effect of ROS on T-cell function is well-described (15). For MDSCs, this suppression is caused by decreased expression of T-cell receptor (TCR) ζ-chain and is abrogated by inhibiting ROS production (14).

Studies have identified peroxynitrite (PNT), a potent oxidant produced by reaction between NO and superoxide anion ($O_2^{\cdot-}$), as a crucial effector molecule of MDSCs. Local production of PNT in the tumor microenvironment is responsible for the non-responsiveness of tumor-infiltrating cytotoxic T lymphocytes (CTLs), and consistently, these CTLs are associated with a high level of nitrotyrosine, a marker of PNT activity (16). PNT suppresses T cells by nitrating the TCR complex, leading to loss of response to specific antigen presented by MDSCs (see below) (17). In addition to the TCR complex, it has recently been shown that MDSCs inhibit T-cell activation by nitrating Tyr394 of lymphocyte-specific protein tyrosine kinase (LCK), an initiating tyrosine kinase in the TCR-mediated signaling cascade (18).

Interference With the Trafficking of T Cells

MDSCs impede the access of T cells to target sites by interfering with their trafficking (19). Expression of a disintegrin and metalloproteinase 17 (ADAM17), a major sheddase of L-selectin (CD62L), by MDSCs cleaves the ectodomain of L-selectin and consequently reduces L-selectin on the surface of naïve $CD4^{+}$ and $CD8^{+}$ T cells, therefore limiting their homing to peripheral lymph nodes and tumor sites (20). In another study, this MDSC-mediated decreased L-selectin level on T cells is regulated by high mobility group box protein 1 (HMGB1) in the tumor microenvironment (21).

Besides directly interfering with T-cell trafficking, MDSC-derived NO reduces E-selectin expression on endothelial cells, and PNT causes nitration and inactivation of CCL2 chemokine, both of which indirectly hamper the migration of T cells to the tumor site (22, 23).

Depletion of Amino Acids Necessary for T-Cell Response

MDSCs are able to deplete amino acids required for T-cell activation and proliferation. A high level of arginase 1 (ARG1) expression by MDSCs depletes L-arginine in the tumor

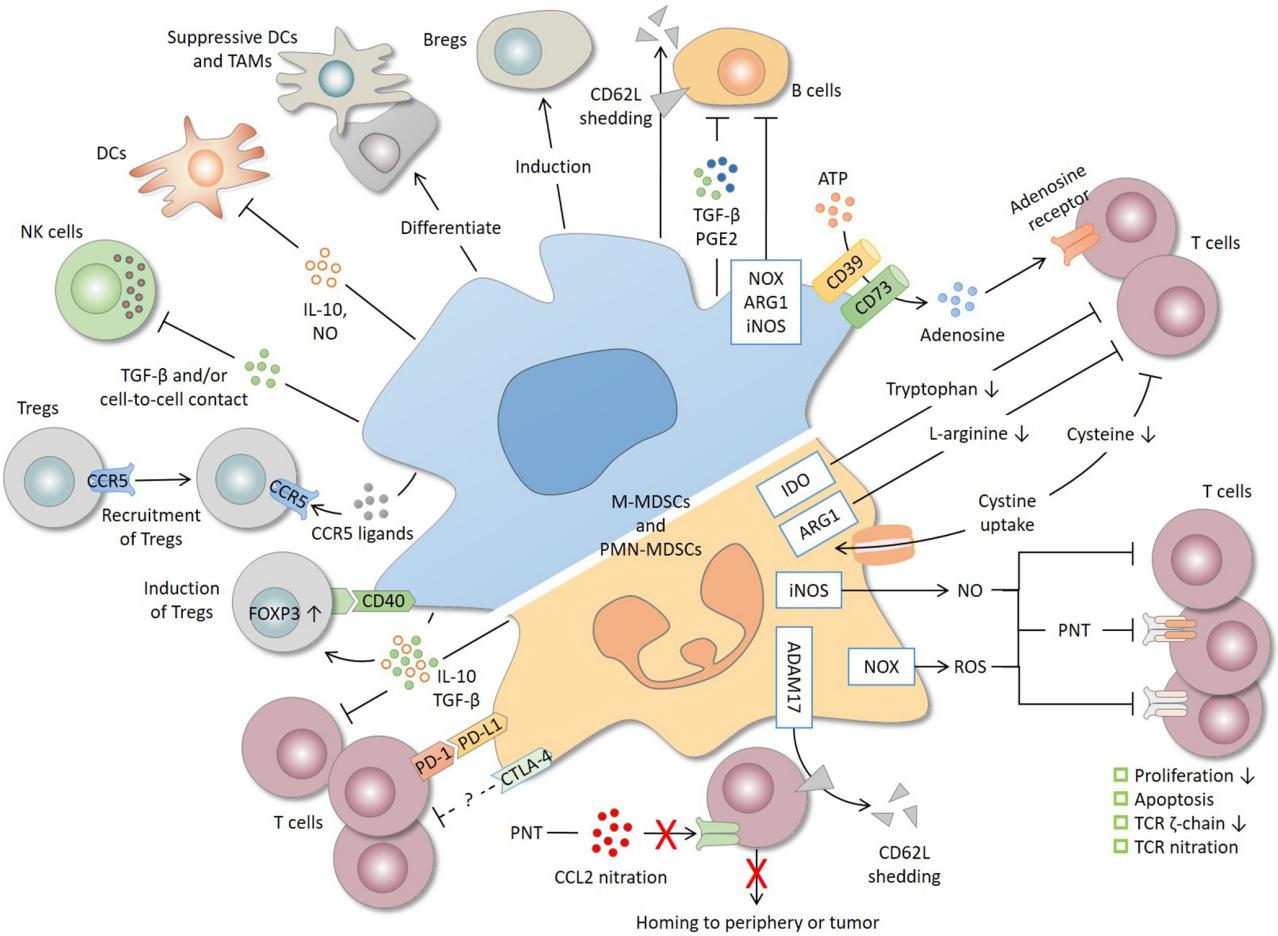

FIGURE 1 | Immunosuppressive mechanisms and cell targets of MDSCs. T cells are the primary target of MDSCs. MDSCs produce a high level of nitric oxide (NO), reactive oxygen species (ROS), and peroxynitrite (PNT), which suppress T cells by inhibiting proliferation, inducing apoptosis, decreasing the TCR ζ-chain and nitrating the TCR complex. MDSCs deplete amino acids essential for T-cell response. For instance, MDSCs decrease L-arginine and tryptophan level through arginase 1 (ARG1) and indoleamine 2, 3-dioxygenase (IDO), respectively, and reduce the cysteine availability through cystine uptake. CD39/CD73 expression by MDSCs produces adenosine that inhibits T cells through adenosine receptors. By shedding CD62L (L-selectin) off the T-cell surface or by nitrating CCL2, MDSCs interrupt T-cell trafficking to the periphery or tumor site. MDSCs express both programmed cell death-ligand 1 (PD-L1), which inhibits T cells through interaction with programmed cell death protein 1 (PD-1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), whose precise role remains unclear. IL-10 and TGF-β, two major immunosuppressive cytokines produced by MDSCs, are implicated in T-cell suppression and regulatory T cell (Treg) induction. MDSCs also induce Tregs through CD40 in a contact-dependent manner and recruit Tregs through the production of various chemokines. In addition to T cells, MDSCs suppress natural killer (NK) cells and dendritic cells (DCs), inhibit B cells, and induce regulatory B cells (Bregs). Lastly, tumor-infiltrating MDSCs, mostly M-MDSCs, may differentiate into suppressive DCs and tumor-associated macrophages (TAMs). ADAM17, a disintegrin and metalloproteinase 17; iNOS, inducible nitric oxide synthase; NOX2, NADPH oxidase 2.

microenvironment, leading to downregulation of the CD3 ζ-chain of the TCR complex and proliferative arrest of T cells (24).

On the other hand, MDSCs deprive T cells of cysteine, an essential amino acid for T-cell activation, by uptaking cystine and not exporting cysteine. Since T cells depend on exogenously generated cysteine, the decreased availability of cysteine in the tumor milieu results in impaired T-cell activation (25). Furthermore, it is also reported that indoleamine 2, 3-dioxygenase (IDO) expression is upregulated in MDSCs isolated from fresh breast cancer tissue and is responsible for MDSC-mediated inhibition on T-cell proliferation and Th1 polarization (26).

Adenosine and Adenosine Receptors

Recent studies have identified adenosine, a purine nucleoside, as a novel effector molecule of MDSCs. Extracellular ATP or ADP is hydrolyzed by CD39 (nucleoside triphosphate diphosphohydrolase) into AMP, which is in turn cleaved by CD73 (ecto-5'-nucleotidase) into adenosine (27). Both CD39 and CD73 are expressed by MDSCs from tumor-bearing mice and cancer patients, suggesting that MDSCs are capable of producing adenosine (28–30). TGF-β promotes the differentiation of MDSCs into $CD39^{+}CD73^{+}$ terminally differentiated myeloid cells with high adenosine production in tumor-bearing mice (31). Consistently, another recent study has demonstrated that tumor-derived TGF-β induces CD39/CD73 expression on MDSCs from

lung cancer patients through the mammalian target of rapamycin (mTOR)-hypoxia-inducible factor 1α (HIF-1α) pathway, and these $CD39^+CD73^+$ MDSCs represent a distinct subpopulation that expresses higher levels of HIF-1α, cyclooxygenase 2 (COX2), IL-10, tumor necrosis factor (TNF)-α, and TGF-β as compared to their counterparts (32).

It is well-studied that adenosine inhibits the activation and effector function of T cells, which signals primarily through A_{2A} and A_3 adenosine receptors (33). In the presence of CD73 substrate 5′-AMP, the inhibition of PMN-MDSCs on anti-CD3/CD28-induced T-cell proliferation is potentiated (28). On the contrary, $CD73^{-/-}$ MDSCs or MDSCs whose CD39 or CD73 enzymatic activity is inhibited show reduced capacity to suppress T cells and natural killer (NK) cells (30, 32, 34). Furthermore, it is reported that MDSCs promote chemoresistance through the activity of CD39 and CD73 (32). Metformin, a biguanide used for type 2 diabetes, reduces the expression and activity of CD39 and CD73 on MDSCs, which leads to reduced MDSC-mediated suppression of $CD8^+$ T cells *in vitro* and *in vivo*, and may partially account for the survival benefit seen in diabetic ovarian cancer patients treated with metformin (30).

The adenosine receptors expressed on MDSCs contribute indirectly to the adenosine-induced immune suppression. Stimulation of A_{2B} receptors preferentially expands PMN-MDSCs (28). In mice with melanoma, blockade of A_{2B} receptors reduces IL-10, monocyte chemoattractant protein 1 (MCP-1), and MDSCs in the tumor site, which is associated with increased frequency of intratumoral $CD8^+$ T cells, elevated levels of TNF-α and IFN-γ, and delayed tumor growth (35). In another murine melanoma model, selective deletion of A_{2A} receptors in myeloid cells leads to significantly reduced IL-10 production by MDSCs, an increase in activated $CD8^+$ T cells and NK cells, and delayed primary tumor growth and metastasis (36).

CD39 and CD73 are also expressed on tumor cells, regulatory T cells (Tregs), effector T cells, Th17 cells, and other stromal cells (33). Ectonucleotidases are supposed to prevent excessive T cell-mediated immune response and to regulate the balance between pro-inflammatory ATP and immunosuppressive adenosine. However, tumor hijacks this network to facilitate immune evasion. In line with the abovementioned findings, Umansky et al. have proposed two modes of adenosine signaling. Firstly, MDSCs, Tregs, and tumor cells may produce extracellular adenosine to suppress T-cell function in a paracrine manner. Secondly, adenosine produced by ectonucleotidase on tumor-infiltrating lymphocytes suppresses their own function in an autocrine manner; the upregulated CD39 and CD73 expression by MDSCs and Tregs also enables autocrine adenosine signaling and potentiates their expansion and/or suppressive activity (33).

MDSC-Derived IL-10

MDSCs are a major source of IL-10 in tumor-bearing host (37–40), and consistently, the frequency of MDSCs is correlated with the IL-10 level in peripheral blood of cancer patients (41). It is becoming clear that IL-10 serves as a non-redundant suppressive mechanism of MDSCs, and accordingly, blockade of IL-10 signaling or neutralization of IL-10 leads to alleviated T-cell suppression, delayed tumor progression, and improved therapeutic efficacy (37, 42). In addition to T-cell inhibition, MDSC-derived IL-10 is implicated in the induction of Tregs and the suppression of DCs (see below).

Recent studies are unraveling the regulation on IL-10 production by MDSCs, which involves cellular and non-cellular participants. For instance, hypoxia significantly upregulates IL-10 secreted by MDSCs (43). Exposure to lipopolysaccharide (LPS), a Toll-like receptor (TLR) ligand, increases IL-10 production by MDSCs, which may require the MyD88 signaling pathway (44). Transmembrane TNF-α (tmTNF-α), but not the secretory form, activates MDSCs to upregulate IL-10 and other immunosuppressive effector molecules through TNFR2 (45). The level of interferon regulatory factor 4 (IRF4), an essential transcription factor required for lymphoid and myeloid cell differentiation, reduces remarkably during the development of MDSCs and modulates the suppression of T cells through IL-10 and ROS production (46). Tumor cells, not surprisingly, participate in the MDSC-derived IL-10 regulation. For instance, knockdown of semaphorin 4D, a pro-angiogenic factor overexpressed in many malignancies, in tumor cells reduces the IL-10 production by MDSCs (47). Glioma stem cell-derived exosomes induce systemic T-cell suppression by polarizing $CD14^+$ monocytes toward M-MDSC phenotype with heightened IL-10 level (48). In another study, the NKG2D ligand RAE-1ε expressed on tumor cells facilitated the expansion and activation of MDSCs that display pronounced ARG1 activity and IL-10 production (49).

Similarly, MDSCs developed in the settings of microbial infection are also capable of producing IL-10 (50–52). In patients with chronic hepatitis B, IL-10 induced by programmed cell death protein 1 (PD-1) signaling is responsible for T-cell suppression by MDSCs (50). In patients with chronic hepatitis C virus infection, M-MDSCs have higher levels of phosphorylated STAT3 and IL-10, while blocking STAT3 signaling reduces hepatitis C virus (HCV)-mediated M-MDSC expansion and IL-10 expression (51).

TGF-β

TGF-β is another well-documented immunosuppressive cytokine secreted by MDSCs in tumor-bearing host (22, 43, 53). MDSCs developed in non-cancer settings are also capable of producing TGF-β (52, 54). Evidence for the regulation of MDSC-derived TGF-β remains elusive. It was shown previously that TGF-β produced by MDSCs is induced *in vivo* by IL-13 and CD1d-restricted T cells that are most likely natural killer T (NKT) cells (55). Recent studies have shown that TGF-β production by MDSCs is regulated by tmTNF-α, ribosomal protein S19, and semaphorin 4D (45, 47, 56). On the contrary, $CD14^+$HLA-$DR^{-/low}$ MDSCs from patients with liver cancer show no TGF-β secretion (57). These findings suggest that TGF-β production by MDSCs may be context-dependent.

MDSC-derived TGF-β contributes to T-cell suppression, although it is probably not the principal mechanism (53). $CD14^+$HLA-$DR^{-/low}$ MDSCs isolated from melanoma patients inhibit T cells via TGF-β with no involvement of ARG1 and iNOS (58). Song et al. have shown that transfer of tumor-derived MDSCs to asthmatic mice leads to reduced pulmonary

recruitment of inflammatory cells, suppressed Th2 response, and decreased IgE production in a TGF-β1-dependent manner (59). Furthermore, TGF-β is essential in Treg induction by MDSCs (see below).

Other immune cells are also inhibited by MDSC-derived TGF-β. For instance, in a murine model of AIDS, M-MDSCs suppressed B-cell response by superoxide, nitric oxide, PNT, and TGF-β (54). $CD14^+HLA\text{-}DR^{-/low}$ MDSCs from melanoma patients inhibit NK cells primarily through TGF-β that is stimulated by tumor-derived PGE2 (60). In addition to soluble TGF-β, MDSCs expanded in tumor-bearing mice express and utilize membrane-bound TGF-β to suppress NK cells and NKT cells in a contact-dependent manner (61, 62).

In addition to immune suppression, TGF-β has been implicated in the regulation of tumor metastasis facilitated by MDSCs. A portion of tumor cells undergoes EMT to disseminate, invade surrounding tissue, and metastasize. In a spontaneous murine model of melanoma, Toh and colleagues have shown for the first time that MDSCs use TGF-β, epidermal growth factor, and hepatocyte growth factor to induce EMT and that depletion of MDSCs results in reduced EMT and fewer metastases (63). In another study, anti-TGF-β treatment in a murine model of mammary tumor inhibited tumor growth and lung metastasis, and depletion of MDSCs diminished this beneficial effect of TGF-β neutralization (64). Another study from the same group later demonstrated that specific deletion of gene encoding TGF-β receptor II in myeloid cells significantly reduces metastasis, which is mediated by decreased TGF-β1 and type 2 cytokine production and by reduced ARG1 and iNOS expression. This effect was largely ascribed to the $CD11b^+Ly6G^+$ myeloid subset (65).

PD-L1 and CTLA-4 Expression by MDSCs

Immune checkpoint pathways act as negative regulators and prevent excessive immune response. MDSCs assist tumor to hijack this mechanism in order to promote T-cell anergy, which signals mostly through the PD-1/programmed cell death-ligand 1 (PD-L1) pathway (66). MDSCs express PD-L1 in various tumor models (43, 67–73). Meanwhile, numerous studies have found PD-L1 expression in MDSCs from cancer patients (29, 42, 53, 72, 74–76). In liver cancer patients, the percentage of $PD\text{-}L1^+$ MDSCs in peripheral blood correlates with disease stage and correlates inversely with clinical outcome (76). On the other hand, MDSCs developed during microbial infection also express PD-L1 (77, 78).

PD-L1 is implicated in MDSC-mediated T-cell suppression. PD-L1 blockade reduces the suppressive capacity of MDSCs on T cells (29, 42, 53, 68, 73, 74, 77–79). In addition to conventional T cells, in a murine model of liver metastasis, PD-L1 expression by MDSCs impairs the proliferation of chimeric antigen receptor cells, while MDSC depletion or PD-L1 blockade improves their therapeutic efficacy (80). Blocking PD-L1 relieves inhibition on DCs by MDSCs as well (81).

Several studies have shown that tumor-infiltrating MDSCs express a higher level of PD-L1 than their peripheral counterparts, suggesting microenvironmental regulation of PD-L1 expression (43, 68, 72, 73, 75). For instance, tumor cells upregulate the PD-L1 expression in MDSCs by interfering with their arachidonic acid metabolism (82). Tumor-derived soluble mediators are also responsible for PD-L1 induction in intratumoral MDSCs (76, 80). Other microenvironmental signals that regulate PD-L1 expression by MDSCs, such as hypoxia, cytokines, and stromal cells, will be discussed in detail in the following sections.

On the other hand, it is reported that MDSCs express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (43, 71). However, unlike PD-L1, the precise role and regulation of CTLA-4 is less well-studied in MDSCs. It is reported that blocking or silencing CTLA-4 reduces the frequency and ARG1 activity of MDSCs (83).

Induction and Recruitment of Regulatory T Cells

MDSCs inhibit effector T cells not only by themselves but also by inducing and recruiting Tregs. The proliferation of Tregs is relatively insensitive to suppression by MDSCs as compared with effector T cells (84). Intratumoral accumulation of Tregs occurs later than that of MDSCs, while depletion of MDSCs reduces infiltrating Tregs, suggesting that MDSCs may facilitate the development of Tregs (85). In non-cancer settings, co-culturing $CD4^+$ T cells with MDSCs from HIV^+ individuals or chronic hepatitis C patients significantly increases the differentiation of $Foxp3^+$ Tregs (51, 86).

The mechanism(s) for Treg induction by MDSCs is not fully understood. During tumor progression, a subset of DCs with an immature myeloid phenotype is licensed by tumor cells to promote proliferation of Tregs by producing TGF-β (87). Huang and colleagues have shown that MDSCs induce Tregs both *in vitro* and *in vivo*, which requires activation of T cells and is dependent on IFN-γ and IL-10. The authors speculated that, in response to IFN-γ produced by activated T cells, MDSCs secret TGF-β and IL-10, both of which participate in the development of Tregs (88). Another study from this same group later demonstrated that CD40 expression on MDSCs is required for Treg induction, since adoptive transfer of CD40-deficient MDSCs or administration of anti-CD40 antibodies fails to induce Tregs (89). Treg induction by MDSCs is attenuated in the Transwell system that separates the two cell types, suggesting the requirement of direct cell-to-cell contact (90). In a murine model of B-cell lymphoma, MDSCs promoted the expansion of Tregs from pre-existing natural Tregs but not conversion from naïve T cells. In that study, MDSCs induced tumor-specific Tregs via antigen uptake, processing, and presentation, which requires ARG1 but not TGF-β (91).

In addition, MDSCs may promote the recruitment of Tregs to the tumor milieu. Tumor-infiltrating M-MDSCs produce CCR5 ligands CCL3, CCL4, and CCL5, and meanwhile, Tregs exhibit high surface expression of CCR5 and are recruited to tumor tissue by CCL4 and CCL5. Accordingly, Tregs from CCR5 knockout mice almost completely lost their ability to migrate toward M-MDSCs *in vitro* (92). In a murine model of glioblastoma multiforme, both M-MDSCs and Tregs were recruited by CCL2 produced by tumor-associated macrophages

(TAMs) and microglia (93). A recent study revealed a closed loop between mast cells, MDSCs, and Tregs in the tumor microenvironment. Mast cells induce infiltration of MDSCs to tumor and induce their IL-17 secretion; MDSC-derived IL-17 attracts Tregs indirectly and potentiates their suppressive activity and IL-9 production; IL-9 in turn promotes the survival and tumor-promoting function of mast cells. In that study, IL-17 promoted Treg recruitment by increasing the level of CCL17 and CCL22 in the tumor microenvironment (94).

Studies on the relation between MDSCs and Tregs in cancer patients are relatively limited. A positive correlation between MDSCs and Tregs in peripheral blood and tumor site has been detected in cancer patients (40, 95). Hoechst and colleagues have shown that $CD14^+HLA\text{-}DR^{-/low}$ M-MDSCs from hepatocellular carcinoma patients induce suppressive $CD4^+CD25^+Foxp3^+$ Tregs in a contact-dependent manner when co-cultured with autologous CD3/CD28-stimulated $CD4^+$ T cells (57). In addition, to induce Tregs from $CD4^+$ T cells, a study from the same group has shown that $CD14^+HLA\text{-}DR^{-/low}$ M-MDSCs are able to convert Th17 cells to $Foxp3^+$ Tregs, which is dependent on MDSC-derived TGF-β and retinoic acid (96). Jitschin et al. have shown that M-MDSCs from chronic lymphocytic leukemia (CLL) patients suppress T-cell activation and promote Treg induction, which is partly dependent on IDO activity (95). Furthermore, the authors have also demonstrated that after co-culture with CLL cells, monocytes from healthy donors resemble the phenotypic, suppressive, and Treg-inducing characteristics of M-MDSCs from CLL patients (95). In patients with lung cancer, a novel tumor-infiltrating $B7\text{-}H3^+CD14^+HLA\text{-}DR^{-/low}$ subset of MDSCs is reported to induce Tregs *in vitro*, which is partly dependent upon IL-10 (40).

Interestingly, there are also reports revealing no clear association between MDSCs and Tregs. In mice bearing T-cell lymphoma, the percentage of intratumoral Tregs is invariably high throughout tumor growth and does not relate to the accumulation kinetics of MDSCs (9). In another study, the T-cell non-responsiveness induced by adoptive transfer of MDSCs was not caused by Treg induction (97). Furthermore, in contrast to the abovementioned Treg-inducing action of M-MDSCs, it is reported that PMN-MDSCs impair TGF-β-mediated generation of inducible Tregs (iTregs) from naïve T cells and inhibit proliferation of naturally occurring Tregs (nTregs) without affecting Foxp3 expression (98). These discrepancies need to be clarified by further study.

Suppression of Natural Killer Cells

NK cells are another major target of MDSCs. The reduced number and impaired function of NK cells in tumor-bearing mice are inversely correlated with the increased level of MDSCs and are restored by depletion of MDSCs (61, 99). A similar inverse correlation is also observed in patients with non-Hodgkin lymphoma (39). It is shown that the enhanced lactate production by tumor cells inhibits NK cells not only directly by inhibiting their cytotoxicity but also indirectly by increasing the number of MDSCs (100). Interestingly, a recent study has demonstrated that a portion of immature NK cells is converted into MDSCs in the presence of GM-CSF and that this conversion is abolished by IL-2 exposure (101). This novel developmental pathway of MDSCs may account, at least partially, for the reduced level of NK cells in tumor-bearing host.

In murine models, the cytotoxicity, NKG2D expression, and IFN-γ production of NK cells are inhibited by MDSCs both *in vitro* and *in vivo* (61, 102). This suppression is contact-dependent and requires membrane-bound TGF-β1 on MDSCs (61, 102). In a recent study, Elkabets et al. identified a novel subset of $Gr\text{-}1^{high}$ PMN-MDSCs that is induced by IL-1β and lacks Ly6C expression ($Ly6C^{neg}$). These $Ly6C^{neg}$ MDSCs produce higher levels of iNOS and ROS than $Ly6C^{low}$ MDSCs and, correspondingly, exhibit stronger suppression of T cells and NK cells (103). The MDSC-mediated NK cell suppression is associated with increased metastasis in mice during gestation (104). In tumor-bearing mice treated with medroxyprogesterone acetate, which is commonly used as hormone replacement therapy and as a contraceptive, MDSCs exhibit higher suppression of NK cells as compared with MDSCs from control mice, implying a potential mechanism for increased breast cancer incidence associated with prolonged medroxyprogesterone acetate administration (105).

In patients with liver cancer or advanced melanoma, $CD14^+HLA\text{-}DR^{-/low}$ MDSCs suppress autologous NK-cell cytotoxicity and IFN-γ production (60, 106). This suppression is independent of ARG1 and iNOS but requires cell-to-cell contact through NK-activating receptor NKp30 on NK cells, suggesting expression of NKp30 ligand(s) by MDSCs (106). In addition, TGF-β produced by MDSCs from melanoma patients, which is stimulated by PGE2, also serves as a major mechanism for NK-cell suppression (60). In addition, MDSCs from cancer patients inhibit Fc receptor-mediated signal transduction and downstream effector function of NK cells, including antibody-dependent cellular cytotoxicity and cytokine production, probably through NO production (107).

As an essential defensive mechanism of the innate immune system, it is not surprising that NK cells are suppressed by MDSCs generated in microbial infection. It is shown that polymorphonuclear neutrophils and PMN-MDSCs dampen the activation and cytotoxic activity of NK cells toward *Aspergillus fumigatus* (108). In another study with mice infected by vaccinia virus, PMN-MDSCs negatively regulated the proliferation, activation, and function of NK cells, which helped to contain excessive NK cell activity (109). In HCV infection, $CD33^+CD11b^{low}HLA\text{-}DR^{low}$ MDSCs suppress the IFN-γ production of NK cells by depleting L-arginine via ARG1 (110). Interestingly, $CD66b^+CD33b^+HLA\text{-}DR^{low}$ PMN-MDSCs increase strikingly in the cord blood of neonates when compared with peripheral blood of healthy children and adults. These cord blood PMN-MDSCs are able to inhibit the function of T cells and NK cells, which may be responsible for the impaired host defense of neonates (111).

Conversely, there are studies showing NK cell activation by MDSCs. For instance, Nausch et al. have found that MDSCs from tumor-bearing mice express NKG2D ligand RAE-1 and activate NK cells to produce IFN-γ, which is partially contact-dependent and requires signaling through NKG2D (112). In mice bearing NK-sensitive tumor, poly I:C treatment allows MDSCs

to prime NK cells and consequently leads to delayed tumor growth. MDSC-derived IFN-α after poly I:C administration activates NK cells, which drives CD69 expression and IFN-γ production but does not induce cytotoxic activity of NK cells (113). A recent study has shown that M-MDSCs infiltrate in the tumor microenvironment prior to NK cells and are required for the tumoricidal activity of NK cells to eradicate galectin-1-deficient GL26 glioma (114). Taken together, the seemingly contradictory findings mentioned above suggest that the effect of MDSCs on NK cells, either inhibitory or stimulatory, is most likely context-dependent.

Impaired Function of Dendritic Cells by MDSCs

Relatively less information is available on the direct impact of MDSCs on DCs. Accumulation of MDSCs in tumor-bearing mice and cancer patients is associated with impaired differentiation and accumulation of DCs (115–117). Unfortunately, the underlying mechanism(s) is not fully understood. In a murine model of allergic airway inflammation, LPS exposure promoted the development of a group of myeloid cells in the lung that resembled MDSCs phenotypically and functionally. These cells inhibited the reactivation of primed Th2 cells by DCs (118). In mice with hepatocellular carcinoma, MDSC-derived IL-10 was found to be responsible for the impaired TLR ligand-induced IL-12 production and T-cell stimulatory activity of DCs (116). Recently, it was shown that MDSC-mediated suppression of antigen presentation from DCs to $CD4^+$ T cells depends on NO, which may cause nitration of STAT1, a key mediator for antigen presentation, and, consistently, this suppression is abrogated by iNOS inhibitors (11). In another recent study, Notch and STAT3 signals were found to be required by MDSCs to suppress the differentiation, maturation, and antigen presentation ability of DCs *in vitro* and *in vivo* (119).

Due to their superior antigen presentation and T-cell activation properties, DCs are utilized as cancer vaccines to prompt immunity against malignant cells. DC vaccines loaded with tumor antigens through various approaches aim to induce and potentiate tumor antigen-specific T-cell response. In line with MDSC-mediated suppression of DCs, favorable therapeutic efficacy of DC vaccination is associated with a reduced level of MDSCs in tumor-bearing mice (120, 121). In cancer patients, when monocyte-derived DCs are used as vaccines, the presence of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs in the starting monocyte population causes impairment of DC maturation, antigen uptake, migration, and T-cell stimulation capacity (122). Therefore, it is reasonable to apply DC-based vaccines in combination with agents that target MDSCs. These agents include chemotherapeutics (e.g., all-trans retinoic acid, gemcitabine, and cyclophosphamide) (123, 124), tyrosine kinase inhibitors (e.g., sunitinib, axitinib, and dasatinib) (125–127), lenalidomide (128), and anti-Gr-1 antibody (120), and these combinations have shown reduced levels of MDSCs and improved efficacy in pre-clinical studies. The initiation of immune response by DC vaccines involves interaction between multiple immune cell types. Therefore, to overcome the immunosuppression mediated by MDSCs and maximize efficacy, further research is still needed to accurately define the action of MDSCs and other immune cells in DC vaccine-induced anti-tumor immunity.

B Cells

In recent years, B cells have emerged as a novel target of MDSCs. In an *in vitro* model of B lymphopoiesis, MDSCs induced by adipocyte-derived factors inhibited B-cell development through IL-1 production (129). PMN-MDSCs inhibited the recruitment, proliferation, and cytokine secretion of B cells in the central nervous system of mice with experimental autoimmune encephalomyelitis (130). In the settings of retroviral infection and autoimmune disease, several animal studies have revealed that MDSCs impair B cell response by many of the mechanisms utilized in T-cell suppression, such as ROS, iNOS, ARG1, TGF-β, and PGE2 (54, 131). MDSCs from mice infected with retrovirus express V-domain Ig-containing suppressor of T-cell activation (VISTA), a negative checkpoint regulator that is homologous to PD-L1 and inhibits T-cell response, and VISTA deficiency in MDSCs or neutralization of VISTA by blocking antibody partially rescues the impaired B-cell proliferation (132). Both contact-dependent and contact-independent inhibition have been implicated in these studies (54, 131).

Whether these suppressive mechanisms are used by MDSCs in cancer settings is less well-elucidated. ROS, ARG1, iNOS PGE2, and TGF-β have recently been suggested to exert suppressive effects on B-cell proliferation and antibody production by tumor-induced MDSCs (133). In a murine model of lung cancer, the impeded B cell differentiation was associated with tumor progression and MDSC infiltration; mechanistically, MDSCs inhibit B cell response by TGF-β-mediated modulation of IL-7 and downstream STAT5 signaling, which are both essential in B-cell differentiation and function (133). In another study, Ku et al. showed that tumor-induced MDSCs reduce L-selectin on naïve $CD4^+$ and $CD8^+$ T cells and that even moderate L-selectin reduction is sufficient to profoundly disrupt homing of T cells to distant lymph nodes. Interestingly, the loss of L-selectin has also been found in B cells. In the study concerned, the shedding of L-selectin from naïve T cells and B cells was contact-dependent and was independent of major L-selectin sheddase ADAM17. Since the trafficking of both naïve B cells and $CD4^+$ precursors of follicular helper T cells was hindered, the authors suggested that the T cell-dependent antibody production in lymph nodes may have been severely impaired (134).

Regulatory B cells (Bregs) are immunosuppressive and inhibit the expansion of pathogenic T cells and other pro-inflammatory lymphocytes through the production of IL-10, IL-35, and TGF-β. In consistence with these properties, Bregs have been shown to suppress anti-tumor immunity and promote tumor growth. In patients with colorectal cancer, the level of Bregs positively correlates with disease stage and with the frequency of MDSCs (135). In a murine model of breast cancer, Shen et al. showed that MDSCs upregulate PD-L1 expression on B cells and dampen their anti-tumor response; more interestingly, MDSCs may transform B cells into a novel subtype of Bregs that possesses higher inhibitory capability on T cells as compared

with other subsets of Bregs (136). In another study, MDSCs induced the expansion of IL-10-producing Bregs, probably through iNOS, and ameliorated autoimmunity in mice with systemic lupus erythematosus (137). Conversely, in mice infected with retrovirus, M-MDSCs inhibited the proliferation of IL-10-producing Bregs in response to LPS stimulation (54).

ANTIGEN-SPECIFIC AND NON-SPECIFIC SUPPRESSION OF CD8$^+$ AND CD4$^+$ T CELLS

Among the various cell targets, suppression of T cells remains the characteristic necessary to define bona fide MDSCs, provided that the phenotypic criteria are met. With the abovementioned mechanisms, MDSCs are capable of suppressing both antigen-specific and non-specific T-cell response (**Figure 2**). It is now generally accepted that ROS, and PNT in particular, are responsible for antigen-specific suppression, provided that MDSCs and T cells are in close contact, since these substances are unstable and short-lived, while iNOS, ARG1, and immunosuppressive cytokines are responsible for antigen-non-specific suppression, since effector molecules of these mechanisms have relatively longer half-lives and require cellular proximity, but not close interaction, to exert inhibition (1).

Early studies have shown that Gr-1$^+$ immature myeloid cells isolated from tumor-bearing mice are able to uptake and process soluble proteins and present the antigenic epitopes on their surface (97). Their suppression of antigen-specific CD8$^+$ T cells requires antigen presentation via MHC class I and ROS production (14, 138). Studies in the last decade have revealed that MDSC-induced antigen-specific T-cell tolerance results from post-translational modification of the TCR complex. MDSCs from gp91$^{phox-/-}$ mice produce little ROS and fail to inhibit CD8$^+$ T cells, and neutralization of PNT abrogates the suppressive activity of MDSCs on T cells (17). Nagaraj et al. demonstrated that the close and prolonged cell-to-cell contact during antigen recognition allows MDSC-derived PNT to cause nitration of tyrosines in the TCR-CD8 complex, which induces conformational changes in these molecules and leads to loss of binding ability to peptide-MHC complex (17). Consistently, using double TCR transgenic CD8$^+$ T cells, the same group later showed that MDSCs induce CD8$^+$ T-cell tolerance only against the peptide presented by themselves, while they do not affect T-cell response to peptide specific for other TCR that is not presented by MDSCs (139). In accordance with previous findings, the authors showed that nitration of surface molecules of T cells is localized to the site of physical interaction between MDSCs and T cells, which may lead to dissociation between TCR and CD3ζ molecules, and consequently, nitrotyrosine positive CD8$^+$ T cells are rendered non-responsive to specific peptide (139). In another study, however, ROS were found not to be involved in antigen-specific T-cell suppression by MDSCs, and MDSCs deficient in MHC class I showed no impairment in antigen-specific suppression, which excludes the necessity of antigen presentation (9).

Interestingly, PNT produced by MDSCs can facilitate immune evasion of tumor cells even in the presence of normal functioning

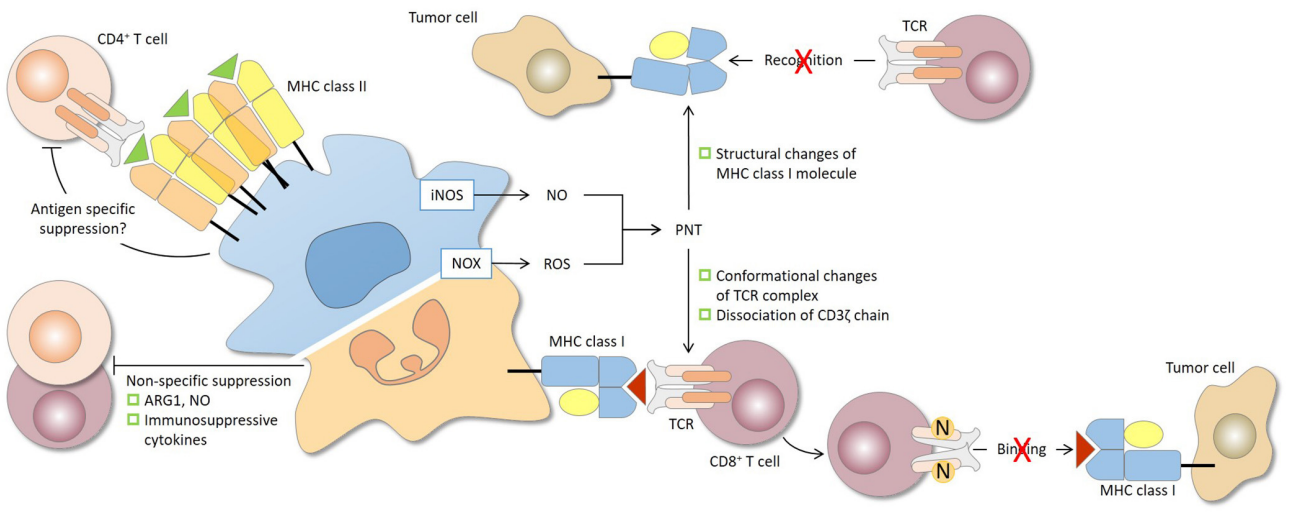

FIGURE 2 | Antigen specificity of MDSC-mediated suppression of CD4$^+$ and CD8$^+$ T cells. The antigen specificity of T-cell suppression by MDSCs is determined largely by the characteristics of the effector molecules involved. The short-lived reactive oxygen species (ROS) and peroxynitrite (PNT) are responsible for antigen-specific suppression, provided that MDSCs and T cells are in close contact, while arginase 1 (ARG1), nitric oxide (NO), and immunosuppressive cytokines, which have relatively longer half-lives, mediate antigen-non-specific suppression. During the close and prolonged interaction between MDSCs and CD8$^+$ T cells in antigen recognition, PNT causes nitration and conformational changes of the TCR complex and dissociation of CD3ζ molecules. CD8$^+$ T cells consequently lose their binding ability to peptide-MHC class I complex and are rendered non-responsive to specific peptide presented by tumor cells. PNT may also induce structural changes of MHC class I on tumor cells, leading to reduced antigenic peptide binding. In this case, antigen-specific CD8$^+$ T cells, even if functional, fail to recognize tumor cells. For CD4$^+$ T cells, antigen-specific suppression by MDSCs has been reported and may require sufficient MHC class II expression by MDSCs. iNOS, nitric oxide synthase; NOX2, NADPH oxidase 2.

T cells. PNT induces nitration and structural changes of MHC class I molecules on tumor cells, which hampers their capacity to bind antigenic peptide and subsequently impairs the recognition by CTLs, therefore affording tumor cells resistance to antigen-specific CTLs (140). These findings collectively suggest the involvement of multiple mechanisms in antigen-specific $CD8^+$ T-cell suppression by MDSCs.

On the other hand, evidence for MDSC-mediated antigen-specific suppression of $CD4^+$ T cells remains elusive, and different results have been reported. It was previously indicated that MDSCs fail to suppress antigen-specific $CD4^+$ T-cell proliferation, which may be due to the low MHC class II expression on MDSCs, which precludes them from forming close contact with $CD4^+$ T cells (91, 138). However, MDSC-mediated suppression of the proliferation of $CD4^+$ T cells exposed to a specific peptide has been reported, which is at least partially due to cysteine deprivation by MDSCs (25, 88). Interestingly, Nagaraj and colleagues have shown that MDSCs are able to suppress antigen-specific $CD4^+$ T-cell response *in vitro* and *in vivo*, as long as their MHC class II expression reaches a sufficient level (141). In different experimental systems, MDSCs are able to blunt IFN-γ production of both tumor-specific $CD8^+$ and $CD4^+$ T cells in the spleen of tumor-bearing mice *in vivo* (142). In patients with liver cancer, depletion of $CD14^+HLA\text{-}DR^{-/low}$ M-MDSCs enhances IFN-γ secreting $CD4^+$ T cells specific to α-fetoprotein (57). These discrepancies might be explained, in part, by the varied MHC class II level of MDSCs that has been described in different tumor models and human studies, and under some experimental conditions, MDSCs could inhibit the proliferation of T cells without affecting the IFN-γ production and vice versa (3, 4).

REGULATION ON THE SUPPRESSIVE NATURE OF MDSCs

In most studies, immunosuppressive activity is detected only in MDSCs derived from tumor-bearing host but not in their control counterparts from tumor-free host, suggesting a tight control over MDSCs by tumor. MDSCs carry out immune suppression principally in the tumor microenvironment, which is a highly dynamic complex and plays a crucial role in tumor development. The constant bi-directional communication between MDSCs and the ever-changing microenvironment shapes the phenotype and function of MDSCs (**Figure 3**). For instance, tumor-derived M-MDSCs show higher suppression of T cells than spleen- or bone marrow-derived M-MDSCs from the same mice. Several cellular and non-cellular components of the tumor microenvironment, including the subset composition of MDSCs, tumor cells, stromal cells, cytokines, metabolic state, and hypoxia, regulate the suppressive nature of MDSCs.

Subset Composition and Antigen Specificity and Capacity of MDSC-Mediated Suppression

It is now clear that the suppressive machineries of MDSCs do not act simultaneously, and subsets of MDSCs use different mechanisms for T-cell suppression (9, 10, 92, 143). For instance, M-MDSCs, whose activity mainly relies on ARG1, NO, and immunosuppressive cytokines, inhibit both antigen-specific and non-specific T-cell response (8, 10, 19, 92, 143, 144), while PMN-MDSCs, whose activity largely depends on high ROS and PNT production, inhibit T cells in an antigen-specific manner (10, 92). In one study, only M-MDSCs, but not PMN-MDSCs, were able to augment the activation-induced Fas upregulation of $CD8^+$ T cells through NO production and sensitize them to Fas-mediated apoptosis and were able to impede the differentiation of mature CTLs (143). Therefore, the suppressive nature of MDSCs is influenced by their subset composition.

PMN-MDSCs is commonly the predominant subpopulation in peripheral lymphoid organs in many murine tumor models, and accordingly, antigen-specific T-cell tolerance is detected at these sites (145, 146). This peripheral antigen-dependent T-cell inhibition may partially explain the findings in some studies that T cells in the periphery retain their responsiveness to other non-specific stimuli (3, 17, 97). On the other hand, the proportion of M-MDSCs is substantially higher in the tumor milieu (144, 145), and in spite of the common findings that PMN-MDSCs may still be the prevalent subpopulation, M-MDSCs are more suppressive than PMN-MDSCs on a per-cell basis (9, 119). As a consequence, tumor-infiltrating MDSCs demonstrate higher immunosuppressive capacity than their peripheral counterparts and are able to inhibit both antigen-specific and non-specific T-cell function (19, 147, 148).

In spite of these findings, it is noteworthy to point out that similar or even stronger inhibitory capacity of peripheral MDSCs has also been reported (140, 149, 150) and that non-specific T-cell suppression is not uncommon in MDSCs derived from peripheral lymphoid organs (149, 151).

It is common that the ratio between subgroups of MDSCs varies in different tumor models. Unfortunately, many of these studies have not addressed the subset composition of intratumoral or peripheral MDSCs in detail, nor have they assessed the suppressive capacity of PMN-MDSCs and M-MDSCs separately. Therefore, the discrepancies on antigen specificity and capacity of MDSC-mediated suppression, on the one hand, should be interpreted with care, and on the other hand, may suggest that subset composition of MDSCs is not likely the sole nor a major determinant that influences their suppressive nature.

Tumor-Derived Mediators

The generation of MDSCs includes two phases. Firstly, aberrant myelopoiesis and blocked differentiation of immature myeloid cells lead to the expansion of MDSCs, mainly driven by various growth factors; secondly, these MDSCs are activated to be fully functional, primarily promoted by pro-inflammatory factors. This two-signal model of expansion and activation may answer the question of why MDSCs are not generated under normal physiological settings or during acute inflammation. In steady state, growth factors stimulate normal hematopoiesis without generating MDSCs due to the absence of pro-inflammatory factors, whereas during acute inflammation, in the absence of sustained growth factors, pro-inflammatory factors alone do not

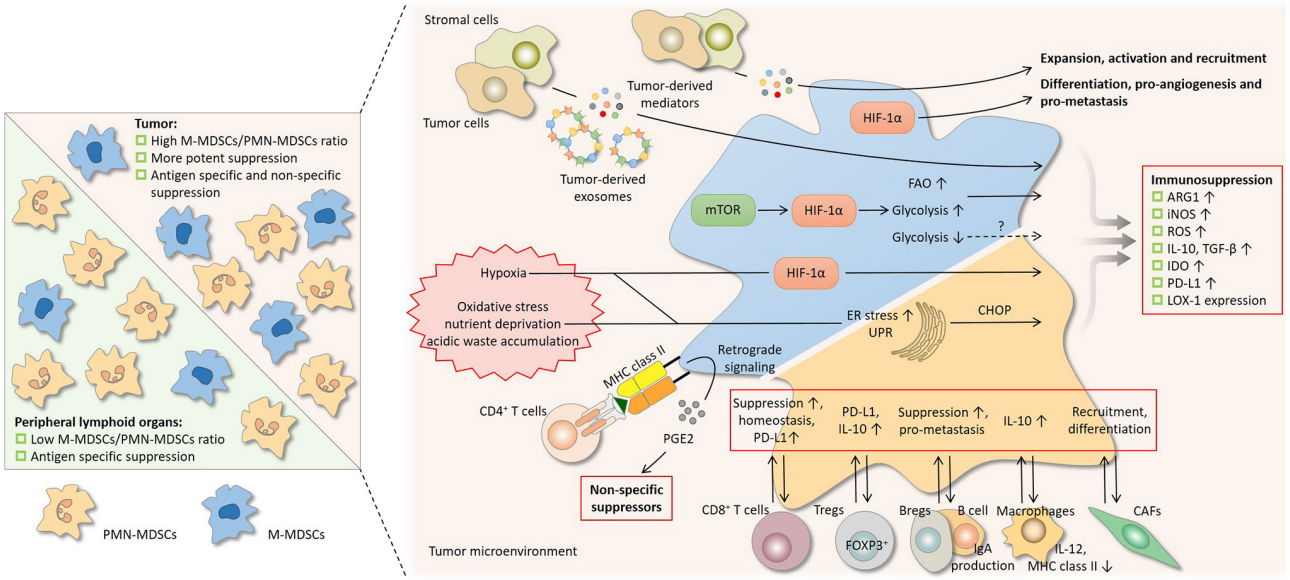

FIGURE 3 | Regulation on the suppressive nature of MDSCs by subset composition and the tumor microenvironment. Differential suppressive capacity and mechanism(s) between PMN-MDSCs and M-MDSCs influence the suppression by MDSCs as a whole population (left). In peripheral lymphoid organs where PMN-MDSCs predominate, suppression by MDSCs is mainly antigen-specific, since the activity of PMN-MDSCs depends largely on reactive oxygen species (ROS) and peroxynitrite (PNT). In tumor where the proportion of M-MDSCs increases, suppression is more potent and is both antigen-specific and non-specific, since M-MDSCs are more suppressive and mainly rely on arginase 1 (ARG1), nitric oxide (NO), and immunosuppressive cytokines. A network of cytokines, hostile physical conditions, and cells in the tumor microenvironment regulates MDSCs in multiple aspects (right). Soluble mediators derived from tumor regulate the suppressive activity of MDSCs and also drive their development. After being taken by MDSCs, the contents of tumor-derived exosomes, which act as intercellular messengers, promote the expansion and potentiate the suppressive capacity of MDSCs. Like tumor cells, MDSCs undergo metabolic reprogramming to adapt to varying surroundings. Hypoxia-inducible factor 1α (HIF-1α) induced by the mammalian target of rapamycin (mTOR) pathway enhances glycolysis and may potentiate suppression by MDSCs, whereas glycolysis has also been reported to be a negative regulator. The heightened fatty acid oxidation (FAO) is associated with upregulated ARG1 and increased NO and PNT production. Hypoxic signaling, primarily through HIF-1α, is another central regulator. HIF-1α promotes many non-immunological activities of MDSCs, including differentiation, pro-angiogenesis, and pro-metastasis. HIF-1α augments MDSC-mediated suppression by upregulating several effector molecules. The hostile conditions in the tumor milieu, such as oxidative stress, nutrient deprivation, and acidic waste accumulation, causes ER stress and induce unfolded protein response (UPR) in MDSCs. ER stress response marker C/EBP homologous protein (CHOP) regulates ARG1, superoxide, and PNT production by MDSCs. The bidirectional communication with stromal cells fine-tunes the induction, homeostasis, differentiation, and suppressive function of MDSCs. Bregs, regulator B cells; CAFs, cancer-associated fibroblasts; DCs, dendritic cells; iNOS, nitric oxide synthase; LOX-1, lectin-type oxidized LDL receptor-1; NK, natural killer; PD-L1, programmed cell death-ligand 1; Tregs, regulatory T cells.

lead to MDSC generation either, since immature myeloid cells may rapidly differentiate into mature myeloid cells.

As discussed in the previous sections, many of the tumor-derived mediators actively regulate the suppressive function of MDSCs. In a murine model of tissue-specific inflammatory response, MDSCs from inflammatory or tumor site are more suppressive than MDSCs from spleen, and splenic MDSCs from inflamed mice are more suppressive than splenic MDSCs from naïve mice (148). Further study from the same group has shown that MDSCs exposed to IFN-γ, IL-13, and GM-CSF *in vitro* or MDSCs localized in inflammatory or tumor site *in vivo* have elevated L-arginine transporter cationic amino acid transporter 2 expression, which parallels the expression of ARG1 and iNOS and is required for optimal suppressive activity of MDSCs (146). These findings suggest a priming effect of tumor-derived pro-inflammatory cytokines. In a more recent study, tumor cells upregulate tumor necrosis factor-α-induced protein 8-like 2 (TIPE2) in MDSCs through ROS, which in turn controls the polarization of MDSCs by increasing pro-tumoral and inhibiting anti-tumoral mediator expression (152).

Several pro-inflammatory factors are reported to enhance the suppressive potency of MDSCs. For instance, PGE2 generated by COX2 in tumor cells upregulates ARG1 expression of MDSCs through the EP4 receptor (153). PGE2 promotes hypermethylation and repression of a cluster of myeloid genes, which is in contrast to the profile from DCs generated *in vitro* or $CD11b^+$ cells from healthy controls. This MDSC-specific gain of methylation requires the upregulation of DNA methyltransferase 3A, while its downregulation abolishes the immunosuppressive properties of MDSCs (154). It another study, PGE2 potentiates the suppressive function of human M-MDSCs induced by GM-CSF/IL-6 from peripheral blood mononuclear cells (155). However, whether these actions of PGE2 occur *in vivo* remains to be determined. IL-17 not only enhances tumor-infiltrating MDSCs, probably by increasing CXCL1 and CXCL5 secretion by tumor cells, but also potentiates their inhibition on T

cells through upregulation of ARG1 and IDO (156). The calcium-binding pro-inflammatory proteins S100A8 and S100A9, which are ubiquitously present in the tumor microenvironment, drive the accumulation of MDSCs through increased recruitment to primary tumor and pre-metastatic niche (150). It was recently reported that S100A8 enhances T-cell suppression by MDSCs (157) and that S100A9 induces IL-6 and IL-10 release by MDSCs (158). Furthermore, MDSCs also express and secret S100A8/A9, thus forming a positive feedback loop that helps to maintain suppressive MDSCs in the tumor microenvironment (150).

Both type I and II interferons upregulate PD-L1 expression in MDSCs. It is well-documented that IFN-γ functions as a master regulator of PD-L1 expression in tumor. IFN-γ neutralization reduces tumor-infiltrating PD-L1$^+$ MDSCs *in vivo*, and mechanistically, IFN-γ upregulates IRF1, which in turn binds to IRF-binding sequence in *cd274* promoter and activates PD-L1 expression (72). The IFN-γ level in the tumor microenvironment may be reduced due to MDSC-mediated suppression of T cells and NK cells, which are important sources of IFN-γ. As a compensatory mechanism, MDSCs may maintain their PD-L1 expression by secreting IFN-α and IFN-β, which bind to IFN receptor type I and upregulate PD-L1 in an autocrine manner (159).

It is noteworthy to point out that many of the tumor-derived mediators influence more than one aspect of MDSCs. For instance, in addition to promoting expansion, GM-CSF alone is able to promote immunosuppression by MDSCs (160). GM-CSF increases IL-4Rα expression on MDSCs, which leads to IL-13-induced ARG1 upregulation (161), and GM-CSF drives PD-L1 and IDO expression of MDSCs through STAT3 activation (69, 80). Tumor-derived migration inhibitory factor has been reported to promote the differentiation, recruitment, and suppressive activity of MDSCs (162, 163). These pleiotropic and redundant effects further complicate the regulatory network of MDSC development.

Tumor-Derived Exosomes

Exosomes are small extracellular vesicles released by nearly all cells and are present in most body fluids. These membrane-bound vesicles contain proteins, DNA, mRNA, and miRNA and act as intercellular messengers (164). Tumor constantly produces and secrets exosomes. Upon contact with target cells, tumor derived-exosomes are able to alter the phenotypic and functional characters of the recipients, reprogramming them into participants in tumor progression. In the early phase of tumor growth, exosomes derived from immune cells in the tumor microenvironment may facilitate anti-tumor response, while in more advanced disease, tumor derived-exosomes promote immune suppression by interfering with the differentiation, maturation, and anti-tumor activity of immune cells (164). Several recent studies have shown that MDSCs also produce exosomes, whose contents are implicated in their own chemotaxis, survival, pro-metastatic, and immunosuppressive activity (165).

Studies have shown that tumor-derived exosomes promote the expansion of MDSCs. Administration of tumor-derived exosomes to healthy mice leads to increased frequency of immature myeloid cells that acquire the phenotypic and functional characters of MDSCs (166). Tumor derived-exosomes induce accumulation of splenic and intratumoral MDSCs that are able to promote tumor growth, which is dependent on exosomal PGE2 and TGF-β (167). In multiple myeloma, exosomes derived from both tumor cells and stromal cells expand MDSCs (168, 169). In addition, tumor derived-exosomes may contribute to metastasis by inducing accumulation of MDSCs, PMN-MDSCs in particular, in the pre-metastatic niche (170, 171).

Many of the suppressive machineries of MDSCs can be potentiated by tumor derived-exosomes, including expression of ARG1 and iNOS, and production of IL-10 and VEGF (48, 167, 169, 172). The suppressive capacity of MDSCs on T cells is accordingly heightened (48, 169). STAT3 is implicated in this exosomal regulation on MDSCs (169). Chalmin et al. have shown that HSP72 expressed on tumor derived-exosomes induces suppressive activity of MDSCs, which activates STAT3 in a TLR2/MyD88-dependent manner through autocrine production of IL-6 (142). Similarly, in another study, MDSCs were expanded and activated by exosomal HSP70, which induced phosphorylation of STAT3 through the TLR2/MyD88 pathway (172). In consistence with these findings, T-cell proliferation is inhibited by MDSCs isolated from mice treated with tumor derived-exosomes but not by MDSCs isolated from MyD88 knockout mice treated with tumor derived-exosomes (171). Furthermore, stromal cell-derived exosomes are also reported to enhance T-cell suppression by MDSCs, probably through the STAT3 pathway as well (168).

Tumor-derived exosomes are able to mediate RNA transfer from tumor cells to recipient cells. Ridder and colleagues have shown that MDSCs are the major recombined cells in the tumor microenvironment after the uptake of exosomes and their RNA content and that MDSCs recombined with exosomal RNA display enhanced ARG1, TGF-β, and PD-L1 expression as compared to the non-recombined counterparts (173). In a recent study, hypoxia increases exosome secretion by glioma cells. Moreover, hypoxia upregulates miR-10a and miR-21 in glioma-derived exosomes, which in turn potentiates the suppressive function of MDSCs (174).

Metabolic Reprogramming of MDSCs

Along with disease progression, malignant cells undergo dramatic alteration in their energy metabolism to meet the demand for rapid tumor growth and to adapt to the varying microenvironment. Meanwhile, it was recently demonstrated that tumor-associated immune cells also experience metabolic changes that help to shape their pro- and/or anti-tumor response (175). In this regard, metabolic reprogramming is emerging as a regulator of MDSCs. Using MSC-1 cells, an immortalized murine MDSC cell line, early *in vitro* studies have revealed two distinct bioenergetic states that coincide with the exponential and stationary growth phases of MSC-1 cells (176) and that their maturation and suppressive potential are accompanied by an increase in the central carbon metabolism activity (177).

MDSCs exhibit a high glycolytic rate (175). The enhanced glycolysis of MDSCs helps to keep their ROS level within a safe range and promotes their survival and accumulation in

tumor-bearing host (178). mTOR-mediated HIF-1α induction is essential in glycolytic activation (175). Inhibiting the mTOR pathway blocks the differentiation of M-MDSCs from precursors by impairing glycolysis. Consistently, 2-deoxyglucose, which inhibits glycolysis, blocks the differentiation of M-MDSCs, while metformin, which promotes glycolysis, rescues the reduction in M-MDSCs caused by mTOR inhibition (179). On the other hand, glycolysis in tumor cells also contributes to the expansion of MDSCs, which is mediated by increased production of G-CSF, GM-CSF, and lactate (100, 180).

In addition to promoting expansion, glycolysis regulates the function of MDSCs. A recent study has found that enhanced glycolysis mediated by the mTOR pathway leads to stronger suppressive capacity of tumor-infiltrating M-MDSCs as compared with splenic M-MDSCs and that mTOR inhibition by rapamycin reduces the glycolysis, intratumoral level, and suppressive activity of M-MDSCs (181). Attenuated iNOS and ARG1 may be responsible for the impaired function caused by rapamycin-mediated glycolysis inhibition (179).

On the contrary, glycolysis as a negative regulator of MDSCs has also been reported. It is shown that mTOR- and HIF-1α-induced glycolytic activation is required for differentiation of MDSCs to a less suppressive M1 phenotype (182). In the settings of transplantation and autoimmune disorder, dexamethasone expands MDSCs and strengthens their function. In a model of immunological hepatic injury, dexamethasone inhibits HIF-1α-dependent glycolysis in MDSCs and promotes their suppressive activity to protect against inflammatory injury (183). In addition, there are studies showing that mTOR inhibition by rapamycin potentiates the suppressive activity of MDSCs, which protects against acute graft-versus-host disease and acute kidney injury (184, 185); yet, unfortunately, the glycolytic or other metabolic characteristics of MDSCs were not determined in these studies. These seemingly conflicting results indicate the complexity and the possibly context-dependent manner in which glycolytic rate determines the function of MDSCs.

Recently, it is shown that tumor-infiltrating MDSCs have increased fatty acid oxidation (FAO), which is accompanied by upregulated ARG1, increased NO, and PNT production, and that FAO inhibition impairs the suppressive activity of MDSCs *in vitro* and *in vivo* (186). Only intratumoral MDSCs, and not splenic MDSCs, have increased FAO, suggesting that the microenvironment is responsible for this metabolic alteration (186). Consistently, a further study from the same group demonstrated that tumor-derived cytokines, such as G-CSF and GM-CSF, induce the expression of lipid transport receptors in intratumoral MDSCs through the activation of STAT3 and STAT5, which leads to increased uptake of lipids that are present at high concentrations in the tumor microenvironment; intracellular accumulation of lipids in turn increases the oxidative metabolism and suppressive activity of MDSCs (187).

Hypoxia and HIF-1α

Hypoxia caused by excessive oxygen consumption by tumor cells and aberrant organization of tumor vasculature is a common feature of the tumor microenvironment and plays a central role in tumor progression, primarily through HIF-dependent signalings. Multiple activities of MDSCs are regulated by hypoxia. For instance, hypoxia facilitates the recruitment of MDSCs to tumor site (188, 189). Intratumoral MDSCs preferentially localize in poorly perfused and hypoxic regions, and their pro-angiogenic capacity is generally enhanced by hypoxia (6). The homeostasis of tumor-infiltrating MDSCs is fine-tuned by the hypoxic microenvironment, since hypoxia promotes the differentiation of intratumoral MDSCs to TAMs (190), while it is also reported that hypoxia promotes the maintenance of MDSCs by upregulating ectonucleoside triphosphate diphosphohydrolas 2 in tumor cells, which forms a 5′-AMP-rich microenvironment and prevents differentiation of MDSCs (191).

It is now generally accepted that microenvironmental hypoxia directly augments the suppressive function of MDSCs (1). In a tumor model with similar PMN-MDSC to M-MDSC ratios in spleen and tumor site, Corzo et al. found that the inhibition on T cells is antigen-specific by splenic MDSCs, which display higher ROS production, while it is both antigen-specific and non-specific by tumor-infiltrating MDSCs, which exhibit upregulated ARG1 and iNOS. Exposure of splenic MDSCs to hypoxia leads to non-specific T-cell suppression, suggesting that the hypoxic microenvironment may convert MDSCs into non-specific suppressors. This conversion is mediated by HIF-1α (190). A similar difference in suppressive mechanisms and antigen specificity is detected in MDSCs obtained from peripheral blood and tumor tissue of patients with head and neck cancer (190). Similarly, it was recently reported that HIF-1α potentiates the immunosuppressive activity of splenic MDSCs in a murine model of chronic *Leishmania* infection (192).

Noman et al. have shown that the PD-L1 level is higher on intratumoral MDSCs than on splenic MDSCs and that hypoxic stress upregulates PD-L1 on splenic MDSCs through HIF-1α. More importantly, hypoxia potentiates the ability of splenic MDSCs to suppress both specific and non-specific stimuli-mediated T-cell proliferation, while PD-L1 blockade abrogates the enhanced suppression under hypoxia, in part by decreasing the production of suppressive cytokines, particularly IL-6 and IL-10, in hypoxic MDSCs (43). The authors have also found that hypoxia increases the secretion of IL-6, IL10, and TGF-β from MDSCs (43). In another study from the same group, tumor-infiltrating MDSCs expressed an increased level of miR-210 as compared with splenic MDSCs, and hypoxia induced miR-210 in splenic MDSCs via HIF-1α. MiR-210 in turn enhanced the suppressive capacity of splenic MDSCs by increasing their ARG1 activity and NO production without affecting ROS, IL-6, or IL-10 production or PD-L1 expression (67). In a more recent study, HIF-1α acted as a transcriptional activator of VISTA, a negative checkpoint regulator in the B7 family, in MDSCs and consistently, antibody blockade or genetic ablation of VISTA abolished MDSC-mediated suppression of T cells under hypoxic but not normoxic conditions (193). These findings suggest that hypoxia regulates MDSC-mediated suppression through multiple pathways.

MDSCs actively participate in tumor metastasis by inducing EMT, increasing the invasiveness and stemness of tumor cells, and stimulating angiogenesis (5, 6). Unfortunately, the precise roles of hypoxia and hypoxic signalings in these

MDSC-driven steps of metastatic cascade are not well-defined. On the other hand, MDSCs actively participate in pre-metastatic niche formation. MDSCs, especially the granulocytic subset, reach the pre-metastatic site prior to the arrival of disseminated tumor cells, which is regulated indirectly by hypoxia in the primary tumor. In a murine mammary tumor model, tumor that grows in pre-irradiated mammary tissue has decreased vascular density and is more hypoxic and metastatic, recapitulating the clinical features of locally relapsed breast cancer after radiation therapy; HIF-1-dependent Kit ligand expression by hypoxic tumor cells mobilizes c-Kit$^+$ PMN-MDSCs to home to pre-metastatic lungs to promote metastasis (194). In other studies, PMN-MDSCs are recruited by MCP-1 or G-CSF derived from hypoxic tumor cells to pre-metastatic lungs, where they may inhibit the cytotoxicity of NK cells (195, 196).

Endoplasmic Reticulum Stress and Unfolded Protein Response

In homeostatic settings, the endoplasmic reticulum (ER) readily handles the folding of secretory and transmembrane proteins. The hostile conditions in the tumor milieu, such as hypoxia, oxidative stress, nutrient deprivation, and acidic waste accumulation, impair the protein-folding capacity of ER, thus provoking a cellular state of ER stress. When the misfolded proteins exceed a tolerable level, PKR-like ER-resident kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6α (ATF6α) detect the presence of ER stress and trigger unfolded protein response (UPR) to improve the folding efficiency in ER (197). These ER-localized sensors are held inactive by chaperone BiP in steady state, while upon ER stress, the dissociation of BiP activates all three sensors: PERK phosphorylates the translation initiation factor eIF2α, which restricts cap-dependent translation and in turn upregulates activating transcription factor 4 (ATF4) and its downstream target C/EBP homologous protein (CHOP); IRE1α cleaves the X-box-binding protein 1 (XBP1) mRNA, and the spliced mRNA is re-ligated to produce highly active XBP1s that regulates gene expression involved in protein folding; ATF6α fine-tunes UPR by regulating the transcription of ER chaperone genes (197).

Unresolvable ER stress often leads to cell death, while tolerable defect in protein-folding capacity may fuel tumor cell survival, metastasis, angiogenesis, and therapeutic resistance. The immunosuppressive effect of ER stress is receiving growing attention (197). Mahadevan et al. have shown that stressed tumor cells actively regulate the function of myeloid cells. For instance, tumor cells undergoing ER stress release yet unidentified soluble mediators that lead to upregulated UPR markers and pro-inflammatory cytokines in responder macrophages (198). This transmissible ER stress also imprints bone marrow-derived DCs with increased ARG1 and decreased ability to cross-present antigen to CD8$^+$ T cells (199). On the other hand, intrinsic ER stress regulates the myeloid cell activity as well. STAT3 synergizes with STAT6 in macrophages to promote cathepsin secretion and tumor invasion through the IRE1α pathway (200). ER stress and XBP1 activation in tumor-infiltrating DCs lead to abnormal lipid accumulation, which impairs their antigen presentation capacity (201).

In line with macrophages and DCs, MDSCs exhibit clear signs of ER stress and UPR. MDSCs isolated from tumor-bearing host have a higher level of ER stress response markers as compared with monocytes and neutrophils from the same host or healthy control (202). Furthermore, the CHOP level in tumor-infiltrating MDSCs is higher than in splenic MDSCs or other tumor-infiltrating immune cells (203).

Recent studies have demonstrated that ER stress response regulates the homeostasis and suppressive function of MDSCs. ER stress induces apoptosis of MDSCs through upregulation of TRAIL-R or through the eIF2α-ATF4-CHOP pathway; though the lifespan of MDSCs is shortened by ER stress, it may stimulate myelopoiesis and the turnover of MDSCs in tumor-bearing host (202, 203). Administration of the ER stress inducer thapsigargin promotes infiltration of MDSCs in tumor and enhances their suppressive capacity through upregulation of ARG1, iNOS, and NOX2 (204).

Thevenot et al. have elaborately shown that the suppressive activity of MDSCs is regulated by ER stress response marker CHOP (203). In CHOP-deficient mice, tumor growth is significantly retarded, while it is partially restored by depletion of MDSCs, suggesting a reversal of the tumor-promoting activity of MDSCs. Consistently, functional assessment of tumor-infiltrating CHOP$^{-/-}$ MDSCs reveals reduced suppression of T cells, which is associated with decreased ARG1, superoxide, and PNT; furthermore, these CHOP$^{-/-}$ MDSCs acquire a DC-like phenotype and are able to stimulate immune response.

In another study, ER stress-related genes were found to be among the most upregulated in PMN-MDSCs, as compared with neutrophils from the same cancer patient or a healthy individual (205). Surface expression of lectin-type oxidized LDL receptor-1 (LOX-1), which is regulated by ER stress, effectively distinguishes immunosuppressive PMN-MDSCs from neutrophils in cancer patients (205). ER stress induced by thapsigargin promotes LOX-1 upregulation in human neutrophils and converts them into immunosuppressive cells, which is prevented by inhibiting the IRE1α-XBP1s pathway (205, 206). However, whether downstream signaling through LOX-1 is responsible for the acquisition of suppressive activity by neutrophils remains undetermined.

Crosstalk Between MDSCs and Stromal Cells in the Tumor Microenvironment

Many of the tumor-promoting roles of MDSCs, such as immune suppression, pro-angiogenesis, and pro-metastasis, are regulated by the surrounding cells in the tumor microenvironment. How tumor cells regulate the immunosuppressive function of MDSCs has been discussed in previous sections. The induction and suppressive capacity of MDSCs are also fine-tuned during the dynamic and mutualistic communication with the non-malignant stromal cells in the tumor milieu. Many of these cells are not merely targets but also regulators of MDSCs.

MDSCs primarily inhibit T-cell response, and on the other way round, T cells influence the suppressive nature of MDSCs.

The antigen-specific $CD4^+$ T cells, and not $CD8^+$ T cells, enhance the immunosuppressive capacity of MDSCs by turning them into non-specific suppressors *in vitro* and *in vivo*. Mechanistically, this effect requires cross-linking of MHC class II on MDSCs during cell-to-cell contact with activated $CD4^+$ T cells, and the subsequent retrograde signaling in MDSCs upregulates COX2 and PGE2 expression (141). In a recent study, IFN-γ produced by T cells was found to be critical in regulating the enhanced suppressive activity of MDSCs induced by TLR2 ligand, which promoted differentiation of MDSCs into $iNOS^+$ macrophages (207).

In addition to immunosuppression, T cells regulate the induction of MDSCs. It has been reported that $FasL^+$-activated T cells may regulate the homeostasis of MDSCs through Fas-FasL interaction, which induces apoptosis of MDSCs (208). In human colorectal cancer, γδT cells promote the recruitment, proliferation, and survival of PMN-MDSCs through secretion of large amounts of IL-17 and other cytokines, including IL-8, GM-CSF, and TNF-α (209). It has been shown in different murine tumor models that TNF-α secreted by $CD4^+$ T cells, and partially by $CD8^+$ T cells, induces myelopoiesis, which increases the frequency of MDSCs (210).

PD-L1 expression on MDSCs is upregulated upon co-culture with T cells (79), and MDSCs are able to induce PD-1 expression on T cells through TGF-β (75, 211). In melanoma-bearing mice receiving IL-2- and TNF-α-coding adenovirus in combination with adoptive T-cell therapy, PD-L1 was upregulated in intratumoral MDSCs, and the frequency of $PD\text{-}1^+$ $CD8^+$ T cells correlated with the PD-L1 expression level on MDSCs in tumor site (70).

Not only do MDSCs promote Treg induction and recruitment: their suppressive function is also modified by Tregs. An earlier study reported that CD80 expression is required for MDSC-mediated antigen-specific T-cell suppression, which is dependent on $CD4^+CD25^+$ Tregs and CTLA-4 and that depletion of $CD4^+CD25^+$ Tregs diminishes the suppression mediated by MDSCs (212). In a more recent study, Treg depletion decreased PD-L1 expression and IL-10 production by MDSCs (73). In a murine model of melanoma, the expansion, recruitment, and activation of MDSCs occurred in a Treg-dependent manner and required the expression of IDO (213). Therefore, it is likely that MDSCs and Tregs do not act separately but rather cooperate reciprocally in immune suppression.

Crosstalk between MDSCs and B cells has been found recently. In one study, MDSCs that accumulated around the germinal center co-localized with B cells in the spleen of tumor-bearing mice, and cell-to-cell interaction through TNFR2 on MDSCs and membranous TNF on B cells promoted the proliferation and differentiation of B cells into IgA-producing plasma cells (214). Both IL-10 and TGF-β are crucial for this MDSC-mediated IgA response. In another study, Bregs from tumor-bearing mice increased the immunosuppressive and pro-metastatic function of MDSCs, partially through the TGF-β type I/II receptor signaling axis (215).

IL-10 is implicated in the interaction between MDSCs and other immune cells. Through cell-to-cell contact, MDSCs produce IL-10 to downregulate IL-12 by macrophages, and macrophages in turn stimulate IL-10 upregulation by MDSCs (216). The increased IL-10 level and reduced IL-12 level consequently skew the immunity toward a tumor-promoting type 2 response. In another recent study, MDSC-derived IL-10 decreased IL-6 and TNF-α while increasing NO produced by macrophages (217). Meanwhile, IL-10 produced by MDSCs may reduce MHC class II molecule expression on macrophages, leading to diminished antigen-presentation capacity (218). This bi-directional crosstalk between MDSCs and macrophages is accentuated by the inflammatory microenvironment. MDSCs isolated from tumor with a heightened IL-1β level produce more IL-10 and downregulate IL-12 by macrophages to a greater degree as compared with MDSCs from less inflammatory tumors (38). This IL-10 elevation by MDSCs requires IL-6 from macrophages and signaling through TLR4 on MDSCs and macrophages (38, 218). This action of inflammation is further corroborated by the findings that pro-inflammatory mediators PGE2 and HMGB1 upregulate IL-10 in MDSCs in the presence of macrophages (21, 218).

It is reported that mast cells not only induce the recruitment but also promote the suppressive function of MDSCs, probably through CD40L-CD40 interaction (219, 220).

Cancer-associated fibroblasts (CAFs) are a heterogeneous group of activated fibroblasts that play pleiotropic roles in tumor development and are able to modulate anti-tumor immunity on various levels. Through secretion of CCL2 and CXCL12, CAFs facilitate the recruitment of MDSCs (221). Meanwhile, pancreatic CAFs produce multiple MDSC-promoting soluble mediators, IL-6 in particular, and favor the differentiation of MDSCs (222). CAFs from hepatic cancer attract monocytes to the tumor microenvironment by CXCL12 and induce their differentiation into MDSCs through IL-6-mediated STAT3 activation (223). The MDSC-promoting effect of CAFs in breast cancer involves epigenetic regulation by histone deacetylase 6 (224). Consistently, inhibiting CAFs leads to reduced *in vivo* induction and intratumoral level of MDSCs (225, 226). On the other hand, MDSCs promote activation and migration of CAFs, suggesting a positive feedback loop that amplifies interaction between them. To further complicate the issue, in recent studies, CAFs show similar phenotypic and immunosuppressive characteristics to the circulating fibrocytes that may arise from MDSCs and may represent a novel MDSC subset (227).

CONCLUSIONS AND PERSPECTIVES

Among the multiple tumor-promoting characteristics of MDSCs, the capacity to suppress T-cell response remains a key hallmark. Given the complexity of the tumor immune microenvironment, it is not surprising that MDSCs are more than a T-cell suppressor and that their function is regulated on multiple levels. With the advances in phenotyping and functional assessment in recent years, a clearer view of the immunosuppressive nature of MDSCs has been achieved. Firstly, several novel suppressive mechanisms have been identified, which makes MDSCs a versatile suppressor. Secondly, the antigenicity of MDSC-mediated T-cell inhibition depends largely on the properties of the effector molecules

utilized, since a different level and duration of intercellular contact is required; furthermore, differential suppressive potency and preferential mechanisms between subsets of MDSCs compartmentalize T-cell suppression in tumor-bearing host: immunosuppression is relatively weak and is antigen-specific in the periphery, while it is strong and is both antigen-specific and non-specific in the tumor milieu. Thirdly, the recognized targets of MDSCs have been extended from T cells to other immune cells, such as NK cells, DCs, and B cells, which broadens their suppressive spectrum and makes them suppressive in both innate and adaptive immunity. Lastly, in addition to clarification of their expansion and activation in the presence of tumor, the development and function of MDSCs are fine-tuned by several microenvironmental factors. With these characteristics unraveled, a pivotal role of MDSCs in the intricate network of immune suppression within the tumor microenvironment has been unveiled.

As a competent accomplice in carcinogenesis and disease progression, the correlation between MDSCs and tumor burden and disease stage is well-documented. For instance, a recent meta-analysis has shown for the first time that a high level of MDSCs is associated with shorter survival outcomes in patients with solid tumors and hematologic malignancies (228). This notion has two therapeutic implications. On the one hand, MDSCs have been regarded as an attractive target in cancer therapy. Various pre-clinical and clinical studies have shown promising benefits by targeting MDSCs, which can be achieved by depleting their quantity, blocking their trafficking, or inhibiting their immunosuppressive activity (5). On the other hand, due to their potent immunosuppressive capacity, MDSCs act as a major obstacle to natural anti-tumor immunity, hinder the efficacy of immunotherapy, and constitute an important mechanism for resistance. Accordingly, a high level of MDSCs predicts poor response to immune checkpoint inhibitor ipilimumab in metastatic melanoma patients (66). More importantly, it is rational to target MDSCs in combination with immunotherapy, which may yield a synergistic effect and delay, or even reverse, the occurrence of resistance (66). For instance, as compared to monotherapy, the efficacy of an immune checkpoint inhibitor or cancer vaccine is enhanced by combining with MDSC-targeted therapy in pre-clinical studies and clinical trials (66), and T cell-based immunotherapy efficacy is enhanced by inhibiting the trafficking of MDSCs (229). These benefits are associated with improved T cell-mediated immune response against tumor or increased antigen presentation capacity of DCs, probably due to the relieved inhibition imposed by MDSCs.

However, approaches to combat with MDSCs are still in their infancy, and there are several conundrums to be addressed. Considering their versatility and the complexity of microenvironmental regulation, the suppressive machineries of MDSCs are not likely to act simultaneously, but most probably function in a context-dependent manner. As a consequence, when we target the suppressive function MDSCs, it would be difficult to identify the most relevant target(s). Meanwhile, taking into account the indispensability of myelopoiesis in physiological processes and the phenotypic similarity between MDSCs and normal myeloid cells, it would be challenging to target MDSCs accurately without affecting the normal myeloid compartment.

Since they were firstly reported in the late 1970s and consensus on their nomenclature was reached in 2007, MDSCs as a group of suppressive myeloid cells have received increasing attention, and research on MDSCs is booming. Their roles in malignant and non-malignant settings are becoming clarified. With the effort in the past decade, an algorithm that includes phenotypic and functional, and, if possible, molecular criteria necessary to identify MDSCs was proposed in 2016 (4). This step-by-step approach aims to minimize ambiguity and helps to dissect the function of MDSCs in future studies. For instance, it may help us to better distinguish MDSCs from normal myeloid cells in the same host. Determining how to target MDSCs more precisely and efficiently relies, hopefully, on a better understanding of the development and suppressive nature of MDSCs. Lastly, more clinical trials are needed to validate the synergistic effect of MDSC-targeted therapy and cancer immunotherapy.

AUTHOR CONTRIBUTIONS

YY conceptualized the study, wrote the manuscript, and approved the final draft for publication. YY and CL prepared the figures. TL, XD, and AB reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Interaction Between microRNAs and Myeloid-Derived Suppressor Cells in Tumor Microenvironment

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells generated during a series of pathologic conditions including cancer. MicroRNA (miRNA) has been considered as a regulator in different tumor microenvironments. Recent studies have begun to unravel the crosstalk between miRNAs and MDSCs. The knowledge of the effect of both miRNAs and MDSCs in tumor may improve our understanding of the tumor immune escape and metastasis. The miRNAs target cellular signal pathways to promote or inhibit the function of MDSCs. On the other hand, MDSCs transfer bioinformation through exosomes containing miRNAs. In this review, we summarized and discussed the bidirectional regulation between miRNAs and MDSCs in the tumor microenvironment.

Keywords: MDSC, miRNA, tumor microenvironment, tumor resistance, exosomes

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INTRODUCTION

Tumor immune escape and metastasis are critical steps in cancer progression, which have been implicated in the failure of cancer immunotherapies. To achieve that, cancer helper cells in the tumor microenvironment (TME), including regulatory T cells (T-regs), tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and myeloid-derived suppressor cells (MDSCs), make a great contribution to protect cancer cells from being recognized and eliminated by the immune system (1).

Among all the immune suppressive cells in TME, MDSCs played a vital role in cancer escape from host immune surveillance (2). MDSCs are a group of immunosuppressive cells differentiated from myeloid cells stimulated by chronic inflammation and other pathological conditions (3). MDSCs were characterized by different phenotypes and functions. In humans, MDSCs were divided into two main groups named monocytic myeloid-derived suppressor cells (M-MDSCs) and polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), also referred to as granulocytic myeloid-derived suppressor cells (G-MDSCs) (4). These two groups of MDSCs were defined as $CD33^+CD11b^+HLA\text{-}DR^{-/lo}CD14^+CD15^-$ and $CD33^+CD11b^+HLA\text{-}DR^{-/lo}CD14^-CD15^+$, respectively. In mice, M-MDSCs and G-MDSCs or PMN-MDSCs were defined as $CD11b^+Ly\text{-}6G^-Ly\text{-}6C^{hi}$ and $CD11b^+Ly\text{-}6G^-Ly\text{-}6C^{lo}$ cells (4–6). Recently, some studies defined early-stage myeloid-derived suppressor cells (e-MDSCs) characterized with the phenotype of $CD3^-CD14^-CD15^-CD19^-CD56^-HLA\text{-}DR^-CD33^{+-}$ and reported their functions and development (7).

MicroRNA (miRNA) has been investigated in different cancers, and the evidence of its involvement in the regulation of the tumor microenvironment has been of much interest. Some

studies found that miRNA expression could be mediated by cancer-derived factors, MDSCs, or through direct miRNA import *via* extracellular vesicles (8). miRNAs have been proven to regulate MDSCs through various ways including disrupting the differentiation of myeloid cells, increasing proliferation, and affecting the immunosuppression and function of immune cells. In the hematopoietic system, microRNAs are treated as important regulators of myeloid lineage induction and differentiation, and recent studies have begun to unravel the crosstalk between miRNAs and MDSCs in TME (9).

Exosomes were first found in 1981 (10) as rubbish carriers to clean degraded or wasted cell components. However, with the deepening of the research, the positive function of exosomes like intracellular communication or immune response was gradually exposed to us (11). Although controversial, thought provoking, studies have revealed that tumor-derived exosomes from MDSCs can carry miRNAs that are parts of the tumor microenvironment and protect tumor cells (9, 12). Furthermore, MDSC-derived exosomes are also delivered to support progression and modulate the expansion and suppressive function of MDSCs themselves (13, 14). MDSC-derived exosomes carrying miRNAs would make MDSCs more convenient to interact with tumor cells. On the other hand, miRNAs transferred by tumor-derived exosomes can make a long-distance travel in body fluid to regulate the expansion and function of MDSCs, which assist tumor angiogenesis and invasion.

To create a suitable microenvironment, tumor cells secrete miRNAs, cytokines, and other molecules to escape from immune surveillance. The expression of miRNAs controls the function of MDSCs and inhibitory immune cells, such as T-regs (15). As an essential component of tumor microenvironment, MDSCs lives in the inflammatory environment, causing tumor progression and helping tumors grow and suppressing immunity as well. MDSCs also regulate miRNAs in the microenvironment. Both MDSC and tumors can regulate miRNA expression to ease their increment and metastasis. Furthermore, the exosomes derived from MDSCs and tumors can transport miRNAs locally and over long distance, so that builds a bridge between MDSCs, tumor cells, and the immune network.

Still, there are challenges remaining. The origin of miRNA is complex and needs further validation, and whether the miRNA secreted by MDSCs or tumor cells influences other immune cells in the microenvironment should be clarified. Solving these questions might help in finding the way blocking miRNAs specifically.

In this review, we focus on the mechanisms of how miRNAs exert an effect on MDSC functions, the intercommunication between miRNAs and MDSCs, their effect on the components of the tumor microenvironment, and progress on miRNAs in the exosomes derived from tumors and MDSCs.

MDSCs REGULATES miRNAs IN THE TUMOR MICROENVIRONMENT

Several studies have shown that not only miRNAs regulate MDSC function and differentiation, but MDSCs could also modulate miRNA expression to promote cancer invasion and metastasis (16). It was reported that MDSCs marked with the myeloid differentiation factor schlafen4 (SLFN4), a regulator of myeloid cell differentiation, were identified in gastric cancer, especially in the preneoplastic changes infected by *Helicobacter* (17). miR-130b from SLFN4$^+$MDSC promoted gastric epithelial cell proliferation and was essential for MDSC expressing the function of T-cell suppression (18). As for papillary thyroid carcinoma (PTC), the PMN-MDSCs showed a great effect on PTC progression. It decreased the expression of miR-486-3p, which targeted the NF-kB pathway directly and thus activated the NF-kB pathway and facilitated PTC invasion and, in turn, increased PMN-MDSC expansion and function of repressing T cells (15). However, the basic mechanism or the key cytokines regulating this axis still need to be further studied.

The progression of ovarian carcinoma was investigated to be highly correlated with MDSCs and cancer stem cells (CSCs), which are dispensable for cancer advancement in TME. MDSCs upregulated miR-101 expression and further repressed C-terminal binding protein-2 (CtBP2), a corepressor gene targeting stem cell core genes directly, and thus promoted the stemness and invasion of cancer cells. Thus, the MDSCs-miR-101-CtBP2-cancer cell core genes axis was therefore considered as a potential target for antitumor immunotherapy (19).

MDSCs-DERIVED EXOSOMAL miRNAs MEDIATE TUMOR PROGRESSION

Studies have shown that not only tumor-derived exosomes or extracellular vehicles can mediate the expansion and suppressive function of MDSCs by delivering miRNAs, but MDSC-derived exosomes can also carry miRNAs, which have been certified using next-generation sequencing (13) and exert influence on tumor invasion and metastasis (14).

miR-143-3p in G-MDSC-derived exosomes inhibited integral membrane protein 2B (ITM2B) and activated the PI3K/AKT pathway, thus promoting the cell proliferation of lung cancer (20). It was reported that MDSCs were involved in the resistance of chemotherapy for breast cancer and identified its underlying mechanism with doxorubicin-induced MDSCs (21). The DOX-MDSC produced exosomal miR-126a and promoted the induction of IL-13$^+$Th2 T cells, which secreted IL-13 to increase the proliferation of DOX-MDSC and exosomal miR-126a. The study also found that the exosomal miR-126a of DOX-MDSC repressed MDSC apoptosis and contributed to tumor angiogenesis in an S100A8/A9-dependent way (22).

Geis-Asteggiante et al. provided evidence that MDSC-derived exosomes carry miRNAs. Four differentially abundant miRNAs (miR-7022, miR-7062, miR-5134, and miR-704) had predicted mRNA targets that were part of the apoptotic pathway-inducing Fas, which was also a validated target of miRNA-98a (14). Another 4 miRNAs in MDSC-derived exosomes included miR-9, miR-494, miR-233, and miR-690, which were capable of affecting the cell cycle, resulting in suppressing the differentiation of myeloid cells and increasing MDSC proliferation (23, 24). miR-155, a key miRNA enriched in MDSC-derived exosomes, increases IL-10 production in MDSC and contributes to the crosstalk between MDSCs and macrophages (25–27). miR-155 mediates the MDSC function of

suppression through at least two ways including SOCS1 and inhibiting the generation of $CD4^+Foxp3^+$ regulatory T cells (28).

THE miRNAs IN THE TUMOR MICROENVIRONMENT REGULATE MDSCs FUNCTION BY DIFFERENT SIGNAL PATHWAYS

JAK/STAT Pathway

The Janus kinase/signal transducers and activators for the transcription (JAK/STAT) pathway show great influence on cell proliferation, differentiation, and inducing inflammatory microenvironment for cancer. The STAT family is composed of seven members including STATs 1, 2, 3, 4, 5a, 5b, and 6 (29). Among all these proteins, STAT3 seems to be a key protein for the creation of cancer microenvironment and be involved in MDSC development modulated by miRNAs (29–31). miRNAs have been proven to interact with MDSCs, and STAT3 could be a crucial target within it. miR-17-5p and miR-20a downregulated the suppressive function of MDSCs by targeting the 3'UTR of STAT3 to block its expression, which remarkably reduced the production of reactive oxygen species (ROS) and H_2O_2 (32). However, only G-MDSCs could be inhibited by miR-17-5p, and miR-20a and M-MDSCs showed less affection. It was also demonstrated that miR-17-5p and miR-20a were regulated by tumor-associated factors and the transfection of these miRNAs could be a possible treatment for tumor immunotherapy. miR-6991-3p was markedly reduced in MDSCs from the tumor microenvironment, which means that miR-6991-3P repressed the MDSC expansion and function of inhibiting T-cell proliferation. STAT3 was proved to be the direct target of mir-66991-3p (33). On the contrary, miR-155 and miR-21 synergistically upregulated STAT3 expression indirectly by targeting SHIP-1 and PTEN, respectively, and eventually enhanced the function and expansion of MDSCs. Both were identified as early indicators for predicting patients' reactions to glucocorticoid treatment. Both monocytic and granulocytic MDSCs were influenced by the upregulation of miR-155 and miR-21 (25). Studies also revealed that tumor environment-associated factors activate STAT3 and C/EBPb to increase the transcription of miR-21a, miR-21b, and miR-181b (34). Increased levels of these miRNAs disrupted the mixed-lineage leukemia (MII1)-complex and allowed the PMN-MDSCs to exert their immunosuppressive function. The STAT3/CEBPb-miR-21a/b/181b-MII1 axis provided an effective immunotherapeutic manner against cancer. The M-MDSC in the colorectal cancer (CRC) microenvironment secreted CCL17. This chemokine was combined with CR2 and activated the JAK/STAT3 pathway, which awakened the dormant cancer cells and promoted cancer progression clinically (35). miR-124-3p was demonstrated to inhibit the PD-L1 pathway and STAT3 signaling in CRC, which might indicate that miR-124-3p mediated the MDSCs of CRC through the PD-L1/STAT3 pathway (36). This might be a potential therapeutic target to prevent MDSC accumulation and CRC recurrence and metastasis.

For other STAT proteins, STAT6 is found to strengthen the expansion of G-MDSCs while it weakens the expansion of M-MDSCs, and STAT6 could be inhibited by the overexpression of miR-449c and increases the accumulation of M-MDSCs (37).

SOCS Signal

Suppressor cytokine signaling (SOCS)1, a member of the SOCS family, is an inhibitor of the JAK/STAT pathway (38), which mediates the expansion and suppressive function of MDSCs. A recent study reported that the expression of miR-155 was required for the suppressive function of MDSCs and was a necessity for the T-reg induced by MDSCs (28). miR-155 mediated MDSCs by targeting SOCS1 directly and eliminated the inhibition of the JAK/STAT pathway conducted by SOCS1, thus contributing to the accumulation of MDSCs and exerting immunosuppressive function.

It is known that SOCS3 negatively mediates the expansion and function of MDSCs *via* inhibiting STAT3 (39). miR-30a was demonstrated to target SOSC3 directly and increased the activation of STAT3, participated in MDSC proliferation and immunosuppression by inducing Arg-1, IL-10, and ROS, thus eventually resulting in B lymphoma deteriorated with upregulating MDSC infiltration and suppression (40). miR-9 was also identified as activating the JAK/STAT pathway *via* targeting SOCS3 and promoted the development of eMDSCs in breast cancer. miR-9 improved and coordinated with miR-181a expression, which was also an inhibitor of the STAT pathway by bounding to PIAS3 (41).

However, in ovarian cancer, miR-101 was reduced while SOCS2 gene expression increased. The transection of miR-101 could remarkably downregulate SOCS2 and thus inhibit the invasion and metastasis of ovarian cancer cells (42).

PTEN and PI3K/Akt Pathway

It is well known that PTEN is a key regulator in neutrophils' spontaneous death (43) and the downregulation of CXCR4-mediated chemotaxis (44). miR-494, induced by tumor-derived factors, such as TGF-β1, is reported as an activator of MDSCs. miR-494 downregulates PTEN and activates the PI3K/Akt pathway to enhance the MDSCs' chemotaxis mediated by CXCR4 and change the normal progress on apoptosis and cell death, which promotes the accumulation of MDSCs in tumors (45). The activation of the Akt pathway also facilitates tumor invasion and metastasis. Studies also found that miR-200c, induced by GM-CSF, showed a positive effect on the proliferation and suppressive function of MDSCs by targeting PTEN and friend of Gata2 (FOG2) and further activated the PI3K/Akt and STAT3 pathways (24). miR-21 is demonstrated to regulate MDSC expansion by targeting PTEN, which increases the activity of the STAT3 pathway (25).

RUNX1/YAP Pathway

The classical myeloid differentiation-related gene runt-related transcription factor 1 (Runx1) is modulated during the differentiation and maturation of MDSCs. RUNX1 is one of the core-binding family transcriptional factors and is essential to

hematopoietic lineage and myeloid expression and differentiation (46, 47). Recently, miR-9 has been demonstrated to be inversely correlated with the expression of RUNX1 in lung cancer and miR-9 would inhibit MDSC differentiation and aggravate the suppressive function of MDSCs. Direct injection of miR-9 successfully repressed tumor development. However, further clinical studies were needed to verify whether the miR-9 inhibitor was an effective anti-tumor immunotherapy (46). It was also found that miR-21 maintains the accumulation of MDSCs in the microenvironment of lung cancer *via* inhibiting the expression of RUNX1 (48). In addition, RUNX1 was found to downregulate the expression of yes-associated protein (YAP) to deteriorate tumor progression (49). Thus, the miR-21/RUNX1/YAP axis could be another underlying mechanism for miR-21 mediating MDSCs and tumor growth.

Targeting CCAAT Enhancer-Binding Protein

CCAAT enhancer-binding protein (CEBP) transcription factors show a significant effect on the proliferation and differentiation of myeloid cells (50). miR-486 was considered as a regulator of myeloid cell differentiation and apoptosis by targeting CEBPα, and the expression between miR-486 and CEBPα was inversely correlated in tumor-induced M-MDSCs (TM-MDSCs). TM-MDSCs are a group of cells involved in tumor angiogenesis and immunity escape by suppressing the function of T cells. However, either miR-486 or CEBPα overexpression would inhibit the differentiation of myeloid cells, indicating that both miR-486 and CEBPα were involved in the expansion of TM-MDSCs in tumors (51). Based on the suppressive function of MDSCs in tumor-bearing mice, $\triangle^9$-tetrahydrocannabinol (THC)-induced MDSCs were used to confirm that miR-690 had great potential on maintaining the immunosuppression of MDSCs *via* decreasing the expression of CEBPα and decaying their terminal differentiation (23). Although some studies utilized miR-155 as a promoter for the induction of MDSCs in tumors and the lack of miR-155 led to the deterioration of solid tumor (52), Kim et al. found that miR-155 negatively correlated with the expression of MDSCs and identified CEBP as a target of the miR-155-mediating recruitment of MDSCs (53).

Other Targets

Hypoxia-inducible factor 1α (HIF-1α) plays a major role in converting MDSC differentiation and function in the tumor microenvironment with hypoxia (54). Under hypoxia, miR-210, elevated by HIF-1α, affected Arg1, IL-16, and CXCL12 expression and further exacerbated the function of MDSCs, promoting the development of tumors (55). HIF-1α, a direct target of miR-155, was upregulated in miR-155-deficient MDSCs, which increased the expression of chemokines and further accelerated MDSC infiltration in TME (56). Other miRNAs also presented the function of tumor-inhibiting, for instance, miR-233 remarkably slowed the progression of the tumor by repressing myeloid cell differentiation to MDSCs *via* targeting myocyte enhancer factor 2C (MEF2C) (57).

miR-34a contributes to the expansion of MDSCs by suppressing the expression of N-myc. Instead of promoting MDSC proliferation, miR-34a reduces the apoptosis of MDSCs without an effect on progenitor cell differentiation to increase their infiltration (58). miR-34a was also demonstrated to be the driver of MUC1, promoting C-Myc expression in AML-related EVs and the expansion of MDSCs (59). Moreover, miR-34 was confirmed to have a synergistic effect on MDSCs with TWIST (60), a transcription factor of the bHLH family, and contributes to cancer progression and immune resistance (61).

It was elaborated that the PEG2/miR-10a/AMPK axis played an undeniable role in chemotherapy-resistant breast cancer. The PEG2 released by doxorubicin-resistant cancer cells stimulated miR-10a expression, which was the activator of the AMPK pathway, thus leading to the upregulation of MDSC immunosuppression (62). Further studies of this axis would provide a silver lining for treating chemotherapy-resistant tumors.

It is known that CXCR4 plays an essential role in recruiting MDSCs and promoting the progression and metastasis of CRC (63). miR-133a-3p was proven to be involved in this process by activating RhoA/ROCK signal and was mediated by lncRNA XIST (64).

Zhao et al. came up with a prognostic model of 4-circulating miRNAs (miR-21, miR-130b, miR-155, and miR-28) to predict the outcome of diffuse large B-cell lymphoma and tested its validity with a cohort study. They also revealed the association between the 4-circulating miRNA model and the RAS signal pathway and how the tumor environment affects lymphoma. In tumor progression, the alteration of these miRNAs led to RAS pathway activation and MDSC upregulation (65).

Tumor-Derived Exosomes and Extracellular Vesicles

Exosomes and extracellular vehicles (EVs) can carry and deliver miRNAs to MDSCs and contribute to the regulation of MDSCs as miRNAs secreted *in situ*. Tumors produce EVs and exosomes as a manner of augmenting the immunosuppression of MDSCs in the tumor microenvironment and assisting their invasion and escape from surveillance of immune cells (66, 67). miR-9 and miR-181a in exosomes derived from breast cancer were identified to target SOCS3 and PIAS3, respectively, and further activated the JAK/STAT pathway, thus promoting the amplification and development of eMDSC (41). The miR-21a in exosomes from Lewis lung carcinoma cells accelerates tumor growth through targeting programmed cell death protein 4 (PDCD4) through activating the autocrine production of IL-6 and phosphorylation of the STAT3 signaling pathway and thus enhances the expansion of MDSCs and tumor growth (68). Furthermore, miR-21 in oral squamous cell carcinoma (OSCC) enhanced the immunosuppressive function of MDSCs through an miR-21/PTEN/PD-L1 axis (69) and in esophageal squamous cell carcinoma (ESCC), miR-21 activated the STAT3 pathway carried by cancer-associated fibroblast (CAF)-secreting exosomes, which upregulated the induction of M-MDSC corporate with IL-6 (70). Has-miR-494-3p and has-miR-1260a

in pancreatic ductal adenocarcinoma (PDAC)-derived exosomes mediated the suppressive function of MDSCs in an Smad4-dependent way (71). miR-10a and miR-21a carried by hypoxia-stimulated glioma-derived exosomes (H-GDEs) showed a more aggressive mediating MDSC suppression on $CD8^+$T cells than N-GNEs did. Both miRNAs in exosomes regulated MDSCs separately through miR-10a/Rora/IκBα/NF-κB and miR-21/PTEN/PI3K/AKT pathways (72). The transfer of miR-29a and miR-92a showed similar effects like miR-10a and miR-21a in glioma with the hypoxia tumor environment. Hypoxia-induced glioma produced exosomes to carry miR-29a and miR-92a and transferred them to promote the differentiation of functional MDSCs (73). MiR-107 in the gastric cancer-derived exosomes was caught by MDSCs and inhibited the expression of *DICER1* and *PTEN* genes, thus increasing the expansion of MDSCs and ARG1 expression, respectively (74). miR-1246 in glioma-derived exosomes was demonstrated to mediate MDSC differentiation and activation in a dual-specificity phosphatase 3 (DUSP3)/extracellular signal-regulated kinase (ERK)-dependent mechanism. The expression of exosomal miR-1246 was correlated with glioma recurrence (75). The main signal pathways of MDSCs that interacted with microRNAs in the tumor microenvironment are illustrated in **Figure 1**.

Tumor-derived extracellular vesicles serve as a communication tool for the crosstalk between cells by carrying proteins, RNAs, and DNAs (76). EV-carried miRNAs could mediate the expansion and suppressive function of MDSCs *via* targeting different points or pathways in the tumor microenvironment (77, 78). CLL-derived EVs contributed to MDSC accumulation by transferring miR-155 and could be inhibited by vitamin D (79). A line of miRNAs (miR-146a, miR-155, miR-125b, miR-100, miR-125a, let-7e, miR-146b, miR-99b) in the EVs derived from melanoma was associated with the accumulation of MDSCs and the immunotherapy of checkpoint inhibitors (67).

PERSPECTIVE

Although MDSCs have been studied for decades, the bidirectional regulation between microRNA and MDSCs still needs further investigation. The first question is where the miRNAs are from. MicroRNA can be secreted by various cells, including MDSCs themselves. The origin of miRNA is complex and needs further validation. The next question is whether the miRNA secreted by MDSCs influences other immune cells in the microenvironment. Immune regulation is a network, regulated by cytokines, miRNAs, and other molecules. It is well known that MDSCs and cancer cells secrete exosomes, which contain many miRNAs, and regulate other immune cell functions. Catherine Fenselau et al. used next-generation sequencing, identifying more than 1,400 miRNAs in MDSC-derived exosomes, and 24% of them were related to MDSC (13). Therefore, using advanced technologies, such as the third-generation sequencing, will help us investigate more information about miRNAs in exosomes. In the future, targeting specific miRNA could block or enhance MDSC function. Through systemic or carrier-loaded delivery, it might regulate MDSC function using miRNA-based drugs.

CONCLUSION

Immune escape and chemotherapy resistance are tough problems for the treatment of tumors. However, with continuous studies of factors in the tumor microenvironment,

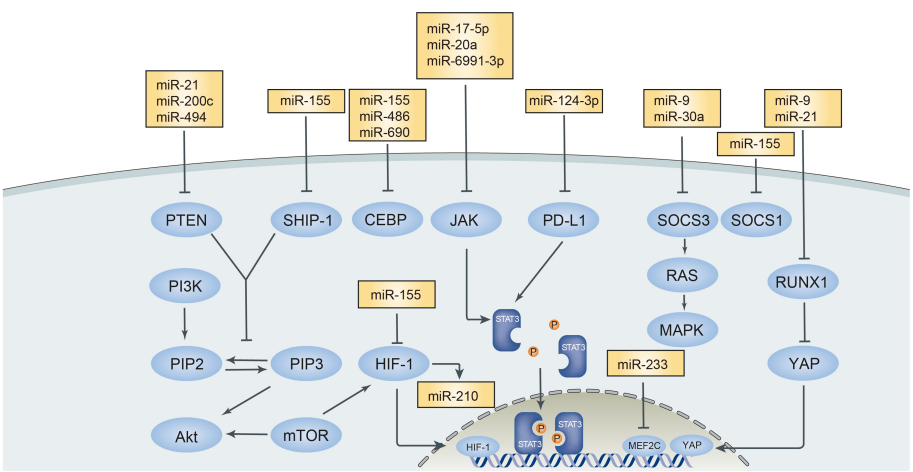

FIGURE 1 | Main signal pathways interacted with microRNAs in tumor microenvironment. MicroRNAs in tumor microenvironment exert positive or negative effect on MDSCs targeting different signal pathways. PTEN, Phosphatase and tensin homolog; SHIP-1, Src Homology 2-containing inositol phosphatase-1; CEBP, CCAAT/enhancer binding protein; JAK-STAT3, Janus kinase-signal transducer and activator of transcription; PD-L1, Programmed death-ligand 1; SOCS3, suppressor cytokine signaling 3; RUNX1, runt-related transcription factor 1; MEF2C, MADS box transcription enhancer factor 2, polypeptide C.

great progress has been made on miRNAs and MDSCs. Multiple studies have elaborated that miRNAs mediate MDSC expansion and function *via* targeting pathways or transcriptional factors including STAT, PTEN, RUNX1, SOCS, CEBP, and other target points. It was also described that MDSCs regulated miRNA expression to facilitate their proliferation and create favorable conditions for tumor growth and invasion. Other than the mechanisms of direct interaction between miRNAs and MDSCs, studies tried to figure out if there were some indirect ways to achieve the same outcome as their counterparts did. The exosomes and extra vehicles secreted from cancer cells and MDSCs carried miRNAs and made a difference in the tumor microenvironment. However, more studies are needed to verify the accuracy and feasibility of the results and data existing.

AUTHOR CONTRIBUTIONS

LL and JL drafted the manuscript. XX and CY revised the manuscript. CY and JL conceived the review design. All authors contributed to the article and approved the submitted version.

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The prognostic value and therapeutic targeting of myeloid-derived suppressor cells in hematological cancers

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The success of immunotherapeutic approaches in hematological cancers is partially hampered by the presence of an immunosuppressive microenvironment. Myeloid-derived suppressor cells (MDSC) are key components of this suppressive environment and are frequently associated with tumor cell survival and drug resistance. Based on their morphology and phenotype, MDSC are commonly subdivided into polymorphonuclear MDSC (PMN-MDSC or G-MDSC) and monocytic MDSC (M-MDSC), both characterized by their immunosuppressive function. The phenotype, function and prognostic value of MDSC in hematological cancers has been intensively studied; however, the therapeutic targeting of this cell population remains challenging and needs further investigation. In this review, we will summarize the prognostic value of MDSC and the different attempts to target MDSC (or subtypes of MDSC) in hematological cancers. We will discuss the benefits, challenges and opportunities of using MDSC-targeting approaches, aiming to enhance anti-tumor immune responses of currently used cellular and non-cellular immunotherapies.

KEYWORDS

hematological cancers, myeloid-derived suppressor cells, immunotherapies, multiple myeloma, leukemia, lymphoma

1 Introduction

The tumor microenvironment is a complex and dynamic network of distinct cell types (1–3). The composition of the environment is variable between different tumor types; however, it typically includes stromal cells, blood vessels, immune cells and extracellular matrix (4). Myeloid-derived suppressor cells (MDSC), tumor associated macrophages (TAM) and regulatory T-cell (Treg) are major components of the microenvironment and are critical drivers of immunosuppression, creating a tumor-promoting and drug resistant niche (5, 6).

MDSC are a heterogeneous population of immature myeloid cells and are generated in the bone marrow (BM) by myelopoiesis (7). Under healthy conditions, the precursor cells can terminally differentiate into mature dendritic cells, granulocytes or macrophages. However, in pathological circumstances including cancer, the differentiation of precursor cells is partially blocked, leading to an accumulation of an immature myeloid cell population, defined as MDSC (8, 9). MDSC are known to accumulate during cancer progression and promote tumor immune escape through multiple mechanisms including (i) the expression of enzymes [e.g., arginase (Arg), nitric oxide synthase (NOS), indoleamine 2,3-dioxygenase (IDO)], (ii) the release of reactive oxygen species (ROS), (iii) sequestering of cystine (↓ extracellular pool of cysteine), (iv) the interaction and stimulation of other immunosuppressive cell types (e.g., Treg) and (v) the secretion of immunosuppressive cytokines (e.g., IL-6, IL-10, TGF-β) (10–13).

In hematological malignancies, the presence and accumulation of MDSC is often correlated with a poor prognosis, however the optimal strategy to specifically eliminate MDSC or alter their suppressive function remains challenging (14). Immunotherapy emerged as one of the most promising treatment options for almost all types of hematological cancers and is primarily focused on the modulation/stimulation of T-cell using monoclonal antibodies, bispecific T-cell engagers, cell therapies, vaccines and immune checkpoint inhibitors (e.g., PD-1-, LAG-3-, CTLA-4-blocking antibodies) (15). In this regard, therapeutic strategies to tackle immunosuppressive cell types (including MDSC, TAM and Tregs) became an interesting option to increase anti-tumor immune responses and overcome the occurrence of drug resistance to currently used or investigated cancer immunotherapies.

2 MDSC phenotype and prognostic value in hematological cancers

MDSC are commonly subdivided into two groups: monocytic MDSC (M-MDSC) and granulocytic (or polymorphonuclear) MDSC (G-MDSC). The phenotype and morphology of M-MDSC is very similar to monocytes, G-MDSC and neutrophils also share common characteristics (e.g., arginase-mediated arginine depletion) (16–18). Despite the morphological and phenotypical similarities, functional differences between steady-state neutrophils and G-MDSC are described including a higher activity of arginase, myeloperoxidase (MPO), and ROS; reduced expression of CD16 and CD62L; and less granules in G-MDSC compared to neutrophils (17, 19). In recent years, it became clear that M-MDSC and G-MDSC also utilize distinct mechanisms to suppress the immune system. M-MDSC hamper T-cell responses in a STAT1/3- and iNOS-dependent manner, which is associated with increased NO and immunosuppressive cytokine production (IL-10, TGF-β). The effect of G-MDSC, on the other hand, is attributed to an antigen-specific induction of T-cell tolerance by STAT3 activity and increased expression of Arg-1, ROS, peroxynitrite and prostaglandin E_2 (8).

In humans, the distinction between MDSC and monocytes/neutrophils can be made based on density gradient and phenotypic markers (e.g., expression HLA-DR), however the distinction between these subtypes in mice is much more challenging and therefore the nature and uniqueness of the MDSC populations continues to be a matter of debate. In murine models, MDSC are phenotypically defined as $CD11b^+GR1^+$ and further subdivided into $CD11b^+Ly6G^-Ly6C^{low}$ for M-MDSC and $CD11b^+Ly6G^+Ly6C^{low}$ for G-MDSC. In humans, both subtypes are distinguished based on the following phenotypic markers: $CD11b^+CD14^+CD15^-CD33^+HLA\text{-}DR^{-/low}$ for M-MDSC and $CD11b^+CD14^-CD15^+CD33^+(CD66b^+)$ for G-MDSC (17, 18). More recently, in humans, a third "early-stage" MDSC subset (eMDSC) has been identified, characterized as Lin^- (CD3/14/15/19/56) $HLA\text{-}DR^-CD33^+$. This subset comprises immature progenitor and precursor cells with colony-forming activity, however its exact function and contribution to immune suppression remains unclear (20). Various reviews described the presence and immunosuppressive function of MDSC in hematological malignancies, however below we aimed to provide a brief and structured overview about the main findings on MDSC subsets and their prognostic value in different hematological cancers as this is particularly important in the context of therapeutic strategies (Table 1) (14, 20, 47–50).

2.1 Leukemia

Acute Myeloid Leukemia (AML) represents the most common myeloid malignancy and is characterized by the expansion of immature myeloid progenitors or blasts in the BM and peripheral blood (PB) (51). In AML, distinct MDSC subsets have been characterized and specifically the circulating M-MDSC subset (defined as $CD14^+HLA\text{-}DR^{low}$) appeared to be elevated and correlated with a poor prognosis in AML patients (21, 22). In addition, eMDSC ($CD33^+CD11b^+HLA\text{-}DR^{-/Low}CD14^-CD15^-$) were also increased in the PB of AML

TABLE 1 Summary of MDSC representative phenotype and their prognostic role in different hematological cancers.

Diseases		Source	MDSC subgroups/phenotype definition	Clinical finding	Ref
Leukemia	AML	PB BM	M-MDSC: $CD11b^+HLA\text{-}DR^-CD14^{-/+}CD33^+CD15^-$ G-MDSC: $CD11b^+HLA\text{-}DR^-CD14^-CD33^+CD15^+$	Higher MDSC level in PB and BM of AML patients VS. HD.	(21)
		PB	M-MDSC: $CD14^+HLA\text{-}DR^{low/-}$	Higher circulating M-MDSC frequencies in $CD14^+$ monocytes and PBMC VS. HD ($p < 0.01$).	(22)
		PB	eMDSC: Lin^-(CD3/14/15/19/56)$HLA\text{-}DR^-CD33^+$	Unknown	(23)
		BM	MDSC: $CD33^+CD11b^+HLA\text{-}DR^{low/neg}$	Significantly increased MDSC in BM ($p < 0.01$).	(24)
	CML	PB	M-MDSC: $CD14^+HLA\text{-}DR^-$ G-MDSC: $CD11b^+CD33^+CD14^-HLA\text{-}DR^-$	MDSC levels were increased at diagnosis and returned to normal levels after therapy ($p < 0.001$, $p < 0.0001$).	(25)
		PB	MDSC: $CD11b^+$ $CD14^-CD33^+$	PB MDSC levels were increased in samples from Sokal high-risk patients ($p < 0.05$).	(26)
	B-ALL	PB BM	M-MDSC: $CD45^+CD19^-HLA\text{-}$ $DR^-CD11b^+CD33^+CD14^+$ G-MDSC: $CD45^+CD19^-HLA\text{-}$ $DR^-CD11b^+CD33^+CD15^+$	G-MDSC were significantly elevated in PB and BM vs. age-matched HD ($p < 0.05$, $p < 0.01$). G-MDSC levels correlated positively with clinical therapeutic responses and B-ALL disease prognostic markers.	(27)
		PB	MDSC: $LinHLA\text{-}DR^-CD33^+CD11b^+$	MDSC levels significantly increased in early diagnosed B-ALL patients VS. HD.	(28)
	CLL	PB	M-MDSC: $CD14^+HLA\text{-}DR^{low/-}$	The M-MDSC were upregulated in patients ($p < 0.0001$) and were correlated with CLL tumor progression, poor prognosis, and correlated with the presence of $CD4^+$ T and $CD5^+CD19^+$ cells.	(29)
		PB	M-MDSC: $CD14^+CD11b^+CD15^-HLA\text{-}DR^{low/-}$	M-MDSC were increased in PB of CLL Patients and correlated with The Rai Stage ($p < 0.001$), and a close association with unfavorable prognostic markers.	(30)
		PB	M-MDSC: $CD14^+CD11b^+CD15^-HLA\text{-}DR^{low/-}$	Higher median percentage of M-MDSC with IL-10 or TGF-1 expression in CLL patients than in HD ($p < 0.001$, $p < 0.0001$).	(31)
		PB	M-MDSC: HLA-DR $CD11b^+CD33^+CD14^+$ G-MDSC: $HLA\text{-}DR^{low}CD11b^+CD33^+CD15^+$	Higher numbers of G-MDSC in patients correlated with different Th- subsets, and were more strongly associated with a poor clinical course than M-MDSC.	(32)
Lymphoma	DLBCL	PB	M-MDSC: $CD14^+HLA\text{-}DR^-$ G-MDSC: $Lin^-CD123^-HLA\text{-}DR^-CD33^+CD11b^+$	Increased M-MDSC and G-MDSC populations in whole blood VS. HD ($p = 0.001$, $p = 0.01$). M-MDSC were correlated with the IPI and EFS ($p = 0.034$, hazard ratio = 0.19).	(33)
		PB	M-MDSC: $CD14^+HLA\text{-}DR^{low/-}$	Increased frequency of M-MDSC was found in ND vs. HD ($p < 0.01$) and associated with tumor progression in patients. (ND vs. Rel VS. Rem, $p < 0.05$, $p < 0.01$).	(34)
	HL	PB	MDSC: $CD11b^+CD33^+CD14^-CD34^+HLA\text{-}DR^-$ M-MDSC: $CD14^+HLA\text{-}DR^{low/-}$ G-MDSC: $CD11b^+CD33^+CD14^-HLA\text{-}DR^-Lin^-$	All MDSC subsets (immature MDSC, G-MDSC, M-MDSC) were higher in patients VS. HD ($p = 0.03$, $p = 0.02$, p 0.04), and higher MDSC percentages were present in non-responders. $CD34^+$ immature MDSC were predictive for a short PFS in HL patients ($p = 0.03$).	(35)
	B-NHL	BM	M-MDSC: $CD14^+CD33^+HLA\text{-}DR^-$ G-MDSC: $CD10^-HLA\text{-}DR^{low/-}$	Differences in M-MDSC (ND, Rem and Rel of B-NHL patients vs. HD, $p < 0.0001$, $P < 0.001$, $p < 0.001$). G-MDSC% was increased in PB (ND and Rem and Rel of B-NHL patients vs. HD, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$).	(36)
Multiple Myeloma		PB	M-MDSC: $CD14^+HLA\text{-}DR^{low/-}$	Increased level of MDSC in patients with MM at diagnosis VS. HD ($p < 0.05$).	(37)
		PB BM	M-MDSC: $CD14^+HLA\text{-}DR^{low/-}$	M-MDSC of ND MM patients were increased in PB and BM vs. HD ($p < 0.01$), and were associated with MM progression and response to therapy (ND and Rem and Rel of MM patients VS. HD, $p < 0.01$).	(38)
		PB BM	M-MDSC: $CD11b^+CD14^+HLA\text{-}DR^{low/-}$ G-MDSC: $CD11b^+CD33^+HLA\text{-}DR^{low/-}CD14^-CD15^+$	PB M-MDSC show correlation with serum creatinine, lactate dehydrogenase, and β-microglobulin and inverse correlation with hemoglobin level PB M-MDSC of patients with progressive disease showed higher levels than those of patients at diagnosis and in complete response ($p = 0.003$ and 0.026, respectively). BM M-MDSC levels were higher in patients with progressive disease than those in patients at diagnosis ($p = 0.007$). PB M-MDSC > 0.3%) at diagnosis had an independent adverse prognostic impact on OS.	(39)

(Continued)

TABLE 1 Continued

Diseases	Source	MDSC subgroups/phenotype definition	Clinical finding	Ref
	PB	M-MDSC: $CD14^+HLA\text{-}DR^{low/-}$ eMDSC: $CD11b^+Lin^-(CD3/14/15/19/56)HLA\text{-}DR^-CD33^+$	In the pre-ASCT analyses, lower M-MDSC (median) were associated with a longer time to progression (TTP) ($p < 0.001$). Pre-ASCT M-MDSC more strongly inhibited the *in vitro* cytotoxic effect of mephalan compared with pre-ASCT eMDSC ($p < 0.01$).	(40)
	PB	M-MDSC: G-MDSC: $CD10^-HLA\text{-}DR^{low/-}$	Higher G-MDSC in PB of ND and Rel VS. HD ($p = 0.03$, $p < 0.001$).	(41)
	PB BM	M-MDSC: $CD11b^+CD33^+CD15^-$ G-MDSC: $CD11b^+CD33^+HLA\text{-}DR^{low/-}CD14^-CD15^+$	G-MDSC are increased in BMMC of MM patients (highest in RRMM) VS. MGUS/SMM patients or HD ($p < 0.05$). G-MDSC in BMMC and PBMC of MM patients expressed higher levels of PD-L1 ($p < 0.05$).	(42)
	PB BM	G-MDSC: $HLA\text{-}DR^{low/-}$ $CD33^+CD11b^+CD15^+CD14$	There is an association between high G-MDSC levels and poor OS in PB and BM of MM patients vs. HD ($p < 0.05$, $p < 0.01$).	(43)
	PB	M-MDSC: $CD33^+CD11b^+HLA\text{-}DR^{low/-}CD14^+CD15$ G-MDSC: $CD33^+CD11b^+HLA\text{-}DR^{low/-}CD14^-CD15^+$	The G-MDSC subpopulation was increased in samples from patients with MM (both patients with progressive disease and patients with stable disease vs. age-matched controls, $p < 0.0001$, $p < 0.0445$.)	(44)
	PB	M-MDSC: $CD66b^+CD15^-CD14^+HLA\text{-}DR^-$ G-MDSC: $CD66b^+CD15^+CD14^-HLA\text{-}DR^-$	G-MDSC and M-MDSC were increased in PB of MM VS. HD ($p < 0.0001$). $Argl^+$G-MDSC percentage was increased in PB of ND MM patients VS. MGUS ($p < 0.0001$), and it was higher in RRMM VS. ND ($p < 0.0001$).	(45)
	BM	G-MDSC: $CD11b^+CD13^+CD16^+$	G-MDSCs are defined as $CD11b^+CD13^+CD16^+$ neutrophils in MM.	(46)

PB, peripheral blood; BM, bone marrow; HD, health donors; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; B-ALL, B-Cell acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; HL, Hodgkin's lymphoma; B-NHL, B-Cell non-Hodgkin lymphoma; M-MDSC, monocytic myeloid derived suppressor cells; G-MDSC, granulocytic myeloid derived suppressor cells; IL-10, interleukin 10; TGF-β1, transforming growth factor beta 1; Th cells, helper T-cells; ND, newly diagnosed; Rel, relapsed; Rem, remission; IPI, international prognostic index; EFS, event-free survival; PFS, progression-free survival; BMMC, bone marrow mononuclear cell; PBMC, peripheral blood mononuclear cell; RRMM, relapsed/refractory multiple myeloma; OS, Overall survival; MGUS, monoclonal gammopathy of undetermined significance.

patients, however its impact of prognosis remains unknown (23). Interestingly, Sun et al. observed a correlation between the total number of MDSC in the BM ($CD33^+CD11b^+HLA\text{-}DR^{low/-}$) and minimal residual disease (MRD) (determined by flow cytometry), as MDSC levels in the high MRD group ($MRD > 1\times10^{-2}$) was significantly higher than that in the middle ($1\times10^{-2} > MRD > 1\times10^{-4}$) and the low ($MRD < 1\times10^{-4}$) MRD groups (24).

Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell malignancy characterized by the acquisition of the t (9, 48) chromosomal translocation leading to expression of the BCR/ABL oncogene (52). Both M-MDSC ($CD14^+HLA\text{-}DR^-$) and G-MDSC ($CD11b^+CD33^+CD14^-HLA\text{-}DR^-$) were increased in the PB of CML patients compared to healthy controls and treatment with the tyrosine kinase inhibitor imatinib decreased the MDSC percentages to normal levels (25, 53). Although higher levels of G-MDSC could be detected in high-risk patients (based on Sokal score) compared to low-risk patients, its impact on prognosis needs to be further elucidated (26).

In precursor B cell Acute Lymphoblastic Leukemia (B-ALL), a malignancy of precursor B cells with the highest incidence among children, elevated levels of G-MDSC ($CD45^+CD19^-HLA\text{-}DR^-CD11b^+CD33^+CD15^+$) in the PB and BM of newly diagnosed patients has been observed (27). Similar to the findings in AML, a correlation could be observed between the G-MDSC levels, in the BM and PB, and MRD status of B-ALL patients at diagnosis. In addition, the frequency of G-MDSC correlated positively with other prognostic indicators including the percentage of $CD20^+$ cells and blast cells (14, 27, 28).

Chronic Lymphocytic Leukemia (CLL) arises from the clonal expansion of $CD5^+$ B lymphocytes in the BM (54). A study of 49 CLL patients demonstrated an upregulation of $CD14^+HLA\text{-}DR^{low/-}$ M-MDSC compared to healthy patients (29). In addition, the elevated levels of M-MDSC were significantly correlated with tumor progression and a poor prognosis of CLL patients (30). The negative impact of M-MDSC ($CD14^+CD11b^+CD15^-HLA\text{-}DR^{-/low}$) on the clinical outcome of CLL patients was also confirmed by Kowalska et al. (31). In contrast, the study by Ferrer et al. found a significant increase in the G-MDSC ($HLA\text{-}DR^{low}CD11b^+CD33^+CD15^+$) number of CLL patients which was associated with a poor clinical outcome. While CLL-derived G-MDSC suppressed T-cell growth *in vitro*, M-MDSC were less immunosuppressive due to the presence of TNFα and were defined as a more immunostimulatory subtype. The authors concluded that the G-MDSC appeared to be the preferred subtype to target, since they more effectively induce immune suppression in CLL patients (32).

2.2 Lymphoma

Diffuse large B cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL) hat develops from the B lymphocytes. Azzaoui et al. observed an increase in M-MDSC ($CD14^+HLA\text{-}DR^{low}$) and G-MDSC ($Lin^-HLA\text{-}DR^-CD33^+CD11b^+$)

populations in DLBCL patients, however the M-MDSC were the only subset that could be correlated with the International Prognostic Index and event-free survival (33). This observation was confirmed by Wang et al. who demonstrated a significant increase in the circulating M-MDSC ($CD14^+CD33^+HLA\text{-}DR^{-/low}$) of newly diagnosed and relapsed DLBCL patients and found that the level of M-MDSC could be used as a biomarker for poor prognosis of DLBCL patients (34).

The presence of Reed-Sternberg cells is a specific hallmark of Hodgkin lymphoma (HL). Romano et al. demonstrated that all circulating MDSC subsets ($CD11b^+CD33^+CD14^-CD34^+HLA\text{-}DR^-$ or immature MDSC, $CD11b^+CD33^+CD14^-HLA\text{-}DR^-$ or G-MDSC, $CD14^+HLA\text{-}DR^{low/-}$ or M-MDSC) were increased in HL patients compared to normal subjects. Higher MDSC percentages were present in non-responders and $CD34^+$ immature MDSC were predictive for a short progression-free survival in HL patients (35).

More recently, a study in B-NHL patients including CLL, DLBCL, marginal zone lymphoma (MZL), high-grade B-cell lymphoma (HGBL), mantle-cell lymphoma (MCL), primary central nervous system lymphoma (PCNSL) and follicular lymphoma (FL) patients was carried out to investigate the impact of MDSC number and subsets ($CD14^+CD33^+HLA\text{-}DR^{-/low}$ for M-MDSC, $CD10^-HLA\text{-}DR^{-/low}$ for G-MDSC) on B-NHL patient's prognosis. A significant increase could be observed in the levels of M-MDSC and G-MDSC in the diverse types of B-NHL compared to healthy donors. MDSC levels were closely associated with disease progression (tumor stage, LDH levels) and both subsets were defined as effective indicators of poor prognosis in B-NHL patients (36, 55).

2.3 Multiple myeloma

Multiple myeloma (MM) is a plasma cell malignancy in which monoclonal plasma cells proliferate in the BM (56). Controversial results were reported regarding the MDSC levels and subtypes present in MM patients. One of the first studies demonstrated elevated levels of M-MDSC ($CD14^+HLA\text{-}DR^{-/low}$) in MM patients at diagnosis compared to healthy controls (57). In addition, M-MDSC levels were correlated with tumor progression and MDSC levels could be considered as an indicator for the efficacy of therapy (37, 38). A study by Bae et al. recently confirmed the independent adverse prognostic impact of PB derived M-MDSC in patients with MM and suggested the analysis of M-MDSC as a prognostic marker in clinical practice (39). In the context of autologous stem cell transplantation (ASCT), lower M-MDSC levels were associated with a longer time to progression. Interestingly, pre-ASCT derived M-MDSC strongly inhibited the *in vitro* cytotoxic effect of melphalan; which could be reduced by the blockade of colony-stimulating factor 1 receptor (CSF1R) (40). However, more recent studies demonstrate a significant increase of G-MDSC ($CD11b^+CD33^+HLA\text{-}DR^{-/low}CD14^-CD15^+$) in BM and PB of MM patients compared to monoclonal gammopathy of undetermined significance (MGUS), smoldering MM patients and healthy controls, while no significance could be observed for M-MDSC (41–44). The increase in G-MDSC was also associated with MM disease activity and could be used to predict the response to immunomodulatory agent lenalidomide (45). Perez et al. also observed a correlation between the clinical significance, immunosuppressive potential, and transcriptional network of well-defined neutrophil subsets. In addition, they suggested a set of optimal markers (CD11b/CD13/CD16) for accurate monitoring of G-MDSC in MM patients (46).

3 Therapeutic approaches to target MDSC in hematological cancers

In past years, some specific and various unspecific strategies have been investigated to either modulate the MDSC suppressive function, affect their differentiation/maturation potential, block MDSC development or deplete this cell population in the tumor microenvironment. Below, and in Figure 1 and Table 2, we will summarize all strategies that have been tested in the context of hematological cancers.

3.1 MDSC depleting agents

3.1.1 Cytotoxic therapies

5-Fluorouracil and Gemcitabine, both chemotherapeutic compounds routinely used in the clinic for the treatment of cancer, have been described to decrease the number of MDSC in preclinical mouse models of hematological cancers (58–60). Due to the low selectivity and dose-dependent toxicity, various encapsulated gemcitabine formulations have been developed and examined for safety and tumor-directed toxicity. Sasso et al. demonstrated that low dose gemcitabine-loaded lipid nanocapsules efficiently targeted the M-MDSC subset and relieved tumor-associated immunosuppression *in vitro* and *in vivo* using the E.G7-OVA lymphoma model. The efficient uptake of the nanocapsules into the M-MDSC subset was attributed to a mechanism called 'macropinocytosis'. Moreover, authors found that preconditioning with low dose gemcitabine-loaded lipid nanocapsules enhanced the efficacy of adoptive T-cell therapy in the E.G7-OVA tumor model, further illustrating its potential as immune modulating therapy in cancer (61).

3.1.2 Monoclonal antibodies

Daratumumab is an anti-CD38 monoclonal antibody, FDA approved in 2015 for the treatment of relapsed/refractory MM patients. Besides the ubiquitous expression of CD38 on MM cells, CD38 antigen is also expressed by other cell types including

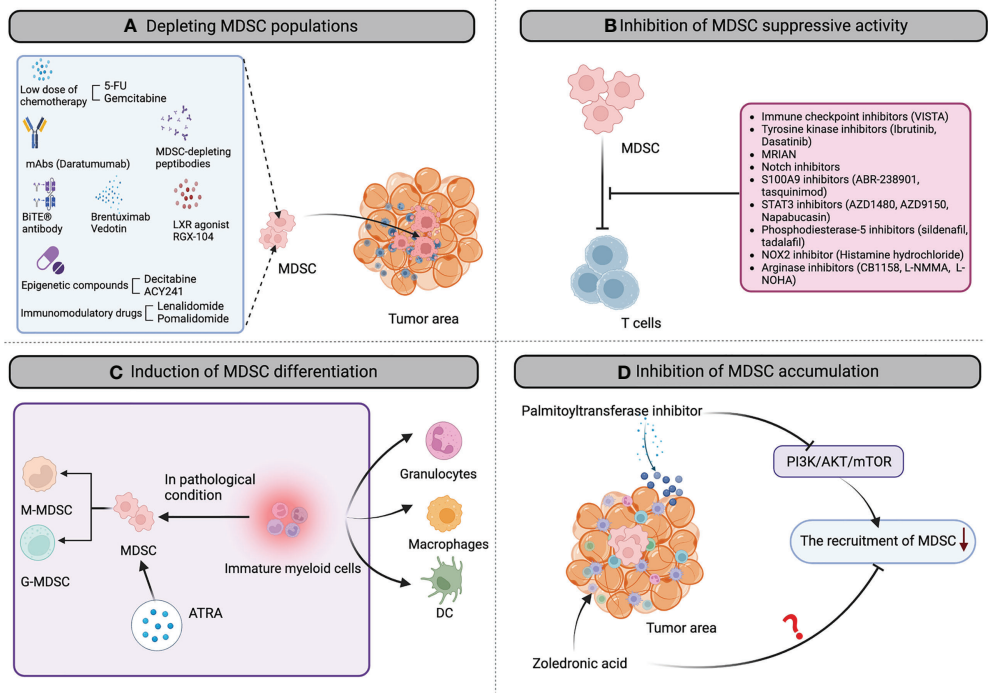

FIGURE 1
The landscape of MDSC-targeting strategies in hematological cancers. Multiple MDSC-targeting approaches were evaluated in hematological cancers to restore the anti-tumor immune response, including: **(A)** depleting MDSC populations through low-dose chemotherapy agents, mAbs, peptibodies, brentuximab vedotin, epigenetic compounds, CD33/CD3-bispecific T-cell engaging (BiTE®) antibody, LXR agonist RGX-104, Immunomodulatory drugs et al; **(B)** attenuating the immunosuppressive mechanisms of MDSC by immune checkpoint inhibitors, tyrosine kinase inhibitors, MRIAN, notch inhibitors, S100A9 inhibitors, STAT3 inhibitors, phosphodiesterase-5 inhibitors, histamine hydrochloride; arginase inhibitors; **(C)** inducing the differentiation of MDSC into mature myeloid cells by all-trans-retinoic acid (ATRA) to reduce MDSC population and remove their immunosuppression; **(D)** inhibiting MDSC accumulation in the tumor microenvironment by palmitoyltransferase inhibitor and zoledronic acid. mAb, monoclonal antibody; BiTE, bi-specific T-cell engagers; 5-FU, 5-fluorouracil; LXR, activation of liver X receptor; STAT3, signal transducer and activator of transcription 3; NOX2, NADPH oxidase 2; ATRA, all-trans-retinoic acid; DC, dendritic cell.

MDSC and regulatory B cells (62). Krejcik et al. demonstrated that *in vitro* generated G-MDSC ($CD11b^+CD14^-HLA–DR^-CD15^+CD33^+$) expressed elevated CD38 and were highly sensitive to daratumumab-mediated ADCC/CDC compared with the isotype control. Findings were confirmed in patients treated with a combination of lenalidomide, dexamethasone with or without daratumumab. Using western blot analysis, a selective reduction of M-MDSC was observed in patients treated with the triple combination compared to patients treated with dexamethasone and lenalidomide (93). Data obtained by Cohen et al. further supported the daratumumab-mediated depletion of M-MDSC using a combination of daratumumab and anti-PD-1 monoclonal antibody cetrelimab in relapsed/refractory MM patients (63).

3.1.3 MDSC-depleting peptibodies

Using a competitive peptide phage display platform, candidate peptides were identified that specifically bind to MDSC derived from EL4 mice, a murine lymphoblastic tumor model. Peptides were fused with the Fc portion of mouse IgG2b to generate MDSC-specific peptibodies. *In vivo* studies in lymphoma models including A20, EL4 and E.G7-OVA demonstrated that the peptibodies were able to deplete intra-tumoral MDSC, without affecting other inflammatory cell types (e.g., dendritic cells and T-cell). In contrast to anti-GR1 depleting antibodies which preferentially eliminate G-MDSC, peptibodies were able to deplete both M-MDSC and G-MDSC subsets. Peptibodies significantly delayed tumor growth in EL4 mice and alarmins S100A8/S100A9 were identified as potential candidate targets expressed by the MDSC (64, 94).

3.1.4 Brentuximab vedotin

Brentuximab Vedotin (BV) is an antibody-drug conjugate designed to selectively deliver monomethylauristatin E, a microtubule-disrupting agent, to CD30-expressing cells. The compound has been FDA approved in 2018 for the treatment of patients with previously untreated stage III or IV classical HL in combination with chemotherapy (95). Although it remains unclear whether CD30 is expressed or not on MDSC subsets, Romano et al. demonstrated that BV reduced the absolute number

of three MDSC subtypes ($CD11b^+CD33^+CD14^-CD34^+HLA\text{-}DR^-$; M-MDSC and G-MDSC) coinciding with reduced soluble Arg-1 levels and restored the entire T-cell populations in HL patients; indicating its therapeutic use as MDSC targeting agent (65).

3.1.5 Epigenetic compounds

5-aza-2′-deoxycytidine, also known as decitabine (DAC), has been shown to act as an irreversible inhibitor of DNA methyltransferases and induces gene-specific DNA demethylation when administered at a low dose (96). Besides the reactivation of tumor suppressor genes through demethylation, DAC exerts pleiotropic effects on the tumor immune microenvironment including the upregulation of MHC-I/MHC-II expression levels, the increased expression of co-stimulatory molecules and the targeting of immunosuppressive cell types. The effect of DAC on MDSC subtypes was analyzed in leukemia (WEHI-3), lymphoma (EL4) and MM (MPC11) models *in vitro* and *in vivo*. DAC treatment induced MDSC apoptosis ($CD11b^+GR1^+$) *in vitro* and increased T-cell activation in leukemia and lymphoma models. In the MCP11 MM model, DAC inhibited MM cell proliferation and induced an autologous T-cell immune response by depleting the M-MDSC subset in the MM BM microenvironment (66, 67).

Histone deacetylase (HDAC) inhibitors (e.g. entinostat, valproic acid, vorinostat) are another class of epigenetic compounds and were also reported to reduce MDSC levels or inhibit MDSC suppressive capacity in solid tumor models (97). Treatment of BM mononuclear cells of MM patients with ACY241, an HDAC6 selective inhibitor, significantly reduced the $HLA\text{-}DR^{low/-}CD11b^+CD33^+$ MDSC population, while it augments the immune response as evidenced by increased perforin/CD107a expression, IFN-γ/IL-2/TNF-α production and antigen-specific central memory cytotoxic T lymphocytes (68).

3.1.6 CD33/CD3-bispecific T-cell engaging (BiTE®) antibody

AMG 330 is the first BiTE® developed against CD33, an antigen that is not only expressed on the majority of AML-blasts, but also on M-MDSC (98). Jitschin et al. observed an increase in the percentage of $HLA\text{-}DR^{low}$ ($CD14^+CD11b^+$) M-MDSC, that co-express CD33, in newly diagnosed AML patients compared to healthy controls. In the presence of AMG 330, T-cell were able to eliminate $CD33^+IDO^+$ *in vitro* generated MDSC. Adding MDSC to co-cultures of T-cell and AML cells resulted in reduced AML-blast killing, while the addition of an IDO inhibitor promoted the AMG 330-mediated clearance of AML-blasts. Data suggest a dual anti-tumor effect of AMG 330 through increased T-cell mediated cytotoxicity against AML blasts and $CD33^+$ MDSC (69). Another study by Cheng et al. evaluated the effects of AMV 564, a novel bivalent CD33/CD3 T-cell engager and showed immunodepletion of MDSC and anti-tumor activity using primary samples of myelodysplastic syndrome (MDS) patients and a disseminated leukemia mouse model (70).

3.1.7 LXR agonist RGX-104

Liver-X nuclear receptors (LXR) are members of the nuclear hormone receptor family that drive, among others, the transcriptional activation of ApoE. Masoud et al. observed that an LXR agonist RGX-104 induces apoptosis of MDSC and enhances T-cell activation in solid tumor models (71). RGX-104 is currently evaluated in an ongoing phase 1 clinical trial in patients with metastatic solid cancers or lymphomas that have progressed on standard therapies (NTC02922764). Blood sample analysis revealed a depletion of G-MDSC and increased T-cell activation after treatment of cancer patients with RGX-104.

3.1.8 Immunomodulatory drugs

Immunomodulatory drugs (IMiDs), including lenalidomide and pomalidomide, are a group of drugs that are derivatives from thalidomide and are routinely used in the treatment of MM (99). Kuwahara-Ota et al. examined the impact of IMiDs on MDSC *in vitro* and found a significant reduction in MDSC level upon coculture of MM-derived PB mononuclear cells and human MM cell lines, with pomalidomide being more potent than lenalidomide (72). However, clinical evidence supporting this hypothesis is missing as lenalidomide-treated patients showed a higher abundance of $CD14^+CD15^+$ MDSC. Moreover, *in vitro* findings by Görgun et al. demonstrated that lenalidomide could not overcome MDSC-mediated T-cell suppression in MM (100). In the A20 lymphoma tumor model, a lenalidomide-associated reduction in systemic MDSC number and increased immune activation has been observed, further illustrating the controversy regarding the impact of lenalidomide on MDSC populations, depending on the used tumor model and type.

3.2 Inhibition of MDSC suppressive activity

3.2.1 Immune checkpoint inhibitors

In MM, the immune checkpoint PD-L1 was significantly higher expressed on the G-MDSC subset of BM and PB-derived MM patients (newly diagnosed and relapsed) compared to G-MDSC of MGUS and healthy individuals (42). Although some studies in solid tumors suggest that PD-L1 blocking could partially restore the MDSC suppressive function, Ahn and colleagues could not observe any effect of a PD-L1 blocking antibody on splenic MDSC number or subsets in the MOPC-315 immunocompetent MM model (101–104). To fully elucidate whether PD-L1 expression on MDSC is linked to its suppressive

TABLE 2 Overview of MDSC-targeting approaches in hematological cancers.

Agents		Disease	Model	Mechanisms/ Functions	Ref
Cytotoxic therapies	5-FU	Lymphoma	EL-4 syngeneic model	Gemcitabine and 5-FU decreased the number of MDSC.	(58)
		MM	5T33MM model	Targeting MDSC by anti-GR1 antibodies and 5-FU reduced tumor load.	(59)
	Gemcitabine	Lymphoma	A20 syngeneic model E.G7-OVA model	Accumulation of MDSC in the spleen of lymphoma-bearing mice. Lipid nanocapsules loaded with a lauroyl-modified form of gemcitabine efficiently target the M-MDSC subset.	(60, 61)
Monoclonal antibodies	Daratumumab	MM	Patient PB, BM samples	G-MDSC expressed elevated CD38 and were highly sensitive to daratumumab-mediated ADCC/CDC. Daratumumab-mediated depletion of M-MDSC using a combination of daratumumab and cetrelimab in RRMM patients.	(62) (63)
MDSC-depleting peptibodies	Peptibodies	Lymphoma	EL-4 syngeneic model	*In vivo*, intravenous peptibodies injection depleted blood, splenic and intra- tumoral MDSC. S100 family proteins were identified as candidate targets.	(64)
Brentuximab Vedotin		HL	Patient PB samples	BV reduced the absolute number of three MDSC subtypes and s-Arg-1 levels. Patients with baseline s-Arg-1 >200 ng/ml had inferior PFS at 36 months.	(65)
Epigenetic compounds	Decitabine	Lymphoma Leukemia MM	EL-4 syngeneic model WEHI-3 model MPC-11 model	DAC treatment depleted MDSC *in vivo*. DAC activated adaptive T-cell response *in vitro* and autologous T-cell response to tumor cells *in vivo* by depleting MDSC. DAC treatment inhibited MPC-11 proliferation *in vivo* by depleting M-MDSC and increasing T-cell infiltration in tumor tissue.	(66) (67)
	ACY241	MM	Patient BM samples	ACY241 decreases the frequency and expression of immune checkpoints on $CD138^+$ MM cells, regulatory T-cells and MDSC.	(68)
CD33/CD3-bispecific BITE® antibody	AMG330	Leukemia	Primary AML-blasts	AMG330 triggers T-cell mediated lysis of AML-blasts that is further enhanced by MDSC depletion.	(69)
	AMV564	MDS	MDS BM primary samples, CD33hi SKM1 xenograft model	AMV 564 showed anti-tumor activity by immunodepletion of MDSC in primary MDS patients and in a disseminated leukemia mouse model.	(70)
LXR agonist RGX-104	RGX-104	Lymphoma		LXR agonist treatment promotes MDSC apoptosis *in vitro* and *in vivo*. Patient blood sample analysis revealed a depletion of G-MDSC after treatment of cancer patients with RGX-104.	(71)
Immunomodulatory drugs	Lenalidomide Pomalidomide	MM	Patient PB, BM samples	LEN and POM prevent MDSC induction through transcriptional expression and production of CCL5 and MIF, and increased the mRNA level of IRF8 (a negative regulator of differentiation towards MDSC) in PBMC.	(72)
Immune checkpoint inhibitors	VISTA-targeting	AML	Patient PB samples C1498 syngeneic PD-1H knockout model	VISTA is highly expressed on MDSC in patients, and increased in ND patients. VISTA knockout/targeting diminished the inhibition of CD8 T-cell activity by MDSC in AML. VISTA on host cells and AML cells induces immune evasion in AML.	(73, 74)
Tyrosine kinase inhibitors	Ibrutinib	CLL	A cohort of previously untreated CLL patients, PBMC samples	Ibrutinib therapy selectively alters the numbers of MDSC, $CD4^+$ and $CD8^+$ T-cells and Th-cell subsets *in vivo*.	(32)
	Dasatinib	CML	Patients and age-matched HD PB samples	The percentage of M-MDSC correlates with MMR in patients treated with dasatinib.	(75, 76)
Metabolic Reprogramming Immunosurveillance Activation Nanomedicine	MRIAN	T-ALL	Activated Notchl mutant driven T-ALL model	MRIAN efficiently penetrates BM and selectively targets leukemic cells and MDSC in T-ALL mice. MRIAN Inhibits mitochondrial metabolism and reduces ROS levels in MDSC.	(77)
Notch inhibitors	ADAM10	T-ALL	ADAM10 transgenic (A10Tg) model	ADAM10 overexpression in transgenic mice resulted in a systemic expansion of MDSC. The accumulation of MDSC was attributed to the differential cleavage of Notch in S2 and S3 products by ADAM10.	(78–80)
			Patient PB samples	Daratumumab-mediated depletion of M-MDSC using a combination of daratumumab and cetrelimab in RRMM patients	
			Notch3-transgenic T-ALL model	Notch-signaling deregulation in immature T-cells promotes $CD11b^+Grl^+$ MDSC in the Notch3-transgenic murine model of T-ALL.	
			Notchl-activated KE-37 cell line and HD PB	Human Notch-Dependent T-ALL cell lines induce MDSC from HD PBMC. Tumors induce Jagged ligands in MDSC through NFκB-p65.	
	Anti-Jagged antibody	Lymphoma	EL-4 syngeneic model	Anti-Jagged therapy induces an anti-tumor effect, and impacts the suppressive activity of tumor-MDSC.	

(Continued)

TABLE 2 Continued

Agents		Disease	Model	Mechanisms/ Functions	Ref
S100A9 inhibitors	ABR-238901	MM	5T33MM model	Blocking S100A9 interactions with ABR-238901 did not directly affect MDSC accumulation but did reduce IL-6 and IL-10 expression by MDSC. ABR-238901 treatment in combination with bortezomib resulted in an increased reduction in tumor load compared with single treatments.	(81)
	Tasquinimod		5T33MM model 5TGM1 model	Tasquinimod has direct anti-tumor effects *in vivo*. Tasquinimod targets M-MDSC and increases serum interferon-gamma.	(82)
STAT3 inhibitors	AZD9150	NHL (primarily DLBCL)	Patient PB	AZD9150 therapy resulted in a decrease of G-MDSC and increased CD4 and CD8 T-cells in three out of four NHL patients.	(83)
Phosphodiesterase-5 inhibitors	Sildenafil	B cell lymphoma	A20 syngeneic model	IL-4Ra expression on MDSC correlates with tumor progression and can be inhibited by sildenafil.	(84)
	Tadalafil	MM	Case report MM patient	Tadalafil, in a patient with end-stage RRMM reduced MDSC function and generated a dramatic and durable anti-myeloma immune and clinical response.	(85)
			Clinical trial of MM patients (refractory to lenalidomide-based regimens	MDSC were not detected in any of the patients at baseline in both blood and BM. No clinical response could be observed.	(86)
NOX2 inhibitor	Histamine hydrochloride	Lymphoma	EL-4 syngeneic model	HDC reduces tumor progression by targeting $NOX2^+$ MDSC. HDC significantly reduced the accumulation of MDSC within EL-4 lymphomas.	(87)
Arginase inhibitor	nor-NOHA CD1158	MM	Patient PB samples	T-cell proliferation and cell cytotoxicity is enhanced by PMN-SN in the presence of arginase inhibition. T-cell cytokine secretion is hyperactivated by PMN-SN in the presence of arginase inhibition.	(16, 88)
		AML	AML mice NOG-SCID mice	The AML mice had significant reductions in plasma arginine compared to controls. The arginine depleting therapy can inhibit antigen-dependent T cell responses *in vitro* and *in vivo*.	(89)
All-trans-retinoic acid		Lymphoma	EL-4 Syngeneic model	ATRA induces expression of GSS and accumulation of GSH in MDSC.	(90)
		APL	Transgenic PML-RARA APL model T-cell depletion in APL B6 model HIS APL model	In PML-RARA mice, the remission following ATRA treatment was accompanied with normalized levels of PGD2, ILC2s, M-MDSC, and a recovery of activated CD8+ T-cells. T-cell depleted APL B6 mice showed a shorter survival and an increase in ILC2 and M-MDSC. The increase in PGD2 and a major accumulation of ILC2 and M-MDSC upon leukemia engraftment were observed in HIS APL mice that were reverted by ATRA therapy.	(91)
PalmitoyItransferase inhibitor	2-BP	AML	Patient PB samples	Palmitoylated proteins on the AML-EVs' surface contribute to the TLR2-dependent MDSC reprogramming	(92)

5-FU, 5-fluorouracil; BV, brentuximab vedotin; DAC, decitabine; LEN, lenalidomide; POM, pomalidomide; VISTA/PD-1H, v-domain immunoglobulin suppressor of T-cell activation; MRIAN, metabolic reprogramming immunosurveillance activation nanomedicine; S100A9, calgranulin B or myeloid-related protein 14, MRP14; LXR, activation of liver X receptor; ADAM10, a disintegrin and metalloprotease 10; HDC, histamine hydrochloride; PMN-SN, polymorphonuclear neutrophil granulocytes supernatants; ATRA, all-trans-retinoic acid; MDS, myelodysplastic syndromes; T-ALL, T-cell acute lymphoblastic leukemia; APL, acute promyelocytic leukemia; HIS, humanized mice; ADCC/CDC, Fc-mediated antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity; CCL5, C-C Motif Chemokine Ligand 5; MIF, macrophage migration inhibitory factor; IFR8, interferon regulatory factor 8; ROS, reactive oxygen species; NFκB-p65, nuclear factor kappa-light-chain-enhancer of activated B cells; IL6, interleukin 6; CTLs, cytotoxic T lymphocyte; GSS, glutathione synthase; GSH, glutathione; PDG2, a receptor for prostaglandin D2; ILC2, group 2 innate lymphoid cells.

function, additional studies are required in MM models that allow BM-derived MDSC investigation as well.

V-domain Ig suppressor of T-cell activation (VISTA) or PD-1H is a novel checkpoint regulator that is predominantly expressed in the hematopoietic compartment, and particularly within the myeloid lineage (105). In solid tumors, inhibition of VISTA resulted in improved anti-tumor immune responses *in vivo* and currently clinical trials are ongoing to assess its therapeutic potential in advanced solid tumor malignancies (NCT05082610, NCT04475523) (106). In AML, VISTA was found to be highly expressed on monocytes ($CD45^{int}CD11b^+$ $CD14^{high/low}$) and myeloid leukemia blasts ($CD45^{in}$ vs. SCC). VISTA expression on PB-derived MDSC ($CD11b^+CD33^+$ $HLA\text{-}DR^-$) was significantly higher in AML patients compared to healthy controls. In addition, siRNA mediated knockdown of VISTA in MDSC resulted in increased T-cell proliferation *in vitro* and diminished the MDSC-mediated suppression of $CD8^+$ T-cell. Strikingly, the authors observed a strong correlation between VISTA-expressing MDSC and PD-1 expressing T-cells (including CD4, CD8 and Treg), indicating a link between both checkpoints to suppress the immune system in AML patients (73). In another study, VISTA-expressing murine myeloid leukemia cells were injected into wild type and PD-1H (VISTA) knock out mice. Authors observed a reduction in AML

cell growth in PD-1H knock out mice, which was further diminished by the administration of PD-1H blocking antibodies. These data suggest that VISTA expression on both the host cells and AML cells are involved in the cancer immune evasion. Moreover, epigenetic modulation using DAC further increased the overall survival of PD-1H knock out mice, indicating the potential of combining both compounds in clinical setting (74).

3.2.2 Tyrosine kinase inhibitors

Ibrutinib is a first-in-class oral irreversible inhibitor of Bruton Tyrosine Kinase (BTK), a critical enzyme in the B-cell receptor signaling cascade, and is highly effective in the treatment of CLL, MCL and Waldenstrom's macroglobulinaemia. BTK has been described to be expressed by MDSC and treatment with ibrutinib was found to affect the MDSC generation and function in solid tumor models, indicating its therapeutic potential to increase immune-based therapies (107, 108). A study by Ferrer et al. demonstrated that G-MDSC were the preferential subset to target in CLL patients to increase T-cell function. Three months ibrutinib therapy of CLL patients resulted in a significant decline of G-MDSC, while M-MDSC and monocytes remained unaffected. While ibrutinib had no direct effect on the T-cell suppressive activity, it skewed the T-cell differentiation to T helper 1 cells in the presence of MDSC, indicating a change from an immunosuppressive towards a more immune effective state (32).

The effect of other tyrosine kinase inhibitors including imatinib, nilotinib and dasatinib on MDSC levels was evaluated in CML patients. All compounds induced a significant reduction in G-MDSC at 3–6 months and 9–12 months of treatment. However, the M-MDSC subset was not significantly changed during imatinib and nilotinib therapy and was only reduced in dasatinib-treated patients. Interestingly, a significant correlation was found between the major molecular response (MMR) values and number of persistent M-MDSC at 12 months of dasatinib treatment, indicating its prognostic value in these patients (75, 76).

3.2.3 Metabolic modifier MRIAN

Metabolic Reprogramming Immunosurveillance Activation Nanomedicine (MRIAN) is an L-phenylalanine polymer, developed to target the immunosuppressive BM micro environment by inhibiting MDSC. MRIAN reduced ROS levels and induced MDSC differentiation towards functional immune cells (e.g., macrophages, natural killer cells, dendritic cells). In T-ALL mice, MRIAN significantly improved the T-cell number and function by inhibiting MDSC. Studies also demonstrated an enhanced cellular uptake of MRIAN in T-ALL cells and MDSC compared to normal hematopoietic cells and progenitors. MRIAN assembled to doxorubicin (MRIAN-Dox) demonstrated an enhanced anti-tumor efficacy and reduced toxicity profile (including cardiotoxicity and myeloablation side effects) in T-ALL mice; indicating its therapeutic potential as metabolic modifier to target MDSC (77).

3.2.4 Notch inhibitors

The Notch signaling pathway has been identified to play a key role in MDSC accumulation (109–111). In transgenic mice overexpressing ADAM10, a Notch processing enzyme, an accumulation of systemic $CD11b^+Gr1^+$ MDSC was found (78). A study by Grazioli et al. observed an expansion of MDSC in a transgenic mouse model of Notch3-dependent T-ALL. Interestingly, using both *in vitro* and *in vivo* experiments, they found that $CD4^+CD8^+$ T-cell (derived from the Notch3-transgenic mice) were the drivers of MDSC accumulation, through a mechanism that was dependent on both Notch and IL6. Conversely, anti-Gr1-mediated depletion of MDSCs in T-ALL-bearing mice significantly reduced the proliferation and expansion of malignant T-cell. These data were confirmed by coculturing human Notch-dependent T-ALL cell lines and healthy donor derived PB mononuclear cells *in vitro*, resulting in increased $CD14^+HLA\text{-}DR^{low/neg}$ MDSC accumulation and T-cell suppression; effects that were not observed with T-ALL cells that did not express Notch1- or Notch3-activated protein (79).

Another therapeutic approach to alter Notch signaling is the use of anti-Jagged blocking antibodies. Sierra et al. assessed the anti-tumor and immunogenic effect of CTX014, a humanized IgG1 blocking antibody, cross-reactive for both mouse and human Jagged1 and 2, in solid and hematological tumor models. Surprisingly, results demonstrated an increase of $CD11b^+GR1^+$ MDSC in tumors of mice treated with anti-Jagged therapy compared to vehicle. Data suggested that anti-Jagged therapy triggered an anti-tumor immune response through induction of immunogenic MDSC-like cells. Anti-tumor and immunogenic effects of anti-Jagged therapy was evaluated in an E.G7-OVA T-cell lymphoma model in combination with adoptive T-cell transfer of OT-I cells. Results showed that anti-Jagged therapy could overcome tumor-induced immune tolerance and increased the effect of the T-cell based immunotherapy (80).

In solid tumors, targeting Notch using γ-secretase inhibitors significantly increased the MDSC number in preclinical cancer models (112). There was a specific increase in the G-MDSC subset and a downregulation of CD80, CD115 and CD124 markers, all associated with MDSC suppressive function. Using short hairpin constructs against *RBP-J*, Notch signaling was attenuated in BM cells and this resulted in reduced MDSC suppressive capacity. In addition, injection of RBP-J-deficient MDSC in tumor-bearing mice significantly reduced the tumor growth compared to controls (79, 113).

Altogether, these studies revealed a role of Notch signaling in the accumulation and suppressive function of MDSC in tumor-bearing mice. However, whether the effect is direct, indirect or a combination of both remains to be elucidated.

3.2.5 S100A9 inhibitors

S100A9 is a calcium-binding protein, mainly secreted by granulocytes and monocytes, and has been reported to be essential for MDSC survival and accumulation in tumor-bearing mice including MM and lymphoma models (114). In MM, our group demonstrated the expression of S100A9 and its receptor TLR4 in both monocytic and granulocytic MDSC subsets. S100A9 acted as a chemoattractant for MM cells *in vitro* and induced the expression of pro-inflammatory cytokines by MDSC (e.g., TNFα, IL-6, IL-10). Targeting the interaction of S100A9 and its receptors using ABR-238901 did not affect MDSC accumulation, but significantly reduced cytokine expression by MDSC. Moreover, anti-angiogenic and anti-MM effects were observed *in vivo* using a combination therapy of ABR-238901 and bortezomib (81). Recently, we also investigated the effects of S100A9 inhibitor tasquinimod, currently evaluated in clinical trial for relapsed/refractory MM patients and observed a clear reduction in the M-MDSC subset *in vivo* (NCT04405167). In addition, tasquinimod abolished the immunosuppressive activity of *in vitro* generated MDSC, illustrating its potential as an immunotherapeutic compound (82).

3.2.6 STAT3 inhibitors

Although STAT3 activation is known to play a pivotal role in MDSC accumulation and function, the effects of STAT3 inhibitors on MDSC activity is rather controversial (115, 116). AZD1480, a small-molecule inhibitor of JAK1/2 kinase, significantly decreased MDSC number and delayed tumor growth in MO4 melanoma-bearing mice. Despite a decrease in MDSC percentage, Maenhout et al. observed an enhanced MDSC-suppressive capacity and impaired T-cell proliferation and IFN-γ secretion upon treatment with AZD1480 (117). AZD9150, a next-generation antisense oligonucleotide inhibitor of STAT3, also demonstrated potent anti-tumor effects of lymphoma cell lines and in preclinical lymphoma models (83). The inhibitor was evaluated in a small group of non-HL patients and three out of four patients showed a decrease in the circulating G-MDSC population and an increase in $CD4^+$ and $CD8^+$ T-cell (118). Napabucasin, another STAT3 inhibitor, was also found to abrogate the MDSC suppressive function in solid tumors and exhibited potent cytotoxicity against NHL cell lines (119, 120). However, napabucasin-mediated MDSC-targeting and modulation has not been investigated in hematological cancers so far.

3.2.7 Phosphodiesterase-5 inhibitors

Phosphodiesterase-5 (PDE5) inhibitors (e.g., sildenafil, tadalafil, vardenafil), particularly used for nonmalignant conditions in the clinic, have been found to increase anti-tumor immune responses by altering the MDSC suppressive function and restoring anti-tumor immunity (121). Using the A20 lymphoma model, it has been found that IL4Rα expression on MDSC correlated with tumor progression and could be inhibited using sildenafil. In addition, sildenafil reduced lymphoma-induced T-cell anergy and expansion of regulatory Treg (84). A case report of a patient with end-stage relapsed/refractory MM showed that the addition of tadalafil to its treatment regimen (lenalidomide, clarithromycin, dexamethasone) reduced the MDSC suppressive activity, as illustrated by a reduction in $IL4R\alpha^+$, iNOS, Arg-1 and ROS. Interestingly, the changes in MDSC function were more pronounced in the BM compared to the blood and were associated with an increase in T-cell function (↑ IFNγ expression). With the administration of tadalafil, the patient could tolerate the combination of lenalidomide and dexamethasone and achieved a very good partial response (+/- 90% reduction in tumor burden) (85). Although a clinical trial was initiated combining tadalafil, dexamethasone and lenalidomide in MM patients who were refractory to lenalidomide-based regimens, the study was terminated at an early stage due to a lack of response. The limited efficacy could be explained by the low number of MDSC present in the patients at the time of enrollment, potentially attributed to the pre-treatment with lenalidomide (86). Further studies are required to investigate the impact of PDE-5 inhibitors in patients with elevated MDSC levels.

3.2.8 Histamine hydrochloride

Histamine hydrochloride (HDC) is a NOX2 inhibitor and is known to inhibit the immunosuppressive function of myeloid cells by reducing ROS production (122). Low-dose IL-2 combined with HDC is approved in Europe for remission maintenance in adult AML patients. Grauers et al. further unraveled the impact of HDC on MDSC number and function using the EL4 lymphoma tumor model. HDC significantly reduced MDSC number *in vivo* and altered the MDSC-induced immunosuppression of T-cells *ex vivo*. Moreover, using Nox2 knock out mice and GR1-depleting antibodies, it has been suggested that HDC exerted its anti-tumor effects by targeting the $NOX2^+$ $GR1^+$ cells *in vivo*. Finally, authors also observed an enhanced anti-tumor efficacy using the combination of HDC and anti-PD-1 antibodies in the EL4 lymphoma model. HDC-mediated effects on MDSC were further evaluated using blood samples of AML patients that received HDC in conjunction with low-dose IL-2 for relapse prevention (NCT01347996) (87). HDC/IL-2 therapy resulted in a significant reduction in the frequency and absolute counts of M-MDSC, and this strong reduction significantly predicted the leukemia-free survival.

3.2.9 Arginase inhibitors

Arginase is a key enzyme involved in the immuno suppressive function of G-MDSC. Romano et al. demonstrated that Arg-1 is mainly expressed by G-MDSC in MM, and that both Arg-1 and G-MDSC are reduced after treatment with lenalidomide *in vivo* (45). Interestingly, Vonwirth et al. demonstrated that arginase inhibition, using nor-NOHA or CB-1158, could reduce T-cell anergy of MM patients in the

presence of supernatant derived from polymorphonuclear neutrophil granulocytes (~G-MDSC). In preclinical solid tumor models, arginase inhibitor CB-1158 inhibited MDSC-mediated immunosuppression, increased T-cell proliferation and activity, and reduced tumor growth *in vivo* (89). A first-in-human phase 1 study in solid tumors demonstrated that CB-1158 was well tolerated and achieved on-target inhibition as illustrated by the increase in plasma arginine (16, 88). Moreover, arginase inhibition has been proposed as an interesting adjuvant therapy by Mussai et al. in leukemia patients. Inhibition of the arginine metabolism by L-NMMA and L-NOHA enhanced the proliferation and cytotoxicity of anti-NY-ESO (AML associated cancer-testis antigen) T-cells against epigenetically-treated AML blasts. In addition, it could also boost the anti-CD33 Chimeric Antigen Receptor T-cell cytotoxicity against AML, further illustrating its potential as adjunct therapy in hematological cancers (89).

3.3 Induction of MDSC differentiation

3.3.1 All-trans-retinoic acid

All-trans-retinoic acid (ATRA), a vitamin A derivative, has been described as an inducer of myeloid cell differentiation and maturation, reducing MDSC number and inducing activation of immune responses in preclinical hematological and solid tumor models (90). In acute promyelocytic leukemia (APL) patients, peripheral 'group 2 innate lymphoid cells' (ILC2s) were found to be increased and hyperactivated, and in turn activated M-MDSC ($CD14^+CD33^+$) through IL-13 secretion. Using patient samples and APL mice, authors demonstrated that ATRA-treatment reversed the increase in ILC2 induced M-MDSC, accompanied by an increase in T-cell function *in vitro* and *in vivo* (91).

Unfortunately, due to a poor solubility and fast drug metabolism, the clinical application of ATRA has been limited. Recently, a drug encapsulated liposome formulation L-ATRA has been developed with sustained release properties. *In vitro* treatment of myeloid leukemia cell lines HL-60 and NB4 resulted in increased expression of myeloid differentiation markers CD11b and CD11c, illustrating its therapeutic potential to target MDSC (123).

3.4 Inhibition of MDSC accumulation

3.4.1 Palmitoyltransferase inhibitor

It has been shown that $CD14^+HLA\text{-}DR^{low}$ M-MDSC accumulate in newly diagnosed AML patients. Tohumeken et al. found that AML-derived extracellular vesicles were taken up by conventional monocytes *in vitro* which subsequently underwent MDSC differentiation. Apparently, the presence of palmitoylated proteins on the surface of AML-derived extracellular vesicles was responsible for the activation of TLR2/Akt/mTOR signaling and accumulation of MDSC. TLR2 neutralizing antibodies, mTOR inhibitor rapamycin or palmitoyltransferases inhibitor 2-BP abolished the generation of MDSC, indicating its potential therapeutic application as MDSC-targeted therapies (92).

3.4.2 Zoledronic acid

Zoledronic acid is a bisphosphonate used for the treatment of MM associated hypercalcemia and bone metastasis in solid tumors (124). Although no information is available on the MDSC-targeting potential of zoledronic acid in hematological malignancies, Porembka et al. observed a reduced MDSC accumulation and improved anti-tumor immune response in pancreatic cancer models (125). These data suggest that zoledronic acid might exert a dual role as anti-MM therapy, impacting on the bone disease and the accumulation of immunosuppressive cell types.

4 Other MDSC-targeting approaches tested in solid tumors

Although not yet tested in hematological malignancies, other specific/unspecific MDSC-targeting approaches might be considered in the future. For example, an indoleamine-pyrrole 2,3-dioxygenase (IDO) peptide vaccine has been developed and significantly decreased IDO-expressing MDSC. The peptide vaccine delayed tumor progression in solid tumors inoculated with either IDO^+ or IDO^- tumor cells, indicating the therapeutic effect was partially mediated by targeting of the immunosuppressive environment (126).

Consistent with the results obtained using AMG 330 and AMV 564, CD33-directed therapy with gemtuzumab ozogamicin demonstrated MDSC depleting capacity in solid tumor models. CD33 was expressed on blood and tissue-derived MDSC of patients across different cancer subtypes, indicating its broad therapeutic potential (127).

5 Combinatorial approaches

The past years, immune checkpoint inhibitors and chimeric antigen receptor (CAR) T-cell therapies emerged as concomitant approaches to treat hematological cancers. However, the presence of immunosuppressive MDSC influences their efficacy. A study in large B-cell lymphoma patients receiving axicabtagene ciloleucel (axi-cel), a CD19-directed CART-cell therapy, demonstrated a clear association between poor CART-cell expansion and PB M-MDSC (128). Combinatorial approaches using CART- therapy or immune checkpoint inhibitors with MDSC-targeting agents (e.g., ATRA, gemtuzumab ozogamicin, AMV 564) clearly enhanced the anti-tumor efficacy in solid tumor models (127–129). These results imply the importance of using a similar approach in the treatment of hematological cancers.

6 Conclusion

Despite the controversy surrounding the nature and uniqueness of MDSC, there is no exists about their value as a therapeutic target in hematological cancers. MDSC contribute to tumor cell survival, immunosuppression and drug resistance; however, strategies to specifically eliminate this cell population or block their development are rather limited. Differences in analysis, tumor models, disease stages and treatment-related changes certainly contributed to the complexity to identify unique markers and specific approaches to tackle this cell type and reverse their immunosuppressive capacity. Further developments and applications of single-cell multi-omics will provide unique insights about the MDSC phenotypical markers and subsets, hopefully leading to a more specific MDSC-targeting approach in future. In addition, as MDSC are key regulators of immunosuppression, they contribute to the reduced effectiveness of current immunotherapeutic approaches including CAR-T therapy and immune checkpoint inhibitors. Specific targeting of these cell types in combination with other immunotherapies should be evaluated in clinical trials as this approach might be the key to increase anti-tumor immune responses and improve patient's outcome.

Author contributions

RF and KDV developed the design and arguments for the paper, drafted the manuscript and designed the figures. AM, NB, EM, EB, KV, KM, and KB revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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The immune suppressive tumor microenvironment in multiple myeloma: The contribution of myeloid-derived suppressor cells

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Myeloid derived suppressors cells (MDSC) play major roles in regulating immune homeostasis and immune responses in many conditions, including cancer. MDSC interact with cancer cells within the tumor microenvironment (TME) with direct and indirect mechanisms: production of soluble factors and cytokines, expression of surface inhibitory molecules, metabolic rewiring and exosome release. The two-way relationship between MDSC and tumor cells results in immune evasion and cancer outgrowth. In multiple myeloma (MM), MDSC play a major role in creating protumoral TME conditions. In this minireview, we will discuss the interplay between MDSC and MM TME and the possible strategies to target MDSC.

KEYWORDS

MDSC (myeloid-derived suppressor cell), TME (tumor microenvironment), multiple myeloma, Immunothearpies, immune suppression

Introduction

Multiple myeloma (MM) is a paradigm disease in which progression is fueled by intrinsic alterations of myeloma cells and tumor-host interactions in the tumor microenvironment (TME) (1). Disease evolution from monoclonal gammopathy of undetermined significance (MGUS) to smoldering myeloma (SMM), and symptomatic disease is characterized by a progressive increase of myeloma cells associated with co-evolving immunological and metabolic changes making the TME unable to hold the disease in check (1). We and others have shown that immune alterations are already detectable in the very early stage of the disease (2, 3) and that they persist in the remission phase (2). The immune MM TME contexture consists of effector cells (i.e, conventional T cells, unconventional T cells like NKT cells, γδ T cells, NK cells etc), professional

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suppressor cells [i.e, regulatory T cells (Tregs), regulatory B cells (Bregs), myeloid derived suppressor cells (MDSC)], and cells that are functionally conditioned by the TME and acquire protumoral functions like bone marrow stromal cells (BMSC), endothelial cells, osteoblasts (OB), and osteoclasts (4). Recently, BM-resident neutrophils have also been reported to contribute to the TME-induced suppressive commitment of MM patients (5). Unbalanced distribution of effector and suppressor cells already detectable in MGUS is induced by the progressive accumulation of myeloma cells driven by genetic and epigenetic drivers. The bone marrow (BM), which is where MM originates and propagates, has the capacity to physiologically host around 2-5% polyclonal plasma cells. When myeloma cell infiltration overcomes this threshold, the TME is immunologically and metabolically shaped to support myeloma cell growth, to induce drug resistance, and to suppress immune recognition. MDSC play a major role in the protumoral reset of MM TME.

We have previously shown that MDSC are significantly increased in the BM of MGUS and MM patients: granulocytic/polymorphonuclear MDSC (PMN-MDSC), and not monocytic MDSC (M-MDSC), are responsible for the increase (2). MDSC frequency is very similar in MGUS, MM at diagnosis, and MM in relapse. Unexpectedly, we have found that MDSC frequency is significantly higher in MM in remission (2), indicating that there is no correlation between the proportion of BM myeloma cells and MDSC expansion. Similar data have been reported in mouse models in which MDSC start to accumulate in the TME as early as one week after tumor inoculation when the frequency of myeloma cells is very low (<10%) as in MGUS individuals (6).

Approximately, 20-40% of MDCS express the Programmed Cell Death-Ligand 1+ (PD-L1+) (2) and therefore are very well-suited to engage and suppress immune effector cells like Vγ9Vδ2 cells and NK cells expressing the Programmed Cell Death-1 (PD-1) receptor (2). MDSC are PD-L1+ in MGUS and MM irrespective of the disease stage, including MM in remission when most myeloma cells have been cleared from BM (2). The persistence of PD-L1+ MDSC can hinder the immunomodulatory activity of drugs like bortezomib or lenalidomide after autologous stem cell transplantation.

In conclusion, MDSC play a major role in the establishment of the immune suppressive TME in MM. The aim of this minireview is to discuss the mechanisms exploited by MDSC in cooperation with myeloma cells, professional immune suppressor cells, and other bystander cells to promote myeloma cell growth in the BM of MM patients. We will also discuss possible interventions to dampen the immune suppression operated by MDSC and other suppressor cells to recover the antimyeloma activity of immune effector cells.

MDSC subsets and differentiation

MDSC play a major role in the regulation of immune homeostasis in healthy individuals, and the regulation of immune responses in infectious diseases, autoimmunity, aging, pregnancy, transplantation, and obesity (7). In cancer, the immune suppressive activity of MDSC is exploited by tumor cells to evade immune surveillance and support their survival and accumulation (7).

MDSC are derived from bone marrow hematopoietic stem cells (7). There are two major subsets of MDSC in humans: PMN-MDSC and M-MDSC. The first one are phenotypically and morphologically similar to neutrophils ($CD15^+$ and/or $CD66b^+$), whereas M-MDSC are similar to monocytes (CD14 +)(7). More recently, a third subset of phenotypically distinct immature early-MDSC (e-MDSC) has been identified in cancer patients (8). In this review we will use the term MDSC to identify both PMN-MDSC and M-MDSC unless otherwise specified.

MDSC development occurs in two partially overlapping waves (9). The first one is driven by cytokines and soluble factors including granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), interleukin 6 (IL-6), and vascular endothelial growth factor (VEGF). These cytokines and soluble factors are produced by tumor cells and/or BMSC in the TME and promote MDSC differentiation from hematopoietic progenitor cells *via* STAT3 and STAT5 activation (10, 11, 12). Mesenchymal stromal cells (MSC) also induce MDSC expansion *via* the hepatocyte growth factor (HGF), c-Met, and STAT3 phosphorylation (10). The second wave is driven by a different set of cytokines and inflammatory soluble factors like interleukin 13 (IL-13), toll-like receptor (TLR) ligands, and prostaglandin E2 (PGE2) yielding to the functional MDSC activation *via* the STAT1 and NF-kB pathways (10–12). The TME is highly predisposed to drive the expansion and activation of MDSC at the expense of other myeloid-derived cells like monocytes, macrophages and dendritic cells (DC) (8).

Immuno suppressive MDSC features

The immune suppressive MDSC activity is dependent on: 1) the depletion of essential $CD8^+$ T- cell nutrients in the TME; 2) the production of immune suppressive cytokines and/or soluble factors; 3) the expression of cell surface inhibitory molecules [i.e., (PD-L1)]; 4) the protumoral metabolic TME rewiring at the expense of immune effector cells.

Amino acid depletion

MDSC express the xc- transporter and import cystine, but, unlike DC and macrophages, they are unable to export cysteine because they lack the ASC neutral amino acid transporter (13). Considering the progressive TME invasion by tumor cells and MDSC at the expense of other cells which can supply extracellular cysteine, the TME becomes depleted of cysteine jeopardizing the activation of CD8+T cells that are unable to convert cystine to cysteine to meet their metabolic requirements (13).

MDSC also deplete the TME of tryptophan *via* the enzyme indoleamine 2, 3-dioxygenase (IDO) (14). T lymphocytes are very susceptible to tryptophan shortage which restrains their proliferative responses by inducing an integrated stress response and the inactivation of the mTOR pathway (15, 16). Tryptophan catabolites can also induce the apoptosis of cytotoxic T cells (17, 18), and the concurrent differentiation of Tregs (16). L-arginine (L-arg) is another essential amino acid which is critical for T-cell immune functions. Arginine metabolism is regulated by the inducible nitric oxide synthase (iNOS) isoenzymes, arginase (Arg 1/2) activity, and proline and polyamines synthesis. MDSC express both iNOS and Arg-1 that induce L-arg depletion in the TME leading to inhibition of CD3-ζ expression in T cells, and induction of apoptosis (7, 9, 19).

Cytokines and soluble factors

The production and release of suppressor cytokines and soluble factors is another mechanism exploited by MDSC to protect tumor cells from immune recognition and killing. Nitric oxide (NO), reactive oxygen species (ROS), peroxynitrite (PNT) (a short-lived product of NO reaction with ROS), interleukin 10 (IL-10), and transforming growth factor-β (TGF-β) are released by MDSC with slightly difference between PMN-MDSC and M-MDSC subsets (7, 9, 20, 21). The hyper-production of ROS and PNT in the TME impairs the ability of CD8+ T cells to bind to peptide–major histocompatibility complexes and to respond to specific peptides (21). NO also hampers the Fc receptor-mediated effector functions of NK cells (22). IL-10 recruits Tregs in the TME and decreases CD8+ T-cell antigen sensitivity by inducing cell surface glycoprotein branching (23). TGF-β is induced by IL-13 (24) and interferon-γ (IFN-γ) (25), and contributes to T-cell suppression through Tregs development (25). Kynurenine is another soluble immune suppressive factor that is generated in the TME as a consequence of tryptophan catabolism by MDSC. Kynurenine can inhibit T-cell and NK cell proliferation and drive the differentiation of naïve T cells into Tregs (16).

Cell surface molecules

The cell surface expression of immune checkpoints ligands (ICP-L) like PD-L1 is another mechanism used by M-MDSC to suppress immune effector cells (2, 7, 9), while PMN-MDSC preferentially exploit the Fas/Fas-ligand pathway to induce T-cell depletion in the TME (26). The V-domain immunoglobulin suppressor of T cell activation (VISTA) is a novel co-inhibitory ligand/receptor highly expressed by MDSC in the TME that suppresses T-cell effector functions and contributes to acquired resistance to PD-1/PD-L1 blockade (27). Lastly, CXCR2 is another cell surface molecule that is critical in mice models and paediatric sarcoma to promote the accumulation of MDSC in the TME and hamper the efficacy of anti-PD-1 treatment (28).

Protumoral metabolic TME rewiring

The TME is a very dynamic ecosystem that is progressively molded by tumor cells to locally create protective conditions to support their growth and resistance to therapy, from conventional chemotherapy to immunotherapy (29, 30). Hypoxia is a major metabolic feature of TME (30), especially in solid tumors, almost always associated with the extracellular acidification induced by lactate accumulation. Tumor cells rewire their metabolism to survive and proliferate in the TME by: 1) increasing glucose and amino acid uptake, glycolytic flux, and lactate production; 2) modifying glutamine metabolism, tricarboxylic acid cycle, and oxidative phosphorylation; 3) increasing the production of mitochondrial ROS; 4) modulating fatty acid synthesis and oxidation (FAO) (30). MDSC partially mimick the metabolic rewiring of tumor cells by adapting their lactate, glucose, and lipid metabolism to the hypoxic and acidic TME conditions (31, 32). As a result, MDSC survive in the TME, contribute to the exacerbation of the protumoral metabolic TME commitment, and maintain unaltered their immune suppressor activity (33–35).

Immune suppressive and metabolic features in MM

MM is a hematologic cancer characterized by the accumulation of malignant plasma cells (myeloma cells) in the BM. Progressive colonization of BM results in a deep remodelling of the BM niche that becomes committed to support myeloma cell growth, immune evasion, and drug resistance (1).

MDSC play a major role in establishing the protumoral TME commitment. We have shown that MDSC accumulation in the

BM is already detectable in MGUS, and their expansion persists throughout the entire period of the disease (2), including the remission phase (2). In our hands, PMN-MDSC was the main subpopulation to be expanded in MGUS and MM (2), while other groups have reported the predominance of M-MDSC in MM at diagnosis and in relapse (36, 37). Immunogenomic characterization identified CD11b+CD13+CD16+ cells as the PMN-MDSC subset with strongest capacity to suppress anti-myeloma activity T-cell immune responses (38). MDSC-like suppressive activity is also exhibited by MM neutrophils (5), suggesting that an accurate characterization of MDSC should be based on phenotypic markers, immunosuppressive potential, and transcriptional network.

Development and suppressor functions of MDSC are supported by myeloma cells and bystander cells *via* direct and indirect mechanisms. Direct mechanisms operated by myeloma cells include: 1) IL-6 production (39, 40) which prevents MDSC differentiation and promotes MDSC accumulation and activation *via* the STAT3 signaling pathway (41); 2) the induction of Mcl-1, an anti-apoptotic protein sustaining MDSC survival (42); 3) the secretion of galectin-1 that targets CD304 on MDSC and enhances their immune suppressive capacity (43); 4) the production of chemokine ligand 5 (CCL5) and macrophage migration inhibitory factor (MIF) (44). MIF has also been reported to potentiate the immune suppressive activity of MDSC *via* CD84-mediated PD-L1 upregulation (45); 5) the release of exosomes that promotes MDSC growth and NO production (46)

Bystander cells in the TME also cooperate with myeloma cells in the induction and activation of immune suppressive MDSC *via* direct mechanisms including: 1) IL-6 release (47, 48); 2) exosome release by BMSC (49); 3) production and release of immune suppressive molecules [i.e. Prostaglandin-Endoperoxide Synthase 2 (PTGS2), TGF-β, Nitric Oxide Synthase 2 (NOS2), IL-10 and IL-6] by MSC and OB (50, 51).

In addition to the direct mechanisms listed above, myeloma cells and bystander cells promote the accumulation and activation of MDSC *via* indirect mechanisms. An example is the metabolic rewiring operated by myeloma cells and bystander cells that creates an hypoxic and nutrient-depleted TME that promotes the accumulation and activation of MDSC at the expense of immune effector cells (52–54). Lactate over-production shifts MDSC differentiation toward PMN-MDSC (55), which is the subset that we and others have shown to be increased in the peripheral blood (PB) and BM of MM patients (2, 56).

The accumulation and activation of MDSC is beneficial to myeloma cells creating a very effective protumoral loop (3, 57). MDSC facilitate the self-renewal of myeloma stem-cells, enhance their tumorigenic potential *via* epigenetic regulation (58), and promote myeloma cell survival *via* AMPK phosphorylation leading to increase β-oxidation, ATP production, and increased NADPH levels (59). MDSC production of S100A9, a calcium-binding protein that promotes the release of TNF-α, IL-6, and IL-10 in autocrine pathway through TLR4 interaction, attracts myeloma cells in the TME (60) and supports myeloma cell growth *via* the activation of the canonical NFκB pathway (61).

Indirect mechanisms operated by MDSC to support myeloma cells are deprivation of nutrients, production of soluble factors, and the expression of cell surface inhibitory molecules. The common denominator is the impairment of anti-myeloma immune responses. In addition, PMN-MDSC are educated to express angiogenesis-related proteins to support tumor angiogenesis (62).

MDSC upregulate enzymes that contribute to the shortage of amino acids essential for immune effector T cells. Arginase 1 (Arg-1) expression and NO production by MDSC limit the availability of L-Arg needed for effective TCR-mediated signaling (63, 64). MDSC can utilize glutamine for anaplerosis like myeloma cells (65, 66), exacerbating glutamine deprivation in the TME (54).

Several soluble factors and cytokines contribute to the immune suppressor activity of MDSC in the TME, like IL-10, IL-6, TGF-β, CD40-CD40 Ligand, and IFN-γ. These cytokines tip the scales in favor of Tregs (44, 67), whose number is directly correlated with MDSC expansion (56). Lastly, CD38 expression on MDSC (68) contributes to the discontinuous multicellular pathway of adenosine (Ado), an immune suppressive nucleoside highly represented in the TME of MM patients (69).

The expression of immune checkpoint (ICP)/ICP-L contributes to the impairment of anti-myeloma immune responses. We have previously demonstrated that PD-L1 is expressed by BM MDSC in all disease states (2) and can contribute to hold in check anti-myeloma activity of PD1+ effector cells such as T cells, NK cells, and Vγ9Vδ2 T cells. Recently, it has been reported in solid tumors that MDSC can boost the immune suppressive activity of Bregs against T cells *via* the PD-1/PD-L1 axis (70, 71).

Lastly, MDSC can trans-differentiate into functional osteoclasts (72) to combine immune suppressive functions and direct protumoral functions (73). In mice models, G-MDSC have also been shown to promote angiogenesis (62), another major protumoral TME disruption occurring in human MM (62).

The direct and indirect mechanisms involved in the cross-talk between MDSC, myeloma cells, immune effector, immune suppressor cells, and other bystander cells in the TME of MM patients are shown in Figure 1.

Therapeutic interventions

The correlation between the frequency of MDSC and the clinical outcome identifies these cells as potential targets of immune-based therapeutic interventions (74). However, the

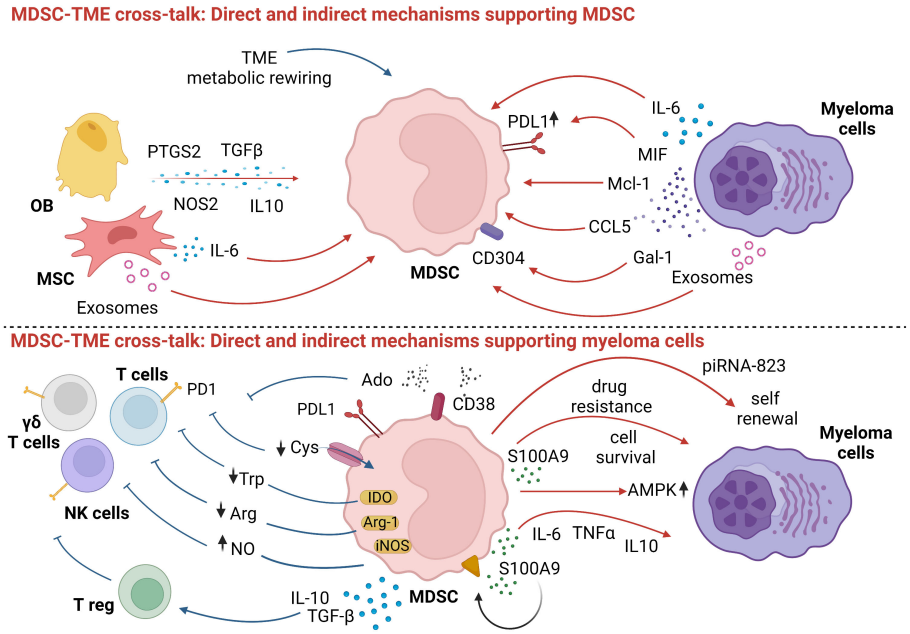

FIGURE 1
Myeloma cell and surrounding cells promote MDSC differentiation and suppressive functions. In turn, MDSC undermine anti-tumor immune responses and guarantee myeloma cells survival and growth. Red arrows: direct mechanisms; blue arrows: indirect mechanisms. Created by BioRender.com.

therapeutic targeting of MDSC is not easy given their multifaceted biological functions and multiple interactions in the TME. Possible strategies are: 1) to restrain their accumulation in the PB and TME; 2) to prevent their functional activation in the TME; 3) to block their protumoral interactions with myeloma cells and bystander cells.

MDSC accumulation can be restrained by immunomodulatory drugs (IMiDs) (44) and proteasome inhibitors (PI) (59). A cereblon (CRBN)-dependent and -independent down-regulation of CCL5 and MIF is a possible mechanism of IMiDs activity on MDSC (44) that can be improved by Arg-1 inhibitors (75). Clinical data confirm the capacity of IMiDs to restrain MDSC *in vivo* as shown by the decrease of PB MDSC in MM patients treated with pomalidomide, dexamethasone, and daratumumab (76). Daratumumab can also exert a favourable immune modulatory activity in the TME of MM patients by depleting CD38+ MDSC *via* antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytoxicity (CDC) (68). Data from mice models indicate that demethylating agents like decitabine (DAC), IL-18, and zoledronic acid (ZA) can also affect MDSC survival in the TME (72, 77, 78). ZA is currently used in MM and other solid cancers to prevent osteoclast activation and bone lesions, but this molecule is also endowed with pleiotropic immune modulatory activity (79), including the capacity in murine models to reduce the numbers of MDSC and prevent their differentiation into osteoclasts (72). Targeting CD84 and the CD304-Gal1 axis are other strategies used in myeloma mouse models to restore anti-myeloma T-cell responses by reducing MDSC accumulation and PD-L1 expression (45).

The immune suppressive activity of MM MDSC has also been inhibited *in vitro* using ABR-238901, a small molecule inhibiting S100A9 interactions (60), and tasquinimod (74). Anti-estrogen therapy may also restrain MDSC suppressive activity, since 17β-estradiol increases their ability to suppress T-cell proliferation (80). iNOS and Arg-1 activities have been down-modulated in mice models with tadalafil (81), a PDE5 inhibitor that has been used with some evidence of clinical efficacy in relapsed/refractory MM patients in combination with lenalidomide (82). Protumoral MDSC cellular interactions in the TME can also be limited by interrupting ICP/ICP-L interactions (2). Daratumumab in combination with the anti-PD1 monoclonal antibody cetrelimab has been reported to decrease the number of circulating MDSC and increase that of CD8+ T cells in the PB of MM patients in relapse (83). In acute myeloid leukemia (AML), knockdown of VISTA, a negative checkpoint regulator in the B7 family, reduced the MDSC-mediated inhibition of T cells (84). Data are not available in MM yet, but VISTA up-regulation is also expected in the BM of MM given the hypoxia and low pH as reported in solid cancer (85).

In conclusion, understanding the mechanisms underlying the immune suppressive activity of MDSC in MM will pave the

ground to the therapeutic targeting of these cells to improve the efficacy of immune-based treatments in MM.

Author contributions

CG, FA and MM contributed to the writing of the manuscript, CG designed the figure, MM revised the manuscript.

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Conflict of interest

MM reports advisory boards for AbbVie, Janssen-Cilag, Sanofi, and research funding from Sanofi.

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Publisher's note

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LncRNAs has been identified as regulators of Myeloid-derived suppressor cells in lung cancer

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Lung tumours are widespread pathological conditions that attract much attention due to their high incidence of death. The immune system contributes to the progression of these diseases, especially non-small cell lung cancer, resulting in the fast evolution of immune-targeted therapy. Myeloid-derived suppressor cells (MDSCs) have been suggested to promote the progression of cancer in the lungs by suppressing the immune response through various mechanisms. Herein, we summarized the clinical studies on lung cancer related to MDSCs. However, it is noteworthy to mention the discovery of long non-coding RNAs (lncRNAs) that had different phenotypes and could regulate MDSCs in lung cancer. Therefore, by reviewing the different phenotypes of lncRNAs and their regulation on MDSCs, we summarized the lncRNAs' impact on the progression of lung tumours. Data highlight LncRNAs as anti-cancer agents. Hence, we aim to discuss their possibilities to inhibit tumour growth and trigger the development of immunosuppressive factors such as MDSCs in lung cancer through the regulation of lncRNAs. The ultimate purpose is to propose novel and efficient therapy methods for curing patients with lung tumours.

KEYWORDS

lncRNA, MDSC, lung cancer, targeted therapy, immunotherapy

1 Introduction

Concerning cancer deaths, lung tumours are recognized as one of the leading causes globally. They are further classified in the category as conditions whose rates of morbidity and mortality, as well as the degrees of malignancy, are the highest. Along with the industrialization progress and environmental changes, the aetiology of lung cancer has become even more complex (1). According to sources, in 2018, many patients diagnosed with non-small cell lung cancer (NSCLC) in stage III were recorded, corresponding to 80% of all cases (2). Despite the ongoing efforts to develop efficient anti-cancer therapies, NSCLC has become among the most lethal cancers worldwide (3). In recent years, tumour-related

immunosuppression has become the focal point in targeted therapy for lung tumours. In this respect, bone marrow-derived suppressor cells (MDSCs) have attracted much attention. As early as 1970, studies have pointed out the MDSCs relation to tumour development (4). Its role in the tumour microenvironment has been pivotal and turned into a new target for cancer therapy (5). In a study by Gabrilovich and Nagaraj, the authors confirmed that MDSCs are heterogeneous cell populations derivative from the bone marrow precursor and immature cells (IMC). Under standard conditions, IMC can quickly specialize into dendritic cells (DC), mature granulocytes, and macrophages. On the occasions of cancer, infection, inflammation or other illnesses, the authors have reported an increase in the number of MDSCs and inhibition of IMC differentiation into developed cells of the bone marrow (6).

Moreover, MDSCs are known for their potent immunosuppressive function. Immunotherapy with immune checkpoint inhibitors has been reported to control the long-term effects of tumours to a certain extent. However, due to the potential repercussions of MDSCs' massive expansion, the MDSCs-induced immunosuppression has been considered the mechanism that effectively hinders the immune checkpoint blockade (7) Regarding lncRNAs, they have been implicated in the progression of tumours and various roles depending on the different expression types. Importantly, lncRNAs have multiple functions that are not limited to regulating the MDSCs generation, recruitment and immunosuppression. They also target multiple pathways simultaneously. The latter allows lncRNAs to act as markers in diagnosing tumours and are highly valued for targeted therapy (8). The modulatory effect of lncRNAs on MDSCs and the regulation of lung tumour tissue may open new horizons in treating individuals with lung tumours. The current paper is mainly based on the targeted therapy of MDSCs that lncRNAs have regulated to change the survival rate of patients with lung tumours. First, to elaborate on the importance of MDSCs in patients diagnosed with lung tumours, we have analyzed the MDSCs mechanism and investigated the clinical studies focused on the matter. Secondly, we studied the role of differential lncRNA expression in these cells and the impact on tumour progression. Finally, we have included clinical studies in which lncRNAs regulated various tumours, justifying their potential therapeutic value in tumours.

2 The roles of myeloid-derived suppressor cells (MDSCs) in lung tumours

2.1 Phenotype of MDSCs

Cell heterogeneity is evident in MDSCs as they include two major subpopulations, those of granulocytes (G-MDSCs) and monocytes (M-MDSCs) in both human and animal (mice) models. They are derived from granulocytes or monocytes and represent relatively stable forms of pathologic activation of these blood cell populations (9). In mice, $CD11b^+$ $Ly6G\text{-}Ly6C^{high}$ and $CD11b^+Ly6G^+Ly6C^{low}$ stand for the phenotype of M-MDSCs and G-MDSCs, respectively. Related studies have confirmed $CD11b^+$ $GR\text{-}1^{low}$cells' ability as the most efficient in suppressing the immune system in contrast to $CD11b^+$ $GR\text{-}1^{high}$ cells, regarded as the least effective. In humans, the MDSCs' complexity is even greater and constitutes $CD11b^+CD14^+HLA\text{-}DR^{low/neg}$ M-MDSCs and $CD11b^+CD14^-CD15^+$ G-MDSCs populations. The MDSCs in cancer patients express granulocyte markers and bone marrow cell markers like CD11b and CD33, which are the most common. However, there is still a need for a profound exploration of the surface markers of M-MDSCs and G-MDSCs due to the differentiation of the MDSCs' phenotype in various diseases (10, 11).Besides, a small group of bone marrow progenitors with MDSCs characteristics is identified only in humans and is named the "early MDSC" group. The group is mainly composed of bone marrow progenitors and precursors that account for less than 5% of the total number of MDSCs (12). However, many ongoing efforts have been made to report the surface markers of MDSCs. With fluorescence-activated cell sorting (FACS) for evaluating the multicolour immunofluorescence staining, several phenotypes of MDSCs in lung cancer have been reported (13).

2.2 Mechanism of action of MDSCs

The potent immunosuppressive effects of MDSCs, are present in most cancers (14). For example, the tumour microenvironment (TME) comprises different cell populations in a complex matrix. Cellular components of TME are markers that can regulate cancer processes like tumour proliferation, angiogenesis, invasion and metastasis, chemotherapy resistance, etc. Therefore, TME has become a new target for cancer treatment (15, 16) Furthermore, during tumour progression, the cells undergo alternations in their metabolism to satisfy their energy needs, with the ultimate goal of achieving proliferation and differentiation of the tumour—the last results in nutrition competition between the immune cells and immune modulators in the TME of MDSCs. Thus, increased glycolysis, fatty acid metabolism, and up-regulation of enzymes that are essential metabolites of catabolism are observed in the MDSCs tumour microenvironment. The last grants MDSCs immunosuppressive function (17). Other investigations have demonstrated that the MDSCs' immunomodulatory role is mainly based on the inhibition of T cells (18).This happens by various mechanisms. The first one is described by the production of reactive oxygen species (ROS), and reactive nitrogen species (RNS) can result in blocking T cells activation and function by MDSCs. As reported by Wang et al., G-MDSCs are mainly responsible for producing ROS and arginase-1 (ARG-1).

In contrast, M-MDSCs mainly produce ARG-1 and nitric oxide (NO) to exert immunosuppressive effects (19). The half-life of the produced NO is more extended, while it requires cell entry. Nonetheless, there is no requirement for close contact between M-MDSCs and T cells, enabling M-MDSCs effectively inhibit non-specific T cell responses (20). One prominent feature of MDSCs is the up-regulation of ROS produced by G-MDSCs in mice and individuals diagnosed with cancer. The immunosuppressive effect of MDSCs can be significantly enhanced by the expression of ROS in cancer patients and mice (21). Concretely, superoxide anion ($O2^-$), hydrogen peroxide (H_2O_2) and peroxynitrite (PNT) represent the abovementioned family of ROS. However, NO reacts with $O2^-$ to form PNT, which then prevents the recognition of the antigen/major histocompatibility complex (MHC) peptide by nitrification of the MHC class I

molecules and the T cell receptors (TCRs). They reduce the TCR affinity for antigen-MHC complex and block T cell migration by nitrification of T cell-specific chemokines (22, 23). The second mechanism providing MDSCs with immunomodulatory roles includes the depletion of L-cysteine and arginine by MDSCs, which are required for the proliferation and activation of T cell (24). The ARG-1 produced by G-MDSCs and M-MDSCs metabolizes L-arginine to L-ornithine in the urea cycle, making L-arginine induce its exhaustion in the TME. Other studies have revealed that PEGylated forms of Arg I (PEG-ARG I) in mice promote tumour growth. This is accompanied by augmented amounts of MDSCs (25).On the other hand, cysteine's role in T cell activation is indispensable. Factually, macrophages and DCs deliver to T cells the obtained cysteine, which the DCs and macrophages metabolise to methionine during the standard antigen processing and presentation. Moreover, in TME is observed a reduction in the activation of T cells and cysteine production by DCs as a result of a deprivation of macrophages of cysteine and DCs. The above is due to a large number of cysteine depletion by MDSCs (26).

The third mechanism includes the interaction between MDSCs with T cells that migrate to the lymph glands and T-cell activity *via* the expression of ADAM17 (metalloprotease structural domain 17 and a disintegrin) on their cell membrane. The MDSCs interfere. It has been reported that the latter leads to the downregulation of the homing receptor CD62L (L-selectin) on T cells (24).When it comes to effective anti-cancer immunity, and for purposes of action, it requires the transportation to the tumour of activated T cells and the activation of tumour-reactive T cells. Meanwhile, CD62L has been regarded as an essential molecule in this process, directing primitive lymphocytes to lymph glands in the periphery.

Furthermore, CD62L has proven necessary for housing the primitive T cells in the lymph glands, activating molecules on and in the cell membrane. Conversely, the suppressed activity of CD62L on primitive T cells by MDSCs decreases the chance of primitive T cells being delivered to the activation site. The last functions as MDSCs' suppressive anti-tumour immune response (27).

The other mechanisms for MDSCs' reduced immunity are explained in findings demonstrating that the regulatory T cells (Tregs) impact tumour immunosuppression. Tregs are considered to be immunosuppressive cells that promote cancer growth, and they are significantly increased in the peripheral blood of NSCLC patients (28). Although there are no proofs to relate MDSCs with the induction of Tregs, they produce a series of cytokines that allow the differentiation of Tregs (29, 30). MDSCs promote the amplification of natural Tregs through the production of IL-10, TGFβ, IFNγ and CD40-CD40L interactions and drive the development of the induced Tregs (24). Sinha et al. have reported that downregulation of the macrophage IL-12 and the IL-10 production is the outcome of T cell polarization to a type 2 pro-tumour phenotype (31). According to sources, the immune cells, specifically the B cells, NK cells, macrophages (Mø) and Treg cells, interact and can be regulated by MDSCs (13). The literature shows evidence for the interaction between DCs, tumour-associated macrophages (TAMS) and MDSCs in the TME. The outcome of this interaction is the enhancement of each cell population's immunosuppressive activity (24).Thus, in addition to T cell inhibition, MDSCs interfere with the innate immune response by affecting various cells like the NKT, NK, and macrophages (Figure 1). Factually, the MDSCs' impact on NK cells is intricate. One part can inhibit the death of the NK cell by hindering the production of IFN-γ. Importantly, due to the interaction of RAE-1 with NKG2D that takes place on the NK cells' surface, the other part *via* the expression of RAE-1 can result in NK cells' activation and provoke their death (24, 32). However, MDSCs' profile is multi-dimensional. Indeed, they promote tumour cell invasion, drug resistance, tumour angiogenesis, pre-metastatic niche formation, and tumour metastasis while participating in tumour immunosuppressive response (33, 34) .It has been suggested by Yang et al. that the MDSCs' non-immunosuppressive effects occur mainly *via* the promotion of tumour angiogenesis (35). Epithelial-mesenchymal transition (EMT) has been shown to play a key role in the process of tumor initiation and even metastasis, which can enhance the ability of cancer cells to enter the circulatory system to promote the metastasis of tumor cells. In the process of EMT, tumor cells lose polarity and cell-cell connection, and then enter a state of low proliferation, migration and invasion are enhanced (36). Studies have shown that EMT is related to the number of MDSCs, and EMT transcription factors can attract immunosuppressive cells MDSCs, leading to tumor immunosuppressive microenvironment. In turn, immunosuppressive factors induce EMT in tumor cells (37).The main mechanism is due to the fact that MDSCs and TAM further enhance chronic inflammation in the inflammatory microenvironment, which leads to EMT and enrichment of cancer stem cell-like cells (CSCs) and immune suppression may be the cause of drug resistance and metastasis (38), It is this feedback loop between EMT and immune suppression that promotes tumor progression. Therefore, the combination of immunotherapies targeting immunosuppressive cells such as MDSCs may be a promising treatment for EMT.

2.3 Clinical studies confirming the roles of MDSCs in lung cancer

MDSCs represent the most significant immunosuppressive cellular population in individuals with lung tumours. In the stroma of the tumour, these cells limit the healing efficacy of anti-cancer curing approaches through their metabolic pathways and other modalities in response to complex TME (39).There are many clinical studies on MDSCs in lung tumours, and therapeutic strategies targeting MDSCs are also gradually emerging. We have summarized these clinical studies and presented them in Table 1.

Some data show a significant accumulation of M-MDSCs in NSCLC patients, especially in stage IV patients, compared to stage III, with the expected lower survival rate of patients with a higher accumulation of M-MDSCs (40).Other authors found that individuals with NSCLC exhibited an advanced proportion of G-MDSCs, linked with improved survival (41). At the same time, other work has reported on a new human MDSCs subpopulation, CD14 (+) HLA-DR (-/low), in another cohort of individuals with NSCLC. Among those individuals, the incidence and total number of CD14 (+) HLA-DR (-/low) cells in the peripheral blood were considerably augmented compared with those in healthy NSCLC patients (42). The authors hypothesized that this was related to tumour metastasis, adverse reactions to chemotherapy, and tumour immunosuppression. Other work reported the results of different clinical trials with patients with

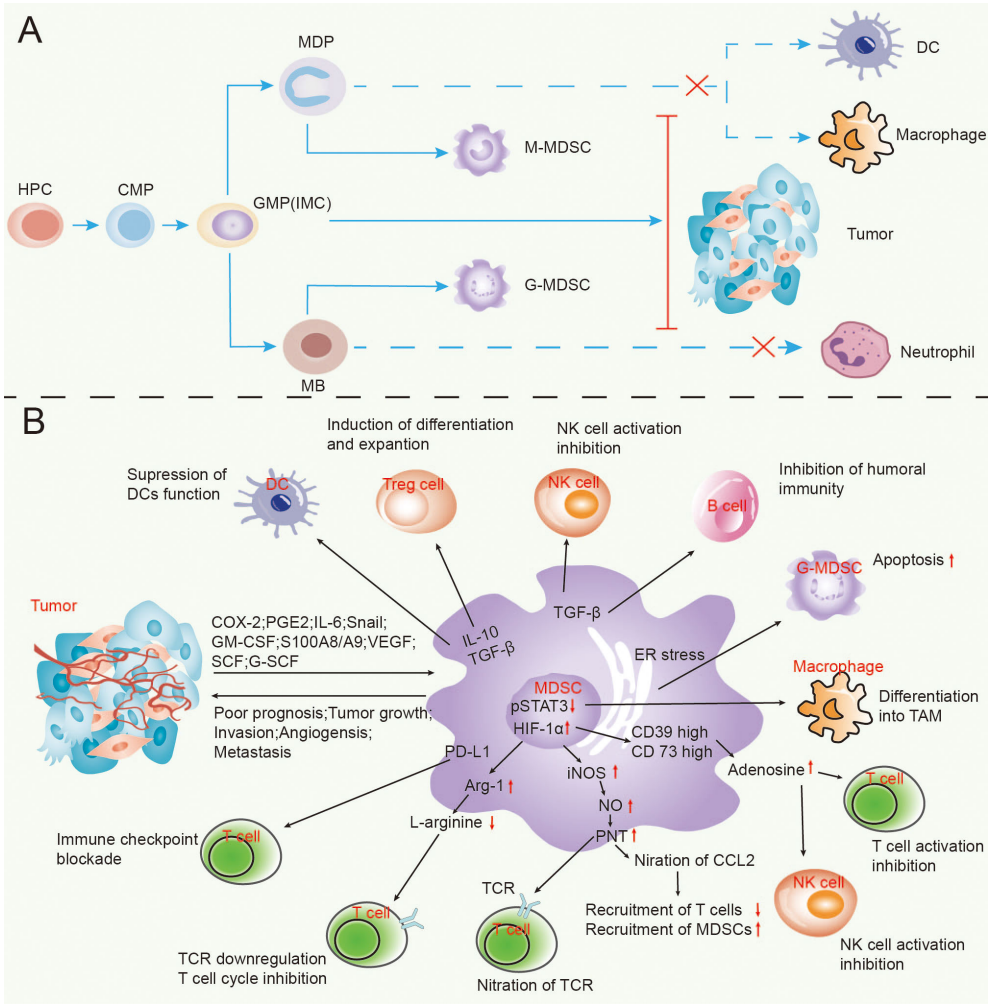

FIGURE 1
The process of MDSCs' formation in lung cancer **(A)** and the appliance of MDSCs exert tumour immunosuppressive effects **(B)**.

small cell lung cancer (SCLC), in which the depletion of MDSCs improved the immune response to vaccination and further enhanced the effect of immune interventions on cancer (43). It was shown that M-MDSCs from NSCLC patients demonstrated a worse prognosis (44).Seyed Sajjad Zadian et al. demonstrated that the frequency of M-MDSCs in NSCLC individuals was significantly more advanced than that in SCLC and healthy people, which impacted the differential diagnosis of NSCLC (45). Chien-Ying Liu et al. provided evidence of increased CD11b +/CD14 ɤ/CD15 +/CD33 + MDSC in the peripheral blood of NSCLC individuals, which played an essential role in mediating immunosuppression of NSCLC (46).Other authors recognized a unique subgroup of MDSCs in NSCLC patients, namely CD14 (+) S100A9 (+), which inhibited T cells through arginase, iNOS, and IL-13/IL-4Rα axis and were closely associated with adverse effects of chemotherapy (47). Some of the reported studies in Table 1 measured the amount of MDSCs in the peripheral blood mononuclear cells (PBMCS in untreated NSCLC individuals and made a comparison with the healthy ones (48). Their studies proved that the number of G-MDSC increased in NSCLC individuals, whereas further studies showed that G-MDSCs blocked T cell proliferation *in vitro*. The report by Tian Tian et al. who applied FACS sorting to analyze the peripheral blood of SCLC patients proved that the amount of CD14 (+) HLA-DR (-/low) MDSCs in the peripheral blood of SCLC individuals were considerably augmented. The authors further provided a clue that the amount of these MDSCs were predictors of poor prognosis of SCLC (49).

Interestingly, other authors analyzed tumour resections from NSCLC patients. The results showed that the occurrence of M-MDSCs in individuals with lung tumours was higher and that the accumulation of M-MDSCs and G-MDSCs in the tumour site was higher than in peripheral blood. The authors further showed that the MDSCs amount in the peripheral blood could predict recurrence after surgery (50).The effect of the first-line treatment on peripheral blood MDSCs in NSCLC patients was analyzed by other authors. The obtained data presented that chemotherapy with Bevacizumab significantly decreased the levels of MDSCs in the peripheral blood of NSCLC individuals (51). Other authors proposed that the novel targets for immunotherapeutic drug combinations and the treatment of NSCLC through the galectin-9/TIM-3 pathway and mMDSCs were key to anti-PD-1 primary or secondary resistance (52). In patients with Nivolumab treatment, the reported data in another study showed that G-MDSCs could be used as potential immune biomarkers in NSCLC treated with Nivolumab and other second-line therapies (53). In another study by Pauline L de Goeje et al., it was

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TABLE 1 Lung cancer clinical studies of MDSCs.

MDSCs	Research population	No. of people	Biological specimens	Results	Reference
M-MDSCs	Advanced NSCLC individuals Healthy people	40 NSCLC patients 20 Healthy People	Peripheral blood	Peripheral blood with stage IV accumulates more than in people with stage III; Individuals with higher accumulation of M-MDSCs have lower survival rates	(40)
G- MDSCs	Individuals with NSCLC Healthy people	90 NSCLC patients 25 Healthy People	Peripheral blood	Patients with low G-MDSCs have better OS	(41)
CD14(+)HLA-DR(-/low)	Individuals with NSCLC	89	Peripheral blood	CD14(+) HLA-DR(-/low) is a novel MDSCs-mediated tumor immunosuppression in NSCLC	(42)
MDSCs	Individuals with SCLC	41	Peripheral blood	Reduction of MDSCs improves the immune reaction to the injection and can enhance the effectiveness of immune interventions against cancer	(43)
M-MDSCs	Individuals with NSCLC	22	Peripheral blood	An increase in M-MDSCs is strongly linked with primary opposition to immunotherapy	(44)
M-MDSCs/G-MDSCs	Individuals with NSCLC Individuals with SCLC Healthy people	26NSCLC patients 16 SCLC patients 8 Healthy People	Peripheral blood	The incidence of M-MDSCs is expressively developed in NSCLC individuals than in SCLC and healthy populations	(45)
CD11b +/CD14^{-}/CD15 +/CD33 + MDSC	Individuals with NSCLC Healthy people	173 NSCLC patients 42 Healthy People	Peripheral blood	CD11b +/CD14-/CD15 +/CD33 + MDSCs express their crucial participation in facilitating immunosuppression in NSCLC	(46)
CD14(+) S100A9(+)	Patients with NSCLC	101	Peripheral blood	CD14(+)S100A9(+) is a unique subpopulation of MDSCs that inhibits T cells by arginase, iNOS and the IL-13/IL-4Rα axis	(47)
G- MDSCs	Individuals with NSCLC Healthy people	185 NSCLC patients 20 Healthy People	Peripheral blood	G-MDSCs block T cell proliferation *in vitro*	(48)
CD14(+)HLA-DR(-/low)	Individuals with SCLC Healthy people	42 SCLC patients 37 Healthy People	Peripheral blood	Necessary escalation in the number and incidence of CD14(+) HLA-DR(-/low) MDSCs in the peripheral blood of SCLC individuals, respectively, whose frequency can be considered as a forecaster of meagre prediction in SCLC	(49)
M-MDSCs/G-MDSCs	Individuals with NSCLC	42	Peripheral blood/tumour tissue	Higher frequency of M-MDSCs in tumour tissues compared to normal subjects An increase of M-MDSCs and G-MDSCs in tumours than in peripheral blood Levels of MDSCs in peripheral blood predict recurrence after surgery	(50)
MDSCs	Individuals with NSCLC	46	Peripheral blood	Significantly lower levels of MDSCs after chemotherapy with Bevacizumab	(51)
M-MDSCs	Individuals with NSCLC	176	Peripheral blood	Galactoglucose-9/Tim-3 pathway and mMDSCs for NSCLC are critical to anti-PD-1 primary or secondary resistance	(52)
G- MDSCs	Individuals with advanced NSCLC treated with Nivolumab	53	Peripheral blood	G-MDSCs play as immune biomarkers in NSCLC after the second-line treatment, such as with Nabumab	(53)
MDSCs	Individuals with NSCLC Healthy people	105 NSCLC patients 20 Healthy People	Peripheral blood	Augmented amounts of MDSCs relate to decreased vitality	(54)

demonstrated that immunoglobulin-like transcript 3 (ILT3) was expressed on MDSCs in fresh peripheral blood mononuclear cells (PBMCS) from individuals with NSCLC. The authors demonstrated that ILT3 cooperated with ligands on the T cells to inhibit T cells, thus augmenting the amount of MDSCs and decreasing survival (54).

Through the above clinical studies, it is not difficult to find that MDSCs are closely related to the prognosis of patients with lung cancer. Compared with normal people, patients with cancer have higher levels of MDSCs, and most of them achieve immunosuppression by inhibiting T cells. When the level of MDSCs in patients is higher, the OS of patients is lower. Lourdes Barrera et al. showed that the OS of patients with low G-MDSCs is better than that of patients with high G-MDSCs through a series of data studies, and the level of G-MDSCs is a potential prognosis of NSCLC disease progression (41). More MDSCs are found in patients with advanced disease, which is associated with poor prognosis. At the same time, primary drug resistance occurs during immunotherapy due to the presence of MDSCs. There are not many studies on the clinical relevance of MDSCs in human cancer, which mainly focus on the correlation between high levels of MDSCS and shorter OS or PFS in different cancers.

Indeed, it is interesting to note that the incidence of M-MDSC and G-MDSC is increased not only in the peripheral blood of patients but also in the neoplastic lesions. Both tumor-infiltrating MDSCs subsets were significantly elevated compared with circulating subsets, confirming that the tumor site had the strongest immunosuppressive effect. In particular, the frequency of tumor infiltration and circulating G-MDSCs correlated with tumor progression (55). In the study by Yoshikane Yamauchi et al., it was noted that the frequency of MDSCs in tumors was higher than that in peripheral blood of the same patients, and this accumulation was associated with increased concentrations of inflammatory mediators involved in MDSC migration to the tumor microenvironment and activation. Moreover, tumor G-MDSCs showed higher expression level of programmed death ligand 1 than the same cells in peripheral blood (50). Xinyu Tian et al. isolated MDSCs from tumor tissues of lung cancer patients by FCM and showed that RUNXOR was significantly associated with MDSCs-induced immunosuppression in lung cancer patients and may be a target for anti-tumor therapy (56). It has been suggested that in a mouse model, monocytic MDSCs can further mature into Tams in the tumor microenvironment, thereby allowing Tams to induce chemotherapy resistance through various mechanisms. Tumor-infiltrating CD68 Tams were analyzed and compared with blood S100A9 MDSCs from the same patients. Indicating their origin from S100A9 MDSCs, it was also found that the percentage of blood S100A9 MDSCs was closely correlated with the counts of S100A9 cells and CD68 TAM in tumor tissue, and patients with higher S100A9 and CD68 cell numbers also showed worse PFS (57).

In addition to clinical studies, several animal trials of MDSCs on lung cancer progression also exist, through which the key role of MDSCs in lung cancer can be supported. In the study of Mi So Park et al., it was found that the main mechanism by which the polypeptide N-acetyl-galactosaminyltransferase (GALNT3) inhibited the development and progression of lung cancer in xenograft and syngeneic mouse models was the ability of MDSCs to infiltrate the tumor site and subsequent angiogenesis, thereby inhibiting the development of lung cancer (58). Although treatment with immune checkpoint inhibitors (ICIs) improves overall survival in a subset of patients with NSCLC, co-occurring KRAS/LKB1 mutations can drive primary resistance to ICIs. Rui Li et al. therefore targeted G-MDSC enrichment as a potential mediator of immunosuppression in LKB1-deficient NSCLC and sensitized tumors to immunotherapy by overcoming MDSCs accumulation with all-trans retinoic acid in a LKB1-deficient NSCLC mouse model (59). Dickson Adah implanted tumors in mice, obtained whole tumors and tumor-derived sorted cells of tumor-bearing mice, and found that malaria infection significantly reduced the proportion of MDSCs and Treg in the lung tumor tissues of treated mice, and inhibited the expansion and activation of MDSCs and Treg in the tumor microenvironment (60). Xiaosan Su et al. also demonstrated that dexmedetomidine (DEX) induced the proliferation of M-MDSCs during the postoperative period in lung cancer patients by inducing spontaneous tumor metastasis in C57BL/6 mice and had a significant pro-angiogenic ability (61). Liran Levy et al. evaluated the effect of splenectomy in several mouse lung cancer models and found that the effect of splenectomy on tumor growth is essentially cell-mediated by MDSCs, which can be used to inhibit the growth of non-small cell lung cancer by depleting MDSCs (62).

Traditional therapeutic approaches for lung tumours are surgery, radio- and chemotherapy. Although some of these treatments are used as first-line therapy, the clinical studies mentioned above have shown that MDSCs can not only exert be immunosuppressive in the TME but could directly promote tumour advancement but also interfere with the prognosis of conventional treatments, thus making it more critical to treat lung cancer by targeting MDSCs. MDSCs were proposed as potential targets for the advance of anti-cancer lung treatment based on the following five aspects (1): promotion of myeloid differentiation (2); blockage of MDSC propagation (3); removal of MDSCs (4); functional decay of MDSCs, and (5) blockade of immune checkpoints (63).Further research on MDSCs showed that miRNAs/lncRNAs could regulate the specialization, propagation, and immunosuppressive roles of MDSCs in TME (64). Therefore, targeting miRNAs and lncRNAs to stop the development and expansion of MDSCs suppressor cells in the tumour environment appears more promising.

In the review's subsequent chapters, we will focus on lncRNAs and their regulation on the generation, recruitment and immunosuppression of MDSCs.

3 Expression and roles of lncRNAs in MDSCs

LncRNAs are a diverse family of non-coding RNAs (ncRNAs). They encompass different ncRNAs like miRNAs, LncRNAs, snRNAs and CircRNAs (65).LncRNAs' transcripts are longer than 200 nucleotides and are involved in the pathophysiology of many diseases (66). Relevant studies have pointed out that the number of human lncRNAs exceeds the number of protein-coding genes (67). The ENCODE project projected that the human genome contained over 28,000 different lncRNAs, most yet undiscovered (68).Some studies show that lncRNA categories have a high degree of diversity (69), ranging in number from a few hundred to several thousand nucleotides (70).RNA polymerase II transcribes lncRNAs, and according to their genomic localization, mode of action and function can be classified into intronic lncRNAs, intergenic lncRNAs (lincRNAs), enhancer lncRNAs (ELNcRNAs), bidirectional lncRNAs, and sense overlapping lncRNAs (64, 71). Some authors have proven that lncRNAs are mRNA

precursors, and compared with mRNAs, ncRNAs show moderate sequence conservation, while lncRNA-pre-mRNA has a significant part in alternative splicing (72, 73). The expression of a variety of lncRNAs is abnormal in various diseases, especially malignant tumours. Some data show that lncRNAs regulate the bone marrow and immune cells. Their regulatory mechanisms are complex and diverse, and they have become vital regulators mediating cell activation, proliferation, differentiation, apoptosis and autophagy (74). Therefore, lncRNAs may have potential diagnostic, prognostic or therapeutic importance. Table 2 demonstrates the expression of lncRNAs and their regulatory effect on MDSCs.

3.1 The lncRNA PVT1 as a potential oncogene in a variety of cancer types

The mouse plasmacytoma variant 1(Pvt1) gene represents a long non-coding RNA located on chromosome 15 (Ch 15) that was reported for the first time in 1985 (82). It is a candidate oncogene coding for a homologous lncRNA to the human Pvt1 gene, localized on Ch 8, specifically near the c-Myc locus on 8Q24. It encodes 52 ncRNA variants, including 26 linear and 26 circular isoforms and six microRNAs and is long 1.9 KB (83, 84). LncRNA PVT1 is recognized as an oncogene in many tumours. Its overexpression is associated with hepatocellular carcinoma, gastric, oesophagal, cervical, and bladder cancer and acute myeloid leukaemia (85–87), Yu Zheng et al. proves that Pvt1 is highly expressed in tumour-expanding G-MDSCs. The results show that lncRNA Pvt1 downregulation considerably blocked the immunosuppressive function of G-MDSC *in vitro*, reducing tumour development and suppressing anti-tumour immune responses. Since Pvt1 expression is augmented in tumour-infiltrated G-MDSCs more than in splenic G-MDSCs, the hypoxic conditions in TME are considered to trigger such a phenomenon. Therefore exposure of splenic G-MDSCs to hypoxic environments reveals an upregulation of both mRNA and protein levels of HIF-1α in these cells. HIF-1α role in the process is clarified by blocking its upregulation by its specific inhibitor YC-1. The results show restored upregulation of Pvt1 and c-myc in hypoxic environments, thus indicating that HIF-1α augmented Pvt1 expression in G-MDSCs cells under hypoxia (75).

TABLE 2 Expression of lncRNA and its regulatory effect on MDSCs.

lncRNA	Length	Time of the first report	Mechanism	Impact MDSCs	Related diseases	Reference
PVT1	1.9kb	1985	Hif-1α up-regulates the expression of PVT1 in MDSCs under hypoxia stress	To promote the immunosuppressive effect of MDSCs	Hepatocellular carcinoma, gastric cancer, oesophagal, cervical, bladder, acute myeloid leukopathy	(75)
RUNXOR	260kb	2014	RUNXOR regulates RUNX1 expression by recruiting RUNX1 protein at the 3' end and binding to promoters and enhancers	Promote the production of MDSCs and immunosuppressive effect	Lung cancer, acute myeloid leukaemia	(56)
lnc-CHOP	1800 bases	2018	Inc-chop binds to CHOP and the C/EBPβ isoform LIP to induce the activity of the C/EBPβ isoform LAP	Promote the production of MDSCs and immunosuppressive effect	Lung cancer, breast cancer, murine melanoma, murine ovarian tumour	(76)
RNCR3	unknown	unknown	RNCR3 binds to Mir-185-5p and releases Chop	Promote the production of MDSCs and immunosuppressive effect	Colorectal cancer, glioma, prostate,	(77)
Olfr29-ps1	963bp	unknown	Olfr29-ps1 promotes the immunosuppressive role and specialization of MDSCs by forming Mir-214-3p after mbA modification	It promotes the immunosuppressive function and differentiation of MDSCs	Lung, breast, pancreatic cancer, urothelial carcinoma	(78)
HOTAIR	2200 bases	2007	HOTAIR induces more CCL2 secretion and promotes the proliferation of MDSCs	It promotes the immunosuppressive function and differentiation of MDSCs	Nasopharyngeal, breast, pancreatic, liver, stomach cancer, non-small cell lung cancer	(79)
HOTAIRM1	1052bp	2009	HOTAIRM1 enhances the expression of HOXA1 in MDSCs	The immunosuppressive function of MDSCs was weakened	Hepatocellular carcinoma, colorectal cancer, Gastric cancer, head and neck neoplasms, Ovarian, Thyroid cancer	(80)
lnc-C/EBPβ	unknown	2018	The binding of LNC-C/EBPβ to C/EBPβ homotypes LIP and WDR5 downregulates IL4il	The immunosuppressive function of MDSCs was weakened	Melanoma, colon cancer, ovarian, breast cancer	(81)
MALAT1	8kb	2003	It acts directly on MDSCs	The immunosuppressive function of MDSCs was weakened	Hepatocellular carcinoma, Endometrial stromal sarcoma, Cervical, Breast cancer, Osteosarcoma, Colorectal cancer	(8)

3.2 The expression of RUNXOR is closely related to MDSC induced immunosuppression in lung tumors

RUNX1 overlapping RNA (RUNXOR) is a lncRNA transcribed by an upstream promoter and overlapping with RUNX1. It significantly controls bone marrow cells' growth by targeting RUNT-associated transcription factor 1 (RUNX1) (88). LncRNA RUNXOR is about 260 KB long (89). As less research has been done on RUNXOR, dating back as far as 2014, RUNX1 is located on chromosome 21 and is usually disrupted by chromosomal translocations in haematopoietic malignancies. The most common observed translocation is t (8, 21), which is common in acute myeloid leukaemia (90). RUNXOR can regulate RUNX1 expression by recruiting the RUNX1 protein at its 3' end, and upon binding to promoters and enhancers, it makes it participate in chromosomal translocations in malignant cells tumours (90).

Furthermore, by binding straight to chromatin, RUNXOR orchestrates the long-range chromosomal inner loops. Xinyu Tian et al. proves that the RUNXOR and RUNX1 in MDSCs from the peripheral blood of lung cancer patients are differentially expressed in the tissues around the lung cancer compared to the normal tissues. The study results show that lncRNA RUNXOR is augmented in the lung cancer blood samples, while RUNX1 activity is reversely connected with immunosuppression in MDSCs. Moreover, the activity of RUNXOR is advanced in MDSCs in the lung tumour samples than in the adjacent tissues. The knockdown of RUNXOR also decreased arg1 activity in MDSCs. This suggest that RUNXOR expression is considerably related to MDSC-induced immunosuppression in lung tumours and may be a good aim for anti-tumour therapeutic approaches (56).

3.3 lnc -chop regulated MDSCs specialization into M-MDSCs

Lnc-chop is a novel lncRNA identified in MDSCs. It is positioned in the intronic region of the gene on Ch 11. Relevant data indicate that transcription factor C/EBPβ, C/EBP-homologous protein (CHOP) and phosphorylated STAT3 directly influence MDSCs growth (91).CHOP is coded by Ddit3 and takes part in the diminished production of significant factors associated with MDSCs functions, including ARG-1, PNT (peroxynitrite), and superoxide, thereby inhibiting MDSCs activity (92). In addition, lnc-CHOP is associated with lung, breast cancer, murine melanoma, and murine ovarian tumours. The results from the 2018 Yunhuan Gao's trial notes that lnc-chop would be expressed in MDSCs mediated by factors involved in inflammation and tumour development, while lnc-chop potentiates MDSCs immunosuppressive activity *in vivo* and *in vitro*. These data indicates that lnc -chop regulated MDSCs specialization into M-MDSCs and that M-MDSCs have a more potent immunosuppressive effect. The mechanism behind this is that lnc-chop interacts with CHOP and the C/EBPβ isoform LIP to activate the C/EBPβ isoform LAP, thus lnc-chop triggers enhancement of H3K4me3 in the promoters of Arg-1, NOS2, NOX2 and COX2, which are implicated in MDSCs role in suppressing the immune system in TEM (76).

3.4 RNCR3 has potential immunomodulatory functions

RNCR3, also known as LINC00599, is a lncRNA, which is highly conserved in mammals. In tumours, RNCR3 has oncogenic functions and promotes the progression of colorectal, prostate and brain cancers (93).RNCR3 is a crucial regulator of cell propagation, specialization, cell death, metastasis and atherosclerosis (94). Knockdown of RNCR3 leads to increased plasma amounts of TNF-α, CCL2 and IL-6, signifying its potential immunomodulatory functions. Wencong Shang's trial confirms that RNCR3by acted as a competing endogenous RNA (ceRNA) during MDSC differentiation. Tying to miR-185-5p and releasing Chop stimulated their specialization and immunosuppressive activity. There is a close relationship between RNCR3, miR-185-5p and Chop, and the relationship between the three and how they regulated MDSCs is further elaborated in their subsequent experiments. As mentioned earlier, Chop triggers MDSCs' specialization and activity *in vivo*, whereas its reduced activity blocks the activity of Arg-1 and iNOS. Therefore, the authors conclude that RNCR3 triggers MDSCs' specialization by interacting with spongy miR-185-5p to free its target gene Chop. In addition, tumour microenvironmental molecules such as IL-6 triggered RNCR3 expression during MDSCs specialization and further promote their immunosuppressive activity (77).

3.5 Olfr29-ps1 can promote the immunosuppressive effect of MDSCs

Olfr29-ps1, a lncRNA distributes in the cytoplasm and nucleus, is a pseudogene, 963 bp in length, located on mouse Ch 4. Its sequence is preserved in vertebrates and is significantly overexpressed in peripheral blood mononuclear cells from individuals with colon and rectal cancer. It is linked with lung, breast, pancreatic, and uroepithelial cancer. Olfr29-ps1 is regulated by the pro-inflammatory cytokine IL6 and tumour-associated factors. IL6 up-regulates Olfr29-ps1 expression in MDSCs, while Olfr29-ps1 is considerably reduced in MDSCs in B16 tumour mice after IL6 knockdown. The Olfr29-ps1 knockdown results in lower NO, H_2O_2 and ROS in the cells, whereas increased NO, H_2O_2 and ROS are detected in MDSCs that overexpressed Olfr29-ps. Moreover, it is further confirmed that Olfr29-ps1 silencing diminished the protein levels of Arg-1, iNOS, Cox2 and Nox2, while the protein levels of Arg-1, iNOS, Cox2 and Nox2 are augmented in MDSCs that overexpressed Olfr29-ps1. These results signify that Olfr29-ps1 promoted Arg-1 production in M-MDSCs, and H_2O_2 in G-MDSCs, contributing to the MDSCs specialization and suppression of the immune system. MDSC overexpressing Olfr29-ps1 in murine models of melanoma resulted in larger, faster and heavier tumour growth. The mechanism by which Olfr29-ps1 affect MDSCs was further investigated. LncRNA pseudogene Olfr29-ps1 can directly sponge mir-214-3p and promote the differentiation and immunosuppressive function of M-MDSC *in vitro* and *in vivo*, which may be achieved by targeting MyD88. Data show that miR-214-3p diminished MyD88 mRNA and protein levels. Furthermore, the interaction between Olfr29-ps1 and miR-214-3p is reliant on the modification of m6A

by Olfr29-ps1. Data show that lncRNA Olfr29-ps1 have seven conserved GGAC sequences, the most shared m6A sequences. Therefore, RIP-PCR Olfr29-ps1 is modified by m6A in IL6-induced MDSC. These results confirm that Olfr29-ps1 promoted MDSCs' functions by forming miR-214-3p after mbA modification (78).

3.6 HOTAIR activity was significantly enhanced in lung cancer patients

HOTAIR was discovered in 2007 by Rinn et al. (95). It is a 2.2-kilobase ncRNA located at the HOXC site, specifically in the intergenic place between HOXC11 and HOXC12 genes in the HOXC cluster on Ch 12. HOTAIR recruits a transcriptional corepressor polycombsin Complex 2 (PRC2) to repress the HOXD (homeobox gene cluster D) expression (96). In individuals diagnosed with lung tumours, HOTAIR activity is considerably augmented. Therefore, it is considered a new controller of lung tumours, which has great significance in the possible therapeutic approaches to lung cancer (97). HOTAIR is also overexpressed in nasopharyngeal carcinoma, breast, pancreatic, liver, gastric and non-small cell lung cancer (98).HOTAIR was proven to be linked with MDSCs functions in hepatocellular carcinoma lines. In HCC patients, MDSCs exerted their immunosuppressive functions by inducing regulatory T cells. Other authors demonstrated that HOTAIR induced the secretion of CCL2, which recruited TAM and MDSCs, in PBMCs co-cultured with HOTAIR overexpressing cells. It was also shown that HOTAIR played a crucial role in promoting macrophages and MDSCs by secreting cytokines and chemokines from HCC cells (79).

3.7 HOTAIRM1 is highly active in different tumors

HOTAIRM1 (HOXA transcribed antisense RNA bone marrow specific 1) was discovered by (Xueqing Zhang et al., 2009). It is 1052bp in length and is located in the HOXA gene cluster between HOXA1 and HOXA2 on human Ch 7 (99). Initially, it was considered the most prominent intergenic transcript of granulocyte differentiation and up-regulation in NB4 promyelocytic leukaemia. Other authors proved that it was overexpressed in specific myeloid lines (100). Recent data show that HOTAIRM1 is a lncRNA that is abnormally active in different tumours and is related to hepatocellular carcinoma, colorectal, gastric, head and neck, ovarian, thyroid cancers, etc (101).Some authors detected that HOTAIRM1 expression was significantly reduced in tumour tissues. In addition, overexpression of HOTAIRM1 downregulated Arg1 expression levels and inhibited the effect of MDSCs. However, when HOTAIRM1 was overexpressed, the HOXA1, a target gene of HOTAIRM1, was significantly enhanced, suggesting that HOTAIRM1 could trigger HOXA1 expression in MDSCs. In contrast, its silencing or HOXA1 expression in MDSCs significantly reduced the frequency of MDSCs and their inhibitory function. It was further found that HOXA1 overexpression downregulated the activity of the immunosuppressive molecule Arg1 and ROS production in MDSCs and that HOXA1 overexpression enhanced CD4+ Th1 and CD8+ CTL cell initiation so that HOXA1 overexpression enhanced the anti-tumour T-cell response and suppressed the immunosuppressive effect of MDSCs, thereby delaying tumour progression (80).

3.8 Lnc C/EBPβ was significantly increased in G-MDSC

Lnc-c/EBPβ is located on Ch 1 and 4. It is represented by three subtypes of C/EBPβ: liver-rich activator protein (LAP) and liver-rich repressor protein (LIP) (102). Studies on LNC-C/EBPβ are scarce. In 2018 some authors reported on the isolation of MDSCs from mice carrying melanoma, colon, ovarian and breast cancer. MDSCs subsets were classified to analyze the expression of RNC-C/EBPβ in different MDSC subsets. The results demonstrated that lnc-C/EBPβ was found in G-MDSC, M-MDSC and macrophages, and the amount of lnc-C/EBPβ was significantly augmented in G-MDSCs. It has been suggested that ARG-1, NOS2, NOX2 and COX2 activity was controlled by C/EBPβ, which lessened MDSCs' immunosuppressive functions (103). The underlying mechanism included LNC-C/EBPβ knockdown, which changed the transcriptional activity of several genes, like interleukin4-induced gene-1 (IL4i1). However, LNC-C/EBPβ binding to C/EBPβ homotypes LIP and WDR5 was necessary for LNC-C/EBPβ-mediated IL4il silencing. The results showed that the expression of LNC-C/EBPβ was controlled by IL 6, while LNC-C/EBPβ potentially promoted the differentiation of PMN-MDSC. Furthermore, LNC-C/EBPβ hindered the specialization of MDSCs into M-MDSCs (81).

3.9 MALAT1 regulates the differentiation of MDSCs

MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is an extensively investigated lncRNA, especially in tumour biology. It is also recognized as NEAT2 (a nuclear-rich transcript 2), mainly localized in the nucleus and highly preserved among animals. In humans, it is found on chromosome 11q13. Its main transcript length is about 8kb in humans and 6.7kb in mice (104). It was one of the earliest lncRNA genes discovered, and some data linked it with metastasis in NSCLC individuals. It was overexpressed in NSCLC patients and appeared predictive of early-stage NSCLC in individuals with a high risk of metastasis. However, MALAT1 was not only highly expressed in lung cancer but participated in the progression and expansion of hepatocellular carcinoma, endometrial stromal sarcoma, cervical, breast cancer, osteosarcoma, colorectal cancer and others (105). Furthermore, MALAT1 regulated the molecular signalling pathways that drove cell division, apoptosis, cell cycle, metastasis, invasion, and immune response. It was linked to tumour site, size, salinization and cancer stage, so the MALAT1 abnormal expression in tumour tissues or body fluids could be used for diagnostic and prognostic purposes (106).Other authors proved that the average transcription activity of MALAT1 in PBMCs isolated from individuals with lung tumours was considerably reduced and was adversely linked with the amount MDSCs. These results proved that MDSCs and CTL were negatively correlated in PBMCs of individuals with lung tumours. However, according to Qinfeng Zhou et al., MALAT1 levels were not directly correlated with MDSCs and CTLs

in PBMCs of patients with lung tumours. Therefore, it could be concluded that MALAT1 regulated the differentiation of MDSCs. This study also demonstrated that MALAT1 knockdown increased the number of MDSCs by regulating their differentiation, which provided a new understanding of the development of lung tumours and proposed a possible target for supplementary diagnosis and management of lung tumours (8).

Tumor microenvironment, such as hypoxia, the presence of IL-6 and other inflammatory factors, may promote the expression of lncRNA in MDSCs. For example, IL-6 or tumor-related factors can induce the overexpression of lnc C/EBPβ, LNC-CHOP, Olfr29-ps1, Pvt1 and RNCR3 in MDSCs, while chronic and low-dose stimulation of inflammation and tumor factors can also promote the down-regulation of lncRNAs. For example, HOTAIRM1 and MALAT1 are down-regulated in MDSCs from lung cancer patients. These microenvironmental factors produce different effects. From the above introduction, we can know that lnc-CHOP, Olfr29-ps1, Pvt1, RUNXOR, HOTAIR and RNCR3 can promote the immunosuppressive function and differentiation of MDSCs. lnc-C/EBPβ, HTOAIRM1 and MALAT1 blocked the immunosuppressive and differentiation functions of MDSCs, resulting in completely different effects. At the same time, some studies have shown that lncRNAs(such as Olfr29-ps1, lnc-CHOP, RNCR3 and RUNXOR) can participate in the first stage of MDSCs expansion and then participate in the second stage of MDSCS activation due to the identification of different targets, while Pvt1 can only participate in the second stage. In addition, lncrnas can also play cell-to-cell immunosuppressive and tumor-promoting roles through exosomes secreted by MDSCs.

4 The roles of lncRNAs in lung cancer

LncRNAs control the immune cells' functions by various mechanisms. The regulation of MDSCs in various tumour tissues by lncRNAs can promote their application in immunoregulatory anti-cancer therapy or as biomarkers. LncRNAs have dual roles in cancer. They can block or trigger its development. For example, the transmission of lncRNAs in exosomes triggers drug non-responsiveness. Data show that LncRNA H19 encapsulated into exosomes and unambiguously facilitated by hnRNPA2B1 is moved to non-resistant NSCLC cells, resulting in non-responsiveness to Gefitinib (36).In our study, we explored and summarized the direct regulation of lung cancer by lncRNAs and further investigated the indirect regulation of lncRNAs on lung cancer by regulating MDSCs (Figure 2).

4.1 LncRNAs directly regulate lung cancer

Taking lncRNAs as an entry point holds the promise of further improving the survival of individuals with lung tumours. Unfortunately, there is a lack of lncRNA-related lung cancer studies. Here, we summarize the available information on lncRNAs' regulation of lung cancer and provide the findings in Table 3.

The roles of lncRNA PITPNA antisense RNA 1 (PITPNA-AS1) were studied in individuals with NSCLC. The results showed that the transcription levels of PitPNA-AS1 in NSCLC tissues were overexpressed in NSCLC tissues. However, its silencing blocked NSCLC propagation and metastasis. An interesting connection between PitPNA-AS1 and microRNA (miR)-32-5p was detected, proving that PITPNA-AS1 downregulation inhibited the progression of NSCLCs by directing Mir-32-5p. This suggested PITPNA-AS1 as a diagnostic and prognostic biomarker of NSCLC (107). Other authors proved that the lncRNA ZEB2-AS1 was overexpressed in NSCLC patients, which stimulated the epithelial-mesenchymal transition (EMT) in these patients, proving that lncRNA ZEB2-AS1 may become a new diagnostic, prognostic and therapeutic factor (108). Hongxia Wu et al. proposed that targeting the lncRNA NEAT1 could be a possible treatment for NSCLC (109). Some studies have pointed out that the zinc finger protein (ZNF) 281 could be a tumour suppressor lncRNA in glioma. Xin Lu et al. followed up on patients for 5 years to analyze the role of ZNF281 in NSCLC. They found that ZNF281 up-regulated the phosphatase and tensin homolog (PTEN) by down-regulating Mir-221 in NSCLC, thus constraining cancer cell propagation and death (110). The lncRNA LINC00473 was overexpressed in lung tumour tissues and NSCLC cells (A549 and H1299), resulting in a low 5-year patients' OS (overall survival). Studies on lung tumour tissues demonstrated that LINC00473 interacted with Mir-497-5p and blocked its activity, thus promoting the propagation of NSCLC cells (111). To explore the relationship between lncRNA transforming associated RNA (PTAR) and Mir-101 in NSCLC, Wenjun Yu et al. conducted a series of studies during which they proved that lncRNA PTAR was up-regulated in NSCLC cells and could be combined with Mir-101 to inactivate it to stimulate the growth of NSCLC cells (112). GACAT1 (the lncRNA gastric cancer-associated transcript 1) plays a carcinogenic role in different types of cancer. It is overexpressed in NSCLC tissues, which may be associated with the adverse outcome of NSCLC patients. This finding delivers new knowledge for developing novel therapeutic approaches for NSCLC (113). AWPPH is a recently revealed lncRNA, which can be highly expressed in NSCLC tissues, thus stimulating the propagation of NSCLC cells, and considerably inhibiting the survival rate of patients with high AWPPH expression (114).LncRNA prostate cancer-associated transcript (PCAT) 19 also has a certain effect on the progression of NSCLC, PCAT19 was found overexpressed in NSCLC, which increased NSCLC cell proliferation and promoted the progression of NSCLC (115).Ting Wang et al. discovered the role of lncRNA-ATB in NSCLC using the *in vitro* cultured NSCLC NCI-H838 cell line. Their findings revealed that lncRNA-ATB promoted lung cancer progression by inhibiting miR-200a expression and reversed the promotion of β-linked protein expression to promote apoptosis in NSCLC cells (116).The newly discovered lncRNA SET binding factor 2 antisense RNA 1 (LncRNA SBF2-AS1) is involved in the progression of many cancers like lung, breast, hepatocellular carcinoma, thyroid, gastric, colorectal cancers and others (118). Interestingly, Weilong Ye et al. identified 13 lncRNAs related to Gefitinib metabolism and applied them to build the prognostic model of NSCLC patients (119). This proves that lncRNA studies are not limited to particular tumour tissue, and more and more studies have proposed new ideas for the diagnosis, treatment and prognosis of lncRNAs.

Ghada Mohamed Gamal El-din et al. verified the expression of serum markers RAB27A mRNA and RNA-RP11-510m2 in 20 individuals with lung tumours, 10 individuals with COPD and 10

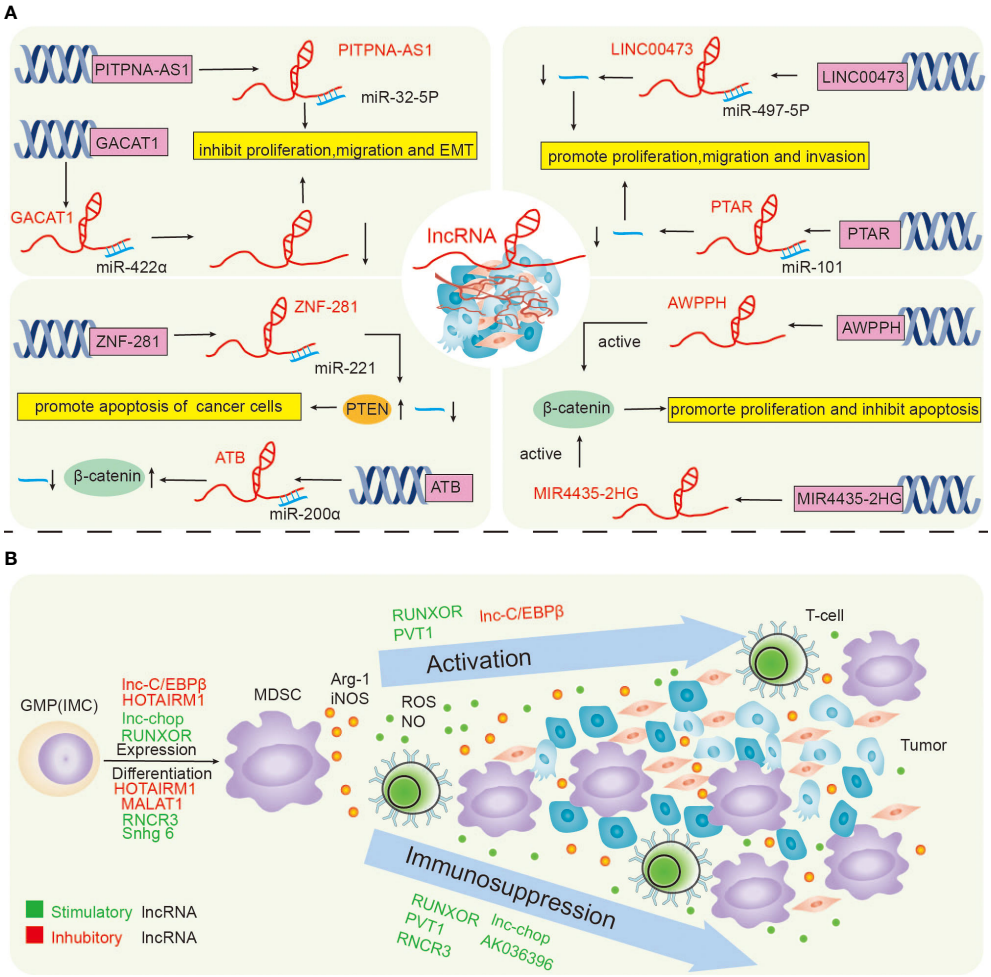

FIGURE 2
LncRNAs directly regulate lung cancer **(A)**, and the lncRNAs regulate lung cancer by regulating MDSCs **(B)**.

controls in good physical shape. The results showed that the serum exosome RAB27A mRNA was positively correlated with lung cancer, whereas NRC-RNA-RP11-510m2 was negatively correlated, which could be developed as biomarkers for diagnosis and prognosis of lung tumours (120).LncRNAs HOTAIR has been shown to induce tumorigenesis of several cancer types. Chunlin Ke et al. explored the relationship between the four types of lncRNA HOTAIR and the susceptibility to lung cancer. One thousand seven hundred fifteen individuals with lung tumours and 2745 healthy subjects were recruited. The results proved that HOTAIR was important in lung cancer screening and prognosis prediction, especially for people with high-risk factors (121). Sik1-lnc is another lncRNA adjacent to salt-inducible kinase 1 (SIK1) and can be abnormally expressed in lung cancer. Other authors (Liu Yang et al.) studied the expression of SIK1 and SIK1-LNC in samples from lung cancer patients and established that the transcription activity of SIK1 and SIK1-LNC was decreased.

Moreover, SIK1-LNC considerably repressed the propagation of lung cancer cells, signifying that SIK1-LNC served as a novel biomarker and target for lung cancer therapeutic approaches (122).TSLC1, as a tumour suppressor gene in various cancers, is considerably repressed in NSCLC tissues and cell lines. The increased expression of TSLC1 inhibited the viability, migration and invasion of NSCLC cells. Other authors explored the biological mechanism of the antisense RNA of TSLC1, namely lncRNA RP11-713B9.1, in the development and progression of NSCLC. They analyzed the tumour tissues of 46 NSCLC patients. They established that the activity of RP11-713B9.1 was undoubtedly connected with TSLC1 (a tumour suppressor gene), while the overexpression of RP11-713B9.1 led to a substantial overexpression of TSLC1. In other words, the inhibition of RNA RP11-713B9.1 transcription backed NSCLC cell survival (123).

4.2 LncRNAs regulate lung cancer by modulating the functions of MDSCs

MDSCs are indispensable for the occurrence and progression of lung tumors. Therefore it is very logical to assume that lncRNAs could regulate lung tumors indirectly b modulating MDSCs' activity. In a study by Qinfeng Zhou et al., the levels of MDSCs and ARG-1 were significantly increased in PBMCs of lung cancer patients. Simultaneously, the relative expression of lncRNA MALAT1 PBMCs of individuals with lung tumors was considerably reduced. The direct effect of MALAT1 on MDSCs was further confirmed by siRNA interference of MALAT1 expression, which resulted in the inhibition

TABLE 3 Regulation of lncRNAs in lung cancer.

lncRNA	Lung cancer subtypes	Effects on lung cancer	Mechanism	Reference
PITPNA-AS1	NSCLC	Silencing inhibits NSCLC cell proliferation, metastasis, and epithelial-mesenchymal transition	To inhibit NSCLC progression by silencing PitPNA-AS1 by targeting Mir-32-5p	(107)
ZEB2-AS1	NSCLC (A549)	It is significantly expressed in NSCLC tissues and triggers metastasis and epithelial-mesenchymal transition of NSCLC tumour cells	unknown	(108)
NEAT1	NSCLC	Modulates sensitivity to iron death in NSCLC cells	unknown	(109)
ZNF281	NSCLC	Blocks proliferation of cancer cells and triggers cell death	Overexpression of ZNF281 and PTEN can accelerate cell apoptosis and inhibit cancer cell proliferation. ZNF281 can down-regulate Mir-221 in NSCLC to up-regulate PTEN	(110)
LINC00473	NSCLC (A549、 H1299)	It can promote cell propagation and metastasis and inhibit cell death in NSCLC	LINC00473 promotes the progression of NSCLC by regulating the ERK/P38 and MAPK signalling pathways and the expression of Mir-497-5p	(111)
PTAR	NSCLC(A549)	It indorses cell division and metastasis of NSCLC	LncRNA PTAR triggers the growth of NSCLC cells by inactivating Mir-101	(112)
GACAT1	NSCLC	Down-regulation blocks cell division and triggers cell death in NSCLC	The expression of GACAT1 in NSCLC was decreased by sponging Mir-422a to inhibit the progression of NSCLC	(113)
AWPPH	NSCLC	Overexpression triggers cell propagation and blocks cell death in NSCLC	LcRNA AWPPH triggers NSCLCs propagation by stimulating the Wnt/β-catenin signalling pathway	(114)
PCAT 19	NSCLC	Overexpression resulted in increased proliferation of NSCLC cancer cells	Overexpression of PCAT 19 can down-regulate p53 and promote the progression of NSCLC	(115)
LncRNA ATB	NSCLC	Promote apoptosis of NSCLC cancer cells	LncRNA ATB inhibited the activity of Mir-200a and promoted the β-catenin transcription in reverse	(116)
lncRNA MIR4435-2HG	adenocarcinoma of lung	Mir4435-2HG knockdown considerably blocked the propagation and metastasis of lung tumour cells	Mir4435-2hg binds to β-catenin to stop its destruction controlled by the proteasome system, thereby controlling the EMT and cancer stem cell properties in lung tumours	(117)

of MDSCs expansion (8). RUNXOR lncRNA is significantly related to MDSCS-induced immunosuppression in individuals with lung tumors and could serve as therapeutic targets. In another study, the expression of lncRNA RUNXOR in PBMCs isolated from individuals with lung tumors was studied by qRT-PCR. The level of RUNXOR in individuals with lung tumors was found to be augmented. Moreover, the authors detected a differential expression of RUNXOR in squamous cell lung and lung adenocarcinoma, thus suggesting that RUNXOR could be used to differentiate lung tumor types. Furthermore, the analysis of the peripheral blood of individuals with lung tumors showed that RUNXOR transcription was augmented with the increase of MDSCs percentage and Arg1 levels but was decreased with the increase of the Th1/CTL cell ratio. This suggested that the transcriptional activity of this lncRNA controlled the immunosuppressive function of MDSCs in these individuals (56). In another study, it was shown that lncRNA Snhg6 mainly exists in the cytoplasm.Furthermore, they proved that Snhg6 controlled MDSCs specialization by decreasing the stability of EZH2 without affecting their immunosuppressive functions (124). As mentioned above, the up-regulation of the lncRNA HOTAIRM1 down-regulated the inhibitory molecules in MDSCs. Other authors proved that HOTAIRM1 was conveyed in different types of lung cancer, especially in lung adenocarcinoma, and its transcription activity was considerably diminished in MDSCs from tumor tissues. Moreover, they showed that when HOTAIRM1 was overexpressed, Arg1 expression levels in MDSCs were down-regulated, thus inhibiting the propagation of MDSCs in lung tumors (125). In another study, the authors established that lncRNA F730016J06Rik (AK036396) was overexpressed in G-MDSCs, whereas its knockdown promoted the maturation of G-MDSCs and reduced their immunosuppression functions. Data show that the Fcnb protein can bind to some proteases to promote the production of ROS and Arg1 by MDSCs through the lectin pathway in granulocyte. The last accelerates the migration of MDSCs to tumor sites. Therefore, AK036396 can enhance the stability of Fcnb protein through the ubiquitin-proteasome system. Thus, the maturation and function of G-MDSCs can be regulated to accelerate immune suppression (126). Yu Zheng et al. implanted Lewis lung cancer (LLC) cells into mice, isolated MDSCs by microbeads and flow cytometry, and measured the transcriptional activity of Pvt1. The authors detected that Pvt1 was overexpressed in G-MDSCs derived from cancer samples. The immunosuppressive effect of tumor-infiltrating MDSCs was mainly due to increased Arg1 transcription and production of nitric oxide by HIF-1α. Coincidentally, Yu Zheng et al. first identified Pvt1 as a HIF-1α target in G-MDSCs of LLC mice cells under hypoxia. RNA interference of Arg1 diminished its activity, ROS production in G-MDSCs decreased, and the antitumor T-cell response was restored. From this perspective, the authors concluded that targeting Pvt1 attenuated G-MDSCS-mediated immune suppression (75). LncRNAs regulate tumors far beyond lung cancer. The proof comes from Zohreh Khodaii et al., who explored the LncRNA-Mir-mRNA complex to find new targets in a rectal tumor. The depletion of Lactobacillus acidophilus in individuals with rectal tumors induced the transcription of the lncRNA-Mir-mRNA network, which delivers new observing and treatment methods for rectal cancer patients (127). Other authors performed biopsies of tumor and non-

tumor tissues from patients with gastric cancer. They showed that lncRNA PVT1 expression was increased, whereas the lncRNA ZFAS1 expression was decreased compared with non-tumor parts. PVT1 and ZFAS1 were biomarkers for detecting and treating gastric cancer cases (128). Furthermore, the lncRNA MEG3 was reported as a tumor suppressor in breast cancer. Battseren Bayarmaa et al. evaluated the impact of MEG3 polymorphism on neoadjuvant therapy in144 patients with breast cancer. The results showed that MEG3 polymorphism was associated with the chemotherapy response and toxicity of paclitaxel and cisplatin. This indicated that MEG3 polymorphism has the potential as a prognostic marker for breast cancer individuals (129). Xianmin Guan et al. obtained bone marrow samples from 146 pediatric patients with acute myeloid leukaemia (AML) and 73 patients with non-hematologic malignancies. They measured lncRNA-SOX6-1 expression to examine the association between lncRNA-SOX6-1 and AML. SOX6-1 transcription was augmented in the AML patients compared to the healthy volunteers, which promoted cell propagation while inhibiting cell death and was related to worse risk diversification and poorer treatment outcomes (130). Similarly, Zhenqing Tan et al. studied the relationship between INK4 expression and the clinical characteristics and prediction of AML in patients, they studied the transcription of ANRIL in bone marrow mononuclear cells (BMMCs) in 178 patients with initial AML and 30 healthy donors. Compared with healthy people, lncRNA ANRIL levels were increased in AML patients, and those with augmented ANRIL transcription had smaller event-free survival (EFS) and OS. Therefore, ANRIL was proposed as a biomarker for AML. Moreover, it has clinical relevance in assisting the diagnosis, treatment and prognosis prediction of AML and identifying potential drug targets of AML (131).

5 Summary and outlook

The development mechanisms of tumour cells are diverse, and the tumour microenvironment changes are also complex and miscellaneous. To successfully implement tumour immunotherapy, tumour suppressors must be removed. Recent data have shown that MDSCs are the chief controllers of cancer immune responses and inflammation in individuals with tumours as they intensely constrain the antitumor immune response of CD4+ T cells, CD8+ T cells and NK cells, thus triggering tumour growth. MDSCs play critical roles not only in lung cancer carcinogenesis but also in its progression and prognosis. Therefore, MDSCs are an attractive therapeutic target because they are carefully related to adverse effects. With the discovery of many novel lncRNAs and the general studies on their roles in different pathologies, particularly cancer, lncRNA research has become a new trend. Factors generated by the tumour and its accompanying hypoxia or inflammatory TME may sustain the expression of some non-coding RNAs. Based on lncRNAs, treatment strategies targeting MDSCs can recover our understanding of MDSCs and disclose new tumour propagation instruments.

Furthermore, the study of novel lncRNAs that regulate the activity of MDSCs is expected to enable their application in immunoregulatory therapy or as biomarkers. In particular, recent data proved that lncRNAs play a significant part in NSCLC development, which will provide a new direction for our subsequent research on lung cancer. However, the complex biological mechanism of MDSCs also poses new challenges for targeted therapy. In addition, the functional connection between lncRNAs and MDSCs does not seem strong enough, the investigations on the way the lncRNAs regulate MDSCs are yet in their infancy, and the clinical research on lncRNA-related lung cancer is very little. Nevertheless, it is hoped that with the development of social science and the progress of medical technology, more clinical studies in this field can be piloted to approve the possibility of regulating MDSCs in lung cancer by lncRNAs. The last will deliver novel ideas for lung cancer treatment and bring more benefits to lung cancer patients.

Author contributions

XP and JH designed and supervised the study. YL and YH reviewed the references. YL and XP wrote the manuscript. YL, YZ and TL contributed to tables and figures, XP and JH revised the manuscript. XP acquired funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Tumor–associated macrophage polarization in the inflammatory tumor microenvironment

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The chronic inflammation of tumor continues to recruit TAMs (tumor–associated macrophages) to the TME (tumor microenvironment) and promote polarization. Pro-inflammatory signals polarize macrophages to the M1 phenotype to enhance inflammation against pathogens. Tumor inflammatory development changes the pro-inflammatory response to an anti-inflammatory response, resulting in the alteration of macrophages from M1 to M2 to promote tumor progression. Additionally, hypoxia activates HIF (hypoxia-inducible factors) in the TME, which reprograms macrophages to the M2 phenotype to support tumor development. Here, we discuss the factors that drive phenotypic changes in TAMs in the inflammatory TME, which will help in the development of cancer immunotherapy of macrophages.

KEYWORDS

tumor-associated macrophages, polarization, inflammatory, tumor microenvironment, cancer immunotherapy of macrophages

1 Introduction

Macrophages are innate immune cells that play a key role in inflammation. At the initiation of inflammation, the number of neutrophils in the circulation increases, followed by monocytes that differentiate to macrophages to promote inflammation against invading pathogens (1). Further inflammation or chronic inflammation can result in tissue damage, and macrophages can also assist in preventing excess inflammation from occurring to protect the body (1). Macrophages in the inflammatory microenvironment eliminate invading pathogens, damaged tissue and apoptotic host cells, which further lead to the resolution of inflammation and tissue reparation (2). It was found that infected tissue without macrophages had an increased apoptotic neutrophil population and prolonged inflammation and tissue damage (3).

Because inflammation has the potential to cause harm, the inflammatory process is typically tightly regulated by macrophages. Pro-inflammatory or activity signals, such as interferon-γ (IFN-γ), colony-stimulating factor-1 (CSF-1), and lipopolysaccharide (LPS), polarize macrophages to the M1 phenotype to promote inflammatory development. Because non-resolving inflammation damages tissue, inflammation should be shut down by anti-inflammatory signals, such as IL-10 and transforming growth factor beta (TGF-β), that

activate macrophages to resolve the inflammatory process (4). An abnormal regulation between pro-inflammatory and anti-inflammatory signals drives many diseases (4).

In the inflammatory TME, the proportion of macrophages can be as high as 30%-50%, and their function has been considered as the 'soil' for tumor growth. At the earliest stage of the tumor, macrophages polarize to M1 to generate an antitumor response. However, once tumors progress past the initial state, the macrophages polarize to M2 to promote tumor progression and malignancy (4–6). Tumors are also considered 'wounds that do not heal' that lead to chronic inflammatory and imbalanced polarization of macrophages (7–9). The present review provides an overview of macrophage polarization in inflammatory TME and proposes a therapeutic strategy for treating cancer.

2 Distribution of macrophages in the TME

Macrophages polarize to different phenotypes in response to signals and cytokines in their environment. Many factors affect TAM polarization and distribution, such as inflammatory signals and cytokines in the TME. The distribution of polarized TAMs in the tumor microenvironment is shown in Figure 1 (9). As an important component of leukocytes, TAMs are mainly derived from circulating monocytes, tissue residue macrophages and myeloid-derived cells (MDSCs). Under specific conditional stimulation and an unequal distribution of nutrients created by the TME, macrophages can be polarized to the M1 type (classically activated phenotype, with markers such as CD80/86) and M2 type (alternatively activated phenotype, with markers such as CD206, CD163, CD204, and stabilin-1), which play an important role in carcinogenesis and metastasis (10). The unequal distribution of oxygen and nutrients in TME affects macrophage polarization. Macrophages are near perfused vessel areas, where nutrients such as glucose, glutamine, and oxygen are high, which induces macrophage polarization to the M1 type. Macrophages residing away from vessels in an environment of chronic hypoxia and a high concentration of lactate are induced to polarize to the M2 type (Figure 1) (1, 9). Both M1 and M2 macrophages are found in TME, whereupon cells in hypoxic areas show more dominant M2 activation. For example, TAMs can express both the M1 marker CD80 and the M2 marker CD206. However, in hypoxic areas, the M1 marker CD80 is expressed at lower levels, and the M2 marker CD206 is expressed at higher levels than in high oxygen areas (11).

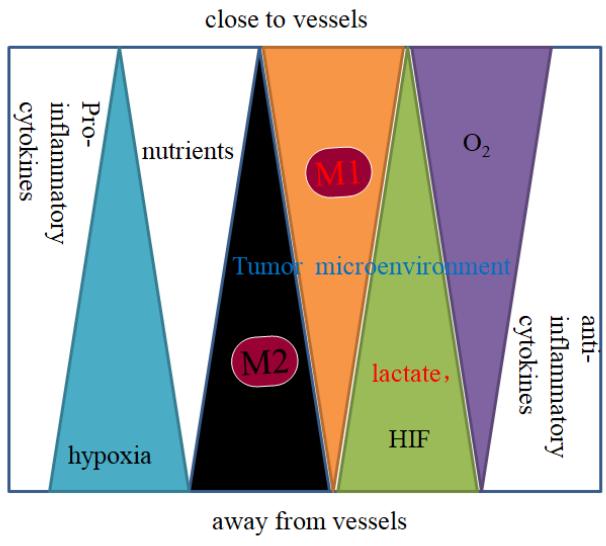

FIGURE 1
Distribution of macrophages in the tumor microenvironment.

Initiation of inflammation in the TME and pro-inflammatory cytokines, such as interleukin (IL)-1-β, IL-6, and IL-8, are produced by tumor cells, immune cells and nonmalignant cells that promote M1 polarization (12). IL-4, macrophage colony-stimulating factor (M-CSF), or granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as IL-10, TGF-β and HIF-1a produced by the hypoxic TME, can skew macrophages to the M2 phenotype. For example, HIF-1a in hypoxic melanoma cells induced translocation and secretion of IL-10, which induced macrophage activation to the alternative M2 phenotype (13).

3 Factors affecting the polarization of tumor macrophages in TME

3.1 Inflammation

In TME, cells face hypoxia, nutrient deprivation and metabolic stress that cause sustained apoptosis and death. In the chronic inflammatory TME, apoptotic cells produce 'find-me' signals to recruit lymphocyte cells that produce inflammatory signals to keep out damaged tissue and prevent their own clearance (9), and the recruitment of neutrophils followed by monocytes that initiate inflammatory signals activates macrophages to the M1 phenotype (8).

But the chronic inflammation leads to the production of anti-inflammatory signals and the transition of macrophages from the M1 to anti-inflammatory M2 phenotype to prevent excess inflammation from occurring (14). M2 macrophages further secrete anti-inflammatory signals, such as IL-10 and TGF-β, to promote angiogenesis, remodeling, and immune suppression, which increase cancer cell proliferation, metastasis and resistance to therapy (15).

Chronic cancer-associated inflammation also contributes to the TME producing cytokines and chemokines, such as IL-4, IL-6, IL-10, IFN-γ, CCL2, CCL5, CD40L, and TNF (8, 16–18). The cytokine and chemokine balance of pro- and anti-inflammatory mediators is a key factor in the progression of macrophage polarization and tumor development. An abundance of pro-inflammatory cytokines and chemokines in the TME recruit and polarize macrophages to the M1 phenotype and promote inflammation. With the development of inflammation, non-resolving cancer inflammation also produces an anti-inflammatory signal to inhibit inflammation that alters macrophages from the M1 to M2 type (9). As mentioned before, The chronic inflammation of tumors continue to cause pro-and anti-inflammatory response occurring, result in sustained polarization macrophages from the M1 to M2 type in the TME (Figure 2) (9).

In the clinic, macrophage polarization is strongly related to tumor stage; in the early phases of tumor inflammation, the TME recruits and polarizes more macrophages to the M1 phenotype, and in the

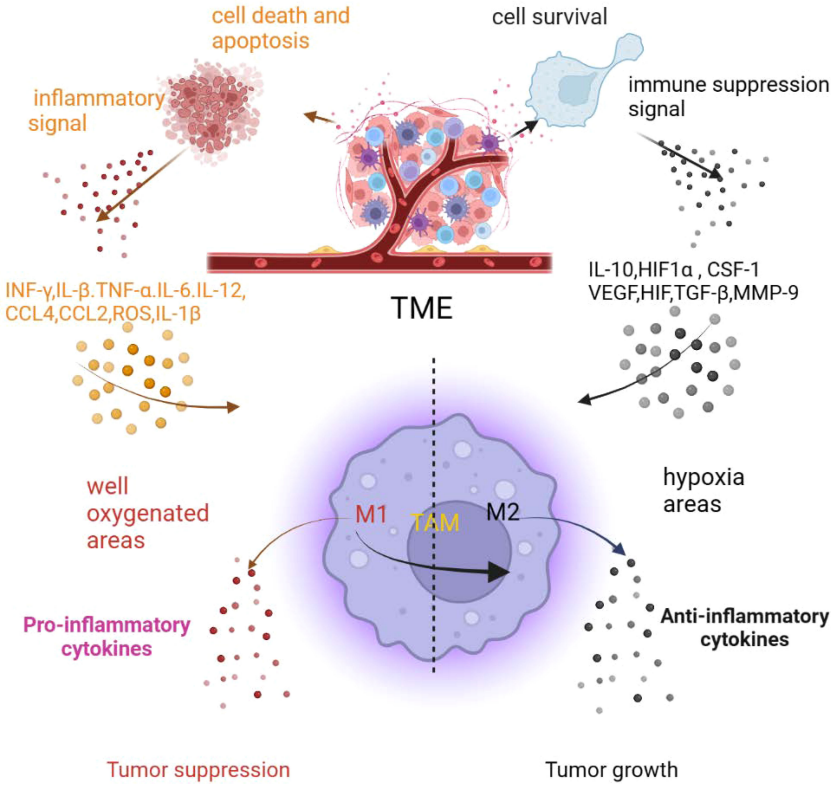

FIGURE 2
The inflammatory tumor microenvironment affects macrophage polarization (created in BioRender.com) (9).

tumor advanced state, more M2 macrophages are found, suggesting a dynamic switch from the M1 to the M2 phenotype (19). Moreover, several studies in murine and human tumors also observed a "mixed" macrophage phenotype in the TME, and the phenotype of macrophages also differs from tumor to tumor or within different areas of the same tumor (20, 21), and macrophages in an advanced state of tumors show a more dominant M2 marker expression pattern (11). Cytokines and chemokines secreted by pro- and anti-inflammatory signals can alter the physiological development of macrophages. It is known that at the earliest stage of the tumor, pro-inflammatory M1 macrophages are activated, and with tumor development, macrophages will convert to the immunosuppressive M2 phenotype in cancer nests to promote tumor growth (4–9, 15, 22, 23).

3.2 Hypoxia

The TME creates an unequal distribution of oxygen and nutrients that affect TAM polarization. In well oxygenated areas of the TME, macrophages show some qualities of classical (M1) activation. In contrast, in hypoxic areas, the TME produces HIF, TGF-β, or IL-6, which provokes alternative (M2) activation of macrophages to promote tumor progression.

Hypoxic stress in the TME not only alters the metabolism of macrophages but also alters their phenotype (9). Hypoxia activates HIF transcription factors to enhance HIF-dependent gene expression and promote the accumulation of the HIF-1/2 protein to adapt to oxygen shortage and metabolic stress. The pathways regulated by HIF can increase glycolysis and suppress O2 consumption. In hypoxic areas, nutrients also become scarce, and HIF enhances the Otto Warburg effect and alters metabolites to express more lactate and kynurenine to promote tumor cell proliferation. Due to the high concentrations of lactate, chemokines, and HIF-1/2 secreted from the hypoxic TME, macrophages are drawn to hypoxic areas and polarize to the M2 phenotype. HIF activation in the hypoxic TME also induces the expression of a number of genes, such as VEGF or matrix metalloprotease 9 (MMP9), that affect macrophage polarization and drive tumor progression (24). In the hypoxic TME of melanoma, tumor cells accumulate HIF-1 and also release high mobility group box 1(HMGB-1), which induces macrophages to produce IL-10 driving them to an M2-like phenotype that promotes proliferation and metastasis (13).

Hypoxic and nutrient stresses not only alter the phenotype of macrophages but also reprogram them. Hypoxic and nutrient stresses also provoke cell apoptosis, necroptosis, and autophagic death. To survive in oxygen- and nutrient-deprived TME, cells promote autophagy signaling pathways, but this promotion is always excessive and causes cell apoptosis and death. Apoptotic and dead cells are recognized by phagocytes that recruit and polarize macrophages to the TME. As mentioned before, the inflammation of tumors cause sustained cell apoptosis and death occurring in the TME, resulting in recruitment of macrophages and direct polarization of macrophages to the M1 phenotype; then, the hypoxic TME

promotes the transition of macrophages from the M1 phenotype to the M2 phenotype or directly polarizes macrophages to the M2 phenotype to support tumor development (Figure 2) (9).

3.3 Tumor cells

In the TME, tumor cells recruit and reeducate macrophages to adopt a special phenotype by secreting vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), TGF-β, CCL2, or M-CSF (17, 25, 26). Hypoxic tumor cells also produce high amounts of lactate and HIF to polarize macrophages to the M2 phenotype to block effective antitumor immune responses by inhibiting tumor surveillance by T and NK cells (9, 27, 28). High lactate levels produced by tumor cells also evoke HIF-1a and HIF-2a accumulation in macrophages, which changes the pro-inflammatory environment to an anti-inflammatory environment by reducing NF-kB activity, in turn reducing T and NK-cell activation (9, 29). Tumor cells also promote membrane cholesterol efflux induces IL-4-mediated signaling in macrophages and alters their phenotype to promote tumor invasion and metastasis (30, 31). It was found that co-culturing macrophages with tumor cells increased HIF-1 and VEGF expression, which induced the dysregulation of arginase and Fizz1, and this was correlated with a gene signature found in alternatively activated macrophages that promote tumor development (9, 32).

The influence of tumor cells and macrophages is interactive. For example, when TAMs are cocultured with hepatoma cells, macrophage-derived IL-6 and IL-8 activate JAK kinase, which phosphorylates STAT3 activating STAT3 signaling in tumor cells and promotes the epithelial mesenchymal transition(EMT), thus enhancing tumor invasion and metastasis (33, 34). M2 TAMs can also induce high expression of both PD-L1 and CTLA4 in cancer cells, which promotes immune escape through limiting activationof cytotoxic T cells in the TME. TAMs induce high levels of PDL1 expression that correlate with poorer clinical outcomes in hepatocellular carcinoma (HCC) (8, 35–39).

3.4 Immune cells

Regulatory immune cells, such as Treg cells, MDSCs and B cells, can also regulate macrophage polarization. Treg cells inhibit CD8+ T-cells secreted IFN-γ, that maintain macrophages in the M2-like phenotype, which also reduces fatty acid oxidation and induces lipid accumulation in macrophages by increasing the expression of sterol regulatory element binding protein 1 (SREBP1) (40). Inflammatory interleukin-17-positive (IL-171) T cells can recruit and promote maturation of chemokine receptor 3–positive (CXCR3) B cells, which induce M2b macrophage polarization in human HCC (41). Tumor-infiltrating lymphocytic B cells program macrophages to the M2 phenotype *via* Bruton tyrosine kinase (BTK) activation in a PI3K-Y manner and inhibit B-cell infiltration. Inhibition of BTK in the pancreatic TME reduced tumor growth and enhanced antitumor activation (42).

MDSCs are heterogeneous immune cells that consist of myeloid progenitor cells and immature myeloid cells (IMCs). They can differentiate into TAMs and can affect macrophage polarization within TME. MDSCs can suppress the immune response by abnormally regulating STAT3 to promote anti-inflammatory (M2-like) macrophage polarization (31, 43). MDSCs are usually recruited to the TME and produce IL-10, which inhibits macrophage expression of IL-12 and alters the macrophage phenotype to M2. MDSCs also express high levels of arginase-1, which promote macrophage polarization and contribute to immune suppression (8, 43, 44).

3.5 Chemokines and cytokines

Macrophage polarization in the TME is dynamic and dependent on the balance of chemokines and cytokines. Numerous chemokines (such as CCL2, CCL5, CCL15, and CCL20) and cytokines (such as TGF–β, CSF-1 and TNF) have been demonstrated to participate in the mechanism of monocyte-derived macrophage recruitment, migration and polarization (45–49). The representative molecules are discussed below.

3.5.1 CCL2

CCL2 is a small chemokine which is mainly produced by tumor cells and surrounding stromal cells. CCL2 recruit CCR2+ inflammatory monocytes from the bone marrow to the peripheral blood that lead to cancer metastases and poor clinical outcomes (50). CCL2 elevation in the TME is essential for the recruitment and education of monocyte-derived macrophage polarization. Macrophages express CCR2 were recruited by CCL2 that result in up regulating their expression levels of angiogenic factors, such as IL–6, VEGF, and MMP9, which contributed to tumor vascularization. Inhibition of the CCL2/CCR2 signaling pathway can block monocyte recruitment and suppress the polarization of macrophages toward the M2 phenotype (51–53).

3.5.2 CSF-1

Colony-stimulating factor 1 (CSF-1) involve in macrophage recruitment, differentiation, mature, and survival. CSF-1 receptor (CSF-1R) is a tyrosine kinase receptor which mainly expressed on monocytic lineages which will differentiate into TAMs. CSF-1 and IL-34 bind to CSF-1R active cascade of signaling in monocytes will increase recruitment of M2-like phenotype and promote immunosuppression (54). Tumor-derived CSF-1 promotes tumor growth and enhances M2 polarization and infiltration. Targeting CSF-1/CSF-1R signaling in combination with CXCR2 antagonists can prevent M2 polarization and shows a strong antitumor effect (55). It was found that CSF-1/CSF-1R signaling inhibition can reduce TAM infiltration and enhance the CD8+/CD4+ T-cell ratio to kill tumor cells. In a transgenic mouse model, targeting TAMs by CSF-1R blockade enhanced the anticancer efficacy of platinum-based chemotherapies (56, 57). It was also found that combination treatments of CSF-1/CSF-1R inhibitors with PD1-PDL1 inhibitors are promising candidates for effective elimination of TAMs (54).

3.5.3 IL-6

IL-6 is an important cytokine, which is closely related to the malignant behavior, such as promotion of inflammation, proliferation, angiogenesis, invasion, metastasis of tumor in TME. It

was found that IL-6 was a risk factor that highly expressed in chronic inflammatory tumor tissue that lead to poor prognosis (58). In inflammatory TME, IL-6 secreted by TAMs resulting in a vicious cycle that further promote macrophages polarization to TAMS and increase IL-6 expression which can lead to a smoldering inflammatory state, and enhance tumor cell metastasis (46, 59). Chen S,et al. showed that IL-6 was responsible for TAMs induced renal cell carcinoma cells migration, invasion, EMT by activating I L-6/STAT3 signaling (60). IL-6 acts on IL-6R/gp130 receptors and active STAT3 signaling which can promote epithelial-mesenchymal transition (EMT), angiogenesis and immunosuppression in cancers (61). Han IH, et al. found that IL-6 induces M2 polarization and promotes proliferation of prostate cancer cells (62). And Zhang W, et al. showed that IL-6 promotes PD-L1 expression in monocytes and macrophages through JAK2/STAT1 and JAK2/STAT3/c-MYC signaling and induces immunosuppression in an orthotopic tumor transplantation model (63). Activated STAT3 by IL-6 also promotes the secretion of IL-10 and maintain the immunosuppressive function of Tregs (64).

3.5.4 IL-10

IL-10 is a immunosuppressive cytokines secreted by immune cells, such as monocytes, macrophages and B cells. IL-10 induces the TAM M2 polarization that further secrete high IL-10, IL-6, TGF-β, which can promoting fibrosis and enhance tumor growth (65, 66). Patients with high level expression of IL-10 in both the serum and peritoneal effusions are correlated with advanced stage disease (67). By contact with its receptor, IL-10 can also activate the IL-10/STAT3 signaling pathway which skew macrophages to TAM M2 and promote high expression of various antiapoptosis, pro-tumorigenic and immunosuppression related genes (68). IL-10 also through TLR4/IL-10 signaling pathway alter macrophages to TAM M2 to promote epithelial-mesenchymal transition in pancreatic cancer cells (69). IL-10 expressed by TAMs suppresses IL-12 production by DCs, thus limit cytotoxic CD8+ T cell responses and resist chemotherapy. It could improve chemotherapy by blocking IL-10 receptor to enhance primary tumor response in breast cancer with paclitaxel and carboplatin treatment (70). It was also found that macrophages exposed to tumor culture supernatants secreting more IL-10 that may trigger a rise of the intratumoral forkhead/winged helix scurfy $(FoxP3)^+$ Tregs population, which are associated with HCC aggressive (71).

3.5.5 TNF

TNF mainly positively regulates M1 polarization by activating tumor necrosis factor receptor (TNFR) and the NF-kB signaling pathway to suppress M2 polarization. Other cytokines, such as myeloid differentiation primary response 88 (MyD88), can also inhibit M2 gene expression in TAMs, leading to an M1 phenotype (8, 72).

3.5.6 TGF-β

TGF-β is a growth regulatory protein that shows both antitumoral and pro-tumoral activities. In the precancerous state, TGF-β inhibits cell proliferation, whereas in the established tumor stage, TGF-β enhances macrophage secretion of IL−10, which promotes macrophage polarization and induces immune evasion and metastasis. TGF−β secreted by TAMs promotes macrophage alteration to the pro−tumor M2 type (73).

4 Cancer immunotherapy of macrophages

Macrophages are trapped in the TME and promote the development and progression of tumors. Inflammation and cell death result in recruitment and maturation of macrophages into M1 TAMs. Hypoxia that enhances HIF genetic expression promotesre polarization of M2-type macrophages (9). The mode of tumors is considered 'wounds never heal', and the non-resolving tumor inflammatory response continues to recruit macrophages and mature them into the M1 phenotype, and with tumor development, hypoxia and anti-inflammatory cytokines transform macrophages to the M2 phenotype to promote tumor growth (9). Depending on the mode, improving tumor therapy should therefore consider blocking inflammation and blocking macrophage recruitment and eliminating preexisting TAMs (74). Because of diversity and heterogeneity of tumors, here, we use solid tumor therapeutic strategies as paradigm to explain as following (Table 1).

4.1 blocking CCL2-CCR2 and CXCR4-CXCL12 signaling

Blocking the CCL2-CCR2 axis and CXCR4-CXCL12 signaling pathway can prevent TAM recruitment and infiltration into the TME (74), which has shown potential therapeutic value for solid tumors in preclinical and clinical studies (Table 1). For example, an anti-CCL2 antibody, carlumab (CNTO888), can inhibitor macrophage infiltration to the tumor in mice, which has been applied in clinical trials to treat solid tumors and metastatic castrate-resistant prostate cancer (75). Clinical studies indicated that single-agent carlumab only temporarily repressed serum CCL2, resulting in no significant antitumor effects (76). However, combination of carlumab with several conventional chemotherapy regimens such as paclitaxel and carboplatin, significantly enhance the antitumor response (77)

Also, inhibition CXCR4-CXCL12 signaling can more specifically promote TAM exclusion. CXCL12 is a cancer-associated fibroblast derived factor which recruit CXCR4-expressing monocytes toTME and skew to M2-like macrophages to promote tumor growth (78). It was found that targeting the CXCR4-CXCL12 signaling could effective treat for solid tumors in the clinic trails. For example, a CXCR4 antagonist Plerixafor (AMD3100), which can inhibit the secretion of VEGF-A from TAMs and lead to reduce tumor angiogenesis, has been used in clinical trials for treating solid tumors and children cancer (73). Other CXCR4 antagonist, such as LY2510924 (CXCR4 antagonist peptide) also use in clinical trials for treating solid tumors (74, 79).

4.2 blocking CSF-1/CSF-1R signaling

As mention before, the CSF-1/CSF-1R signaling pathway also plays a key role in TAM recruitment and polarization. Therefore, blocking the signal in TAMs has been developed in clinical trials for solid tumor therapy (80). For example, a monoclonal antibody Emactuzumab (RG7155) could effectively inhibit CSF-1R activation. Emactuzumab treatment significantly reduces CSF-1R+/CD163+ macrophages in diffuse-type giant cell tumor and increases the ratio

of CD8+/CD4+. Emactuzumab in combination with chemotherapy and immunotherapy are underway in clinical trials of solid tumor treatment (74). CSF-1R specific inhibitors, such as PLX3397, PXL7486, AMG820, BLZ945, et al., also have been used in clinical trials for treatment of solid tumor. It was found that both CSF-1R antibodies and inhibitors could improve therapy in preclinical and clinical trial. For example, the CSF-1R inhibitor BLZ945, alone or in combination with anti-PD1 antibody immunotherapy could block macrophage recruitment and alter macrophage polarization to antitumor type that currently was being assessed in clinical trials for advanced-stage solid tumors treatment (74).

4.3 blocking CD47-SIRPα signaling

Although eliminating and inhibiting recruitment TAM strategies can delay tumor progression, these therapeutic approaches may have systemic toxicities as they target all macrophages without specific, and

TABLE 1 Clinical trials of solid tumors associate of macrophage-targeting compounds.

Action	Compounds	Clinical phase	Tumor type and combination agent	Clinical trials
Elimination and blocking recruitment of M1 and M2 TAMs	Carlumab (CNTO888,CCL2 inhibitor)	I	Solid tumors + doxorubicin liposome injection; +gemcitabine;+ Paclitaxel and carboplatin;+ docetaxel	NCT01204996
	plerixafora (AMD-3100, CXCR4 antagonist)	II	Solid tumors	NCT01225419
	LY2510924 (CXCR4 antagonist peptide)	I	Solid tumors	NCT02737072
	Emactuzumab (RG7155, CSF-1R antibody)	I	Advanced Solid Tumors + RO5509554	NCT01494688
	Emactuzumab (RG7155, CSF-1R antibody)	I	Advanced solid tumors + Atezolizumab	NCT02323191
	PLX3397(Plexxikon, CSF-1R inhibitor),	Ib/II	Advanced solid tumors + paclitaxel	NCT01596751
	ARRY-382(CSF-1R inhibitor)	II	Advanced solid tumors + pembrolizumab	NCT02880371
	Pexidartinib (CSF-1R inhibitor)	I	Advanced solid tumors	NCT02734433
	BLZ945(CSF-1R inhibitor)	I	Advanced solid tumors	NCT02829723
	JNJ-40346527(CSF-1R inhibitor)	I	Prostate cancer	NCT03177460
	IMC-CS4(CSF-1R inhibitor)	I	Advanced solid tumors	NCT01346358
	FPA008 (Cabiralizumab, CSF-1R antibody)	I	Advanced solid tumors + nivolumab	NCT02526017
	PXL7486(CSF-1R inhibitor)	I	Advanced solid tumors	NCT01804530
	AMG820 (Amgen, CSF-1R inhibitor)	I	Solid tumors	NCT01444404
	SNFX-6352 (CSF-1R antagonists)	I	Advanced solid tumors + Durvalumab	NCT03238027
	Trabectedin	I	Solid tumor, Adult + Durvalumab	NCT03496519
Reprogramming TAM M2 to M1 to antitumor	Hu5F9-G4(CD47-SIRPα inhibitor)	I	Advanced solid malignancies	NCT02216409
	Hu5F9-G4(CD47-SIRPα inhibitor)	I	Advanced solid malignancies and colorectal carcinoma + cetuximab	NCT02953782
	TTI-621 (SIRPα-IgG1 Fc)	I	Solid tumors + Rituximab or Nivolumab	NCT02663518
	Selicrelumab(CD40 agonist)	I	Solid tumors + Atezolizumab	NCT02304393
	Selicrelumab(CD40 agonist)	I	Advanced solid tumors + Vanucizumab or Bevacizumab	NCT02665416
	SEA-CD40(CD40 agonist)	I	Solid tumors + pembrolizumab	NCT02376699
	IMO-2125(TLR9 agonist)	I	Refractory solid tumors, metastatic melanoma	NCT03052205
	SD101(TLR9 agonist)	I/II	Solid tumors + SBRT + pembrolizumab	NCT03007732
	Anakinra (IL-1R antagonist)	I	Advanced solid tumors + everolimus	NCT01624766
	GSK1795091(TLR4 agonist)	I	Advanced solid tumors + GSK3174998 anti- OX40) or (GSK3359609 anti- ICOS) or pembrolizumab	NCT03447314

(Continued)

TABLE 1 Continued

Action	Compounds	Clinical phase	Tumor type and combination agent	Clinical trials
	Telratolimod (MEDI91973, TLR7/8 agonist)	I	Solid tumors + Durvalumab and/or Palliative Radiation	NCT02556463
	IPI-549(PI3Kγ inhibitor)	Ib	Advanced solid tumors + nivolumab	NCT02637531
	Vanucizumab(Vasculature-modulating agent Ang2/VEGF)	I	Advanced/metastatic solid tumors	NCT02665416
	EF-022(Efranat, vitamin-D-binding protein, macrophage-activating factor)	I	Solid tumors	NCT02052492

Data were obtained from http://clinicaltrials.gov.
CCL2, C-C motif chemokine ligand 2; CCL5, C-C motif chemokine ligand 5; CCR2, C-C motif chemokine receptor 2; CXCR2, C-X-C chemokine receptor type 2;CSF-1,colony-stimulating factor-1; CSF-1R, Colony stimulating factor 1 receptor; CARMs, chimeric antigen receptor macrophages; EMT, epithelial mesenchymal transition; Fizz1, found in inflammatory zone 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; HIF, hypoxia-inducible factors; HCC, hepatocellular carcinoma; HMGB-1, high mobility group box 1; IFN-γ, interferon-γ; IL-6, interleukin-6; IMCs, immature myeloid cells; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MDSCs, myeloid-derived suppressor cells; MMP9, matrix metalloprotease 9; MyD88, myeloid differentiation primary response 88; PD-L1: programmed cell death ligand 1; PD-1, programmed cell death protein 1; PDGF, platelet-derived growth factor; RP-182, a synthetic 10-mer amphipathic analog of host defense peptides; ROS, reactive oxygen species; SREBP1,sterol regulatory element binding protein 1; SIRPa, signal regulatory protein alpha;TAMs, tumor–associated macrophages; TME,tumor microenvironment; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TGF-β: transforming growth factor beta; VEGF, vascular endothelial growth factor.

eliminating TAMs can be rapid compensation by tumor-associated neutrophils (TANs). It was found that withdrawal of CCL2/CCR2 inhibitors might accelerate metastasis in breast cancer by dramatically releasing of monocytes which were trapped in the bone marrow (81). So, it is appealing new strategies such as re-educating macrophages to anti-tumor phenotypes to overcome these limitations.

One method of re-educating macrophages is using inhibitors to block receptor signals on macrophages that modulate phagocytosis. Tumor cells overexpress the "don't eat me" signaling molecule CD47, which suppresses macrophage phagocytic capacity by interacting with signal regulatory protein alpha (SIRPa). Using anti-CD47 antibodies to disrupt the CD47-SIRPα axis can restore the ability of macrophages to engulf tumors (74). Many conventional anti-CD47 antibodies have been demonstrated to be successful in preclinical and clinical trials. For example, it was found Hu5F9-G4, an anti-CD47 antibody, could inhibit the interaction of CD47 with SIRPα and promoted macrophage-mediated phagocytosis to kill cancer cells. Hu5F9-G4 has been used in clinical trials to treat solid tumors and various hematological malignancies (82).

Also, the polypeptides or recombinant proteins including engineered high-affinity SIRPα protein which derived from SIRPα can act as decoy bind to CD47 to disrupt the CD47-SIRPα signaling. Studies showed that recombinant protein TTI-621 which composed of the N-terminal domain of SIRPα fused to human IgG1 could suppress tumor growth by increasing macrophage-mediated phagocytosis of solid tumor cells (83). TTI-621 is now in clinical investigation to treat solid tumors.

4.4 CD40 agonists

CD40 is a superfamily member of TNF receptor and expresse on many antigen-presenting cells (APCs) as well as some tumor cells. It was found that agonistic anti-CD40 antibodies could stimulate TAMs to promote the secretion of the proinflammatory cytokines such as NO and TNF-α to activate effector T cells to reestablish tumor immune surveillance. It was found that many agonistic anti-CD40 antibodies such as Selicrelumab (CD40 agonist) in combination with immunotherapy significantly promoted macrophages phagocytic activity to antitumor. Selicrelumab combination with immunotherapy such as atezolizumab has been use in clinical trials to treat solid tumors (74).

4.5 Toll-like receptor agonist

Toll-like receptors (TLRs) play critical roles in activating the innate immune reaction of macrophages toward antitumor M1 phenotype. Activation of multiple TLR signals promotes phagocytic activity of macrophages and enhances antitumor responses. For example, it was found that TLR4 and TLR5 agonists could polarize more CD206+ M2 TAMs to CD86+ M1 phenotype and suppressed tumor growth without obvious toxicity. Other TLR agonists have also been found to alter f M2 TAMs to pro-inflammatory M1 phenotype and promote tumor regression in mouse models (84). TLR9 agonist IMO-2125, which can induce tumor regression by promoting macrophage polarization to antitumor type, has been evaluated in clinical trials to treat refractory solid tumors and metastatic melanoma (74). However, TLR stimulation by agonist always lead to PD-L1 expressed level elevation in macrophages, resulting in limiting antitumor responds. To overcome this setback, IMO-2125 combined with immunotherapy such as iplimumab to treat cancer more effective. Recently, MO-2125 combined with iplimumab was approved by FDA to treatment of melanoma. Others TLR9 agonist, such as SD101 was also investigation along with PD-1 blockade in clinical trials to enhance therapeutic efficacy (74, 85).

4.6 PI3Kγ inhibitor and other treatments promoting macrophage reprogramming

Phosphatidylinositide 3-kinases (PI3K), which can specifically phosphorylate the 3′ position in the inositol moiety of phospholipids, play crucial roles in inflammatory, immunosuppression associated with cancer or autoimmune diseases. PI3Kγ is the class IB PI3K member which playing significant roles in immunosuppressive transcriptional programming by contacting with G protein (86). PI3Kγ promotes transcription of genes and enhance immunosuppressive factors Arg1,

TGF-β, and IL-10 expression that links to the M2 immunosuppressive macrophage phenotype (87).

PI3Kγ inhibitors can alter macrophages toward proinflammatory phenotype and block recruitment of macrophages and neutrophils from peripheral blood (88, 89). IPI-549, the PI3Kγ-selective inhibitor, has been reported to promote macrophage polarization to M1 states and enhancing immunotherapy by increasing CD8+ T-cell activation and cytotoxicity (90). IPI-549 combination with nivolumab has been investigated in phase I clinical trials for several advanced solid tumors.

Other classical treatments such as blocking the function of TAM-expressed PD-L1to promote macrophage reprogramming to enhance antitumor effects. TAMs expressing the checkpoint molecule PD-L1 negatively regulate the phagocytic ability of TAMs and suppress cytotoxic T-cell immunity against tumor cells. Blocking the PD-1/PD-L1 pathway can enhance the phagocytosis of macrophages and prolong the survival of mice in cancer models (91). It also improved therapy in clinical treatment by blocking the function of TAM-expressed PD-L1 (92).

Recently, a new technique of re-educating macrophages to generate chimeric antigen receptor macrophages (CARMs) has emerged for cell-based cancer immunotherapy. It was found that CARMs encoding the CD3ζ intracellular domain can target the tumor antigen mesothelin or HER2 and kill antigen-positive solid tumor cells (93, 94). A huge breakthrough was shown in CARM immunotherapy on July 27, 2020, and the FDA approved the investigational new drug application for anti-human HER2-CARM (CT-0508) to treat recurrent or metastatic HER2-overexpressing solid tumors (79).

5 Conclusions and perspectives

Inflammation is a double-edged sword in tumor treatment. It should distinguish 'antitumor inflammation (acute inflammation)' and 'pro-tumor inflammation (chronic inflammation)' for precision tumor therapy. 'Antitumor inflammation' can active the immune system that recognize and cause tumor cell death by immune surveillance process. But chronic inflammation promotes immunosuppression and tumor progression (95).TAMs polarize and orchestrate tumor-related inflammation in TME. M1 phenotype secrete pro-inflammatory cytokines (TNF-α, IL-1β, IL-12, e.g.), and co-stimulatory molecules to present antigen efficiently and promote Th1 response to destroy tumor cells. However, M2 TAM secrete anti-inflammatory and immunosuppressive molecules (IL-4, IL-10, TGF-β, e.g.), to promote chronic inflammation that lead to sustained recruit and polarize TAMs to the TME and promote tumor malignant transformation.

Targeting TAM therapeutic protocols, such as eliminating and inhibiting recruitment, switching the M2 phenotype to the M1 phenotype, enhancing phagocytosis and increasing antigen presentation to kill tumor cells, and new CARM technology have also greatly improved cancer treatment. However, these cancer treatment technologies are still a long way off. The biggest difficulty is how to precisely promote the 'antitumor inflammation' inducing by macrophage to kill tumor cells and eliminate pro-tumor chronic inflammation in tumor therapy. For example, eliminating and inhibiting recruitment TAM strategies to treat inflammatory tumor may have systemic toxicities as they target all macrophages including M1and tissue-resident macrophages without specific that will leads to increased bacterial infections, metastasis and accelerated death. And switching the M2 to the M1 phenotype may only result in temporary and limited antitumor efficacy. Because of tumor heterogeneity, it is difficult to definite TAMs subpopulations in different human tumors. Also, TAMs are not stably inherited and they can change in TME. By contact with tumor cells, M1may sustained polarize to M2 to promote malignancy progression. In addition, although anti-tumor inflammation producing by TAM M1 can cause tumor cell death, it can also create a mutagenic microenvironment which may lead to TAM polarization to M2 resulting in promoting tumor progress.

So, it need to explore new strategies that not only renovate the inflammatory tumor "soil" that consist by the TAMs to construct a anti-tumor microenvironment, but also kill the tumor "seeds" in the "soil". Thus, continuous studies are needed to elucidate the mechanisms that drive phenotypic changes in TAMs in the inflammatory TME, which will help in the development of cancer immunotherapy of macrophages.

Author contributions

BL, ZZ gathered the related literature, prepared the figures and drafted the manuscript. HL and ML participated in the design of the review and drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

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

Publisher's note

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Carbon dot-based nanomaterials: a promising future nano-platform for targeting tumor-associated macrophages

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The tumor microenvironment (TME) is the internal environment that tumors depend on for survival and development. Tumor-associated macrophages (TAMs), as an important part of the tumor microenvironment, which plays a crucial role in the occurrence, development, invasion and metastasis of various malignant tumors and has immunosuppressant ability. With the development of immunotherapy, eradicating cancer cells by activating the innate immune system has yielded encouraging results, however only a minority of patients show a lasting response. Therefore, *in vivo* imaging of dynamic TAMs is crucial in patient-tailored immunotherapy to identify patients who will benefit from immunotherapy, monitor efficacy after treatment, and identify alternative strategies for non-responders. Meanwhile, developing nanomedicines based on TAMs-related antitumor mechanisms to effectively inhibit tumor growth is expected to become a promising research field. Carbon dots (CDs), as an emerging member of the carbon material family, exhibit unexpected superiority in fluorescence imaging/sensing, such as near infrared imaging, photostability, biocompatibility and low toxicity. Their characteristics naturally integrate therapy and diagnosis, and when CDs are combined with targeted chemical/genetic/photodynamic/photothermal therapeutic moieties, they are good candidates for targeting TAMs. We concentrate our discussion on the current learn of TAMs and describe recent examples of macrophage modulation based on carbon dot-associated nanoparticles, emphasizing the advantages of their multifunctional platform and their potential for TAMs theranostics.

KEYWORDS

TAMs, carbon dots (CDs), bioimaging, theranostics, macrophage modulation

Introduction

Overall, 1,918,030 new cancer cases are expected to be diagnosed in the United States in 2022, equivalent to 5,250 new cancer patients per day. Men have a lifetime cancer probability of 40.2%, slightly higher than women (38.5%), and it is also the leading cause of death. In recent years, immunotherapy has emerged as a rising star in the cancer therapeutics spectrum and is a promising strategy for cancer treatment (1). Regrettably, definitive durable therapeutic effects are observed in a small proportion of patients. But the majority show limited clinical benefit or no response at all (2). In order to overcome the resistance of immunotherapy, the mechanism of immunosuppression has been studied in depth in recent years. Numerous studies have shown that the tumor microenvironment (TME) plays an important role in immunosuppression. Multiple inhibitors in the tumor microenvironment (TME) have been identified through analysis. Cell populations, among which tumor-associated macrophages (TAMs) stand out, are promising new targets for tumor immunotherapy (3). TAMs are a prevalent type of inflammatory cell found in the stroma of various tumors. They exhibit a diverse range of phenotypic characteristics and contribute to tumor growth, metastasis, and recurrence by facilitating an immunosuppressive environment. In solid tumors, TAMs are closely associated with poor prognosis.

Despite the complex phenotype, macrophages can be divided into two subtypes based on function: M1 (anti-tumor immunity) and M2 (immunosuppression and tumor immune evasion through suppression of T cell function). M1 macrophages secrete pro-inflammatory cytokines and chemokines, present antigens professionally, participate in positive immune responses, and play a role in immune surveillance. In contrast, M2 macrophages have weaker antigen presentation abilities and primarily inhibit immune responses through their secretions. Cytokines such as IL-10 and TGF-β can down-regulate immune responses, with M2 macrophages as the central players. When combined with other immunosuppressive cells in the tumor microenvironment (TME), these factors not only cannot exert anti-tumor activity but can also create a favorable environment for tumor growth and metastasis. Therefore, evaluating the balance between M1 and M2 macrophages can be a useful strategy for characterizing the immune landscape of the tumor microenvironment. Higher levels of tumor-infiltrating M2 were significantly associated with shorter survival, while higher proportions of M1 with pan-macrophages (% M1) showed a positive correlation with longer overall survival (4). At present, a variety of related small molecule drugs have been developed targeting TAMs (5). Nevertheless, the lack of targeting of these small molecule drugs and the complex microenvironment of solid tumors in clinical trials have limited the efficacy of these small molecule drugs to a certain extent (6). Nanomaterials possess a diverse range of physicochemical properties that enable them to function as both delivery carriers and immunomodulators, making them a promising avenue for improving the immunosuppressive microenvironment of tumors. Research on polarization induced by nanomaterials has focused on a variety of materials, including carbon-based materials, iron oxide nanoparticles, gold particles, zinc oxide particles, and more (7).

As a new member of the family of carbon nanomaterials, CDs are small carbon-based nanoparticles that have gained a lot of attention in recent years due to their unique optical, electrical, and chemical properties, such as small-scale morphology, easily functionalized surface, and tunable optical properties (8). These properties make CDs attractive for a range of applications, including in medicine.

One of the most promising applications of CDs in medicine is in the field of bioimaging (9, 10). CDs can be easily conjugated with biomolecules such as proteins, antibodies, or nucleic acids, and can be used as fluorescent probes to visualize cells, tissues, and organs (11).

Carbon dots offer several advantages compared to traditional organic dyes or semiconductor quantum dots, such as low toxicity, good biocompatibility, and high photostability. They can be used in various bioimaging applications, including fluorescence imaging, intracellular imaging, and biosensors. Carbon dots have a high quantum yield, making them effective fluorescent probes for detecting cancer cells, pathogens, and other biological targets (12, 13). Their small size and ability to penetrate cell membranes make them ideal for imaging intracellular structures and studying cellular processes such as endocytosis, exocytosis, and cell division (14–16). In addition, carbon dots can be used as biosensors to detect specific biomolecules or environmental factors, such as glucose, heavy metals, and other chemicals in biological and environmental samples (17, 18).

Another potential application of CDs in medicine is in drug delivery (19). CDs can be functionalized with different types of molecules such as drugs, peptides, or nucleic acids, and can be used to deliver these molecules to specific cells or tissues. CDs have shown promise for delivering drugs to cancer cells, for example, by targeting tumor-associated macrophages or by enhancing the therapeutic efficacy of chemotherapy drugs (20, 21).

In addition to their potential applications in bioimaging and immunotherapy, carbon dots have also been investigated for their antibacterial and antiviral properties (22–24). These nanoparticles have been found to inhibit the growth of various types of bacteria and viruses, including drug-resistant strains, and have been proposed as a potential alternative to traditional antibiotics or antiviral drugs (25–27).

Overall, the theranostic potential of carbon dots (CDs) and associated nanoparticles is rapidly advancing due to their unique optical properties and versatility in preparation and functionalization. CDs have been utilized for imaging macrophages and tracking their movement in tissues, due to their high quantum yield and photostability (28). Given the intrinsic physicochemical properties and multifunctionality of CDs, their interactions with TAMs offer exciting possibilities that are worth exploring. Currently, there is limited research on carbon dot-targeted TAM imaging, diagnosis, and treatment, although studies have demonstrated their potential in inflammation and antibacterial applications. CDs have also shown promise in immunotherapy, where they can stimulate the immune system to fight diseases. This review paper primarily focuses on analyzing the potential of CD-associated nanoparticles in targeting TAMs, summarizing their application in monitoring and regulating macrophages, and highlighting current challenges in this field.

Characteristics of tumor-associated macrophages

Origin, phenotypes, and function of TAMS

Macrophages are distributed throughout body tissues with the functions of phagocytosis and in response to inflammatory signals strategically. Tissue macrophages are derived from embryonic or adult hematopoietic stem cell (HSC) progenitors, and the relative contribution of these cell populations varies from tissue under homeostasis conditions (29). A monocyte is a kind of white blood cell that is made in the marrow and travels through the blood to tissues in the body where it becomes a peripheral monocyte reservoir or non-classical patrolling monocyte or tissue-resident macrophage in the steady state (30). Macrophages respond to the combined stimulation of the origin and resident tissue which contribute the polarization responses (31).

In most human solid malignancies, tumor-associated macrophages (TAMs) and their precursors occupy the most significant portion of bone marrow infiltration, which can account for up to 50% of the total solid tumor volume (32). A large number of current studies show that the localization and density of TAMs are related with poor clinical outcomes in some kinds of solid cancers, including bladder, breast, liver, renal, prostate, and gastric cancer (33–40). Monocyte-derived TAMs take a large part of tissue-resident macrophages in tumors, except for a small part of TAMs derived from tissue-resident macrophages (41). Monocytes are recruited by chemokines (CCL1, CCL2, and CCL5), VEGF, PDGF, TGF-β and CSF -1. Among these cytokines, CCL2 plays a major role in the recruitment of monocytes (42–48). Studies have shown that targeting the CCL2-CCR2 axis could effectively reduce tumor growth and metastasis in mouse models (49). After being recruited to the TME, monocytes can differentiate into M1-like macrophages (pro-inflammatory and usually anti-tumor) and M2-like macrophages (anti-inflammatory and pro-tumor) due to the heterogeneity of the microenvironment (50–52). More and more evidence suggests that TAMs are similar to normal macrophages in their capacity for adopting a broad range of intermediate activation states, reflecting the diverse microenvironmental conditions and rich plasticity according to different signals in the tumor microenvironment (5, 53).

The phenotype of tumor-associated macrophages (TAM) is driven by both the tumor microenvironment (TME) and the tumor immune microenvironment (TIME). Under the influence of TIME, adaptive and innate immune cells provide chemical messengers for regulating the functional phenotype of macrophages, such as immunoglobulin secreted by B cells, IL4 and IL13 secreted by TH2 cells, Treg cells secreted IL10 and TGFβ, as well as IFNγ and TNF secreted by NK cells, CTL and TH1 cells. In the TME, cytokines secreted by tumor cells, tumor-associated fibroblasts, directly affect the phenotype of TAMs, while oxygen deficiency, fibrosis, and cellular stress also customize the phenotype of TAMs. Thus, immune-related and non-immune-related factors jointly drive functional or dysfunctional antitumor immune. TAMs are programmed to drive inflammation when the microenvironment has functional vasculature, normoxia, low extracellular matrix density, more TH1 cells than TH2 cells, and high cytotoxic T cell (CTL) infiltration. Macrophages exhibit a robust antitumor adaptive immune response. In contrast, tumor hypoxia and fibrosis are combined with infiltration of large amounts of cancer-associated fibroblasts (CAFs) and immunosuppressive cells, and macrophages are programmed to promote a pro-tumor phenotype of immunosuppression and tissue remodeling, resulting in cytotoxicity T lymphocyte (CTL) rejection and suppression.

Studies have shown that M1 phenotype macrophage is stimulated by cytokines such as IL12, TNF, and IFNγ, microbe-associated molecular patterns (MAMPs) such as bacterial lipopolysaccharide (LPS), or other Toll-like receptors (TLR) agonists (54–57). In contrast, anti-inflammatory M2 macrophages are polarized by the stimulation of some of cytokines such as IL4, IL5, IL10, IL13, CSF1, TFGβ1 and PGE2 (58, 59).

M1-type TAMs can express factors such as nitric oxide synthase (iNOS), reactive oxygen species (ROS), and IL-12 that have the functions of phagocytosis and killing target cells (60). M2-type TAMs are associated with high expression of IL-10, IL-1β and VEGF *in vivo*. They can also express a large amount of scavenger receptors, which have the functions of clearing debris, promoting angiogenesis, tissue reconstruction, and injury repair and promote the function of tumorigenesis and development (61–63). Patients with more M2 TAMs infiltration have a lower survival rate and an increased lymph node metastasis rate (64). In general, both M1 and M2 TAMs exhibit strong intrinsic plasticity, can cross-regulate each other's functions, and do not represent a fixed, frozen phenotype; M1 and M2 TAMs can co-exist in the same tumor microenvironment; therefore, molecular targets that control polarization balance may be important avenues for tumor immunotherapy. Polarization biomarkers for M1-type macrophages include CD86 and CD80, and for M2-like macrophages include CD163, CD204, CD206, CD115, and CD301 (65).

The induction of monocytes into the tumor microenvironment into M1/M2 macrophages also changes dynamically with the development of tumors. In the early stages, macrophages can recognize and present malignant cells to lymphocytes. Early stages of tumors exhibit a limited degree of hypoxia, at which time the immune microenvironment exhibits an immunostimulatory state, such as a massive infiltration of effector T cells and polarization of tumor-associated macrophages (TAMs) to an M1-like state (66). As the tumor progresses, cancer cells consume a large amount of glucose. They produce more lactate, which promotes the generation of a hypoxic environment, and the secretion of cytokines also facilitates the recruitment of hematogenous monocytes. It promotes them to an immunosuppressive M2-like state of polarization (67).

TAMs are the center of inhibiting the ability of T cells in tumors to respond, and the current limitations of various immunotherapies are closely related to this, especially those related to immune checkpoints. One study showed that TAM and CD8+ T cells engage in specific, persistent, antigen-specific synaptic interactions that not only fail to activate T cells but actually exhaust them and accelerate the process under hypoxic conditions (68). The current findings indicate that TAMs can

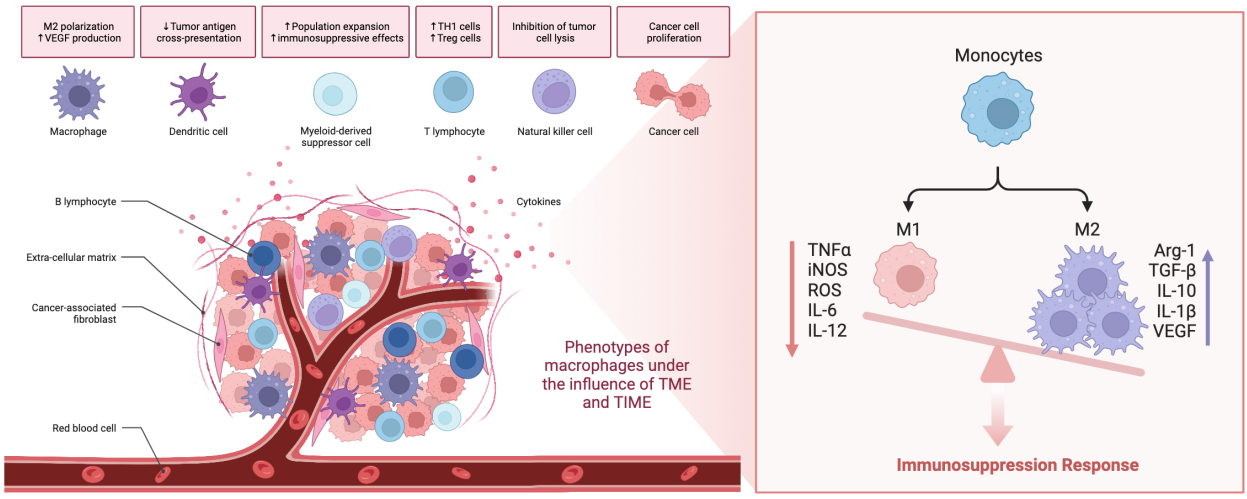
regulate T cells through direct and indirect pathways, respectively (69). Tumor-associated macrophages (TAMs) can directly inhibit cytotoxic T cells through three pathways. Macrophages are involved in immunosuppression through a variety of mechanisms, such as expressing immune checkpoint molecules, including programmed cell death 1 ligand 1 (PDL1) (51), producing inhibitory cytokines like IL-10 and transforming growth factor-β (TGF-β) (52), and modulating their metabolic activity by consuming metabolites (such as L-arginine) and producing reactive oxygen species (ROS) (70, 71). In this summary, we highlight the effects of immune cells and the alterations in macrophage phenotype that occur within the tumor microenvironment and immune microenvironment (Scheme 1).

Modulating TAMs for tumor immunotherapy

Depending on the different sources and phenotypes of TAMs, tumor immunotherapy targeting macrophages can be divided into four categories (1): inhibiting the migration of monocytes or M-MDSCs to tumors (2), depleting TAMs (3), repolarizes TAMs (4), altering TAM metabolism (72), as shown in (Scheme 2). Since conventional modulators of TAMs face challenges such as non-specific targeting, limited drug delivery efficiency, rapid blood clearance, and systemic toxicity, nanoparticles are rationally designed to deliver them or directly participate in regulation, as they can be designed with tunable dimension and surface charge, Moreover, nanoparticles can be easily internalized by the phagocytosis inherent in macrophages, which promotes the effective accumulation of nanoparticles and their payloads in tumors to enhance their tumor penetration (73). Therefore, engineered nanoparticles for targeted delivery of TAMs to tumors or direct modulation of TAMs have enormous potential to strengthen tumor-specific accumulation and modulator blood circulation time and thus reduce side effects, which can enhance TAMs modulatory efficacy (7). Here, we will analyze whether CDs-associated nanoparticles can regulate the possibility of TAMs based on the above four strategies.

Mechanism and importance of carbon materials in TAMs

The remarkable physicochemical properties and biocompatibility of carbon-based materials have sparked significant interest in their potential applications in cancer immunotherapy. These nanomaterials exhibit distinctive characteristics that make them highly promising for biomedical imaging and therapy. They have been extensively investigated for their ability to facilitate one-photon and two-photon imaging, which makes them ideal for both shallow and deep-tissue imaging. Additionally, their ease of functionalization and biocompatibility allow for targeted delivery of therapeutic agents and imaging agents. As research progresses, carbon-based nanomaterials have the potential to become valuable tools in the diagnosis and treatment of a range of diseases, including cancer (74–78). Rajendra K. Singh and Hae-Won Kim and their team have developed a novel type of nanoparticles called fluorescent mesoporous bioglass nanoparticles (fBGn) that can be used for cancer diagnosis and treatment. These nanoparticles are based on carbon dots (CD) and possess a variety of beneficial properties, including triple-mode imaging, photodynamic and photothermal therapeutic effects, and the ability to deliver anticancer drugs in a pH-dependent manner. The researchers were able to demonstrate the effectiveness and biocompatibility of fBGn *in vivo* using a nude mouse model. The authors suggest that fBGn hold great promise for cancer theranostics due to their multifunctional capabilities for imaging, drug delivery, and therapy (79). Singh and Kim have developed a novel nanoplatform called C-dot bioactive organosilica nanosphere (C-BON) that has the potential for therapeutic and



SCHEME 1
The role of TAMs in the tumor microenvironment.

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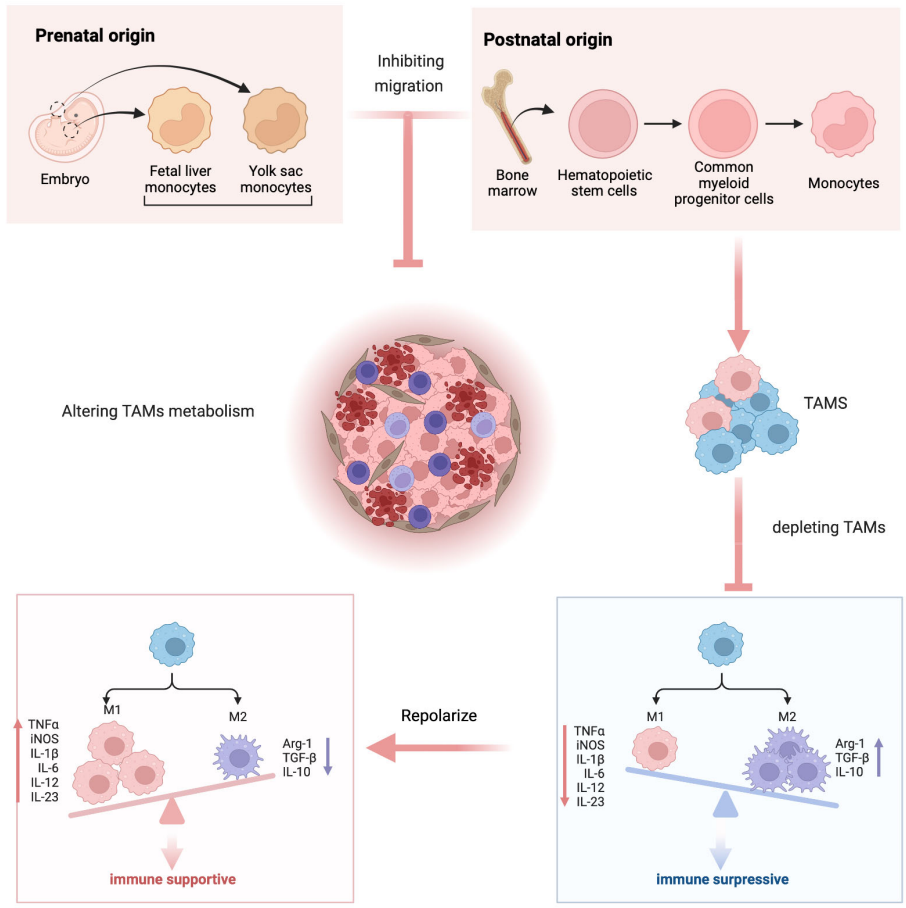


SCHEME 2
Schematic representation of modulating tumor-associated macrophages as immunotherapy.

diagnostic purposes in tissue repair and disease treatment. This platform has several advantages, including its ability to label cells and tissues, load and deliver drug molecules, and exhibit photothermal activity. Additionally, the C-BON has demonstrated excellent bioactivity and cell compatibility, making it a promising candidate for future applications in theranostics. Overall, this innovative technology offers a multifunctional approach to chemotherapy and photothermal therapy with optical imaging, paving the way for improved treatments in the future (80).

Carbon-based materials, such as carbon nanotubes, graphene oxide, and fullerenes, have been shown to modulate TAMs' activation state and promote an anti-tumor immune response (78, 81, 82). One of the mechanisms by which carbon-based materials can achieve this is through the regulation of TAMs' phagocytic activity. Carbon-based materials have been shown to enhance TAMs' phagocytosis of tumor cells, leading to their subsequent destruction and increased activation of the immune system against the tumor (83).

In addition, carbon-based materials can also promote the polarization of TAMs towards an M1-like phenotype, which is associated with an anti-tumor immune response (82). This is achieved through the activation of toll-like receptors (TLRs), which are involved in the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) on the surface of cancer cells (77, 84, 85).

Moreover, carbon-based materials can also act as a drug delivery platform for targeted delivery of anti-cancer agents to TAMs (86). This targeted delivery can increase the efficacy of anti-cancer agents and minimize their off-target effects.

Overall, carbon-based materials have emerged as a promising strategy for modulating TAMs' activation state and promoting an anti-tumor immune response. The unique properties of carbon-based materials make them an attractive candidate for further development in cancer immunotherapy.

Characteristics of CDs

Carbon-based nanostructured substances, such as graphene, carbon nanotubes and fullerenes, have attracted wide attention due to their unique physical and chemical properties and diverse applications. Compared to the above carbon nanostructures, carbon dots (CDs) exhibit excellent dispersion, low toxicity, biocompatibility, biodegradation, abundant raw materials, low cost, and abundant photoluminescence (PL) and photoelectrochemical properties (8, 87). Historically, In 2004 CDs

was discovered in arc emission carbon soot, whose PL emission attracted the attention of researchers (88). In 2006, polymers (i.e., PEG, etc.) were used for surface passivation to enhance the PL emission of CDs (89). In 2010, well-crystallized CDs were synthesized and purified, showing size-dependent photoluminescence (90). In general, CDs can be thought of as spherical carbon particles (graphitic fragments) less than 10 nm in size (91). The chemical structure of carbon dots can be a hybrid carbon structure of sp2 and sp3, with a single-layer or multi-layer graphite structure, or it can be aggregated particles of polymers. Specifically, carbon dots include graphene quantum dots (GQDs), carbon quantum (CQDs), and polymer dots (CPDs). GQDs refer to a carbon core structure with a single layer or less than 5 layers of graphene and chemical groups bonded to the edges. The size of graphene quantum dots has a typical anisotropy and carbon lattice structure, and the lateral dimension is larger than the vertical height; CQDs are spherical and have a clear lattice, and the surface has abundant chemical groups CQDs have an intrinsic state luminescence mechanism and a quantum confinement effect of particle size. CPDs are usually cross-linked flexible aggregates formed from non-conjugated polymers through dehydration and partial carbonization, and there is no carbon lattice structure. Currently, four fluorescence mechanisms have been reported as follows (1) quantum confinement effect (QCE) (2), defect state (3), molecular (fluorophore) state, and (4) crosslink-enhanced emission state (92). The characteristics of carbon dots have attracted widespread attention in the field of biomedicine. Currently, CPDs are the core of research and development of carbon dot materials. The excellent properties of CPDs, such as photostability, excellent biocompatibility, simple synthetic route, flexible designability, deep red/NIR emission, and two-photon/multiphoton fluorescence, make CPDs an ideal candidate for fluorescent probes for *in vitro* and *in vivo* bioimaging (19, 93).

Synthesis strategy of carbon dots for bioimaging and therapy

There are many methods for preparing carbon dots, which can be generally divided into top-down method (Top-down) and bottom-up method (Bottom-up). The top-down synthesis method is mainly to thoroughly pulverize the carbon skeleton to generate CDs, while the bottom-up method uses some organic molecules as precursors (carbon sources) to synthesize CDs (94). In the history of carbon dots, the top-down strategy was first used to prepare carbon dots, which refers to the synthesis of carbon dots by physically or chemically stripping carbon nanoparticles from large carbon skeletons, including discharge methods, electrochemical methods, etc. method, laser ablation method, etc. (95, 96). Although these methods can generate CDs in relatively large quantities, they often suffer from expensive instrumentation, complex synthesis procedures, long synthesis times, low yields, high impurities, complex purification procedures, and still require post-synthesis procedures to tune optoelectronic properties (97). From the perspective of fluorescence properties of CDs, the oxidative cleavage of carbon sources leads to more structural defects, which leads to the degradation of photoluminescence performance, which is the most restrictive issue for their biomedical applications (98).

Bottom-up synthesis is more prevalent now (99). The advantage of this strategy is the availability of a large number of molecular precursors, among other benefits including multiple heat treatment options, faster reaction times and more uniform properties of the final material. The selection of precursors and synthesis procedure (i.e., pre-synthesis control) affects the physicochemical properties of CDs in terms of size, degree of graphitization, surface functional groups, and doping. However, some structural and functional features of the precursors can be retained in the nanoparticles, which allows a certain degree of predictability in the designed nanoparticles. At the same time, the strategy of using heteroatom doping can enrich the functional properties of carbon dots and adjust the range of photoluminescence.

Bottom-up synthetic strategies can obtain nanoparticles emitting from the blue to the near-infrared (NIR) region (100). The bottom-up method mainly uses some organic molecules as precursors to prepare CDs through a series of chemical reactions, including template method, microwave digestion synthesis method, ultrasonic oscillation method, solvothermal method, strong acid oxidation method and hydrothermal method, etc. Among these methods, hydrothermal method, solvothermal method and template method are widely used (101). CDs can be functionalized by surface passivation and heteroatom doping (102). With proper functionalization, carbon dots have promising applications in biomedical fields such as biosensors, bioimaging, and photodynamic therapy; magnetic resonance imaging of chemical exchange saturation transfer; photodynamic and photothermal therapy; PH and ROS in microenvironments monitor and treatment (103–109).

Application of carbon dot-associated nanoparticles in monitoring macrophages

Currently, cancer treatment response is routinely assessed with the Response Evaluation Criteria in Solid Tumors (RECIST), based on changes in tumor size and the presence or absence of new tumors (110). However, in immunotherapy, pseudoprogression has emerged as a distinct response mode in which activated immune cells infiltrate the tumor environment leading to increased tumor volume and delayed treatment response (111). Because TAMs are the highest proportion of immune-infiltrating cells in tumors and their substantial impact on immunotherapy, immunoimaging of TAMs is essential to evaluate changes in tumor burden, allow early treatment intervention, reflect the dynamic shift in immune markers during immunotherapy, and avoid early termination of effective therapy according to RECIST criteria (112).

Thanks to carbon dots' inherent fluorescence characteristics and physical and chemical properties, it has the intrinsic advantage of being a macrophage imaging agent. Raja S and co-workers synthesized a carbon dot derived from curauá that exhibited a

graphitic-like structure with an average diameter of 2.4 nm, good water solubility, sophisticated carboxyl and hydroxyl functional groups, excitation-dependent multicolor fluorescence emission (in the range of 450 nm to 560 nm) and excellent photostability. Cell experiments show that carbon dots tolerate the J774.A1 mouse macrophage cell line, can effectively internalize carbon dots into its cytoplasmic compartment and is an excellent nanoprobe for effective long-range cell imaging (113).

Xiaowei Xu and colleagues aimed to develop a carbon nanoparticle incorporating aspirin. They synthesized fluorescent aspirin-based carbon dots (FACD) through a one-step microwave-assisted method, condensing aspirin and hydrazine. Imaging data revealed that FACD effectively penetrated mouse monocyte-macrophage cells *in vitro* (114).

Shi Y et al. synthesized highly fluorescent and ultra biocompatible N-doped carbon quantum dots derived from aminated alkali lignin green precursors for cellular imaging and intracellular irons detection of RAW 264.7 cells. AL-CQDs produced in the 4–10 nm range exhibited excitation-dependent and pH-stable fluorescence properties. They were used to detect iron ions ranging from 100 nm to 1 mm with a detection limit as low as 8 nm, where Fe3+ ions could be detected by the AL-CQDs. The amine group is trapped, forming an absorbing complex that results in significant fluorescence quenching (115).

Yawei Li and colleagues fabricated stable nanoparticles composed of the supramolecular assembly of carbon dots (CDs) and RTBs, which could be taken up and visualized by macrophages. Notably, the CDs-RTB nanoparticles were found to promote macrophage proliferation, as well as the production of NO, IL-6, and TNF-α in RAW264.7 cells, and increase mRNA expression, indicating enhanced immunomodulatory activity. These findings highlight the potential of CDs as a simple and stable platform for assembling RTB, thereby facilitating the application of RTB as an immunostimulant (116).

The photoluminescent properties, low toxicity, and biocompatibility characteristics of these carbon dots exhibit excellent properties in bioanalysis and bioimaging. However, fabricating stable highly near-infrared (NIR) fluorescent GQDs using facile methods remains a challenging task. Reagen S and a co-worker developed NIR CDs from the biomass-derived organic molecule cis-cyclobutane-1,2-dicarboxylic acid *via* one-step pyrolysis. The prepared GQDs exhibit excellent photostability and stability over a wide pH range. Using biomass as raw material to prepare carbon dots is a very convenient and economical method. Most importantly, there were two peaks in the fluorescence emission spectra of GQDs, one in the NIR region around 860 nm. The results of cell experiments on the mouse macrophage cell line RAW 246.7 showed that GQDs entered cells by endocytosis on fluorescence images and were nontoxic to cells at concentrations up to 200 μg/mL (117).

At the same time, the CDs-based composite material has more prosperous functions, which can significantly improve CDs' cellular uptake and imaging potential. It was shown that nanocomposite formulations of carbon dots (<5 nm) encapsulated in lipid-based lyotropic liquid crystal nanoparticles (~250 nm) enhanced the bioimaging potential of carbon dots by improving cellular uptake efficiency and converging carbon dot light emission (118). Carbon dot-associated nanoparticles enable multimodal imaging by doping with heteroatoms or forming assemblies. Sun S and co-workers anchored a small amount of photosensitizer chlorin e6 (Ce6) (0.56% by mass) on amino-rich red-emitting carbon dots (RCD). They synthesized Ce6-modified RCD (named Ce6-RCD) multimodal imaging capability (i.e., fluorescence (FL), photoacoustic (PA), and PT) (119).

Saladino GM et al. synthesized metallic rhodium (Rh) nanoparticles conjugated and cross-linked with nitrogen-doped carbon quantum dots, which combine optical and X-ray fluorescence as multimodal bioimaging contrast agents. CQDs confer optically fluorescent properties to Rh NPs and improve their biocompatibility, as demonstrated *in vitro* by real-time cell analysis (RTCA) on a macrophage cell line (RAW 264.7) (120).

Su Y and colleagues developed Hafnium-doped carbon dots (HfCDs) using a simple one-pot pyrolysis method. This innovative nanoparticle exhibited remarkable capabilities for CT/fluorescence imaging (9).

By doping Gd (iii) into CQDs *via* one-pot pyrolysis, Pan Y et al. reported an efficient and mild method for the facile synthesis of carbon quantum dots (CQDs)-based bimodal fluorescent (FL)/ Magnetic resonance (MR) imaging probe cryogenic process. Nanoparticles doped with heavy N elements can significantly improve the quantum yield. Gd3+ is stably captured and sequestered by the carbon dot framework, maximizing its role in shortening the longitudinal relaxation time. Therefore, the synthesized nanoparticles have the advantages of strong fluorescence brightness and high MR response with minimal Gd3+ extravasation, making them an ideal dual-modality imaging probe (121).

He X et al. prepared novel carbon dots (CDs) L-CD/C-CD from Gd (iii) salt/complexes, cationic polymers, and citric acid, which combine the abilities of gene delivery and multi-modal (MR/FL) imaging (122).

Weng Y and co-workers et al. report a multifunctional nanocarrier (CDs/ICG-uLDHs) prepared by simple self-assembly of red-emitting carbon point (CDs) and indocyanine green (ICG), which can be used for three-mode fluorescence/photoacoustic/two-photon bioimaging and high-efficiency photothermal therapy (123). By doping rare earth ions, carbon dot composites can obtain excellent UCL imaging, magnetic resonance imaging (MRI), and computed tomography (CT) imaging performance (124).

The multifunctional hybrid nanoparticles prepared by Wang H et al. have fluorescence/MRI dual-mode imaging capabilities, which are made by embedding a magnetic Fe3O4 core into a mesoporous silica shell of carbon point (CD) and paclitaxel (PTX), covered by another layer of silica (21).

In addition to direct cell imaging, carbon dots also serve as sensitive sensors to rapidly image reactive oxygen species (ROS) and reactive nitrogen species (RNS) signals involved in various biological processes and many pathologies with high selectivity and contrast. Gong Y et al. developed phosphorus and nitrogen co-doped carbon dots (PC-NDs). ROS and RNS can sensitively and selectively quench the strong fluorescence of PN-CD *in vitro* and *in vivo*. It can be used for live-cell imaging of reactive oxygen species

(ROS) and reactive nitrogen species (RNS) in macrophages. The carbon dots prepared by Yu C et al. are highly selective for NO and can be operated in an utterly aqueous medium, which can track exogenous NO levels in various cell lines such as Raw 264.7, L929 and Hela cells; it is also used to visualize endogenously produced NO stories in the Raw 264.7 macrophage cell line (125).

Studies as shown in Figure 1 illustrate that different protocols for multimodal imaging in monitoring macrophages can be achieved with appropriate surface functionalization, heteroatom doping, and assembly of carbon dot-associated nanoparticles (Figure 1).

Application of carbon dot-associated nanoparticles in regulating macrophages

CDs are generally soft and nontoxic *in vitro* and *in vivo*. However, due to their efficient light harvesting in an extensive spectral range from ultraviolet to near-infrared, CDs exhibit strong photodynamic effects, and photoexcited CDs can generate reactive oxygen species (ROS). ROS is a crucial mediator of oxidative stress and redox signal transduction in immune cells (126–128). The regulation of ROS by CDs may have a profound impact on the immune response. Yu Jin et al. found that CDs can reprogram macrophages by eliminating ROS to suppress pro-inflammatory responses and promote pro-reparative M2 conversion (129). Huibo Wang and colleagues found that carbon dots (CDs) produced through a one-step hydrothermal process using citric acid and glutathione exhibited excellent intracellular reactive oxygen species (ROS) scavenging activity in macrophages. This scavenging activity was effective in reducing inflammation caused by lipopolysaccharide (LPS) induction in macrophages, suggesting that CDs have potential as a therapeutic agent for inflammatory conditions. Studies have found that CDs can effectively remove up to 98% of intracellular ROS, especially inhibit the nuclear factor kappa-light chain enhancer (NF-κB) signaling pathway of activated B cells, reduce the expression level of inflammatory factor IL-12, thereby regulating Macrophage phenotype (130). At the same time, many studies have shown that carbon dots can induce autophagy (131–133). Mitochondrial ROS plays a key role in promoting macrophage polarization into an inflammatory phenotype by damaging the autophagolysosome system (134). Therefore, carbon dots may regulate immune responses through these two aspects and impact on macrophages (135–137). A study shows that degradable carbon dots (CDs-1) prepared from L-ascorbic acid can up-regulate the expression of HMOX1 in animal cells and tissues, and can increase the expression of HMOX1 by 5 times in a short period of time, thereby reducing cell inflammation ROS levels in models with therapeutic effects on LPS-induced acute lung injury in mice (138).

In another study, researchers synthesized highly biocompatible CDs (Gly-CDs) by hydrothermal method using glycyrrhizic acid, an active ingredient of Chinese herbal medicine, as a raw material. The results indicated that Gly-CDs inhibited the invasion and replication of PRRSV, stimulated the antiviral innate immune response, and inhibited the accumulation of intracellular reactive oxygen species (ROS) caused by PRRSV infection (139).

Osteoclasts, specialized cells derived from the fusion of monocyte/macrophage hematopoietic lineage precursors, are the primary cells involved in normal bone remodeling and pathological bone destruction *in vivo*. One of the main causes of hyperactivation of osteoclasts is the overproduction of reactive oxygen species. Chitosan-derived nitrogen-doped carbon dots (N-CDs) synthesized by Chen Runfeng et al. have the ability to scavenge reactive oxygen species (ROS). Experiments showed that N-CD effectively abolished the RANKL-induced increase in ROS generation, thereby attenuating the activation of NF-κB and MAPK pathways, whereby osteoclast genesis and bone resorption were effectively attenuated *in vitro*. Furthermore, N-CD protected mice from lipopolysaccharide (LPS)-induced calvarial destruction and breast cancer cell-induced tibial bone loss. Based on the excellent biocompatibility and efficient ROS scavenging ability of

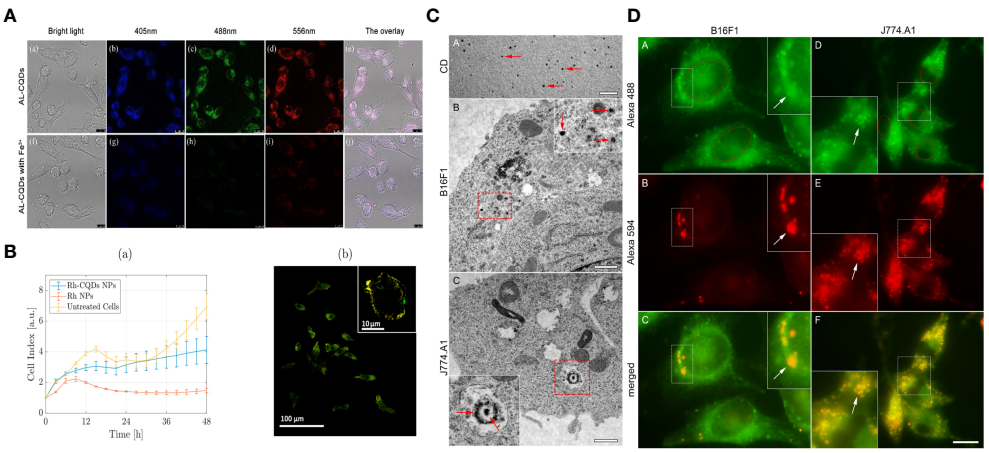


FIGURE 1
Bioimaging of macrophages with various CDs :**(A)** Fluorescence images indicated AL-CQDs could detect iron ions of RAW 264.7 cells. **(B)** The RTCA assay demonstrated that Rh-CQDs NPs can enable multimodal imaging in the RAW 264.7 cell line. **(C, D)** Transmission electron microscopy and Fluorescence microscopy analysis shows that C-dots can be stably imaged in B16F1 and J774.A1 cytoplasm.

N-CDs, for the first time, it provides a nanomaterial treatment plan for the clinical treatment of osteolytic diseases (140).

Cai H et al. synthesized a carbon dot capable of simultaneously achieving cell labeling and regulating mesenchymal stem cell (MSC) behavior. Bifunction CDs were prepared with D-glucosamine hydrochloride and sodium p-styrene sulfonate as raw materials by one pot hydrothermal method. The synthesized CDs had uniform particle size (about 4 nm), was well dispersed in aqueous solution, and showed excellent fluorescence stability under other conditions. More importantly, CDs can effectively promote osteogenic and chondrogenic differentiation of rBMSCs through the production of reactive oxygen species (ROS), without affecting their pluripotency (141). Shao D et al. also had similar results with citrate-based carbon dots, which significantly provided long-term tracking and promoted the differentiation of rBMSCs into osteoblasts through the ROS-mediated MAPK pathway (142).

Injection of GQDs was able to penetrate the blood-brain barrier, inhibited the loss of cerebellar Purkinje cells, and demonstrated reduced microglial activation. Microglia are macrophages in the brain, suggesting that carbon dots can regulate macrophages through autophagy (143). Another study shows that electrochemically produced CDs irradiated with blue light (470 nm, 1W) produce reactive oxygen species, including singlet oxygen. Light-excited CD-induced cell death is manifested by apoptosis (externalization of phosphatidylserine, activation of caspases, DNA fragmentation) and autophagy (autophagy vesicles formation, LC3-I/LC3-II transformation, morphological and/or biochemical characterization of autophagy target p62) (144).

The results of Yiru Qin et al. revealed that CDs slightly affected the cell viability and membrane integrity of macrophages, while CDs significantly increased reactive oxygen species (ROS) production as well as apoptotic and autophagic cell death, while Bax, Bad, caspase 3, caspase 9 increased expression levels of beclin 1 and LC3-I/II and decreased Bcl-2. In addition, low concentrations of CDs significantly increased the expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-8. In contrast, high concentrations of CDs had a negative effect on cytokine production opposite effect. SB202190 is a selective inhibitor of p38 mitogen-activated protein kinase (MAPK), which abolishes the cytokine induction of CD in macrophages. Furthermore, CDs significantly increased the phosphorylation of p38 MAPK and p65 and promoted the nuclear translocation of nuclear factor-κB (NF-κB). These results suggest that CDs induce ROS production, apoptosis, autophagy, and inflammatory responses in THP-1-activated macrophages through p38MAPK and NF-κB-mediated signaling pathways. This indicates that carbon dots have the function of regulating stimulatory factors in macrophages (145).

Carbon dots also offer enormous potential due to their enzymatic properties compared to natural enzymes. Yao L et al. report a carbon dot-based nanozyme prepared from chlorogenic acid (ChA), a primary bioactive natural product in coffee. The study found that ChA CDs exhibited significant GSH oxidase-like activity, which recruited a large number of tumor-infiltrating immune cells, including T cells, NK cells, and macrophages, thereby transforming "cold" tumors into "hot" tumors, activating systemic anti-tumor immune response (146).

Although ricin-binding subunit B (RTB) can promote the activation of macrophages and regulate cell-mediated immunity, its application is severely limited due to the inherent properties of the protein, such as poor stability and low cellular uptake efficiency. In the work of Li Y et al., stable nanoparticles were prepared by supramolecular assembly of carbon dots (CDs) and RTBs. The formed CDs-RTB are highly durable and can protect RTB from enzymatic hydrolysis. More importantly, CDs-RTB could promote the proliferation of macrophages, increase the production of NO, IL-6, and TNF-α in RAW264.7 cells, and increase the expression of mRNA, indicating that CDs-RTB enhanced the immunomodulatory activity. This work highlights the potential of CD as a simple and stable assembly platform that effectively facilitates the application of RTB as an immunostimulatory agent (147). At the same time, it suggested that CD has the potential to be an excellent immune adjuvant.

Sun Q et al. have developed a novel nanocomposite to target activated macrophages in the colon with real-time imaging and therapeutic capabilities. The nanocomposite was formed by covalent conjugating mannosylated NPs (Man-NPs) with carbon dots (CDs). Cellular experiments showed greater uptake of nanocomposites by inflamed macrophages compared to untreated macrophages and the mannose receptor-negative cell line 4T1. This indicates that carbon dots can target and recognize M2 macrophages after functionalization (148).

The above studies indicate that carbon dots have the ability to influence macrophage plasticity through several mechanisms. Firstly, they can induce ROS production and autophagy, which can alter macrophage phenotype from M2 to M1-like, resulting in an enhanced immune response against tumors. Secondly, carbon dots can modulate macrophage polarization by inhibiting the expression of cytokines such as IL-10 and TGF-β, leading to an increase in the M1/M2 ratio and improving the characterization of the tumor immune microenvironment. Additionally, carbon dots can act as immunomodulators and delivery vehicles, improving the uptake of therapeutic agents by macrophages and potentially improving the immunosuppressive microenvironment of tumors. These findings suggest that carbon dots may hold promise as a therapeutic approach for targeting TAMs. A relevant mechanism is illustrated in Figure 2.

Concluding remarks and future perspectives

TAMs contribute to tumor initiation, progression, and metastasis. Therapeutic agents that eliminate TAMs, inhibit TAM infiltration, and/or activate TAM polarization toward the M1 phenotype have shown remarkable clinical potential. Considering the critical role of TAMs in tumor immune suppression, various macrophage-targeting nano theranostics formulations have been developed in recent years. As a new type of nanomaterial, CDs have evolved from a single functional capability of diagnosis (or treatment) in nanomedicine theranostics by their inherent photoluminescence characteristics, excellent physical and chemical properties, and rich tunability. It is an intelligent

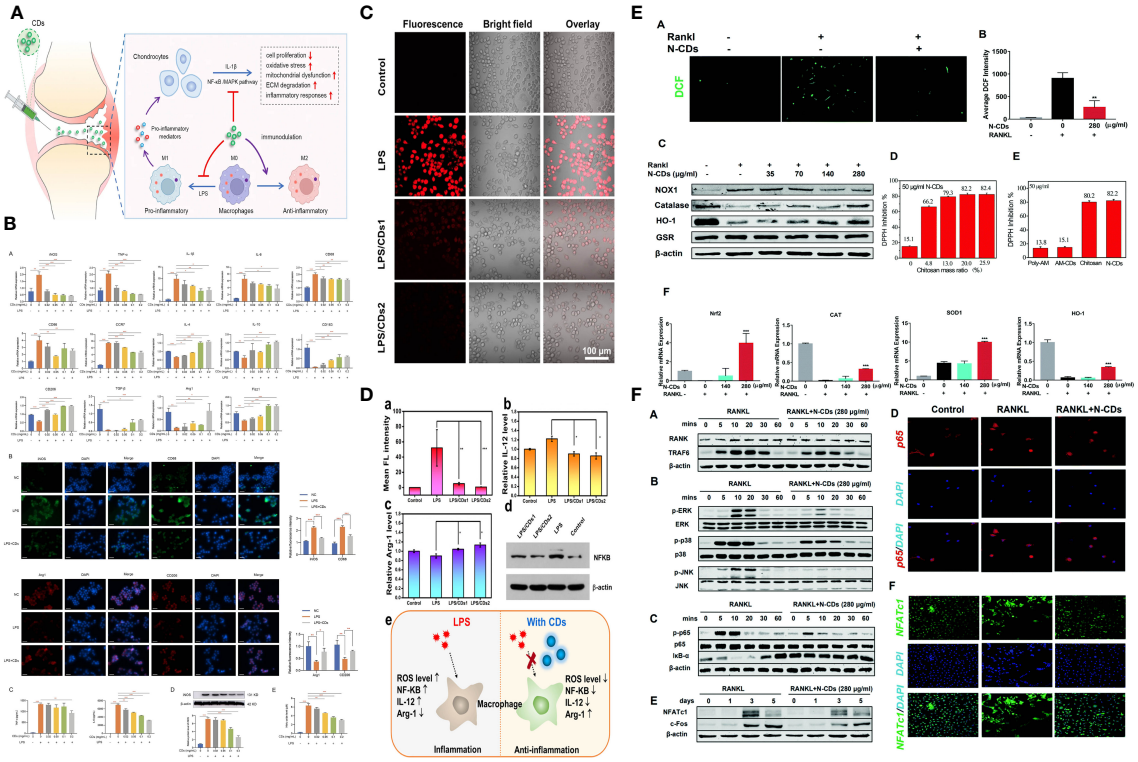
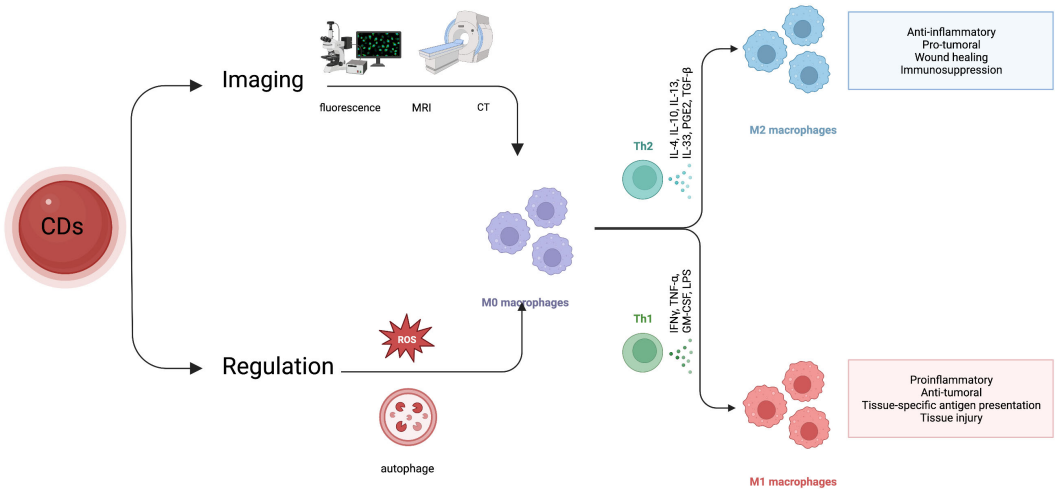


FIGURE 2
Regulation ROS and autophage with CDs. **(A, B)** CDs orchestrated macrophage repolarization in vitro and indicating the immunomodulatory mechanism of CDs mediated OA therapy. **(C, D)** CDs with radical-scavenging activity in alleviating the LPS-induced inflammation in macrophages. **(E, F)** N-CDs downregulated ROS with suppressed ROS downstream signaling pathway.

treatment and diagnosis system. So far, there are few studies on the application of carbon dots in evaluating and regulating TAMs. However, through literature analysis, this review found that CDs have apparent advantages in the imaging and regulation of macrophages. Here, we illustrate the potential of carbon dots in macrophage imaging and regulation (Scheme 3). The fluorescence visible in the whole range provides a basis for monitoring macrophage distribution, polarization state, and functional changes. At the same time, the carbon dots exhibited the role of nanozyme and immune adjuvant, which can regulate the polarization state of macrophages and promote the infiltration of immune cells through the ROS generated by photoluminescence



SCHEME 3
Application of carbon dots in macrophage imaging and modulation.

and the induction of autophagy. In addition, TAMs are highly enriched in tumor hypoxic sites. This shows that CDs have inherent advantages and great potential for monitoring and regulating TAMs. However, compared with other nanomaterials that have been applied for a long time, the application of CDs in diagnosis and therapy needs to solve more difficulties.

First, further theoretical breakthroughs are required to fine-tune the properties of carbon dots. On this basis, the demand for near-infrared photoluminescence can be stably realized. Second, the tumor microenvironment is complex, and how to achieve safe and efficient target recognition of TAMs is a crucial point that needs to be studied. Third, the current application of carbon dots in macrophages shows a bidirectional effect of ROS and autophagy. Therefore, how to correctly evaluate the state of TAMs and change the immunosuppressive effect of TAMs is very important in the future. Developing multimodal CDs with synergistic strategies may be feasible to achieve this maximal theranostic purpose.

Therefore, with nanomedicine development, CDs are a suitable carrier and a promising reagent for nanomedicine theranostics. If scientists and engineers adequately resolve the above problems, CDs are expected to make outstanding contributions to the development of immunotherapy.

Author contributions

YM: preparation, creation, and presentation of the published work, specifically writing the initial draft (including substantive translation). BZ: preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre-or post-publication stages. LL: acquisition of the financial support for the project leading to this publication. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Function of reactive oxygen species in myeloid-derived suppressor cells

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous myeloid cell population and serve as a vital contributor to the tumor microenvironment. Reactive oxygen species (ROS) are byproducts of aerobic respiration and are involved in regulating normal biological activities and disease progression. MDSCs can produce ROS to fulfill their immunosuppressive activity and eliminate excessive ROS to survive comfily through the redox system. This review focuses on how MDSCs survive and function in high levels of ROS and summarizes immunotherapy targeting ROS in MDSCs. The distinctive role of ROS in MDSCs will inspire us to widely apply the blocked oxidative stress strategy in targeting MDSC therapy to future clinical therapeutics.

KEYWORDS

MDSCs, ROS, immunotherapy, tumor, tumor micro environment (TME)

1 Introduction

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells with immunosuppressive activity. MDSCs play a crucial role in tumorigenesis and inhibit antitumor immune responses to promote tumor development (1, 2). In addition to cancer, MDSCs are also involved in autoimmune diseases, sepsis, bone marrow transplantation and infection diseases (1, 3).Reactive oxygen species (ROS) have miscellaneous effects and are involved in both cell biological activities and oxidative stress disease (4). Notably, ROS are one of the dominant immunosuppressive functional effector molecules of MDSCs, and MDSCs can also adjust the ROS level to a proper level to maintain the state of MDSCs. Currently, immunotherapy that targets MDSCs has achieved significant results, but targeting ROS in MDSCs has not yet become a therapeutic focus that will be worth further investigation.

This paper summarizes the distinctive regulation, scavenging and effects of ROS in MDSCs. In addition, we generalize immunotherapy that targets ROS in MDSCs. This will provide novel potential insight for targeting MDSC immunotherapy.

2 MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population composed of immature myeloid cells (IMCs). In pathological conditions such as cancer, infectious diseases, trauma, and some autoimmune disorders, IMCs cannot differentiate into mature myeloid cells, which causes the activation and expansion of MDSCs (1, 5). At present, we can identify MDSCs by phenotype and immunosuppressive function. The phenotype of mouse MDSCs is $CD11b^{+}Gr\text{-}1^{+}$. According to different epitopes (Ly6G and Ly6C) in Gr-1, mouse MDSCs can be further divided into two subgroups: $CD11b^{+}Gr\text{-}1^{+}Ly6G^{high}Ly6C^{low}$granulocyte/polymorphonuclear MDSCs (G-MDSCs/PMN-MDSCs) and $CD11b^{+}Gr\text{-}1^{+}Ly6G^{low}Ly6C^{high}$monocytic MDSCs (M-MDSCs) (6). More importantly, we can use different antiapoptotic molecules to discriminate PMN-MDSCs and M-MDSCs. The antiapoptotic molecule MCL-1 is required for the development of PMN-MDSCs, while M-MDSCs require another antiapoptotic molecule, c-FLIP (7). The phenotype of human MDSCs and their subsets is different from that of mice. Human MDSCs express $CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}$. Human PMN-MDSCs express CD15, while human M-MDSCs express CD14 (8). In most types of cancer, PMN-MDSCs are the predominant population, while M-MDSCs have stronger immunosuppressive activity than PMN-MDSCs (1). Except for phenotypic identification, many original methods are being exploited to identify MDSCs. Single-cell RNA sequencing (scRNAseq) technology could describe MDSCs by novel surface markers (CD84, JAML) and definite PMN-MDSCs with enrichment genes (Ngp, Ltf, Anxa1, Mmp8 and Cybb) (9, 10). IHC staining analysis showed that MDSCs are located in the tumor epithelial border (11). Moreover, metabolite and lipid analyses of MDSCs also demonstrated that MDSCs have a specific response to high glucose concentrations (12).

The immunosuppressive activity of MDSCs relies on the expansion and activation of MDSCs. There are a variety of factors accounting for the expansion of the MDSCs, such as cyclooxygenase 2 (COX2), prostaglandin, stem-cell factor (SCF), macrophage CSF (M-CSF), granulocyte/macrophage CSF (GM-CSF), vascular endothelial growth factor (VEGF), TNF-α, polyunsaturated fatty acids, MyD88 and HIF-1α (13–15). Most of these factors advance the expansion of the MDSCs by triggering the STAT3, IRF8, C/EBP-β and NOTCH signaling (16). Among them, STAT3 is a vital regulator of the expansion of MDSCs, which can also upregulate the proinflammatory protein S100A8/9 expression and induce the expression of the downstream targets of STAT3 including survivin, BCL-XL and cyclin-D1 (13, 14). Endoplasmic reticulum (ER) stress can promote the accumulation of MDSCs by activating TNF-related ligand receptors which induce the apoptosis (15). Last but not least, the metabolites adenosine, IDO and lactic acid accumulated in the TME can also contribute to the expansion of MDSCs (17). Other factors, such as IFN-γ, IL-1β, IL-4, IL-6, IL-13, TNF and high mobility group Box 1(HMGB1), could influence the MDSCs suppressive activity by activating STAT1, STAT3, STAT6 and NF-κB signaling pathways (18).

Several major molecules contribute to MDSC-mediated immune suppression, including arginase 1 (Arg-1), inducible nitric oxide synthase (iNOS), COX2, TGF-β, IL-10 and ROS. Many factors, such as STAT3, C/EBPβ, p50 NF-κB, and IDO1, play a critical role in MDSC function by regulating these functional effector molecules (16, 19, 20). Arg-1, which converts L-arginine to urea and L-ornithine, inhibits T-cell function by decreasing the expression of the CD3ζ chain and impairing the expression of cyclin D3 and cyclin-dependent kinase 4 (cdk4) (21). ROS are the characteristic molecules of PMN-MDSCs, while M-MDSCs mainly produce NO (8). NO produced by MDSCs leads to the suppression of T-cell responses by reducing the tyrosine phosphorylation of JAK3 and STAT5, preventing MHC II transcription and triggering T-cell apoptosis (22). The interaction between ROS and NO can promote the formation of peroxynitrite, which leads to the desensitization of T-cell receptors and T-cell tolerance. Treatment of cancer with AT38 ([3-(aminocarbonyl)furoxan-4-yl] methyl salicylate) could increase antitumor immunity by interfering with the expression of ARG1 and NOS2 enzymes in myeloid cells (23).

In addition, MDSCs can recruit and expand Treg cells *via* the immunosuppressive cytokines IL-10 and TGF-β. MDSCs can also reduce the secretion of IL-6 and TNF-α by macrophages and shape them into the M2-type phenotype, which promotes tumor progression (24). In turn, Treg cells induce the expression of B7 homolog 1 (B7-H1), B7-H3 and B7-H4 on the cell surface of MDSCs, which causes an increase in IL-10 production and immunosuppressive activity of MDSCs (25). In addition, MDSCs can produce adenosine due to the high expression of CD73 and CD39, which hydrolyze ATP into adenosine, and adenosine can inhibit the immune responses of both T cells and NK cells in the tumor microenvironment (26).

3 ROS

Reactive oxygen species (ROS), oxygen-containing derivatives, include a range of species such as superoxide ($O^{2\cdot-}$), hydrogen peroxide (H_2O_2), nitric oxide, peroxynitrite, hypochlorous acid, singlet oxygen and hydroxyl radicals (27). Among them, the three most common forms of ROS are superoxide, H_2O_2 and hydroxyl. Different forms of ROS can have different targets. To illustrate, H_2O_2 takes effect through the modification of specific cysteine, selenocysteine, methionine and histidine residues in targeted proteins (28, 29), but $O^{2\cdot-}$, hydroxyl radicals and peroxynitrite can irreversibly undermine intracellular proteins, DNA and lipids (30). In cancer, the most studied ROS components are $O^{2\cdot-}$and H_2O_2 (31). However, the main increased pool of ROS released by MDSCs is primarily H_2O_2 under pathological conditions (32).

ROS are byproducts of aerobic respiration that can be produced by many cells, including hematopoietic stem cells (HSCs), tumor cells, cancer stem cells (CSCs) and immune cells (33). The production of ROS relies on cell type. Tumor cells, MDSCs and professional phagocytes can produce abundant ROS. However, HSCs and CSCs have low ROS content (34, 35).

ROS are short-lived, strong-effect and short reaction distance compounds that serve as a double-edged sword that elicits both beneficial and harmful effects in cells. The most common influence is the toxic side effects of ROS. Elevated levels of ROS can damage cells and intracellular components, cause DNA hydroxylation, protein denaturation and tissue damage, and ultimately lead to cell cycle G2/M arrest, apoptosis, senescence and death, and ROS can also participate in mitochondria, death receptors, and endoplasmic reticulum-mediated apoptosis (36). However, ROS also serve as the second messenger of cell signal transmission to play a regulatory role in many crucial biological activities of normal cells (4).

4 Sources of ROS in MDSCs

NADPH oxidase (NOX) enzymes and mitochondria are major sources of endogenous ROS. In addition, there are numerous cellular sources of ROS, including xanthine oxidase, cyclooxygenases, cytochrome p450 enzymes, lipoxygenases and the endoplasmic reticulum (28).

Two major sources of ROS in MDSCs are NOX2 and mitochondria. Compared with MDSCs, cancer cells and macrophages also utilize mitochondria and NADPH oxidase to produce ROS. However, T cells express no or very low levels of NADPH oxidase (37).

Mitochondria have ten sites to generate $O^{2\cdot-}$, particularly those derived from mitochondrial electron transport chain (ETC) complexes. Complex I and III of the ETC generate $O^{2\cdot-}$, which is rapidly converted to H_2O_2 *via* mitochondrial SOD2, while the $O^{2\cdot-}$ from the complex can be converted into H_2O_2 by cytosolic SOD1 (38). Mitochondrial ROS are implicated in diverse diseases, including cancer, diabetes and inflammatory disorders, and regulate healthy cell physiological function (39).

The NOX family has seven members: NOX1–5, DUOX1 and DUOX2 (40). NOX2 is a multicomponent complex that is made up of a transmembrane heterodimer that contains NOX2 and p22phox. Other components are cytosolic protein factors, including p47phox, p67phox, p40phox and small GTP-binding proteins such as G proteins RAC1 or RAC2. Under basal conditions, gp91phox and p22phox are transmembrane proteins, while the cytosolic subunits p47phox, p67phox and p40phox are connected together, and RAC combined with GDP forms a complex with its inhibitor Rho-GDI and does not interact with the other three cytosolic subunits. When exposed to stimulus, NOX2 is activated. Upon activation, p47phox is phosphorylated and then migrates to the membrane, where it combines with gp91phox and p22phox. Rho-GDI separates from the complex, and then RAC binding with GDP combines with gp91phox to form a multicomponent complex (41). NOX2 catalyzes the conversion of oxygen molecules into superoxide anions, which generates H_2O_2 by SOD. Deficiency or dysfunction of NOX2 in phagocytes may reduce ROS production, resulting in chronic granulomatous disease (CGD) (42). Comparably, MDSCs in NOX2-deficient mice produced less ROS, which lose the ability to inhibit the $CD8^+$ T-cell immune response (43). Rats and mice with decreased ROS caused by allelic polymorphisms of p47phox were more susceptible to developing severe arthritis (44).

5 Regulation of ROS production in MDSCs

Many factors can regulate ROS production, such as GM-CSF, interleukin, TGF, TNF, FGF, platelet-derived growth factor, TLR agonists, protease, nucleotide receptors, TCR stimulation and peroxynitrite (32, 45, 46).

Various types of cells and survival environments possess different ROS regulatory mechanisms. In terms of MDSCs, it has been proven that multiple molecules can govern intracellular ROS, such as STAT3, fatty acid transport protein 2 (FATP2) and noncoding RNAs. STAT3 is an important transcription factor related to the expansion, differentiation and function of MDSCs. STAT3 directly increases the expression of p47phox, which belongs to the NOX2 complex, by binding to the promoter region of p47phox. Blocking STAT3 could downregulate the expression of gp91phox and p47phox to decrease ROS production (43, 47, 48). In addition, tumor-derived GM-CSF activated STAT3 signaling to induce the expression of FATP2 in MDSCs. Subsequently, FATP2 in MDSCs can take up abundant lipids that cause elevated ROS levels (49). Furthermore, noncoding RNAs (lincRNAs and miRNAs) that have been upregulated during bacterial and viral infection are reported to influence ROS generation in MDSCs (50). During virus infection, lncRNA RUNXOR and HOTAIRM1 are upregulated and are responsible for elevated levels of ROS, Arg-1 and iNOS in MDSCs (51, 52). MiRNA-10a and miRNA-21, which are also upregulated in hypoxia-induced glioma-derived exosomes, could strengthen ROS and NO production in MDSCs with the potential to enhance the suppressive activity of MDSCs (53).

In addition, cancer-associated fibroblasts (CAFs) can polarize monocytes to MDSCs, which suppress $CD8^+$ T-cell proliferation and function by generating ROS (54). Murine olfactory ecto-mesenchymal stem cell-derived exosomes could also enhance the suppressive activity of MDSCs by upregulating ROS and NO levels (55).

In contrast to MDSCs, other myeloid cells, such as macrophages, can stimulate NADPH oxidase expression and activity to elevate the level of ROS by other disparate factors, such as P2X7, brain-specific angiogenesis inhibitor 1 (BAI1), beryllium, myocardin-related transcription Factor A (MRTF-A) and TLRs (56–60). However, mitochondrial uncoupling protein 2 (UCP2), paraoxonase 1 (PON1) and IL-10 negatively regulate the ROS level in macrophages (61–63).

6 ROS scavenging in MDSCs

In general, the cell needs an appropriate level of ROS to maintain normal physiological function. Either too few or too many ROS are harmful. Normally, ROS production is controlled in a safe range, and superfluous ROS can be neutralized by the antioxidant system to maintain cell homeostasis. The antioxidant

system contains antioxidant enzymes and nonenzymatic molecules. Common antioxidant enzymes include superoxide dismutase (SODs), catalase, peroxidase (PRDXs), peroxiredoxins (Prxs) and glutathione peroxidase (GPXs) (64). Other nonenzymatic antioxidant molecules are glutathione, flavonoids, thioredoxin, and vitamins A, C and E (65, 66). If the redox system is out of balance, the rising ROS will lead to oxidative stress. Oxidative stress is considered a vital inducer of many pathological diseases, such as cancer, atherosclerosis, multiple sclerosis, ischemia and reperfusion injury, Alzheimer's disease, cardiovascular diseases and traumatic brain injury (67–71).

For example, the antioxidant system of tumor cells can cope with the production of ROS properly *via* antioxidant enzymes and autophagy (72). The overproduction of ROS in tumor cells could maintain the pro-tumourigenic signaling, which results from the upregulation of SOD expression, local inactivation of a H_2O_2-degrading enzyme, oxidative inactivation of phosphatase and tension homolog (PTEN) and mutations in Nrf2 and P53 transcription factors (4, 73–75).

Surprisingly, MDSCs could still survive and function excellently by producing high levels of ROS. How can MDSCs scavenge superfluous ROS? This can be ascribed to some essential factors, such as Nrf2, HMGB1, IDO1, calcium-calmodulin kinase 2 (CaMKK2), HIF-1α, pyruvate dehydrogenase kinase 1 (PDK1) and phosphoenolpyruvate (PEP) (Figure 1)

The Nrf2 transcription factor plays a crucial role in regulating the antioxidative response and inducing the expression of antioxidant and detoxification enzyme genes, including heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), catalase and SOD (76). Under normal circumstances, Nrf2 combined with Kelch-like ECH-associated protein 1 (Keap1) is limited to degradation in the cytoplasm. However, under oxidative stress conditions, Keap1 is modified at a specific cysteine position to disable its E3 ligase adaptor and release Nrf2. The released Nrf2 translocates into the nucleus and binds to the small musculoaponeurotic fibrosarcoma (sMaf) protein to form active heterodimers that transactivate downstream antioxidant response elements (AREs) and induce their transcription to exert antioxidant effects (77). Nrf2 is greatly applied to reduce intracellular oxidative stress and apoptosis. Compared to wild-type MDSCs, Nrf2-deficient MDSCs display a greater accumulation of intracellular ROS and attenuated antioxidant enzyme induction (78). MDSCs in the host expressing Nrf2 reduce oxidative stress and cell apoptosis; thus, MDSCs can survive longer (79, 80).

With the exception of Nrf2, the existence of HMGB1 in MDSCs cannot be underestimated. HMGB1, a damage-associated molecular pattern (DAMP) molecule, is a vital driver of MDSC accumulation and immunosuppressive function, as reported in early studies. In the tumor microenvironment, elevated ROS can increase

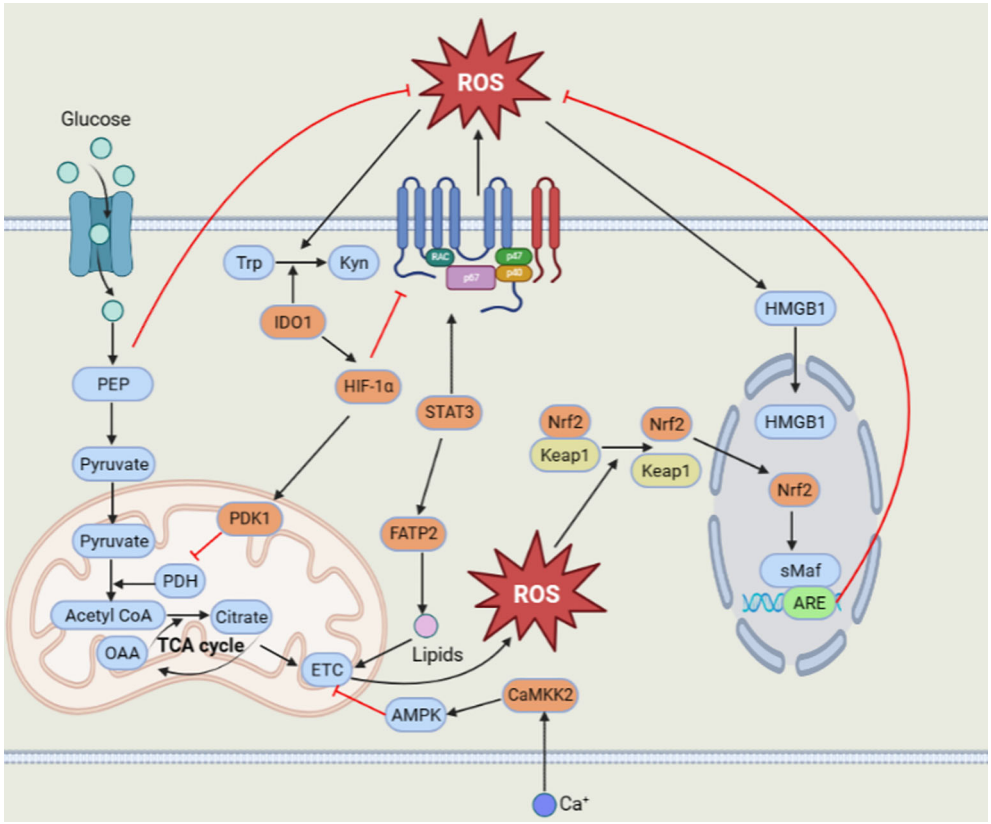

FIGURE 1
Regulation of ROS in MDSCs.

cytoplasmic translocation and release HMGB1 (81). Subsequently, HMGB1 promotes the survival and viability of MDSCs by inducing autophagy (80, 82).

MDSCs also express some enzymes, such as IDO1 and CaMKK2, to negatively modulate the generation of ROS. IDO1, a heme-binding metabolic enzyme, consumes superoxide anion radicals and peroxides to catalyze tryptophan (Trp) into kynurenine (Kyn) (83). $CD11b^{+}Gr1^{+}$ MDSCs from IDO-KO hosts enhanced ROS generation and downregulated the expression of ROS scavenging genes (84, 85). Moreover, CaMKK2 could upregulate the transcription level of Nrf2, not NOX1 and NOX2, to decrease the ROS level by phosphorylating and activating its downstream target AMPK (86, 87).

In addition, hypoxia plays a crucial role in regulating the function of tumor derived MDSCs. HIF-1α could decrease NOX2 expression and excessive ROS production, which may give rise to the preferable survival of MDSCs in the tumor microenvironment (88). In turn, ROS could facilitate HIF-1α accumulation, and then HIF-1α activates PDK1, which could prevent the persistence of potentially harmful and superfluous mitochondrial ROS by inhibiting pyruvate dehydrogenase to restrain the conversion of pyruvate to acetyl-CoA, resulting in a lessened tricarboxylic acid (TCA) cycle (89, 90). Apart from hypoxia, tumor cells can increase the glycolysis of MDSCs in the tumor microenvironment. Tumor derived MDSCs displayed higher glycolysis to prevent the cell apoptosis by restraining excess ROS production. Most importantly, the glycolytic metabolite phosphoenolpyruvate (PEP) is a crucial antioxidant agent that averts MDSC apoptosis and contributes to MDSC survival by hindering excessive ROS production (91).

In contrast to MDSCs, HSCs and CSCs have low ROS content. Several signaling molecules, such as ataxia telangiectasia mutated (TAM), PI3K/Akt, FoxO3 (FoxO transcription Factors 3), phosphatase and tensin homology (PTEN), p53, Prdm16 (PR domain-containing 16), HIF-1α, p38MAPK, and Nrf2, account for the low ROS level to maintain stemness and quiescence in HSCs (33). For example, neural stem cells have a high level of ROS (92). CSC cells also have reduced levels of ROS, which may be attributed to the variant isoform CD44v of the adhesion molecules CD44 and CD13 that boosts the activity of the free radical scavenging system (93, 94).

MDSCs can produce ROS by mitochondria and NOX2. MDSCs can take up lipids through FATP2 to promote mitochondrial ROS production. Moreover, the transcription factor STAT3 can increase NOX2 expression to upregulate ROS levels in MDSCs. Then, the elevated ROS level can activate the antioxidant system to eliminate excessive ROS. Nrf2 could be transcriptionally activated to initiate the expression of its downstream antioxidant genes. The high level of ROS can also induce nuclear heterotopic HMGB1 to promote the survival of MDSCs by autophagy. In addition, HIF-1α can activate PDK1 to inhibit mitochondrial ROS production. IDO1 can scavenge ROS with its metabolic characteristics. Another enzyme, CaMKK2, can activate AMPK to decrease ROS production. The glycolytic metabolite PEP could also prevent massive ROS production to keep the ROS level in a suitable range.

7 Effects of MDSC-derived ROS

ROS signaling can activate cellular signaling pathways, such as NF-κB, mitogen-activated protein kinase (MAPK), JAK/STAT and phosphoinositide 3-kinase (PI3K)/AKT (4, 95). Furthermore, ROS also enhance the activity of activator protein-1 (AP-1) by stimulating MAPK cascades to dominate a wide range of cellular processes and trigger P53 transactivation that mediates apoptosis, and ROS can induce the expression of redox factor-1 (Ref-1), leading to the transcriptional activity of HIF-1α (96, 97). Generally, ROS are considered to have proinflammatory effects, but it has also been reported that ROS derived from NOX2 have anti-inflammatory functions (45). In the murine arthritis (CIA) model, NADPH-deficient dendritic cells can produce more proinflammatory cytokines and induce both Th1 and Th17 responses to promote autoimmune arthritis (98). In addition, ROS derived from NOX2 could inhibit the NLRP3 inflammasome *via* the PI3K/Akt/NF-κB pathway at 3 days after stroke (99).

ROS produced by MDSCs could have distinct impacts on different cells (Figure 2). In the tumor microenvironment, PMN-MDSCs release ROS into the extracellular space to directly and indirectly support tumor progression. ROS produced by PMN-MDSCs inhibited T-cell responses through p-STAT3 signaling. ROS have an impact on the activation, proliferation and effect of T cells by regulating cell surface thiol levels (44, 100). Specifically, peroxynitrite could nitrate the TCR/CD8 complex, which prevented it from combining with pMHC, and H_2O_2 reduced the TCRζ chain and IFN-γ secretion of T cells to destroy T-cell function (43, 48). In addition, when encountering circulating tumor cells (CTCs), PMN-MDSCs can produce excessive levels of ROS to upregulate Notch1 expression in CTCs *via* the Nrf-2-ARE axis. Notch1 could bind to the ligand jagged on the surface of PMN-MDSCs. In addition, Nodal, the downstream target gene of Notch1 in CTCs, can bind to Noda1 recptor cripto in PMN-MDSCs in turn, and the interaction between these signals eventually promotes the survival and proliferation of CTCs (101). Likewise, ROS derived from macrophages and granulocytes can inhibit the activation, proliferation and effect of T cells, and macrophages and activated T cells produce ROS to induce regulatory T cells (102–105).

In addition to inhibiting T cells, ROS released by MDSCs have immunosuppressive activities on B cells and NK cells under infection pathological conditions. During virus infection, two subsets of MDSCs rapidly accumulate at the infected site. In detail, PMN-MDSCs inhibit the activation, proliferation and function of NK cells and reduce the secretion of IFN-γ and granzyme B *via* ROS (106, 107), while M-MDSCs release ROS, including superoxide, peroxynitrite, and nitric oxide, but not H_2O_2, to suppress B-cell responses (108). Similarly, human PMN-MDSCs isolated from buffy coats could also produce ROS and other soluble mediators to suppress B-cell proliferation and antibody production (109).

However, professional phagocytes, tumor cells and CSCs are distinct from MDSCs. Professional phagocytes generate ROS to effectively jeopardize pathogens by interacting with microbial

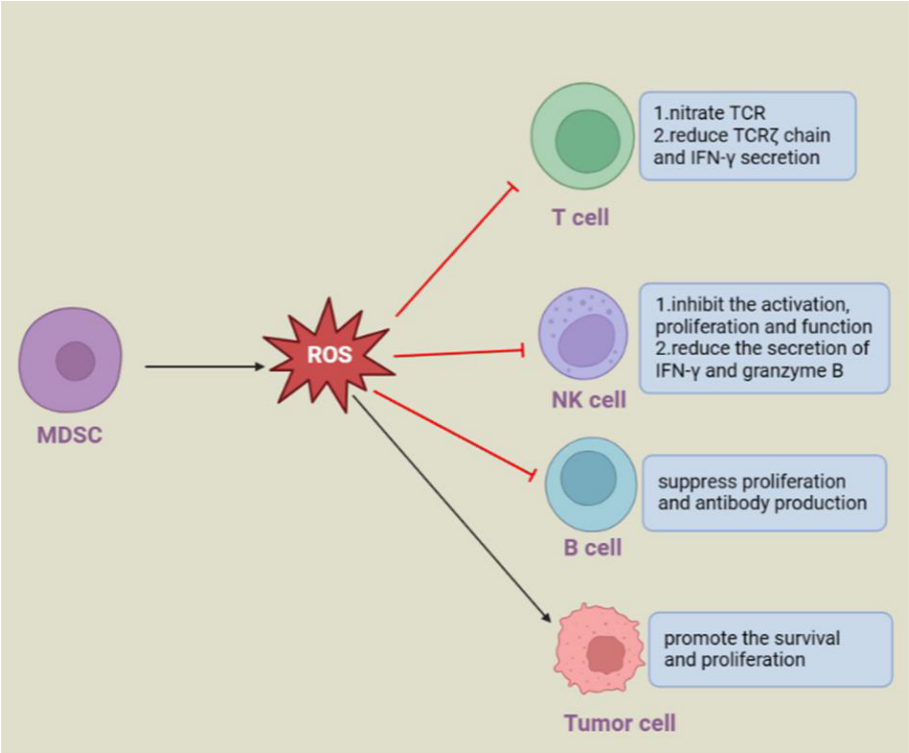

FIGURE 2
Regulation of other cells by MDSC-derived ROS.

components to impair bacterial metabolism (110). ROS in tumor cells have dualistic impacts on the initiation, promotion, progression and metastasis of tumor cells (111). Increased ROS levels in tumor cells could facilitate tumorigenicity by enhancing the proliferation, growth, survival, invasion and metastasis of tumor cells. In contrast to these effects, ROS can suppress tumor growth by inducing apoptosis, autophagy, necrosis and ferroptosis. Both normal stem cells and CSCs exhibit low levels of intracellular ROS content to maintain stemness (112).

In summary, MDSCs and ROS are interactive and mutually beneficial. MDSCs can produce ROS to inhibit antigen-specific T cells (32, 47). In turn, ROS could regulate the differentiation and immunosuppressive activity of MDSCs. In the absence of ROS, the function of MDSCs could be lost to suppress adaptive T-cell responses (43). Additionally, ROS can affect the differentiation of myeloid cells by regulating related gene expression. High levels of ROS can prevent MDSCs from differentiating into mature myeloid cells, while low levels of ROS resulting from catalase and a lack of NOX2 activity enable MDSCs to differentiate into TAMs and DCs (113). How to control ROS levels in MDSCs is a priority and needs further investigation.

ROS produced by MDSCs can have diverse effects on different kinds of cells. MDSCs-derived ROS can promote the proliferation and metastasis of circulating tumor cells by Nrf2/Notch1/Nodal signaling. MDSCs-derived ROS have an inhibitory effect on other immune cells, such as T, B and NK cells, and promote disease progression by inhibiting their function.

8 Targeting ROS therapy for MDSCs

Currently, a variety of immune therapies to target MDSCs are being exploited to improve the efficacy of cancer immunotherapy. MDSCs mediated immuno- suppressive function could be abrogated when ROS production is inhibited (114). Remarkable achievements have been made in strategies to lessen the ROS production and block the induction of oxidative stress in MDSCs (115) (Table 1).

The most representative molecules of anti-inflammatory and antitumor drugs are bardoxolone methyl (CDDO-Me), nitroaspirin and Embelin. On account of its capacity to upregulate several antioxidant genes, including NAD(P)H: quinone oxidoreductase 1 (NQO1), thioredoxin, catalase, superoxide dismutase and heme oxygenase, CDDO-Me could efficiently abrogate the immune suppressive effect of MDSCs and enhance T-cell function by activating the target gene NQO1 to decrease MDSC-mediated ROS production, while CDDO-Me did not affect the NO level in MDSCs (116). Nitroaspirin has also been proven to inhibit ROS production and limit the activity of Arg-1 and iNOS in MDSCs (18). Treatment combining vaccination against gp70 with nitroaspirin could inhibit MDSC function and enhance antitumor activity (117). Embelin has anti-inflammatory and antitumor effects in previous studies. It could impair the immunosuppressive activity of MDSCs by reducing the generation of ROS through STAT3 signaling to improve the antitumor immune response in colitis-associated cancer mice (129).

TABLE 1 Effect of ROS-targeted drugs on MDSCs.

Drug	Type	Disease	Mechanism	References
CDDO-Me	Synthetic triterpenoid	Renal cell carcinoma or soft tissue sarcoma patients EL-4 thymoma, MC38 colorectal carcinoma and Lewis lung cancer mouse model	Activate the target gene NQO1 to decrease ROS level	(116)
Nitroaspirin	Nitro derivative	CT26 colon carcinoma mouse model	Decrease ROS level	(18, 117)
Sanguinarine (SNG)	Benzophenone alkaloid	Lewis lung cancer mouse model	Decrease ROS level	(118)
Baicalein	Traditional Chinese medicine	Systemic lupus erythematosus mouse model	Enhance Nrf2 activation to decrease ROS level	(119)
Jianpi Huayu Decoction (JHD)	Traditional Chinese medicine	H_{22} hepatocellular carcinoma mouse model	Decrease ROS level	(120)
1a,25-Dihydroxyvitamin D3 (calcitriol)	Vitamin D	4-nitroquinoline 1-oxide (4-NQO)–induced esophageal cancer mouse model	Decrease the phosphorylation of STAT3 to decrease ROS level	(121)
Endostatin (ES)	Fragment derived from collagen XVIII	Orthotopic renal cell carcinoma mouse model	Decrease ROS level	(122)
Ferumoxytol	Iron supplement	LPS-induced sepsis mouse model	Decrease ROS level	(123)
L-NIL	iNOS inhibitor	B16 melanoma mouse model	Decrease STAT3 activation to decrease ROS level	(124)
Histamine dihydrochloride (HDC)	NOX2 inhibitor	MC38 colorectal carcinoma and 4T1 mammary carcinoma mouse model	Decrease ROS level in NOX2-dependent way	(125)
Celecoxib	COX-2 inhibitor	AB1 mesothelioma mouse model	Decrease ROS level	(126)
SAHA	Histone deacetylase inhibitor	4T1 mammary tumor mouse model	Increase ROS level	(127)
Alisertib	Aurora-A kinase inhibitor	4T1 mammary tumor mouse model	Downregulate the mRNA expression level of CYBB and NCF1 and inhibit JAK2-STAT3 pathway to decrease ROS level	(128)
Embelin	X-linked inhibitor of apoptosis protein (XIAP) inhibitor	Colitis-associated cancer mouse model	Limit C/EBPβ and STAT3 signaling to decrease ROS level	(129)
Sildenafil	Phosphodiesterase-5 inhibitor	Immunocompetent murine tumor models of major surgery	Decrease ROS level	(130)
N-acetylcysteine (NAC)	ROS inhibitor	P493 B lymphocytoma xenograft mouse model	Stimulate the degradation of HIF-1α to decrease ROS level	(131)
Pam3CSK4	TLR2 agonist	Hepatocellular carcinoma mouse model	Decrease ROS level	(132)
Swertianolin	Isolated from plant gentianella acuta	Sepsis mouse model	Decrease ROS level	(133)
Curcumin	Derived from plant turmeric	Lewis lung cancer mouse model	Decrease ROS level	(134)
Withaferin A (WA)	Natural product	4T1 mammary tumor mouse model	Decrease the phosphorylation of STAT3 to decrease ROS level	(135)
polysaccharide nCKAP-2	Isolated from plant *Curcuma kwangsiensis*	MSC2 cells	Activate TLR4-NF-κB signaling to decrease ROS level	(136)
liposomal doxorubicin and liposomal vaccine containing E75	Liposomal antibiotics and the liposomal peptide	TUBO breast cancer mouse model	Decrease ROS level	(137)
GMI	An immunomodulatory peptide from *Ganoderma microsporum*	*S.aureus*-induced periprosthetic joint infection mouse model	Decrease ROS level	(138)

(Continued)

TABLE 1 Continued

Drug	Type	Disease	Mechanism	References
ApoA-I mimetic peptide 4F (L-4F)	An apolipoprotein A-I (ApoA-I) mimetic peptide	Pancreatic cancer mouse model	Decrease the phosphorylation of STAT3 to decrease ROS level	(139)

In addition, many inhibitors are being exploited to reduce ROS level such as N-acetylcysteine (NAC), L-NIL, histamine dihydrochloride (HDC), celecoxib, alisertib, SAHA and sildenafil. NAC, a well-established antioxidant that had the ability to reduce ROS and increase the extracellular pool of cysteine. Many animal models have verified its antitumor efficacy. NAC could stimulate the degradation of HIF-1 and inhibit its activity by neutralizing ROS (131). Moreover, the iNOS inhibitor L-NIL, NOX2 inhibitor HDC and COX-2 inhibitor celecoxib could weaken MDSCs function by downregulating ROS production, resulting in enhanced antigen-specific cytotoxicity of CTL (124–126).

Along with inhibitors that target the effector molecules of the immunosuppressive activity of MDSCs, enzyme inhibitors can achieve similar outcomes. The selective Aurora-A kinase inhibitor alisertib directly weakened the immunosuppressive function of MDSCs by notably downregulating the mRNA expression levels of associated genes, such as NOS2, S100A8, S100A9, CYBB and NCF1, and compromising ROS production by inhibiting the JAK2-STAT3 pathway (128). Phosphodiesterase-5 (PDE-5) inhibitors reversed surgery-induced PMN-MDSC immunosuppression by downregulating the level of ROS (130).

Out of the ordinary, the histone deacetylase inhibitor SAHA could augment the intracellular ROS to induce apoptosis in MDSCs. That might be a promising and novel MDSCs-targeted therapy (127). In addition, TLR2 agonist Pam3CSK4 could attenuate hepatocellular carcinoma progression by decreasing ROS content and promoting MDSCs polarization (132).

Natural products are increasingly being discovered and researched in tumor therapy. With the deeper comprehension of natural products, many plant extracts have antioxidant impacts. Among them, withaferin A (WA), a component of the root extract of the plant Withania somnifera Dunal (WRE), could decrease ROS production in MDSCs through a STAT3-dependent mechanism (135). The polysaccharide nCKAP-2 contained in native Curcumae Rhizoma (CR) could induce MDSC apoptosis in a dose-dependent manner through the TLR/NF-κB pathway. In addition, nCKAP-2 can significantly relieve the inhibitory effect of MDSCs on T cells by reducing the ROS level (136). Moreover, curcumin has been reported to lessen the production of ROS and the Arg-1 expression level in MDSCs, which not only inhibited the accumulation of MDSCs in spleen and tumor tissue but also weakened the immunosuppressive function of MDSCs (134). Swertianolin isolated from Swertia and sanguinarine (SNG) derived from Sanguinaria canadensis could prominently decrease the secretion of ROS to inhibit the immunosuppressive effect of MDSCs (118, 133).

Traditional Chinese medicines have made enormous achievements in antioxidant activity. Baicalein is a traditional Chinese herbal medicine. Baicalein prevented the expansion and function of MDSCs in lupus mice, which can be attributed to decreased ROS levels and enhanced Nrf2 activation (119). Jianpi Huayu decoction (JHD), another traditional Chinese medicine, is an experienced prescription for tumor therapy. When MDSCs were treated with JHD, MDSCs could differentiate into macrophages and dendritic cells, and ROS levels were reduced (120).

Furthermore, endostatin (ES) derived from collagen XVIII has the potential to target PMN-MDSCs selectively, resulting in obviously reduced ROS production (122). Doxorubicin (Dox), the conventional chemotherapy to reduce the number of MDSCs in tumor tissues and promote antitumor responses, is converted into a liposomal formulation to improve the efficacy of therapy, as well as the peptide named the E75 epitope (Pep) originating from human epidermal growth factor receptor 2 (HER2/neu). Combination therapy with liposomal nonliposomal Dox and liposomal Pep was the best treatment compared to other single therapies, which decreased ROS generation and downregulated multiple genes related to immunosuppressive function, such as S100A8, S100A9, Arg-1 and iNOS (137). 1a,25-Dihydroxyvitamin D3 (calcitriol) supplementation could reverse the increased level of ROS in IL-6-induced MDSCs (121). In the same way, iron supplementation with ferumoxytol could attenuate MDSC function by significantly downregulating ROS production and inhibiting the expansion of MDSCs in LPS-induced septic mice (123). In addition, GMI is a fungal immunomodulatory protein isolated from *Ganoderma microsporum* that reduces MDSC expansion in bone marrow cells (BMCs) stimulated by *S. aureus* biofilms, which was attributed to increased cytokine expression and a reduction in ROS levels (138, 140, 141). L-4F, an apolipoprotein A-I (ApoA-I) mimetic peptide, inhibited the immunosuppressive function of PMN-MDSCs but not M-MDSCs by decreasing ROS and H_2O_2 production (139).

Conclusion

Based on a previously published review, this paper further updated and listed the new molecules found in recent years that can regulate ROS levels in MDSCs and comprehensively summarized the therapeutic drugs that can target ROS levels in MDSCs. This provides a treatment strategy for cancer immunotherapy.

Compared to other myeloid cells, such as macrophages or tumor cells, ROS play an irreplaceable and distinctive role in MDSCs. On the one hand, MDSCs are required to produce ROS to suppress the antitumor immune response. In turn, excessive ROS can be removed to promote MDSC survival comfortably by activating factors, such as Nrf2, HMGB1, HIF-1α, IDO1,

CaMKK2 and PEP. On the other hand, an appropriate level of ROS can prevent further differentiation of MDSCs to better maintain their state and nature. However, other myeloid cells, such as macrophages, have the same sources of ROS as MDSCs and regulate intracellular ROS levels by different factors, such as P2X7 and BAI1. Tumor cells can also induce autophagy to scavenge excessive ROS.

It is widely believed that ROS can promote the development of tumors, but a large number of studies have shown that ROS can promote tumor cell apoptosis and death. At present, studies are emerging that tend to exploit immunotherapies that utilize the ability of ROS to kill tumor cells. Therapy targeting ROS in MDSCs can be therapeutic by impairing MDSC differentiation and function. How to combine targeted ROS therapy in MDSCs and tumor cells requires further consideration.

Author contributions

JH drafted the manuscript. YZ, KZ and KY discussed and revised the manuscript. SW designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Deciphering the mechanism of *Peptostreptococcus anaerobius*-induced chemoresistance in colorectal cancer: the important roles of MDSC recruitment and EMT activation

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Peptostreptococcus anaerobius (*P. anaerobius*, *PA*) in intestinal flora of patients with colorectal cancer (CRC) are associated with poor prognosis. Studies have shown that *P. anaerobius* could promote colorectal carcinogenesis and progression, but whether *P. anaerobius* could induce chemoresistance of colorectal cancer has not been clarified. Here, both *in vitro* and *in vivo* experiments showed that *P. anaerobius* specifically colonized the CRC lesion and enhanced chemoresistance of colorectal cancer to oxaliplatin by recruiting myeloid-derived suppressor cells (MDSCs) into the tumor microenvironment. Furthermore, this study revealed that it was the increased secretion of IL-23 by MDSCs that subsequently facilitated the epithelial–mesenchymal transition (EMT) of tumor cells to induce chemoresistance of CRC by activating the Stat3-EMT pathway. Our results highlight that targeting *P. anaerobius* might be a novel therapeutic strategy to overcome chemoresistance in the treatment of CRC.

KEYWORDS

***Peptostreptococcus anaerobius*, chemoresistance, MDSCs, IL-23, EMT, colorectal cancer**

1 Introduction

Colorectal cancer (CRC) is the third most common diagnosed cancer and the second-leading cause of cancer death worldwide. Moreover, in the past few decades, CRC is shifting to diagnosis at a younger age and a more advanced stage (1–3). Despite the fact that palliative chemotherapy for advanced-stage colorectal cancer has led to substantial improvement of overall survival, over half of CRC patients suffered from

chemoresistance, and the pervasive development of acquired chemoresistance has always been the main cause of cancer relapse and metastasis (4–6). Therefore, uncovering the underlying mechanisms associated with CRC chemoresistance is indispensable for designing novel treatment strategies.

The intestinal flora, representing the largest microbial reservoir in human body, is intimately associated with human growth, nutritional metabolism, and disease onset (7–9). The cecum and colon harbor the most dense and diverse communities of bacteria in gut microhabitats. These bacteria can be found in feces, gut lumen, colon mucus layers, colorectal epithelia, and even tumor stroma (10, 11). Meanwhile, it is remarkable that the intestinal flora has been found to be involved in regulating the onset and progression of CRC by modulating the tumor microenvironment (12). It is reported that some intestinal flora, such as *Streptococcus bovis*, *Enterotoxigenic Bacteroides fragilis*, and *Enterococcus faecalis*, can promote the occurrence, development, and chemoresistance of CRC through inflammatory reaction, genotoxins, oxidative stress, metabolites, and biofilms (11). In particular, certain bacteria such as *Gammaproteobacteria* and *F. nucleatum* can penetrate mucus and lead to chemoresistance by metabolizing chemotherapeutics and activating autophagy in colorectal tumor (13, 14). *Peptostreptococcus anaerobius* (*P. anaerobius*, *PA*), an anaerobic Gram-positive bacterium that commonly exists in human oral and intestinal tracts, has been found in high abundance in intestinal flora of chemoresistant CRC patients (13, 15–18) and *P. anaerobius* could directly educate CRC cells and the corresponding microenvironment to promote cancer progression (13, 17–19). However, whether *P. anaerobius* could induce CRC chemoresistance and, if so, its underlying mechanism, remains unclear.

Recruited from immature myeloid cells by tumor-derived growth factors and inflammatory factors (20–22), MDSCs play important roles in modulating immune responses to promote CRC progression (20). CRC patients with high levels of MDSCs have worse outcomes (23–27) than those with low levels of MDSCs (28–30). Remarkably, a significantly high enrichment of MDSCs in a CRC model with *P. anaerobius*-treated ApcMin/+mice was reported recently (19). Consistently, another prognostic analysis showed that *P. anaerobius* was enriched in high-risk stage III colon cancer samples, and the invaded bacteria activated tumor-associated myeloid cells and caused them to produce the cytokine IL-23, which was significantly characteristic in the high-risk group (31). These findings indicated that both *P. anaerobius* and MDSCs were closely related to the development of CRC, but the relationships among *P. anaerobius*, MDSCs, and chemoresistance still need to be further clarified.

In this study, both *in vitro* and *in vivo* experiments demonstrated that *P. anaerobius* could promote chemoresistance of CRC to oxaliplatin by colonizing colorectal tumor lesion and facilitating the recruitment of MDSCs into the tumor microenvironment, which drove EMT and chemoresistance of tumor cells by releasing IL-23 (Figure 1).

2 Materials and methods

2.1 Bacterial culture

P. anaerobius (ATCC27337) was purchased from Ningbo Mingzhou Biotechnology Co. Ltd (B81243, MingZhouBio). The bacteria were maintained in Modified Reinforced Clostridial Broth Medium (MD039; ATCC Medium 2107, Shandong Topu Biol-Engineering Co. Ltd) in an anaerobic jar (D-110, MITSUBISHI). The anaerobic condition was created by the usage of Anaeropack (D-04, AN0035; MGC AnaeroPackTM Series, MITSUBISHI).

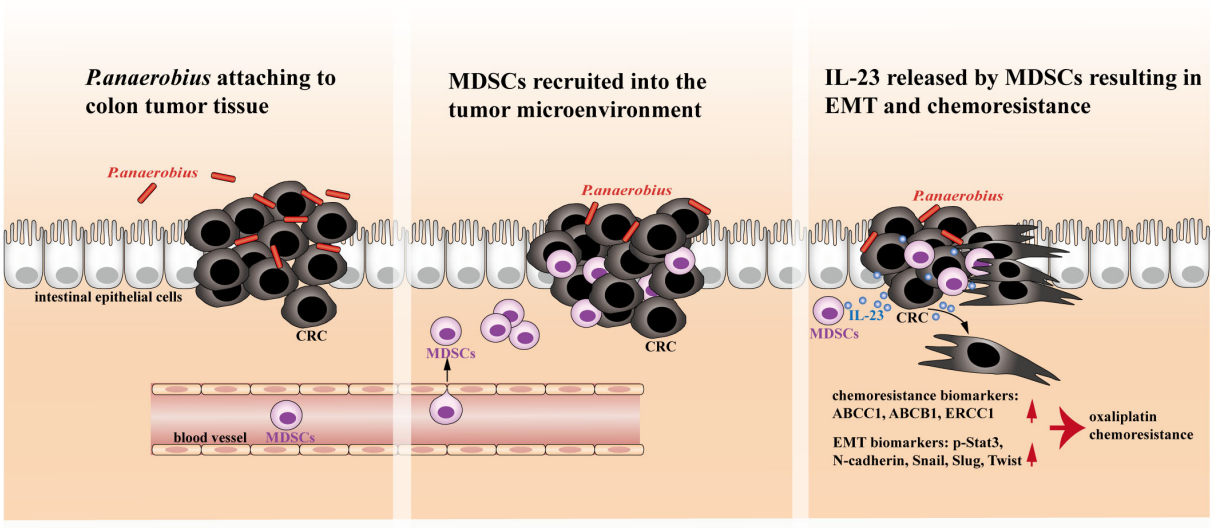

FIGURE 1
The proposed mechanistic scheme of *P. anaerobius* promoting colorectal chemoresistance.

2.2 Animal experiments

MC-38 cells (1×10^6 cells per mouse) were implanted in the cecum of C57BL/6 mice (male, 8 weeks old). Two weeks after implantation, mice were gavaged with *P. anaerobius* suspension (1×10^8 c.f.u.) concurrently with administration of oxaliplatin (5 mg/kg/3 days) intraperitoneally for 3 weeks, and feces were collected weekly for qPCR analysis (Figure 2B). In addition, anti-Gr-1 monoclonal antibody (32) (anti-Gr-1 mAb, 200 μg/mouse, three times/week, BE0075, Bio X cell) or anti-mouse IL-23 monoclonal

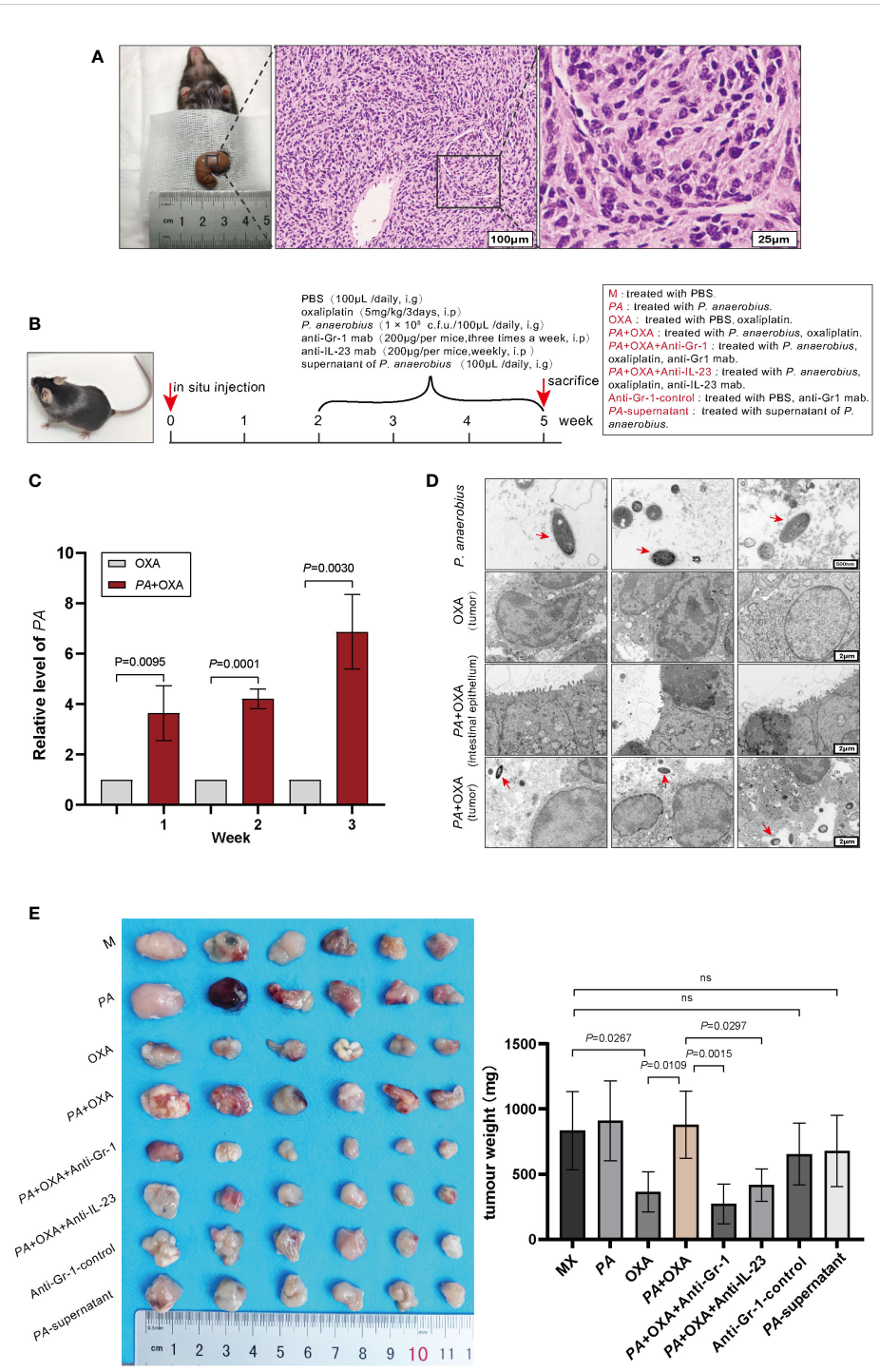

FIGURE 2
P. anaerobius attenuated the therapeutic effect of oxaliplatin in CRC mice. **(A)** Representative morphologies and histological images (H&E) of CRC tissues. Scale bar: 100 μm (left), 25μm (right). **(B)** Schematic diagram of experimental design and timeline of CRC mice model (n = 6). *PA*: *P. anaerobius*; OXA: oxaliplatin; anti-Gr-1mab: anti-mouse Gr-1 monoclonal antibody; anti-IL-23 mAb: anti-mouse IL-23 monoclonal antibody. **(C)** The amount of *P. anaerobius* in stool samples of CRC mice determined by qPCR (mean ± SD, two-tailed unpaired Student's *t*-test). **(D)** Representative TEM images of *P. anaerobius* (red arrows) attaching to colon tumor tissue. Scale bars: 500 nm for *PA* images, 2 μm for colon tumor tissue images. **(E)** Representative tumor images and statistical analysis of tumor weights of CRC in different groups (n = 6, mean ± SD, one-way analysis of variance).

antibody (anti- IL-23 mAb, 200 μg/mouse/week, BE0313, Bio X cell) was intraperitoneally given to the corresponding group of mice respectively for 3 weeks to observe the function of MDSCs and IL-23 in *P. anaerobius*-induced chemoresistance. Then, the mice were sacrificed and colonic tumors were collected and weighed. All animal work was approved by the Animal Experimentation Ethics Committee of Weifang Medical University.

2.3 Assessment of colonic histopathology

Colonic tumor specimens were formalin-fixed and paraffin-embedded for histologic examination. Sections of 5 μm were stained with H&E and reviewed in a blinded manner by an experienced pathologist. Dysplasia was defined according to the latest World Health Organization Classification of Tumors of the Digestive System.

2.4 Microbial DNA extraction and *P. anaerobius* quantification

Stool DNA was extracted by ZR Fecal DNA MiniPrep (D2700, Beijing Solarbio Science & Technology Co., Ltd) from the feces samples of mice. DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Two microliters of DNA (0.5 ng) was used in each 20 μL of 2×SYBR Green qPCR Master Mix (G3320, Servicebio) reaction. The reaction was performed in triplicate and analyzed on a QuantStudio 7 Flex System (CFX Connect, Bio-Rad). The primers for *P. anaerobius* were GTA AAG GGT GCG TAG GTG GTC (forward 5'→3') and CCT CAG TGT CAG TTG CAG TCC (reverse 5'→3'), and primers for total bacteria were GTG STG CAY GGY TGT CGT CA (forward5'→3') and ACG TCR TCC MCA CCT TCC TC (reverse 5'→3').

2.5 Transmission electron microscopy

Tumor tissues were fixed in 1% OsO_4 in 0.1 MPB (pH 7.4) and rinsed three times in 0.1 MPB (pH 7.4). After that, the samples were dehydrated, embedded, cut into 50-nm sections, and stained with 2% uranium acetate and 2.6% lead citrate. A transmission electron microscope (HT7800/HT7700, HITACHI) was used to obtain corresponding images.

2.6 Flow cytometry

Multicolor flow cytometry (FCM) was performed to observe the percentage of MDSCs in the bone marrow of CRC mice. After being freed of muscles and tendons, the femurs and tibiae of mice were placed in 70% ethanol for 2 min and subsequently washed in PBS, then a syringe was used to flush bone marrow cells from the femurs and tibias with PBS. After red blood cells were lysed, flushing fluid was filtered through a 100-μm membrane to obtain suspension of single cells. Cells were incubated with Fc blocking antibody (BioLegend, 101319) for 15 min and then stained with fluorescence-conjugated antibodies of surface markers CD11b (clone M1/70, eBioscience, 11-0112-82) and Gr-1 (clone RB6-8C5, Biogems, 83122-80-25) for 30 min. The samples were detected by a BD FACS Aria Fusion Flow Cytometry Cell Sorter (BD Biosciences), and the data were analyzed using FlowJo v.9 software (FlowJo LLC).

2.7 Immunofluorescence

Slides (3–4 μm thick) of colonic tumor specimens were prepared and incubated with FITC-conjugated anti-mouse CD11b (BioLegend, USA, 101205) and APC-conjugated anti-mouse Gr1 (BioLegend, USA, 101211) overnight at 4°C. Images were acquired using a fluorescence microscope (Olympus, Japan). Quantification of fluorescent signals was performed using ImageJ software. The density of infiltrated MDSC in the tumor microenvironment was evaluated by averaged $CD11b^+Gr1^+$ co-positive (red and green) area from at least three random 0.42 mm^2 fields within the tumors.

2.8 Cell culture

The colon cancer cell line MC-38 was obtained from ATCC and cultured in the usual culture medium composed of RPMI-1640 medium (GIBCO, Carlsbad, CA) and 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere. For bacterial co-culture, MC-38 cells were exposed to *P. anaerobius* with a multiplicity of infection (MOI) of 200 for 6 h under anaerobic conditions. Then, the medium containing *P. anaerobius* was replaced with the usual medium supplemented with 2% penicillin/streptomycin and 10 mg/mL gentamicin. After 24 h, conditioned medium was collected and named *PA*+MC-38-CM for further research.

2.9 Wright's Giemsa staining

Naive MDSCs collected through flow cytometry were prepared by cytospin to perform morphological assessment using Wright–Giemsa (Leagene, Beijing, China) staining according to the manufacturer's protocol.

2.10 Migration and invasion assay

Transwell assays for evaluating migration and the invasion ability of cells were conducted using 24-well Millicell Hanging Cell Culture Insert 8.0 μm PET (Merck Millipore, Darmstadt, Germany). For migration assay of MDSCs, 2×10^5 cells per well were incubated in serum-free 1640 in the upper chamber with usual culture medium in lower wells supplemented with *PA*-CM (*PA* supernatant), MC-38-CM (MC-38 supernatant), or *PA*+MC-38-CM, respectively. For invasion assay of MC-38 cells (1×10^5 cells

per well), 8.0-μm PETs were coated with 10% Matrigel matrix to imitate extracellular matrix and the usual culture medium in lower wells was supplemented with MDSCs-CM (supernatant of MDSCs cultured with *PA*-MC-38-CM), IL-23 (40 ng/mL, ab259423, Abcam), or anti-IL-23 (100 ng/mL, BE0313, Bio X cell) + MDSCs-CM, respectively. After 48 h, migrating/invasion cells on the basolateral side of the chamber membrane were fixed with formaldehyde and stained with crystal violet (Merck Millipore, Darmstadt, Germany). The number of migrating/invading cells was counted under a light microscope at a magnification of ×400 in five random fields. All assays were repeated at least three times independently.

2.11 For wound-healing assay

MC-38 cells were pretreated with MDSCs-CM, IL-23 (40 ng/mL, ab259423, Abcam) or anti-IL-23 (100 ng/mL, BE0313, Bio X cell) + MDSCs-CM for 24 h respectively. The wound-healing assay was performed by scratching a single cell layer with a pipette tip. Images of the scratch area were recorded at five random spots at 0 and 48 h. The migration distance of the wound edge was measured using a standard size field for each image. The mean migration distances of the five spots were calculated in triplicate and all data were statistically analyzed.

2.12 ELISA

Proteins were extracted from the tumor tissues as described previously (33). The expression levels of VEGF, HGF, IL-6, and IL-23 in MDSCs culture supernatant with corresponding stimulations and the IL-23 level in tumor tissues were analyzed by commercial ELISA kits (R&D Systems) according to the manufacturer's protocol. The color reaction was measured as OD_{450} units on the microplate reader (Model 550; Bio-Rad). The concentration of cytokines was determined via a standard curve that was obtained using the kit's standards. Experiments were performed in triplicate.

2.13 Cell viability assay

The viability of MC-38 cells was evaluated by the CCK-8 (BS350B, Biosharp) assay. Co-culture of *P. anaerobius* and MC-38 cells or *PA*-CM treatment were conducted to observe the influence of *P. anaerobius* on the efficacy of OXA (0.1360 μM) to MC-38 cells (5×10^3 cells per well). In addition, the efficacy of OXA (0.1360 μM) to MC-38 cells treated with MDSCs-CM, IL-23, and anti-IL-23+MDSCs-CM (100 ng/mL, BE0313, Bio X cell) respectively, were also detected. After cells (5×10^3 cells per well) were incubated for 24 h, CCK-8 assay was conducted by adding 10 μL of CCK-8 reagent to each well and incubating for 3 h. Finally, the optical density was determined at 450 nm using the microplate reader (Model 550; Bio-Rad). Experiments were performed at least in triplicate.

2.14 Western blot

The proteins were isolated from cells, separated by 10% SDS–PAGE, and transferred onto PVDF membranes. Then, the membranes were blocked with 5% BSA for 2 h and incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 h at room temperature, respectively. Anti-ABCC1 (1:1,000, bs-24241R), Anti-ABCB1 (1:1,000, bs-0563R), Anti-ERCC1 (1:1,000, bs-1726R), Snail (1:2,000, bs-1371R), and Twist (1:2,000, bs-2441R) were obtained from Bioss (Bioss, BeiJing); N-cadherin (1:1,000, 14215S), E-cadherin (1:1,000, 14472S), Slug (1:2,000, 9585T), Stat3 (1:1,000, 9139T), and p-Stat3 (1:1,000, 4113S) were obtained from Cell Signaling Technology (MA); β-actin (1:5,000, 81115-1-RR) was obtained from Proteintech (MA); Goat-anti-mouse IgG (1:5,000, A0216) was obtained from beyotime (Shanghai). Membranes were exposed to Pierce ECL Western Blotting Substrate (GE Healthcare). Band intensities were determined using ImageJ (National Institutes of Health). The band intensities were represented by the averages of three independent experiments.

2.15 Statistical analysis

A Student's *t*-test was performed to compare the variables of the two sample groups. Multiple group comparisons were made by one-way analysis of variance (ANOVA) followed by Tukey's test. *p*-value less than 0.05 was considered statistically significant. Data were expressed as mean ± SD from three independent experiments. All tests were performed using GraphPad Prism, version 8.0 (GraphPad, La Jolla, CA) or SPSS, version 20 (SPSS Inc, Chicago, IL).

3 Results

3.1 *Peptostreptococcus anaerobius* accumulated in implanted colon cancer lesion and attenuated the therapeutic effect of oxaliplatin

To investigate the roles of *P. anaerobius* in CRC chemoresistance, a colorectal cancer model in C57 mice was constructed by implanting MC-38 cells *in situ*. Two weeks later, three randomly selected mice were dissected to observe tumor growth and all reached 80–100 mm^3 tumor volume (Figure 2A). Then, *P. anaerobius* (1×10^8 c.f.u.) were gavaged to CRC mice daily accompanied with oxaliplatin treatment (5 mg/kg/3 days) for 3 weeks (Figure 2B). Quantitative PCR proved that *P. anaerobius* were successfully colonized in intestinal flora (Figure 2C) and transmission electron microscopy (TEM) showed that *P. anaerobius* was more likely to accumulate in the colon cancer lesion than in normal intestinal epithelium (Figure 2D). In addition, it seemed that the accumulation of *P. anaerobius* could promote the growth of implanted colon cancer since the weight of CRC treated with *P. anaerobius* was higher than that without *P.*

anaerobius treatment. Interestingly, the supernatant of *P. anaerobius* had no obvious influence on tumor proliferation (Figure 2E). Furthermore, *P. anaerobius* significantly hindered the effectiveness of oxaliplatin while the growth of implanted colon cancer without *P. anaerobius* gavage could be effectively inhibited by oxaliplatin (Figure 2E), indicating that *P. anaerobius* could attenuate the therapeutic effect of oxaliplatin in CRC mice.

3.2 *Peptostreptococcus anaerobius* promoted drug resistance by recruiting MDSCs into colorectal cancer microenvironment

As having been reported that the accumulation of *P. anaerobius* in colorectal cancer lesion was closely related to chemoresistance (19, 31), this study also found that *P. anaerobius* could attenuate the therapeutic effect of oxaliplatin in a mouse model. Meanwhile, *in vitro* cell experiments showed that either the co-culture of MC-38 and *PA* or *PA* supernatant stimulation did not affect the sensitivity of MC-38 to oxaliplatin (Figure 3A). Since MDSCs have been reported to be modulated by *P. anaerobius* and be responsible for developing chemoresistance (21), the MDSCs in bone marrow of CRC mice were analyzed by flow cytometry. The result showed that the amount of MDSCs was significantly higher in bone marrow of CRC mice treated with *P. anaerobius* (Figure 3B). In addition, immunofluorescence showed a significant increase of MDSC infiltration in colorectal tumor lesions of mice treated with *P. anaerobius* (Figures 3C, D). These findings are consistent with previously reported studies that found increasing proportion of MDSCs in CRC with *P. anaerobius* infection (19). Furthermore, MDSCs collected from the bone marrow of CRC mice (Figure 3E) were incubated by *PA*-CM, MC-38-CM, or *PA*+MC-38-CM, respectively. Interestingly, the chemotaxis ability of MDSCs treated with *PA*+MC-38-CM increased significantly while compared with those treated with MC-38-CM and *PA*-CM (Figure 3F), indicating that it was the interaction between *P. anaerobius* and MC-38 but not the metabolites of *P. anaerobius* that induced the infiltration of MDSCs into the tumor microenvironment.

Anti-Gr-1 mAb can selectively cut down MDSCs (34) and has no obvious influence on other immune cells, then anti-Gr-1 mAb (200 μg/mouse, three times/week) was intraperitoneally injected in mice to eliminate MDSCs both in the bone marrow (Figure 3B) and in the tumor microenvironment (Figures 3C, D). It was interesting to find out that the tumor was evidently diminished while MDSCs were eliminated by Anti-Gr-1 in the *PA*+OXA+Anti-Gr-1 group compared with the *PA*+OXA group (Figure 2E), and tumor was also diminished in the Anti-Gr-1-control group compared with the M group; however, there was no statistically significant difference. This could be attributed to the lower levels of MDSCs in the M group, and the fact that Anti-Gr-1 mAb does not directly exert cytotoxic effects on the tumor. Taken together, these data suggested that *P. anaerobius* could facilitate chemoresistance by promoting the recruitment of MDSCs into the colorectal cancer microenvironment.

3.3 IL-23 secreted by MDSCs promoted chemoresistance of CRC cells

It has been reported that elevated MDSCs could contribute to tumor progression by remodeling TME through autocrine and paracrine (35–37), and a number of soluble factors secreted by MDSCs, such as IL-6, IL-23, HGF, and VEGF, in various tumors including CRC were associated with poor chemotherapeutic effect (31). Therefore, IL-6, IL-23, HGF, and VEGF in the culture medium of MDSCs were detected by ELISA, and the results showed that the IL-23 level of MDSCs treated with *PA*+MC-38-CM increased significantly and was the highest in all groups (Figure 4A). In addition, a similar result was obtained *in vivo* that IL-23 in tumor tissues of CRC mice treated with *P. anaerobius* was significantly higher than that of the CRC model mice (Figure 4B). Furthermore, IL-23 in tumor tissues decreased remarkably after MDSCs were eliminated by anti-Gr-1 mAb (Figure 4B).

Next, the role of IL-23 on CRC chemoresistance was investigated. CCK-8 assay was used to test the viability of MC-38 cells treated with oxaliplatin, and the IC_{50} of oxaliplatin to MC-38 cells was 0.136 μM (Figure 4C). As expected, both MDSCs-CM and recombinant IL-23 boosted chemoresistance of MC-38 cells to oxaliplatin, and interestingly, anti-IL-23 antibody attenuated MDSCs-CM induced chemoresistance of MC-38 cells to oxaliplatin (Figure 4D). Consistent with the results of CCK-8 assay, Western blot analysis also showed obviously increased expression of chemoresistance biomarkers (ABCB1, ABCC1, and ERCC1) in MC-38 cells stimulated by MDSCs-CM or IL-23 while anti-IL-23 antibody dramatically diminished the expression of ABCB1, ABCC1, and ERCC1 in MDSCs-CM-treated MC-38 cells (Figure 4E). Moreover, the tumor weight was significantly reduced by intravenous administration of anti-IL-23 antibody (Figure 2E). These results strongly suggested that IL-23 secreted by MDSCs promoted chemoresistance of CRC cells to oxaliplatin.

3.4 IL-23 promotes chemoresistance by activating the EMT in colorectal cancer cells

Subsequently, the underlying mechanism of chemoresistance induced by interaction of *P. anaerobius* and colorectal cancer was investigated. Epithelial–mesenchymal transition (EMT) has always been a major cause of chemoresistance in various kinds of cancers (38) and notable mesenchymal-like fusiform morphological changes were observed in MC-38 cells treated with MDSCs-CM (Figure 5A); thus, wound-healing assay and transwell invasion assay were carried out to observe the migration and invasion ability of MC-38 cells. The results showed that MDSCs-CM and IL-23 could significantly enhance the migration and invasion ability of MC-38 cells, and this enhancement could be inhibited by anti-IL-23 antibody (Figures 5B, C). Furthermore, Western blot analysis showed that N-cadherin, Snail, Slug, Twist, and p-Stat3 expression were significantly upregulated in MDSCs-CM-treated

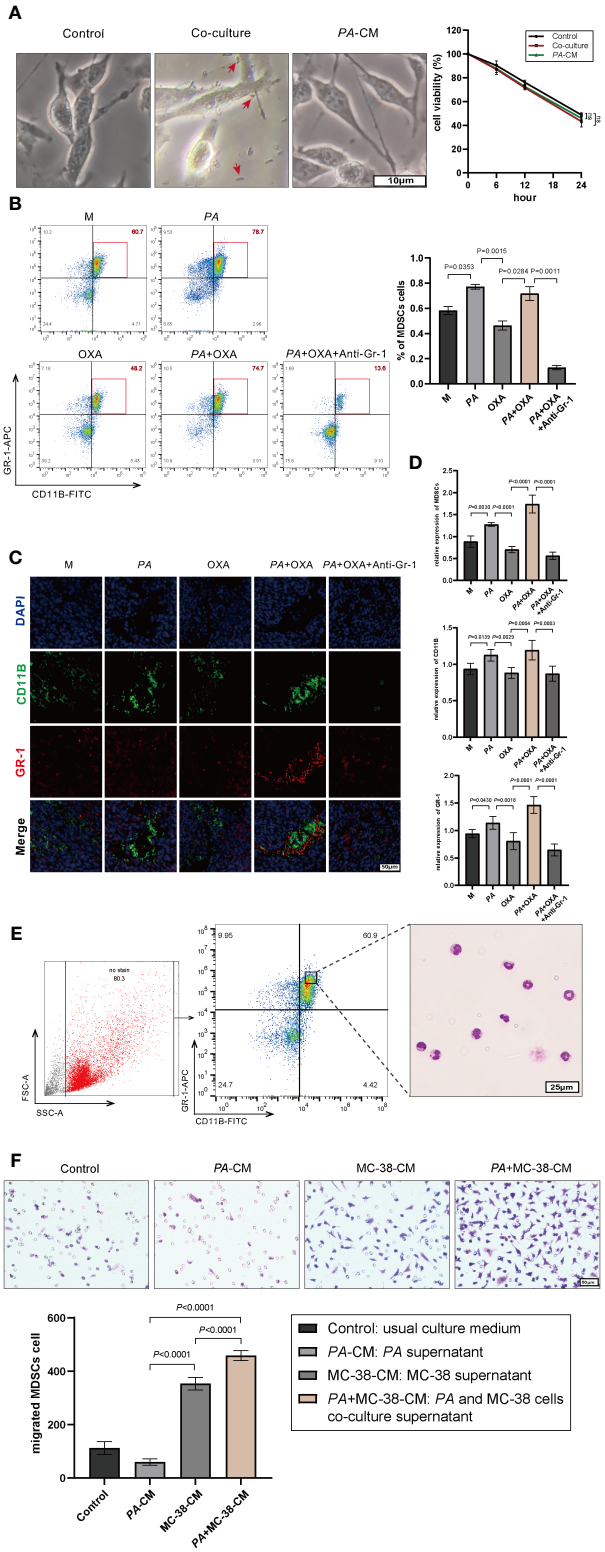

FIGURE 3
P. anaerobius promoted oxaliplatin resistance by recruiting MDSCs into the CRC microenvironment. **(A)** Viability of MC-38 cells co-cultured with *P. anaerobius* or in conditioned medium supplemented with *PA* supernatant (CCK-8 assay, $p > 0.05$, *PA* indicated by red arrow). **(B)** MDSCs ($Gr\text{-}1^{+}CD11B^{+}$) from bone marrow of CRC mice detected by multicolor flow cytometry. **(C, D)** MDSCs infiltrated into the tumor microenvironment detected by fluorescein isothiocyanate (FITC) with anti-mouse CD11b antibody (green), allophycocyanin (APC), anti-mouse Gr-1 antibody (red), and DAPI (blue). **(E)** Morphological feature of MDSCs collected by FCM (Giemsa staining, purple-blue leaf-shaped or round-type nucleus and almost colorless cytoplasm). **(F)** Chemotaxis ability of MDSCs treated with *PA*-CM, MC-38-CM, and *PA*+MC-38-CM (co-culture medium of *PA* and MC-38), respectively. **(A–F)** Data were presented as mean ± SD, *p*-values were determined by one-way analysis of variance. Three independent experiments were performed with consistent results.

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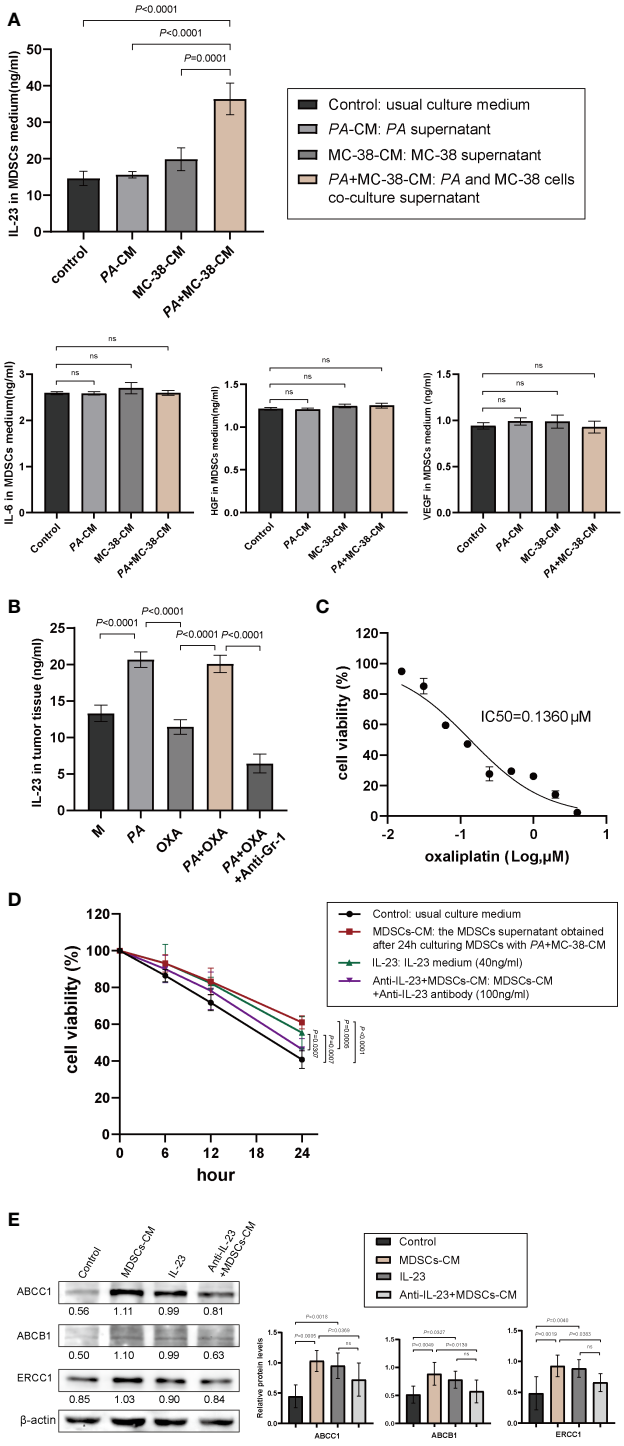

FIGURE 4
IL-23 released by MDSCs promoted chemoresistance of CRC. **(A)** The expression levels of IL-6, VEGF, HGF, and IL-23 in culture medium of MDSCs determined by ELISA. **(B)** IL-23 levels in tumor tissues with different treatments. **(C)** The viability of MC-38 cells treated with different concentrations of oxaliplatin (CCK-8 assay). **(D)** The viability of MC-38 cells treated with oxaliplatin after pretreatment with MDSCs-CM, recombinant IL-23, or anti-IL-23+MDSCs-CM, respectively. **(E)** The expression of chemoresistance biomarkers of ABCB1, ABCC1, and ERCC1 determined by Western blotting (mean ± SD, one-way analysis of variance, triplicated).

MC-38 cells, whereas the expression of E-cadherin had no obvious change (Figure 5D). In conclusion, these findings suggested that IL-23, which is secreted by MDSCs, could promote chemoresistance in colorectal cancer cells by activating EMT.

4 Discussion

Metagenomic profiling of stool and mucosal samples from CRC patients revealed that *P. anaerobius* was an oncogenic bacterial

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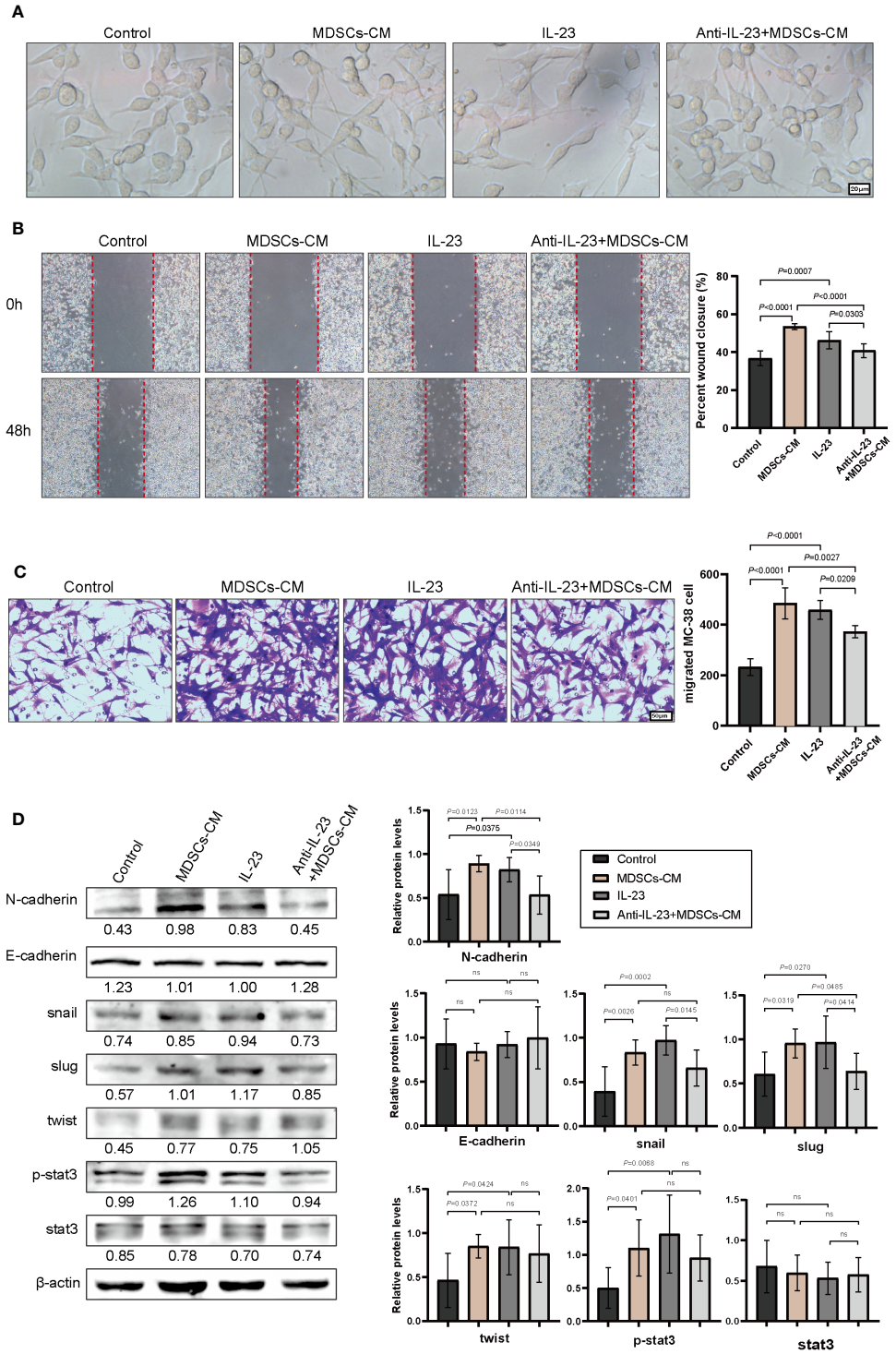

FIGURE 5
IL-23 activated the EMT signaling pathway. **(A)** The morphology of MC-38 cells after incubation (48 h) with MDSCs-CM, IL-23, anti-IL-23+MDSCs-CM, respectively. **(B)** Representative images of wound healing assay of MC-38 cells. **(C)** Representative images of invasive MC-38 cells. **(D)** The expression of N-cadherin, E-cadherin, Snail, Slug, Twist, Stat3, and P-stat3 in MC-38 cells detected by Western blot. **(B–D)** Data were presented as the mean ± SD; *p*-values were determined by one-way analysis of variance. Three independent experiments were performed with consistent results.

candidate enriched in CRC (13, 17, 18) and *P. anaerobius* has been found to be involved in the proliferation and chemoresistance of CRC (19). However, the underlying mechanisms about the contribution of *P. anaerobius* to CRC chemoresistance remains unknown. In this study, we elucidated that the accumulation of *P. anaerobius* in tumor lesion could mediate the recruitment of MDSCs into the CRC microenvironment and promote IL-23 secretion by MDSCs, which led to EMT and chemoresistance of CRC cells.

Bacterial colonization, such as *F. nucleatum* and *γ-proteobacteria*, are often prerequisite steps to tumor malignant progression (14, 39). Consistent with previous reports, *P. anaerobius* avidly colonized the implanted colon tumor lesions in C57 mice and attenuated the effectiveness of oxaliplatin. Considering that *P. anaerobius* has not been found to induce CRC chemoresistance directly and the modification of the tumor immune microenvironment has been reported to play vital roles in intestinal bacteria-related drug resistance, we suspected that *P. anaerobius* might promote chemoresistance by modulating the CRC microenvironment.

Since MDSCs have been reported to be modulated by *P. anaerobius* and responsible for developing chemoresistance (40, 41), the MDSCs in bone marrow and in colorectal tumor lesions of CRC mice were analyzed. The results showed that the amount of MDSCs both in bone marrow and in implanted colon tumor lesions was significantly increased in CRC mice infected with *P. anaerobius*. Furthermore, *in vitro* experiments showed that *PA*+MC-38-CM had the highest ability of enhancing chemotaxis ability of MDSCs among *PA*-CM and MC-38-CM. In addition, the sensitivity of implanted colorectal cancer to oxaliplatin was rescued by MDSC elimination. All these findings indicated that *P. anaerobius* might facilitate chemoresistance by the aggregation of MDSCs into the colorectal cancer microenvironment and the interaction between *P. anaerobius* and colorectal cancer cells contributed to chemoresistance of CRC.

MDSCs promote tumor progression and chemoresistance by remodeling the tumor microenvironment via crosstalk with surrounding cells by expression of pro-inflammatory cytokines, growth factors, and angiogenic factors favoring tumor progression (21). Here, the main tumor-promoting cytokines released by MDSCs, VEGF, HGF, IL-6, and IL-23 were detected, and the results showed that only IL-23 levels were significantly increased in the supernatant of MDSCs cultured with *PA*+MC-38-CM as well as in implanted colorectal cancer loaded with *P. anaerobius*. Meanwhile, it is also important to note that IL-23 in implanted tumor tissues decreased remarkably and the efficacy of oxaliplatin significantly improved after MDSCs were eliminated by anti-Gr-1 mAb, suggesting that IL-23 released by MDSCs facilitated chemoresistance of CRC to oxaliplatin.

As EMT plays important roles in promoting stem cell transformation and chemoresistance (38) and IL-23R was abundantly expressed in colorectal cancer cells (42), we wondered if IL-23 mediated the chemoresistance and EMT of colorectal cancer. As expected, both MDSCs-CM and IL-23 induced increased expression of chemoresistance and mesenchymal biomarkers as well as transcription factors Snail, Slug, and Twist by activating the Stat3-EMT signaling pathway, while this activation could be diminished by anti-IL-23 antibody, supporting the notion that both MDSC recruitment and IL-23 secretion are essential for *P. anaerobius*-related chemoresistance.

There is a complex interaction between tumor microbiome and gut microbiome, which leads to the limited effect of chemotherapy and a negative impact on the host immune system (43). Although *P. anaerobius* had negative roles in colorectal cancer progression, it could augment anti-tumor immune responses in oral squamous cell carcinoma (44). Anyway, the limitations of this study should be addressed. Firstly, the precise mechanisms by which *P. anaerobius* recruited MDSCs into CRC microenvironment were not fully elucidated in the current study. Secondly, a recent study found that MDSCs-derived IL-1β was involved in CRC chemoresistance (45), indicating the heterogeneity and necessity of epigenetic profiling for individualized diagnosis and treatment of cancer. Hence, we cannot dismiss the possibility that there might be additional cytokines contributing to the augmentation of chemoresistance in colorectal cancer. Further investigation is warranted to elucidate the specific mechanism through which MDSCs promote chemoresistance.

In conclusion, this study identified the potential contribution of *P. anaerobius* to colorectal cancer chemoresistance. In particular, the colonization of *P. anaerobius* in CRC lesion mediated the recruitment of MDSCs into the colorectal cancer microenvironment, which secreted IL-23 and subsequently promoted chemoresistance by activating Stat3-EMT of colon cancer cells. These findings provide clinical implications for improving prognostic assessment and designing new targeted treatment for CRC patients.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Laboratory Animal Ethics Committee of Affiliated Hospital of Weifang Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JG and XL performed statistical analysis and drafted the manuscript. WL, GL, and YZ critically revised and finalized the manuscript. JG and XH performed data analysis and interpretation. LS and XZ reviewed and edited the manuscript. JG designed the study and performed all experiments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Potential clinical impact of T-cell lymphocyte kinetics monitoring in patients with B cell precursors acute lymphoblastic leukemia treated with blinatumomab: a single-center experience

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Blinatumomab is a bispecific anti-CD3 and anti-CD19 antibody that acts as a T-cell engager: by binding CD19+ lymphoblasts, blinatumomab recruits cytotoxic CD3+ T-lymphocytes to target the cancer cells. Here we describe seven different patients affected by B-cell precursor acute lymphoblastic leukemia (Bcp-ALL) and treated with blinatumomab, on which we evaluated the potential association between the amount of different T-cells subsets and deep molecular response after the first cycle, identified as a complete remission in the absence of minimal residual disease (CR/MRD). The immune-system effector cells studied were CD3+, CD4+ effector memory (T4-EM), CD8+ effector memory (T8-EM), and T-regulatory (T-reg) lymphocytes, and myeloid-derived suppressor cells (MDSC). Measurements were performed in the peripheral blood using flow cytometry of the peripheral blood at baseline and after the first cycle of blinatumomab. The first results show that patients with a higher proportion of baseline T-lymphocytes achieved MRD negativity more frequently with no statistically significant difference ($p=0.06$) and without differences in the subpopulation count following the first treatment. These extremely preliminary data could potentially pave the way for future studies, including larger and less heterogeneous cohorts, in order to assess the T-cell kinetics in a specific set of patients with potential synergy effects in targeting myeloid-derived suppressor cells (MDSC), commonly known to have an immune evasion mechanism in Bcp-ALL.

KEYWORDS

blinatumomab, T-cell kinetics, minimal residual disease, acute lymphoblastic leukemia, cytokines, MDSCs

1 Introduction

Treating B-cell precursor acute lymphoblastic-B cell leukemia (Bcp-ALL) is historically a challenge, above all in adult-age patients, where the hematopoietic stem cell transplant (HSCT) represented a curative choice. The use of chemotherapic regimens based on pediatric-inspired schemes has changed the landscape, improving the outcomes of these patients (1, 2). Some studies over the years suggested for some categories of patients no survival advantage from HSCT in first complete remission compared to the intensive or pediatric-based chemotherapy regimens alone or combined with tyrosine-kinase inhibitors (TKIs) for Philadelphia-chromosome positive (Ph'+) Bcp-ALL (3, 4). On the other side, the introduction of immunotherapy with monoclonal antibodies opened up a new treatment chapter for Bcp-ALL patients, above all when used as a consolidation treatment for those with measurable residual disease (MRD) after chemotherapy treatment and in case of relapse. In this scenario, blinatumomab represents the first bispecific anti-CD3 and anti-CD19 monoclonal antibody that acts as a T-cell engager recruiting cytotoxic CD3+ T-lymphocytes and directing them to attack CD19+ lymphoblastic cells. In a phase 3 study in patients with relapsed/refractory (R/R) Bcp-ALL, blinatumomab, compared to standard "rescue" chemotherapy, demonstrated superiority both in terms of complete response rate (44% vs. 25%) and overall survival (7.7 months vs. 4 months) (5). Although it is evident that the mechanism of action of blinatumomab involves the patient's immune system, confirmation of the potential role of lymphocyte cytokine kinetics is still lacking. Moreover, it is not well understood which immune mediators (immune-effector cells and cytokines) play a major role in determining the response to blinatumomab. MDSCs cover a central role and are significantly elevated in peripheral blood and bone marrow of Bcp-ALL patients, correlated with the clinical therapeutic responses through an initially well-described mechanism of immune evasion of tumor cells (6).

Starting from these premises, in this case series, we describe the treatment response to blinatumomab in 7 Bcp-ALL patients with unfavorable characteristics and collect data regarding specific immunological markers associated with peripheral blood T-cell lymphocytes as potential predictive factors of deep molecular response to blinatumomab. All patients have provided written informed consent and were evaluated with peripheral blood flow cytometry according to our center's internal guidelines due to the specific targeting mechanism of the bispecific antibody.

2 Case-series presentation

A schematic representation of the cases is reported in Table 1.

2.1 Case n. 1

A 53-year-old female patient diagnosed with Ph+ Bcp-ALL was being treated in another center for the first two years from diagnosis. She received a first-line therapy based on a second-generation tyrosine kinase inhibitor (dasatinib 100 mg daily) and steroids between March 2020 and October 2020, achieving complete hematological remission (CR), i.e., blast cells in the bone marrow (BM) <5% without evidence of extramedullary disease (EMD). MRD was measured by real-time quantitative PCR (RT-qPCR) by determining the levels of the *BCR-ABL*1 fusion transcript, according to international guidelines (7). MRD was defined as the persistence of the *BCR-ABL*1 >0.01% (8). Due to persistently high level of MRD, the patient was started on second-line treatment with Ponatinib for an additional 15 months, failing to achieve MRD negativity. The patient next suffered from a hematological relapse during Ponatinib, with a high rate of blast cells (>60%) in the bone marrow, and was treated with one cycle of chemotherapy according to the hyper-CVAD scheme. Unfortunately, the blast cells were still present in a significant amount (15%). She was then referred to our center and treated with fourth-line therapy with blinatumomab as a bridge to an allogeneic hematopoietic stem cell transplant (HSCT). After the first cycle, the patient achieved a hematological remission withpersistent MRD positivity. After the second cycle, an MRD negativity (<0.01%) was obtained. Therefore, she was referred to receive an allogeneic HSCT from an HLA identical sibling donor. She is incomplete remission (+8 months) and has MRD negativity.

2.2 Case n. 2

A 57-year-old male patient was admitted to the Emergency Department due to the onset of evening fever with chills and sweats and isolated thrombocytopenia. Bone marrow aspirate revealed a clonal population of cells (>30%) showing the following immunophenotype with TdT-pos, PAX5-pos, CD10-pos, and CD33-neg. A diagnosis of Ph-negative Bcp-ALL was performed. Therefore, the patient received first-line pediatric-inspired chemotherapy, including Pegylated Asparaginase (PEG-ASP), according to the GIMEMA LAL1913 protocol (9). The patient achieved complete disease remission after the first induction cycle. However, he was switched to blinatumomab due to MRD persistence after the fifth cycle of therapy. MRD was measured by RT-qPCR, and it is defined as the persistence of clonal IgH-rearrangement $>10^{-4}$ (8). Three consecutive cycles were performed, achieving MRD-negativity after the first one and bridging the patient to allogeneic HSCT from a matched unrelated donor (MUD). The patient is currently in complete molecular remission (CMR +6 months after HSCT).

2.3 Case n. 3

A 13 years-old female patient was diagnosed with Ph negative Bcp-ALL at the Center of Pediatric Hematology Oncology in our Hospital. She was enrolled in an AIEOP-BFM protocol achieving CR after Induction with persistent MRD positivity. MRD negativity was obtained after consolidation therapy. Five years after achieving CR, a molecular relapse (i.e., a reappearance of the identical IgH-rearrangement $>10^{-4}$) was diagnosed. The patient achieved a second

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TABLE 1 A brief summary of the 7 patients described in the text.

Case N.	Patients and ALL's features at diagnosis	Previous treatments for ALL	Status of disease at blinatumomab treatment	Concurrent treatment and n. of cycles	Type of response	Outcome
1	55-year-old female with Ph'+ ALL-B	Dasatinib and CS; ponatinib; chemotherapy based on the hyper-CVAD scheme	Active disease	2 cycles	CR with MRD (1^{st} cycle) CR and MRD- (2^{nd} cycle)	HSCT consolidation after blinatumomab, close follow-up started, without signs of disease after five months
2	57-year-old male with Ph'- ALL-B	Polychemotherapy scheme for 5 cycles	CR with MRD	3 cycles	CR and MRD- (1^{st} cycle)	HSCT consolidation after blinatumomab, complicated by GVHD and maintaining CR after six months
3	18-year-old female with Ph'- ALL-B	Polychemotherapy scheme with CR for 5 years. Vincristine, idarubicin, and chrysantaspase scheme for 1^{st} relapse	CR with MRD	2 cycles	CR and MRD- (1^{st} cycle)	HSCT consolidation after blinatumomab, without signs of relapse after 3 years
4	42-year-old female with Ph'+ ALL-B	Dasatinib and CS; polychemotherapy scheme for MRD, followed by consolidation with HSCT. Ponatinib and DLI for early relapse; 3 cycles of polychemotherapy scheme	Active disease	2 cycles, associated with DLI	CR with MRD (1^{st} cycle) Relapse after 2^{nd} cycle	Treated with inotuzumab ozogamicin, venetoclax, asciminib, until death for disease's progression
5	36-year-old female with Ph'+ ALL-B	Dasatinib and CS; methotrexate and high-dose cytarabine consolidated by HSCT and ponatinib as maintenance therapy. For subsequent relapse, polychemotherapy	CR with MRD	5 cycles, associated with DLI	CR with MRD (1^{st} cycle) CR and MRD- (2^{nd} cycle) Relapse after 5^{th} cycle	Treated with inotuzumab ozogamicin associated with ponatinib, ASP-based polychemotherapy, venetoclax, until death for disease's progression
6	14-years-old male with Ph'- ALL-B	Polychemotherapy protocol for induction of remission; 6-mercaptopurine and MTX as maintenance	CR with MRD	3 cycles	CR and MRD- (1^{st} cycle)	Close follow-up, without signs of relapse after 2 years
7	16-years-old male with Ph'- ALL-B	Polychemotherapy scheme with CR for 8 months. Vincristine, mitoxantrone, and ASP for relapse	CR with MRD	2 cycles	CR and MRD- (1^{st} cycle)	Close follow-up, without signs of relapse after 1 year

ALL-B, acute lymphoblastic B-leukemia; CS, corticosteroids; CR, complete remission; MRD, minimal residual disease; HSCT, hematopoietic stem cell transplant; ASP, asparaginase; DLI, donor lymphocyte infusion.

remission after an induction phase containing vincristine, idarubicin, and chrysantaspase due to a previous allergic reaction to PEG-asparaginase. A blinatumomab-cycle was performed because of a persistent MRD positivity, achieving CR with MRD negativity, bridging the patient to an allogeneic HSCT from a sibling HLA-identical donor. She maintained a complete molecular remission with MRD negativity (i.e., clonal IgH-rearrangement $<10^{-4}$ by RT-qPCR) up to the last follow-up in December 2022 (+3 years).

2.4 Case n. 4

A 40-year-old female was diagnosed with Ph-positive Bcp-ALL at our Institution. At the diagnosis, we performed a CT scan that showed skeletal lesions of the right shoulder compatible with extramedullary disease (EMD). The patient started on induction therapy with dasatinib and corticosteroids, achieving a major molecular response (*BCR-ABL1* transcript ≤0.1%) (10). Therefore, the patient received an allogeneic HSCT from an HLA-identical sibling donor. The conditioning regimen was myeloablative and based on total-body irradiation (TBI) and cyclophosphamide. Right after, the patient achieved an MRD as early as day +30. Approximately 100 days after the HSCT, the patient experienced an overt relapse, with 40% of blast cells in the bone marrow, a *BCR-ABL1* positivity (39%), and a chimerism with 20% of DNA from the recipient (chimerism was measured by short tandem repeat analysis) (11). Salvage therapy was started with ponatinib and donor lymphocyte infusions (DLIs). At the end of the third DLI, a CR with MRD negativity and a full donor chimerism were

achieved. One year later, a new relapse occurred during the maintenance treatment with ponatinib. In the bone marrow, 30% of blast cells was detected, and the mutational analysis by NGS sequencing (12) identified the T315I and the E255V mutation, respectively, in 25% and 60% of *BCR-ABL*1 positive cells. After three cycles of chemotherapy based on the LAL1913 scheme, showing no response, the patient underwent blinatumomab associated with DLI, obtaining a major molecular response after the 1st cycle. After the second cycle, the patient experienced bone pain in the spine and chest, and a CT/PET scan showed lesions compatible with EMD of the ribs and vertebrae. The patient was switched to inotuzumab ozogamcin for 5 cycles without obtaining a response and died of disease progression.

2.5 Case n. 5

A 34-year-old female patient with Ph-positive Bcp-ALL was treated as the first line with dasatinib in association with steroids, achieving both complete hematological and molecular remission. After four months of treatment, she experienced an extramedullary relapse of the mammary gland. Therefore, she received rescue therapy with methotrexate and high-dose cytarabine, achieving a new complete response documented by a PET-CT scan. This response was consolidated with allogeneic HSCT from an HLA-identical donor, followed by maintenance therapy with ponatinib. Twelve months after HSCT, the patient suffered a hematological relapse and was treated with vincristine and idarubicin. Due to the persistence of the *BCR-ABL*1 fusion transcript, she underwent therapy with blinatumomab associated with DLIs "escalated dose". Each DLI was administered after each cycle achieving complete molecular remission after the second DLI course. After the fourth cycle of blinatumomab and DLI, molecular relapse occurred, and the patient was switched to inotuzumab ozogamicin for six cycles reaching CR with MRD negativity. This response was maintained for 6 months, followed by an overt hematologic relapse with 10% leukemic cells in the BM. The patient was enrolled in an experimental trial with CARCIK-CD19 (13), reaching a transient response and, 3 months after the infusion of the cellular product, had an overt hematological relapse and died of disease progression.

2.6 Case n. 6

A 14-year-old male presented to the Center of Pediatric Hematology Oncology in our Hospital, reporting spinal pain and pancytopenia. He was diagnosed with a Ph-negative Bcp-ALL and enrolled in the ongoing protocol AIEOP-BFM ALL 2017. During the induction phase, he experienced a severe adverse event characterized by septicemia due to carbapenemase-producing Klebsiella pneumoniae (KPC-KP) and fungemia due to Candida species. These infections were complicated by pneumonia requiring positive pressure ventilation and a cerebral abscess. The patient received treatment with ceftazidime/avibactam, voriconazole, and supportive therapy until the resolution of the infectious complications. Because of the persistence of MRD after the induction treatment, the patient received therapy with blinatumomab for 4 cycles in an off-label manner because of the patient's age (<18 years), reaching MRD negativity after the first one. The patient was then followed with periodical BM assessments, maintaining complete remission up to two years after the completion of the treatment.

2.7 Case n. 7

A 16-year-old male patient was diagnosed with a Ph-negative Bcp-ALL at the Center of Pediatric Hematology Oncology in our Hospital. He was enrolled in the ongoing protocol (AIEOP-BFM ALL 2017), achieving complete remission with MRD negativity at the end of consolidation (final risk: medium). Two months after the end of the first-line treatment, an early (<30 months from diagnosis) bone marrow isolated relapse was diagnosed. Thus, the patient received a second line therapy based on international protocol IntReALL-2010 HR (including mitoxantrone, ASP, and vincristine), achieving CR with the persistence of MRD positivity. For this reason, because he reached adult age, he received treatment with blinatumomab, completing 2 cycles and achieving MRD clearance after the first cycle. Then, he underwent an allogeneic MUD-HSCT in another Center. One year after the completion of the therapy, the patient is still in CR withMRD negativity.

3 Results: T-cell lymphocyte kinetics at baseline and after one blinatumomab cycle

Patients' peripheral blood samples (PB) were evaluated using flow cytometry at baseline and after the first blinatumomab cycle, according to our center's internal guidelines. Global T-lymphocyte kinetics was assessed by measuring the absolute counts of CD3+, CD4+ effector memory (T4-EM), CD8+ effector memory (T8-EM) T-lymphocytes, and the count of T-regulatory cells (T-reg). The individual lymphocyte kinetic variations were reported for each patient. The T-cell kinetics between different populations is represented in Figure 1. Patients were divided into two groups according to the MRD status following the first blinatumomab cycle (positive in three vs. negative in four patients). MRD status was chosen as the primary endpoint because it has been highlighted as the most powerful prognostic factor for patients with Bcp-ALL (14). The working hypothesis of the present study was that the status of immune-mediators, such as the T-cell subsets, their ability to express cytokines involved in the anti-tumoral response, and the expression of the T-cell exhaustion markers could potentially be correlated to the depth of blinatumomab response.

In order to evaluate any possible correlation and given the heterogeneity of the patients in terms of disease type (Ph-Positive versus Ph-Negative), prior treatment (chemotherapy versus chemo-free, HSCT versus no HSCT), and tumor burden (MRD positive versus hematological relapse), we measured both the absolute

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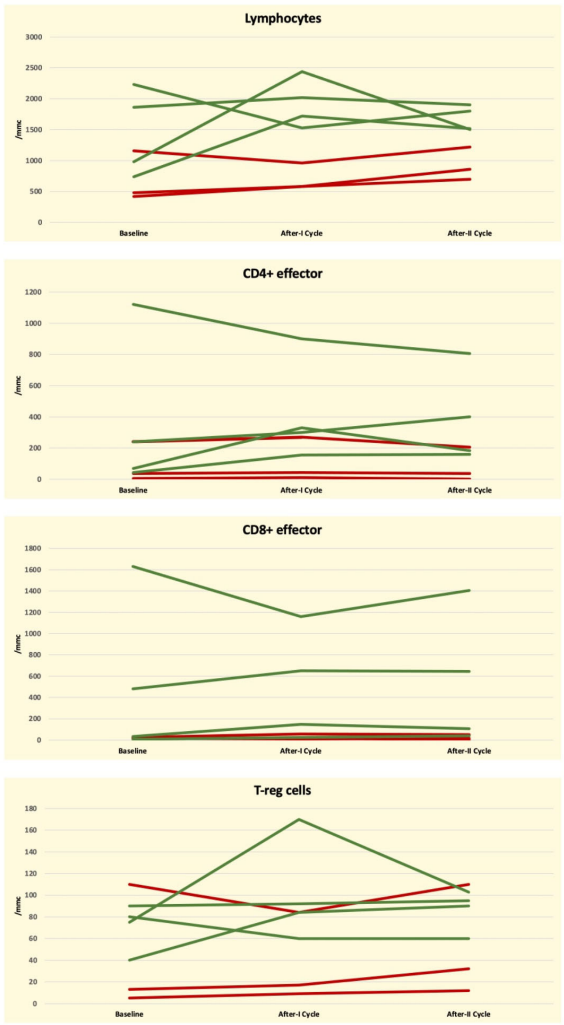

FIGURE 1
Graphical representation of Global T-Cell lymphocyte kinetics at baseline and following the first blinatumomab cycle according to MRD negative (green line) and positive (red line) status in seven ALL-B patients.

counts of the immune-cell subsets and the ratio between the counts after the first cycle of blinatumomab (T0) and at baseline (T1). The absolute values and the ratio T1/T0 of the different classes of lymphocytes are reported in Table 2.

We compared the median absolute cell counts at baseline and after the first cycle and the ratio T1/T0 between MRD-positive and MRD-negative patients (respectively, MRD-responsive and MRD not-responsive patients) at the end of the first cycle of blinatumomab. These unpaired comparisons were performed by the Mann-Whitney U-test, with a $p < 0.05$ considered significant.

The MRD-responsive patients after the 1st cycle had a median baseline count of T- lymphocytes of 1450/mmc, that was higher than that of the MRD not-responsive patients. The statistical comparison of the absolute cell counts between MRD-negative and MRD-positive patients did not reach statistical significance ($p = 0.06$). Similarly, the median values of T4-EM (368 vs 94), T8-EM (537 vs. 17), and T-reg (71 vs. 39) were higher in MRD responsive patients than in MRD-not responsive patients but without reaching statistical significance (0.22, 0.14, and 0.40, respectively). The p-values obtained by comparing the ratio T1/T0 measured in MRD-responsive and in MRD not-responsive patients were, respectively, 0.86 for absolute T-lymphocyte, T8-EM, and T-reg and 0.63 for T4-EM.

To evaluate cytokines involved in the anti-tumoral response, we measured the expression of interferon γ (IFNγ) and programmed death protein-1 (PD1) in T4-EM and T8-EM. These data were collected only from 5 patients, 4 of them were MRD-responsive; thus, an accurate analysis could not be performed. However, the preliminary results are reported in Table 2.

Based on these findings, we assume that the higher value of total lymphocytes can predict a deep and robust response to blinatumomab, despite not achieving a statistically significant correlation. As for the specific subsets of the T-cell population and their expression of IFNγ and PD1, a minimal number of heterogeneous patients makes it difficult to draw conclusions.

4 Discussion

Ever since the development of the first-in-class bispecific T-cell engager antibody blinatumomab, the importance of T-cell kinetics was discussed as a hypothetically predictive factor for MRD response, although limited data are available. In a phase 2 study of blinatumomab, Zugmaier and colleagues assessed the long-term survival of 36 adult Bcp-ALL relapsed/refractory (RR) patients (15). Twenty-five patients (69%) achieved MRD response, and ten were long-term survivors with overall survival (OS) greater than 30 months, including both patients that were consolidated with allogeneic HSCT and blinatumomab treatment alone for a total of 5 cycles. The more significant expansion of CD3+ T-cells and increased numbers of CD3+ effector memory cells were predominant in the long-term survivors in both cycle 1 and cycle 2. The OS was inferior for 30 months in patients with persistent MRD positivity.

Nägele et al, investigated the correlation between immunological biomarkers and the clinical response to blinatumumab in the same study population. In this study the authors, by monitoring serum cytokines before and during the first week of each course of blinatumomab, demonstrated that in patients in complete remission after blinatumomab the serum levels of IL-6, IL-10, and IFNγ reach higher values than in non-responders patients (16).

In a phase 1 dose-escalated study, the same authors demonstrated a correlation between a greater expansion of CD4+ and CD8+ T-cells and the clinical response to blinatumomab (17).

Finally, in the phase-3 trial leading to the approval of blinatumomab in relapsed/refractory Bcp-ALL, the percentage of CD3+ T-cells measured at baseline had a significant impact on MRD-response to blinatumumab and greater values of both CD4+ or CD8+ T-cells at baseline predicted higher rates of hematological remission (18).

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TABLE 2 Reported count of peripheral blood lymphocytes evaluated at baseline and after the first cycle of blinatumomab, expressed through absolute count and ratio (T1/T0).

Case N.	Lymphocytes			CD4			CD4-IFNγ			CD4-PD1			CD8			CD8-IFNγ			CD8-PD1			T-reg			MRD Response after I cycle
	Baseline	I cycle	*Ratio*	Baseline	I cycle	*Ratio*	Baseline	I cycle	*Ratio*	Baseline	I cycle	*Ratio*	Baseline	I cycle	*Ratio*	Baseline	I cycle	*Ratio*	Baseline	I cycle	*Ratio*	Baseline	I cycle	*Ratio*	
1	1160	960	*0.83*	240	270	*1.13*	215	235	*1.09*	4.79	2.74	*0.57*	16	10	*0.63*	267	273	*1.02*	2.25	1.23	*0.55*	110	84	*0.76*	Positive
2	2230	1530	*0.69*	1120	900	*0.80*	320	300	*0.93*	2.4	1.9	*0.79*	1630	1160	*0.71*	1081	879	*0.81*	1.9	1.6	*0.84*	80	60	*0.75*	Negative
3	1860	2020	*1.09*	240	300	*1.25*	51	191	*3.74*	1.54	1.52	*0.99*	480	650	*1.35*	95	212	*2.23*	2	1.1	*0.55*	90	92	*1.02*	Negative
4	480	580	*1.21*	5	11	*2.2*	NA	NA	*NA*	NA	NA	*NA*	10	20	*2*	NA	NA	*NA*	NA	NA	*NA*	5	9	*1.80*	Positive
5	420	580	*1.38*	38	44	*1.16*	NA	NA	*NA*	NA	NA	*NA*	25	57	*2.28*	NA	NA	*NA*	NA	NA	*NA*	13	17	*1.31*	Positive
6	980	2440	*2.49*	70	330	*4.71*	35	166	*4.74*	1.7	1.51	*0.89*	33	146	*4.24*	76	244	*3.21*	2.8	2.64	*0.94*	75	170	*2.27*	Negative
7	740	1720	*2.32*	44	155	*3.52*	48	294	*6.13*	1.52	1.23	*0.81*	6	24	*4*	72	381	*5.29*	2.2	1.3	*0.59*	40	84	*2.10*	Negative

NA stands for "not available".

As regard the potential role of the T-cell exhaustion in hampering the response of Bcp-ALL to blinatumomab, Feucht and co-workers have demonstrated that blast cells of Bcp-ALL highly expressing PD1-ligand are less susceptible to the blinatumomab-induced cell lysis and this phenomenon could be reversed, *in vitro*, by the adjunction of PD1-inhibitors (19).

The role of MDSCs, on the other side, is increasingly discussed and known, also in the setting of Bcp-ALL and the relationship with the T-lymphocyte compartment (20). Zahran et al. reported that the MDSCs correlated to the missed therapeutic response and wished for a future role as a prognostic indicator or a potential therapeutic target (21). The same results were confirmed in pediatric patients, with granulocytic MDSCs levels correlated positively with therapeutic responses and Bcp-ALL disease prognostic markers (among which MRD) (6).

The major limitations of the present study are the very limited number and the heterogeneity of the patients, which makes it impossible to draw conclusions for the clinical practice.

Moreover, although the recognized role of the MDSCs in mediating the immune evasion of Bcp-ALL, the investigation of the MDSCs in hampering the activity of blinatumomab was not included in the initial conceptualization of the present study and was included in a subsequent revision of the study-design that is still ongoing. This knowledge, together with that of the lymphocyte kinetics, will allow us to have a better understanding of the underlying mechanisms of the sensitivity of the Bcp-ALL to immune therapy.

Mainly because of these limitations, the statistical analysis failed to demonstrate an association between the T-cell kinetics and the MRD response after one cycle of blinatumomab. However, the correlation between the absolute lymphocyte counts at baseline and the MRD response to Blinatumomab was close to the statistical significance threshold in such a small sample of patients. This is in line with what has already been reported (18) and is encouraging in continuing the study by expanding the cohort of the patients in order to assess not only the function of the T-cell subsets but also the potential impact of MDSCs in influencing the lymphocytes-compartment and the response to blinatumomab.Given that the T-cell expansion in response to blinatumomab could be a biological pre-requisite for the anti-leukemic activity, we also evaluated the T1/T0 ratio rather than the absolute lymphocyte counts alone. In patients with relapsed/refractory Bcp-ALL, a low pre-treatment lymphocytes count, a non-permissive microenvironment due to MDSCs, or a low T1/T0 ratio during the 1st cycle, could direct the choice toward different drugs (i.e., antiCD22 inotuzumab ozogamicin) (22).

Finally, a strict correlation between MDSCs and PD1/PD1 ligand was reported (23). In different models, MDSCs could contribute to the resistance to immune checkpoint inhibition drugs by inhibiting the anti-neoplastic properties of T and NK cells and stimulating T-regs (24).

Furthermore, IFN-γ could regulate the role and the function of the MDSCs through the modulation of the anti-apoptotic Bcl2 protein by direct interaction with the phosphorylated STAT-1 (25). Based on these findings, monitoring peripheral MDSCs (defined as CD11b+CD14−CD15+ or CD11b+CD14−CD66b+) (26) and

investigation of their potential inhibition in improving the T-lymphocyte role, represents our future aim for the prosecution of the study.

5 Conclusion

In the era of the anticancer immunotherapy the discovery of immunological biomarkers linked to the clinical response to the bi-specific antibodies could potentially lead to a better selection of the patients likely to benefit from the treatment. A large, prospective trial could provide this data, driving the physician toward a choice that should be adapted to the health of the patient's immune system.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Comitato Etico Policlinico "G.Rodolico-San Marco" Catania 1. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

AD, UM, SL, and AR contributed to the conception and design of the study. AD, NP, SL, and UM organized the database. AD performed the statistical analysis. AD, UM, and SL wrote the first draft of the manuscript. EM, MP, CV, GM, PF, LN, and CM collected literature and patient data. AD, UM, AR, SL, and FDR revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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