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The role of tumor-associated macrophages in tumor progression

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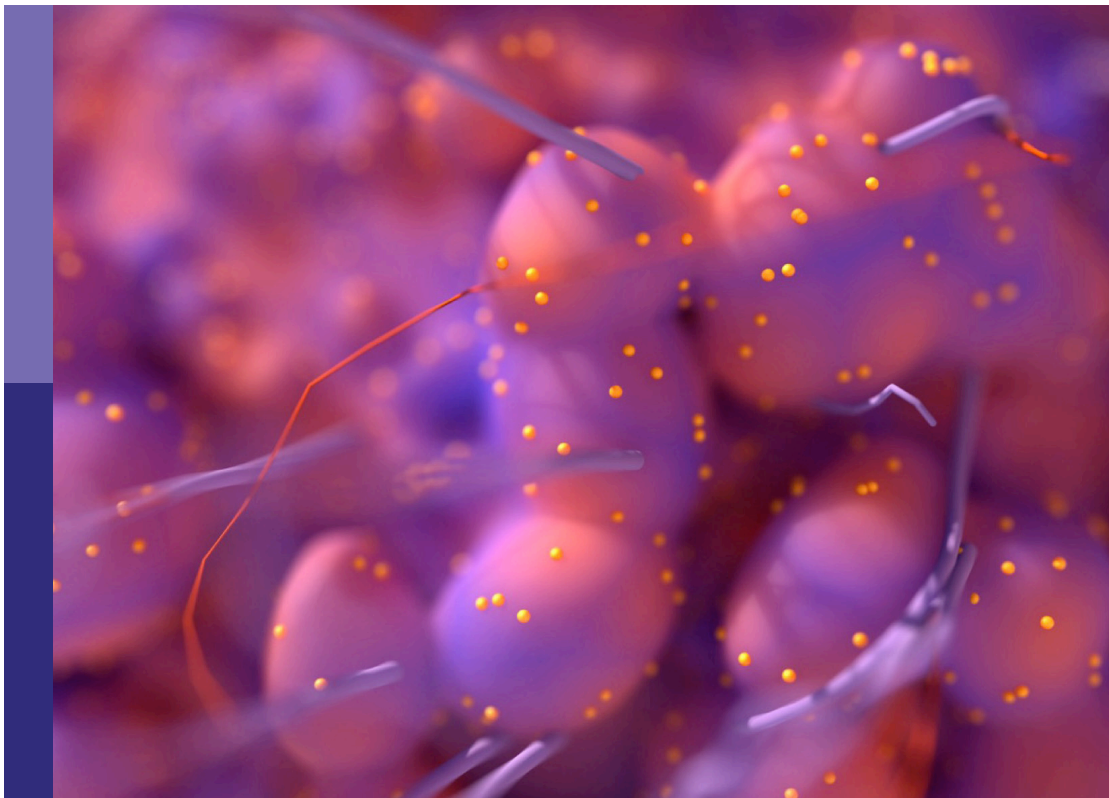

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The role of tumor-associated macrophages in tumor progression

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REVIEW
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Tumor-Associated Macrophages: Recent Insights and Therapies

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Macrophages, which have functions of engulfing and digesting foreign substances, can clear away harmful matter, including cellular debris and tumor cells. Based on the condition of the internal environment, circulating monocytes give rise to mature macrophages, and when they are recruited into the tumor microenvironment and in suitable conditions, they are converted into tumor-associated macrophages (TAMs). Generally, macrophages grow into two main groups called classically activated macrophages (M1) and alternatively activated macrophages (M2). M2 and a small fraction of M1 cells, also known as TAMs, not only lack the function of phagocytizing tumor cells but also help these tumor cells escape from being killed and help them spread to other tissues and organs. In this review, we introduce several mechanisms by which macrophages play a role in the immune regulation of tumor cells, including both killing factors and promoting effects. Furthermore, the targeted therapy for treating tumors based on macrophages is also referred to in our review. We confirm that further studies of macrophage-focused therapeutic strategies and their use in clinical practice are needed to verify their superior efficacy and potential in cancer treatment.

Keywords: macrophages, tumors, tumor-associated macrophages, immunity, immunity therapy

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BACKGROUND

This review is based on the interaction of macrophages and tumor cells, and summarizes the origin, function, and classification of macrophages. Emphasis is placed on the dual role of macrophages in tumor cells and targeted therapy of related binding sites. The existing reviews about macrophages and the interaction with tumor cells are not a few, but the most are focused on one of the recognition mechanisms, specifically illustrating its molecular mechanism in detail. Nevertheless, based on the research findings in recent years, this review summarizes a variety of related mechanisms, sorts out and reintegration them to make them systematic. In the meanwhile, we also provide new ideas about tumor targeted therapy. Regarding tumor-targeted therapy, this review classifies them in treatment methods and sites to make the relevant treatment ideas clearer. There are still some methods that need further research, and this review explains and looks forward to the progress of the new step.

INTRODUCTION

Macrophages, which are a type of white blood cells of the mononuclear phagocyte immune system, play vitally important roles in anti-infective immunity, the maintenance of tissue homeostasis, and the protection of our body through the functions of engulfing and digesting foreign substances (1, 2). Macrophages also clear away harmful matter, including cellular debris and tumor cells *in vivo*. Macrophages mediate non-specific defense (innate immunity) and help initiate specific defense mechanisms (adaptive immunity). In addition to stimulating the immune system, macrophages exert an immune modulatory impact by secreting various cytokines and activating the complement system, which may lead to inflammation.

Based on the conditions of the internal environment, such as the presence of chemokines, cytokines, and other factors secreted by tumor cells, mesenchymal cells, and immune cells, and the presence of local anoxia, inflammation, and high levels of lactic acid, the monocytic series in the blood are recruited to the tumor microenvironment and become tumor-associated macrophages (TAMs) (3, 4). Macrophages roughly develop into two main groups with different functions in immune defense and immune surveillance called classically activated macrophages (M1) and alternatively activated macrophages (M2), both of which can transform into each other with the changes in the internal environment.

Here, we introduce several kinds of mechanisms by which macrophages interact with tumor cells and kill them. Also, we compare these mechanisms with those by which TAMs play a role in promoting the development of tumor cells, in immune evasion and in immunosuppression. Therefore, based on macrophages differentiating into TAMs on cellular and molecular levels, our review shows several therapeutic targets for treating tumors caused by immunosuppression. In addition, we summarize some tumor therapy strategies at present aimed at macrophages, especially the theoretical basis and the feasibility of blocking the CD47-SIRPα pathway to treat tumors. In this way, engineered macrophages would play a significant role in suppressing tumors with potential clinical utility.

Abbreviations: TAMs, tumor-associated macrophages; M1, M1 macrophages; M2, M2 macrophages; LPS, lipopolysaccharides; CCL2, chemokine (C-C motif) ligand 2; STAT3, signal transducer and activator of transcription 3; EMT, epithelial-mesenchymal transition; Bcl-2, B-cell lymphoma-2; JAK, janus kinase; ELMO1, engulfment and cell motility 1; IBC, inflammatory breast cancer; GRO, growth-related oncogene; TGF-β, growth factor-β; PGE2, prostaglandin E2; MMP-7, matrix metalloproteinase-7; TLR4, Toll-like receptor 4; CIP2A, cancerous inhibitor of PP2A; PI3K, phosphatidylinositide 3-kinases; PD-1, programmed cell death protein; PD-L1, programmed cell death-ligand 1; NK, natural killer; CD47, cluster of differentiation 47; SIRPα, signal regulatory protein alpha; ITIM, immunoreceptor tyrosine-based inhibitory motif; MHC, major histocompatibility complex; LILRB1, leukocyte immunoglobulin like receptor subfamily B member 1; Siglc-10, sialic-acid-binding Ig-like lectin 10; ApoE, apolipoprotein E; PDAC, pancreatic ductal adenocarcinoma; MDE, macrophage-derived exosomes; PrCR, programmed cell removal; Btk, Bruton's tyrosine kinase; CRT, calreticulin; MDP, muramyl dipeptide; BCG, bacilli calmette-guerin; sTn, Sialyl-Tn; tEVs, tumor-derived extracellular vesicles; MCP-1, monocyte chemotactic protein-1; CSF-1, colony stimulating factor-1; CREB, cAMP response element binding protein; GCN2, general control nonderepressible 2; MDSCs, myeloid-derived suppressor cells; ATF4, activating transcription factor 4; HAC, an engineered small protein which can block human PD-L1; QPCTL, glutaminyl-peptide cyclotransferase-like protein; ADCP, antibody-dependent cell phagocytosis; AZA, azacytidine; DFMO, difluoromethylornithine; iSNAPS, integrated sensing and activating protein.

A SIMPLE CHARACTERIZATION OF MACROPHAGES

The origin of macrophages is still inconclusive, although it is currently universally believed that the major portion of macrophages is derived from monocytes in the peripheral blood circulation, as the mechanism has been clarified in some studies (5, 6). During the early stages of embryonic development, monocytes are recruited from marrow circulating blood and then travel to various tissues and organs via circulation, thus developing and differentiating into tissue-specific macrophages. Nevertheless, there are still some tissue-resident macrophages that are not derived from blood monocytes, such as alveolar macrophages in the lungs, microglia in the brain, and Kupffer cells in the liver, and the mechanisms of their origin, self-renewal, proliferation, and substitution have not been clarified as well (7). Recent studies confirmed the coexistence of tissue-resident macrophages proliferating *in situ* and those derived from blood monocytes in several tissues, including the lungs, spleen, and brain, and confirmed the phenotype and functions of these tissue-resident macrophages (8).

In macrophage subpopulations, M1 macrophages, which produce proinflammatory cytokines with strong killing effects on pathogens invading the body, play an important role in human immune function and may contribute to tissue destruction. Cytokines, such as INF-γ, GM-CSF secreted by other immune cells and lipopolysaccharides (LPS) of the outer membrane of bacteria, can induce M1 macrophage activation (9, 10). M2 macrophages participate in parasite infection, tissue remodeling, allergic diseases, and angiogenesis, playing an important role in above processes. Previous studies have shown that CSF-1, IL-4, IL-13, IL-10, parasite infections, and other kinds of stimulation can lead macrophages to polarize to M2 macrophages (11, 12) (**Figure 1**). M1 and M2 are only two extreme descriptions of the polarization state of macrophages without covering a wide range of macrophage subpopulations (13). As an example, there are still CD169+ macrophages and TCR+ macrophages, and as is confirmed by present knowledge, in tumor-related studies, a large number of TAMs have been found in tumor-tissues (14). There is not much information about CD169+ macrophages and TCR+ macrophages, but present research has shown that they play certain roles in some respects. Some macrophages in the spleen, liver, bone marrow, lymph nodes, etc., express high levels of CD169 antigen on the surface. Relevant studies have failed to elucidate the relevant functions of CD169+ macrophages, but it is believed that CD169+ macrophages play a certain role in maintaining the homeostasis of the body, in immune regulation, and in immune tolerance (15–17). Concerning TCR+ macrophages, researchers discovered that TCR-αβ complex existed on 5–8% of neutrophils in the circulation (18), and Beham's group found that TCRβ gene rearrangement occurred in the early stage of bone marrow macrophages differentiation. TCR+ macrophages express chemokine (C-C motif) ligand 2

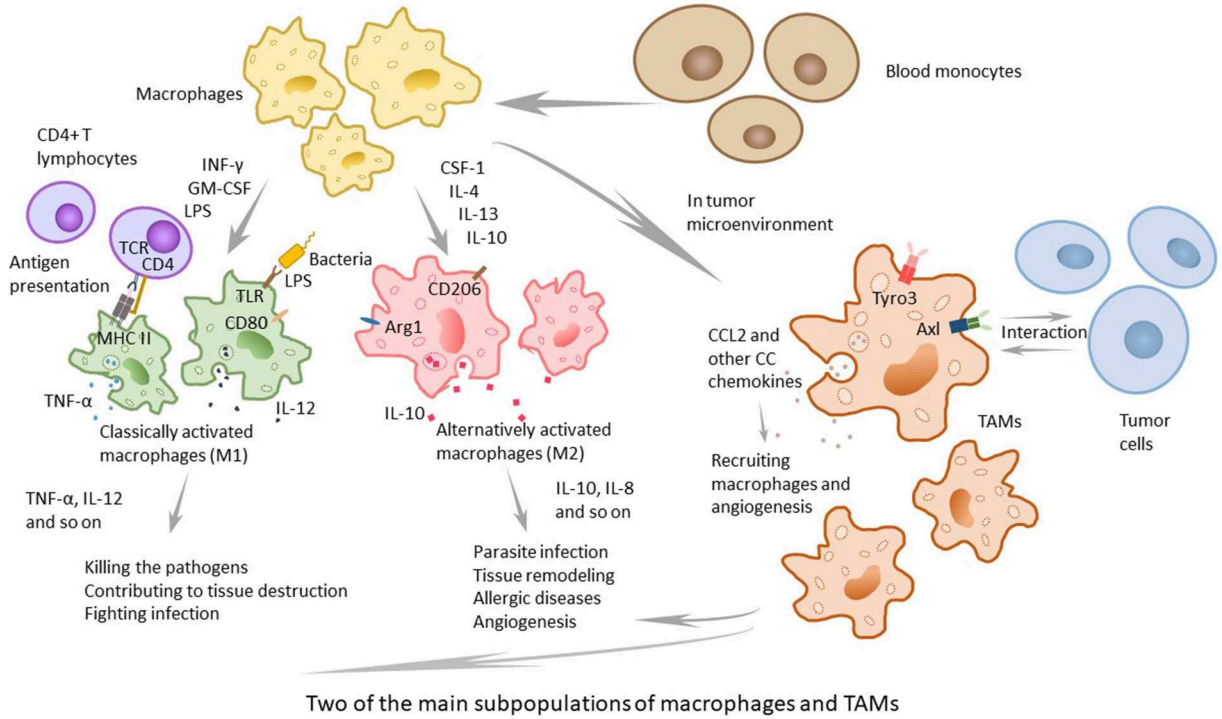

FIGURE 1 | The two main subpopulations of macrophages and TAMs. Macrophages can be classified to several subpopulations, and the two main subpopulations are classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 macrophages, active by IFNγ, GM-CSF, other cytokines and LPS, play an important role in human immune function and contribute to tissue destruction by producing proinflammatory cytokines with strong killing effects on pathogens. M2 macrophages, that can be active by CSF-1, IL-4, IL-13, IL-10, and other stimulation, participate in parasite infection, tissue remodeling, allergic diseases, and angiogenesis, and play an important role in above processes. TAMs, recruited in tumor microenvironment, are not a typical kind of macrophages and different from M1 or M2. They express special TAM receptors on membrane, and are interacted with tumor cells and play the dual role in tumor microenvironment.

(CCL2) and have strong phagocytic ability, which is not the same as the functions of traditional macrophages (19).

TUMOR-ASSOCIATED MACROPHAGES, A SPECIAL KIND OF MACROPHAGES

The solid tumor consists of neoplastic cells and blood-born cells, including granulocytes, macrophages (up to 50%), and mast cells, as well as periphery cells—fibroblasts and epithelia (20, 21). Macrophages are recruited to the tumor site by the microenvironment, which produces cytokines. It has been proposed that the recruitment and differentiation progress are related to local anoxia, inflammation, and high levels of lactic acid. The CC chemokines, such as CCL2, CCL11, CCL16, and CCL21, which are major determinants of macrophage infiltration and angiogenesis, have been demonstrated to function in the cancer of breast, lung, esophagus, ovary and cervix, and CCL2 primarily contributes to the recruitment of macrophages (4, 22). Moreover, TAMs can produce CCL2, which means that they can recruit macrophages in turn. To some extent, TAMs can enlarge the recruitment of macrophages (23). Some studies and human diagnoses have demonstrated that the density of CCL2 is related to the quantity of TAMs, the tumor invasion and the clinical prognosis (**Figure 1**).

Involved in different microenvironments, macrophages acquire different specific phenotypes (3). The phenotypes of TAMs are plastic and regulated by the local microenvironment. Indeed, TAMs have been confirmed in recent studies to be present in large amounts in tumor tissues and to be significantly associated with tumor development progress. Strictly speaking, the division of macrophage types is complex. TAMs are not regarded as a classical subgroup of macrophages because these cells cannot be observed in the steady state but rather related to specific pathologic conditions, such as inflammation and tumors. There are some special receptor tyrosine kinases consisting of TAM receptor family, including Tyro3, Axl, and MerTK, and these receptors not only are of importance in interacting with tumor cells, but also play roles in macrophage polarization, efferocytosis and autoimmune disease (24). Active TAMs have several properties similar to M2. As a consequence, sometimes M2 macrophages are defined as TAMs in a narrow sense (14, 25). However, previous studies have shown that TAMs not only have the characteristics of M2 but also share M1 and M2 signature polarization. Therefore, the view that TAMs are equal to M2 is inaccurate (14). TAMs have profound effects on increases in angiogenesis, tumor invasion and the depression of immunity,

as a result, TAMs can be taken into consideration in tumor immunotherapy (26, 27).

THE DUAL ROLE OF TAMs IN TUMOR MICROENVIRONMENT

Regarding the process of immune cells specifically recognizing and eliminating tumor cells, the mechanism is very complicated since various immune system components are involved, and macrophages are one of the most important members in these processes. TAMs are a key component of the leukocyte infiltrate that is seen broadly in various tumors. Examination of the roles of TAMs in tumor progression, in conjunction with investigations of other cells, has paved the way to eliciting new methods for tumor therapies. It's well-recognized that TAMs infiltrated in malignant metastatic cancers can promote tumor growth and metastasis, but that's not all, few kinds of macrophages subtypes can also have the antineoplastic activity.

TAMs in Promoting Tumor Progression

Cytokines

Several studies have supported that TAMs can secrete chemokines and cytokines that promote the development of tumors, and studies on IL-6, IL-8, and IL-10 (typical examples) have made substantial progress in this respect.

IL-6

IL-6, secreted by tumor-associated endothelial cells and TAMs, is considered to increase the possibility of carcinogenesis and the developmental progress of malignant tumors by regulating the corresponding genes of the cell cycle, promoting tumor angiogenesis, aggravating local inflammation, and helping stem cell self-renewal. Because the major signaling pathway mediated by IL-6 is regulated by signal transducer and activator of transcription 3 (STAT3) phosphorylation and at the same time the epithelial-mesenchymal transition (EMT) is the main characteristic of tumor stem cells, the transcription factor Snail may have an important regulatory function (28). Therefore, researchers detected the expression of STAT3 phosphorylation and Snail in tumor cells interacted with TAMs and tumor-associated endothelial cells expressing or overexpressing B-cell lymphoma-2 (Bcl-2), which could promote the secretion of IL-6. And at the same time, they added a STAT3 suppressor to the group that overexpressed Bcl-2 and contained more IL-6. To obtain the results, the researchers tested the landmarks of the EMT. The results shows that IL-6 promotes STAT3 phosphorylation and the expression of Snail. When the phosphorylation of STAT3 was suppressed, the expression of Snail decreased simultaneously. The experimental results suggest that IL-6 may mediate the EMT by the janus kinase (JAK)/STAT3/Snail pathway (29). Another research also show s that IL-6 combined with IL-6R can activate STAT3 phosphorylation and lead to anti-apoptosis in tumors (30) (**Figure 2**).

IL-8

IL-8 is highly secreted by TAMs and serum IL-8 levels can correctly monitor and predict clinical benefit from immune checkpoint blockade. And experiments also showed that angiogenesis, tumor invasion, and the depression of immunity were more remarkable at higher levels of IL-8 (31, 32). Engulfment and cell motility 1 (ELMO1) is an evolutionarily conservative protein expressed in tumor cells that mainly mediates cell phagocytosis, migration, and morphological changes. Studies have shown that IL-8 can escalate tumor metastasis by upregulating the expression of ELMO1 in tumor cells (33). To a wide extent, the activation of the JAK2/STAT3/Snail pathway is considered to be another mechanism for the capability of IL-8 to promote carcinogenesis. With the increase in exogenous IL-8, the expression of p-JAK2, p-STAT3, and Snail shows extreme improvement. Hence, it is reasonable to speculate that IL-8 can promote EMT via the JAK2/STAT3/Snail pathway (34) (**Figure 2**). In inflammatory breast cancer (IBC), IL-8 and the growth-related oncogene (GRO) chemokines that activate STAT3 are strongly expressed, with monocytes recruitment and high-level expression of macrophage polarizing factors, promoting macrophages recruitment and transformation into M2, causing the highly infiltration. The highly infiltration macrophages also secrete high levels of IL-8 and GRO chemokines, resulting in a feed-forward chemokine loop that further drives the EMT of IBC (35).

IL-10

In the tumor microenvironment, TAMs secrete cytokines such as IL-10, transforming growth factor-β (TGF-β) and inflammatory mediators, including prostaglandin E2 (PGE2) and matrix metalloproteinase-7 (MMP-7), to inhibit the normal process of antigen-presenting, which makes T cells lose their competence in recognizing and even killing tumor cells. It is convinced that IL-10 family cytokines play an essential role during infection and inflammation to maintain tissue homeostasis, through upregulation of innate immunity, restriction of excessive inflammatory responses, and promotion of tissue repairing mechanisms (36). During chronic inflammation, toll-like receptor 4 (TLR4) can stimulate M2 to secrete the cytokine IL-10 (37). Moreover, the activation of TLR4 signaling by lipopolysaccharide profoundly increased the EMT in pancreatic cancer cells (**Figure 2**) and IL-10 increases cancerous the expression of inhibitor of PP2A (CIP2A) via the phosphatidylinositide 3-kinases (PI3K) signaling pathway and promotes tumor aggressiveness in lung adenocarcinoma (38, 39). Additionally, the researchers have found a positive correlation between IL-10 levels in serum and tumor progression, which shows that IL-10 has an important influence on promoting the development of tumors (40).

Immunosuppressive Receptors and Ligands

PD-1/PD-L1 signaling

Programmed cell death protein (PD-1) is a significant molecule in immunosuppression and belongs to the CD28 superfamily. It is of great importance to consider PD-1 as a target for immune regulation to fight tumors, for anti-infection, for autoimmune

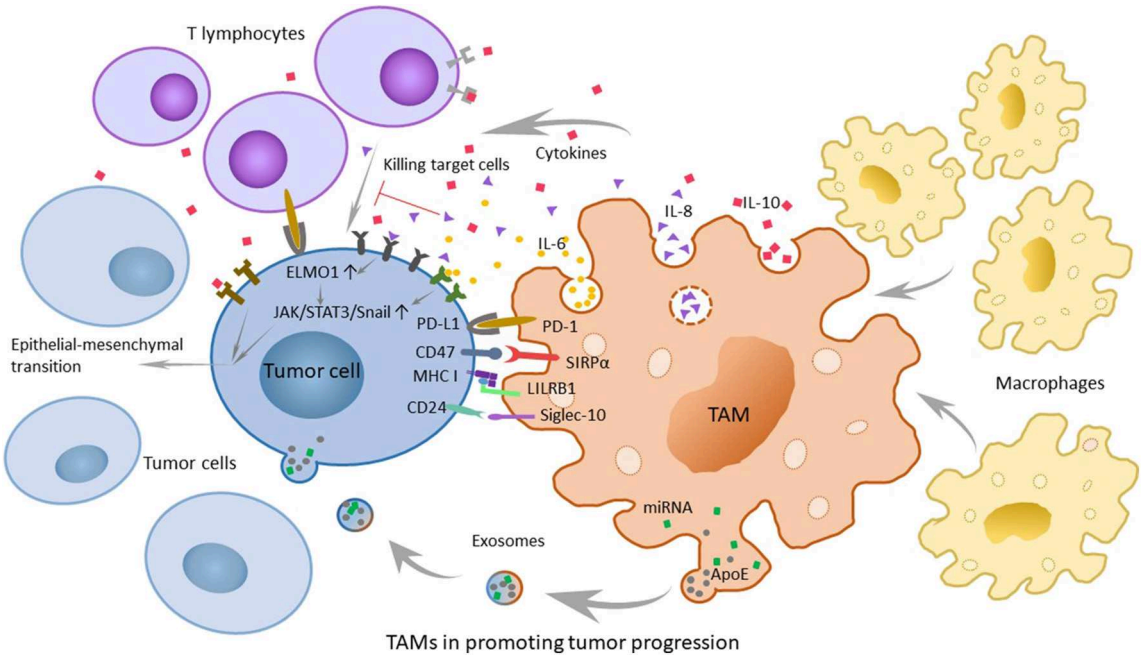

FIGURE 2 | The role of tumor-associated macrophages (TAMs) in promoting tumor progression and related mechanisms. TAMs can secrete chemokines and cytokines that promote tumor development, such as IL-6, IL-8, and IL-10. Furthermore, various molecular mechanisms play a large role in immunosuppression. The PD-1/L1 signaling pathway promotes the possibility of tumor immune escape because it can inhibit the normal function of macrophages. The SIRPα/CD47 pathway is referred to as the "do-not-eat-me" signal, while tumor cells with CD47 expression can be recognized as self-normal cells. LILRB1/MHC class I component β2-microglobulin is also a significant mechanism of tumor escape. In breast cancer and ovarian cancer, CD 24 on tumor cells can promote immune escape through the interaction of Siglec-10. In addition, recent researchers have found that TAMs can promote the development of tumors through exosomes.

diseases and for organ transplantation survival. Its ligand, programmed cell death-ligand 1 (PD-L1), is the first type of transmembrane protein of 40 kDa. When the body is in a healthy condition, PD-L1 is expressed in antigen-presenting cells, which are combined with PD-1 carried by T cells, and the combination with PD-1 indicates that T cells will not launch an attack (41). However, just as tumor cells know the cipher sent to PD-1, PD-L1 can sometimes be expressed on the surface of tumor cells through poorly characterized oncogenic signaling pathways (42). T effector cells make a judgement that tumor cells are part of the "self"; thus, they are unable to kill the shrewd invaders. And in the meanwhile, PD-1 is also expressed on TAMs (43). The PD-1/L1 signaling pathway promotes the possibility of tumor immune escape because it can limit the functions of T effector cells, natural killer (NK) cells, dendritic cells, TAMs, and so on, such as suppressing activation, proliferation and cytokine expression effects on T cells and inhibiting the phagocytosis of TAMs (44) (**Figure 2**).

CD47-SIRPα signaling

The cluster of differentiation 47 (CD47) molecule is a membrane protein widely distributed on membrane surfaces of various cells, including tumor cells. Its corresponding ligand, signal regulatory protein alpha (SIRPα), is a membrane protein mainly expressed on macrophages and bone marrow cells, informing a typical immunoreceptor tyrosine-based inhibitory motif (ITIM). The interaction between the NH2 terminal domain of the ITIM motif and the single domain of CD47 can phosphorylate the ITIM motif, recruit the cytosolic tyrosine phosphatase SHP-1 or SHP-2 and activate it. As a consequence, this interaction can dephosphorylate multiple substrates and regulate downstream signaling pathways, ultimately inhibiting the phagocytosis of macrophages to normal cells. Therefore, CD47 is often referred to as the "do-not-eat-me" signal (45) (**Figures 2**, **3A**).

The combination of these two molecules can produce a variety of physiological functions, and there is a balance between the two molecules. When the expression level of CD47 on the cell surface increases, the balance is upset, because CD47 sends out a "do-not-eat-me" signal to inhibit the phagocytosis of the tumor cells, promoting the occurrence, and development of tumors.

MHC class I component β2-microglobulin/LILRB1 signaling

Researchers have found that there are still some tumor cells escaping from the phagocytosis of macrophages after inhibiting the CD47 molecule. Recently, Weissman and colleagues have found that there is another recognition mechanism between tumor cells and macrophages that protects tumor cells from the phagocytosis of macrophages (46). The signaling molecule is the major histocompatibility complex (MHC) class I component β2-microglobulin on the surface of tumor cells (**Figures 2**, **3A**). When the molecule is blocked or negative expression, macrophages can be awakened *in vivo* to enhance phagocytosis

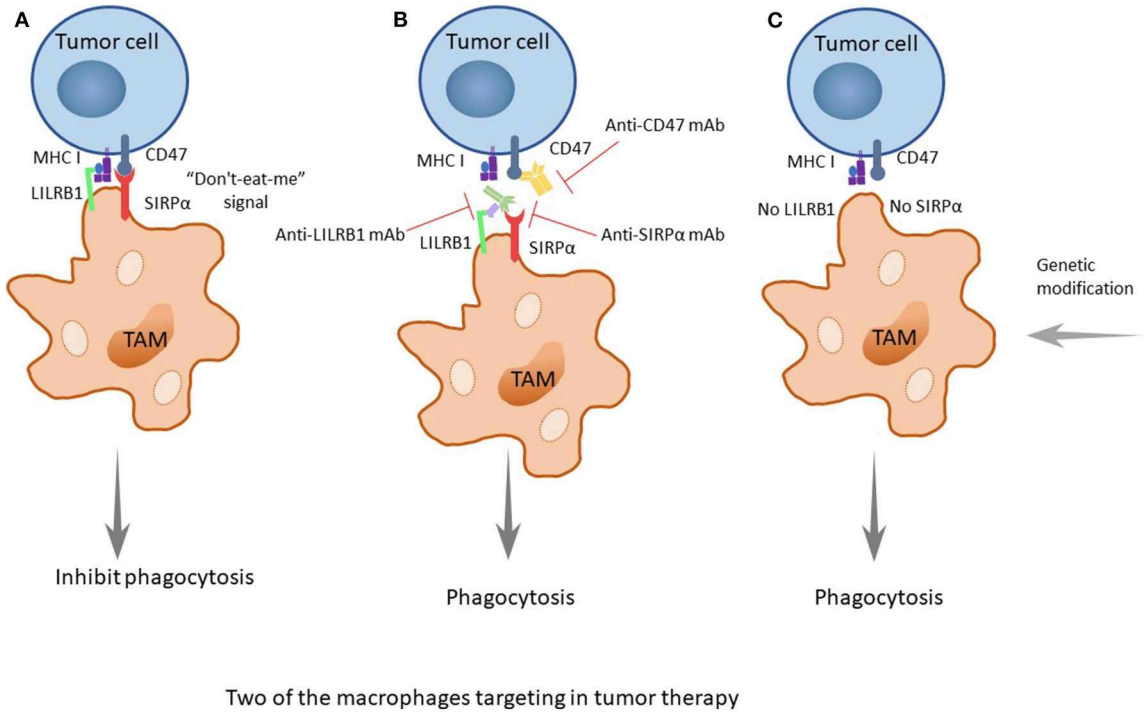

FIGURE 3 | The two important treatment strategies targeting SIRPα/CD47 and LILRB1/MHC I. **(A)** CD47 is identified as a marker of self, or as a signal of "do-not-eat-me," when the CD47 molecule of tumor cells is combined with its receptor, SIRPα, which is expressed on TAMs, and when MHC I on tumors is recognized by LILRB1 on TAMs; the signals will inhibit the phagocytosis of macrophages, promoting the occurrence, and development of tumors. **(B)** When using drugs such as monoclonal antibodies (anti-CD47 mAb, anti-SIRPα mAb, or anti-LILRB1 mAb), the recognition pathways are blocked, and the phagocytosis of macrophages is enhanced. Furthermore, the better treatment effect occurs when both pathways are blocked. **(C)** As it was discovered earlier that knocking out LILRB1 genes can cause macrophages to kill tumor cells, it could be predicted that more genetic modification of macrophages, such as making the SIRPα gene silent to suppress its expression, can achieve a similar effect.

and eliminate tumor cells, extending the survival time of tumor-bearing mice by 70%. In addition, when researchers knockout the receptor leukocyte immunoglobulin like receptor subfamily B member 1 (LILRB1) on the surface of macrophages recognized by MHC class I molecules, macrophages change from promoting tumor growth to inhibiting tumor growth. Current studies have shown that the inhibition of the LILRB1 protein with the simultaneous administration of anti-CD47 monoclonal antibodies can significantly increase the phagocytosis and kill capacity of macrophages on tumor cells (**Figure 3B**), and the inhibition of LILRB1 does not damage normal tissue cells *in vivo*. Nevertheless, this mechanism needs further researches and clinical experiments (46).

CD24-siglec-10 signaling

Regarding the "do-not-eat-me" signal mentioned above, in the study of the magnitude and durability of the response to these agents such as monoclonal antibodies, the researchers found that there were still unclear escape signals. In breast cancer and ovarian cancer, Irving L, and his colleagues found that CD 24 was a dominant innate immune checkpoint and a promising target for tumor immunotherapy. They demonstrated that tumors expressing CD24 could promote immune escape through the interaction of inhibitory receptor sialic-acid-binding Ig-like lectin 10 (Siglec-10), which was expressed on TAMs. Further studies have shown that in addition to breast cancer and ovarian cancer, other tumors can also overexpress CD24, while TAMs express high levels of Siglec-10 (**Figure 2**). Blocking the interaction of CD 24 and Siglec-10 with monoclonal antibodies, or ablating the genes of CD 24 or Siglec-10, can both enhance the phagocytic function of TAMs to all human tumors expressing CD 24. This finding deserves further study and it proposes a new approach to tumor immunotherapy (47).

Exosomes From TAMs

Exosomes are small cell vesicles originating from cells that carry genetic information (proteins, nucleic acids, etc.) and mediate the information transmission and exchange of material between cells, which can affect the functions of target cells. In malignancies, exosomes serve as important carriers for materials and information exchange in the tumor microenvironment and participate in the survival and outgrowth of cancer cells and the different stages of tumor metastasis, which can be used as targets for tumor immunotherapy (48, 49). Previous studies have focused on the secretion of soluble signaling molecules such as cytokines and chemokines (50), while the discovery of exosomes provides a new idea for the correlation study of tumor immunity.

Recently, researchers have discovered that TAMs characterized by an M2-polarized phenotype can promote the metastasis of gastric cancer cells through exosomes (51) (**Figure 2**). TAMs can deliver exosomes to tumor cells, which are rich in miRNA, lncRNA, and specific proteins that can contribute to tumor metastasis. Mass spectrometric analysis reveals that M2-derived exosomes are rich in apolipoprotein E (ApoE), which can activate the PI3K-AKT pathway in tumor cells and induce the EMT and cytoskeleton rearrangement of gastric cancer cells, thus enhancing their metastatic potential as a consequence (51). Coincidentally, another research group studying the resistance of pancreatic ductal adenocarcinoma (PDAC) to gemcitabine have found that the mechanism by which TAMs help gemcitabine resistance may be related to exosomes. Using a genetic mouse model of PDAC and electron microscopy analysis, they found that TAMs secrete vesicles, with selective internalization by tumor cells, which indicated that TAMs and tumor cells communicate with each other. Furthermore, these authors also proved that the sensitivity of PDAC cells to gemcitabine could be significantly reduced by these macrophage-derived exosomes (MDE), which was mediated by the transfer of miR-365 in MDE (52). These discoveries open a new door for the study of the interaction between macrophages and tumor cells, and in quite a few ways, prompt researchers in this field to think about and study relevant mechanisms in greater depth. Perhaps further studies will discover the effects of exosomes on other tumors and their mechanisms for promoting tumor development, which are of great importance in clinical treatment.

Enhancing the Antineoplastic Activity

Macrophage-Mediated Programmed Cell Removal (PrCR)

Macrophage-mediated programmed cell removal plays an important role in tumor elimination and surveillance. The activation of TLR pathways in macrophages induces the activation of Bruton's tyrosine kinase (Btk) signaling pathway (53), which makes the cell surface calreticulin (CRT) in endoplasmic reticulum phosphorylated and dissociated. The dissociated CRT is expressed on the surface of macrophages and then forms the CRT/CD91/C1q compounds to target cancer cells for phagocytosis (54). The induction of PrCR by "eat-me" signals on tumor cells is antagonized by "do-not-eat-me" signals, which bind macrophages SIRPα to inhibit phagocytosis. Blocking CD47 on tumor cells will block "do-not-eat-me" signals. Therefore, the activation of TLR signaling pathways in macrophages can synergize with blocking CD47 of tumor cells to enhance PrCR.

Enhancing the Toxicity

Activated macrophages defend against tumors by directing tumor cytotoxicity and by secreting cytokines. Researchers enhance macrophage cytotoxicity through specificity to stimulate activation, such as by adding M-CSF and muramyl dipeptide (MDP) when macrophages are cultured *in vitro* to enhance macrophage cytotoxicity; by using the adoptive transfer treatment to achieve anti-tumor effects; or by using intravenous liposomes that load immune modulators to enhance the toxicity of macrophages. The molecules of microbial agents and pathogens can stimulate the antitumor cytotoxicity of macrophages, such as using bacilli calmette-guerin (BCG) in the treatment of bladder cancer, through stimulating macrophages to increase the cytotoxicity of macrophages to certain bladder cancer cell lines (55). In addition, there is evidence that the increased levels of IL-6, IL-12, and TNF in the urine of bladder cancer patients treated with BCG may be related to the enhancement of the function of macrophages. Sialyl-Tn (sTn) is a kind of glycan that controls synthesis by sialic acid transferase ST6GALNAC1 and is abnormally expressed in bladder cancer cells. The researchers established a bladder cancer cell lines that expressed sTn (MCRsTn) in the process of study where sTn participated in the BCG treatment of bladder cancer. These researchers found that the secretion of BCG could promote MCRsTn to secrete IL-6 and IL-8. These cytokines further stimulate macrophages to produce large amounts of IL-6, IL-1 and TNF-α to enhance the toxicity of macrophages to tumor cells (56).

Preventing the Diffusion and Metastasis of Cancers

In recent years, researchers have discovered sub-membranous lymphoid sinus macrophages (SCS macrophages), which can form a protective membrane around lymph nodes to prevent the growth and metastasis of tumors (57). Present studies have demonstrated that a potential way in which information transfer can occur between tumor cells and immune cells. Tumor-derived extracellular vesicles (tEVs), especially highly concentrated near lymph nodes, can leave the tumor tissue and migrate to the whole body. They are vital participators in this way (58). As has been found in some studies, tEVs can interact with SCS macrophages, which form a layer of cells in the fibrous capsule surrounding the lymph nodes, thus limiting the spread of tEVs, preventing the entry of tEVs into lymph nodes, and blocking the pathway that causes B cells to produce tumor-promoting growth substances, thereby inhibiting the migration and transformation of melanoma. The specific molecular mechanism remains to be elucidated (59). In this case, the protection of SCS macrophages against tumor growth can be considered in the treatment of tumors; with further development of this research, additional mechanisms and whether this mechanism exists in other tumors will be discovered, and this recommendation needs more clinical experiments and confirmation.

MACROPHAGES TARGETING IN TUMOR THERAPY

In recent years, tumor immunotherapy has been widely concerned and made remarkable progress. By adjusting the immune defense function of the body, tumor immunotherapy can transform immune cells or use various types of immune-active substances to achieve balance between immune system and tumors. CAR-T and PD-1/PD-L1 blockade therapy has achieved significant clinical efficacy. Macrophages, as the important members of tumor microenvironment, become potential hot spots for immunotherapy drug development because of their characters. Next, we will summarize various

TABLE 1 | Macrophages targeting therapies.

Category	Substance	Target site	Mechanisms of action
Inhibitor	Zoledronic acid	CCL2	Suppress the expression of CCL2
	Gefitinib	CCL5	Decrease the secretion of CCL5
	PLX3397	CSF1R	Inhibit the expression of CSF1R
	GW2580	CSF1	Inhibit the expression of CSF1
	Wortmannin	PI3K	Decrease serum cytokine levels by inhibiting PI3K
Monoclonal antibody or blocker	HAC	PD-L1	Block human PD-L1
	BMS-936558	PD-1	Block the interaction between PD-1 and PD-L1
	Hu5F9-G4	CD47	Bock CD47 that induces tumor-cell phagocytosis
	KWAR23	SIRPα	Combined with tumor-opsonizing antibodies to augment neutrophils and TAMs antitumor activity
	GHI/75	LILRB1	Bock the MHC I/LILRB1 signaling way
	Trabectedin	Macrophages	Block the immunosuppressive effect
	Immunomodulator linemode	Macrophages	Block the activity of macrophages in tumor angiogenesis
Biological response modifier	DNMTi 5-Azacytidine (AZA)	Macrophages	Regulate of macrophages polarization
	α-Difluoromethylornithine (DFMO)	Macrophages	Regulate of macrophages polarization
	Dual-inhibitor-loaded nanoparticles (DNTs)	M2 macrophages	Make M2 macrophages repolarize to active M1 macrophages and inhibit CSF1R and SHP-2

In this table, we summarize the relevant macrophages targeting therapies mentioned in chapter 5 about anti-tumor cells. It divides the drugs into inhibitor, monoclonal antibody, or blocker and biological response modifier, and in each category, it contains substance, target site, and mechanisms of action of these drugs.

tumor immunotherapy strategies targeting macrophages and their application prospects.

Macrophages Targeting Therapy (Table 1)

It has been known that the use of non-discriminatory medicine for the whole body in the treatment of tumors has many disadvantages, such as damaging the immune system and upsetting the equilibrium of the microenvironment or even the entire balance. Therefore, in seeking a treatment that damages the tumor only, one concern, the need for targeted therapy and modification of molecules in the expression pathways, has been present for a long time.

CCL2 and CCL5

Stimulated by proinflammatory factors, such as IL-8 and TNF-α, a large secretion of CCL2 (also known as monocyte chemotactic protein-1, MCP-1) occurs by activated macrophages, monocytes and dendritic cells. In other words, the interaction between resident macrophages and newly recruited macrophages is bidirectional because resident TAMs conversely can recruit macrophages to deteriorate tumor metastasis. As a peritumoral function of TAMs, CCL2 is considered a promising target site to prevent the tissue from collecting TAMs (60). Recently, researchers have found that zoledronic acid, a diphosphate compound, can suppress the expression of CCL2/MCP-1, decreasing the number of recruited macrophages and performing an antitumoral function (61). A high concentration of CCL5 can also bring about the recruitment of TAMs by connecting with CCR2 on the surface of monocytes in some cases. Gefitinib, a tyrosine kinase inhibitor that can decrease the secretion of CCL5, inhibits the cross-talk between TAMs and prostate cancer cells, leading to the proliferation of the tumor cells and the inhibition of docetaxel activity (62).

Colony Stimulating Factor-1 (CSF-1)

Many studies on targeted therapy are based on a purposeful strategy of CSF1/CSF1R, that is, to focus on the recruitment of TAMs and the secretion of cytokines, tumor cells secrete CSF1 for the purpose of collecting TAMs by connecting CSF1 with CSF1R on macrophages. CSF1 is related to macrophage recruitment, differentiation and repolarization; thus, it is an effective way to target CSF1/CSF1R. As was shown in a previous study, the tyrosine kinase inhibitor PLX3397 was used for the treatment of melanoma in mouse models driven by BRAFV00E. It shows the ability to inhibit CSF1R, and through its inhibition of the CSF1R, it is currently used as the treatment of patients with glioblastoma, breast cancer, and other cancers in clinical. These researchers found that the number of TAMs was remarkably reduced and that the proportion of M2 also decreased (63). Similarly, in MMTV-Neu transgenic mice, inhibiting the CSF1/CSF1R pathway by a CSF1 inhibitor named GW2580 led to a noticeable decrease of TAMs infiltration in tumor tissue (64). Another study showed that with the assistance of inhibitor PLX3397 or a monoclonal antibody of CSF1, CSF1-deficient mice showed specific changes, such as the decrease number of TAMs (65). It is now generally believed that the loss of the CSF1/CSF1R signal possesses the ability to give absolute control for consuming M2 macrophages, contrary to the uninfluential M1 macrophages (66).

Related Kinase Signaling Blocking

According to the description above, IL-10 promotes the growth and transfer of tumors by increasing CIP2A expression via the PI3K signaling pathway. Studies show that IL-10 secreted in E6-positive lung cancer cells is regulated by the phosphorylation of cAMP response element binding protein (CREB) via the pathway, and the feedback of IL-10-CIP2A-phosphorylated-CREB is likely to affect the progression of tumors. One of the

targeted therapies uses specific inhibitors, such as wortmannin or LY294002 (PI3K inhibitors), to block the signaling transduction pathway. Wortmannin, a commonly used cell biology reagent, has been previously used to suppress DNA repair, receptor-mediated endocytosis and cell proliferation (67). Wortmannin has been confirmed to be effective in decreasing serum cytokine levels by inhibiting PI3K/Akt, which may suppress tumor invasiveness. In recent research, Halaby et al. have discovered serine-threonine kinase general control nonderepressible 2 (GCN2) is important to maturation and polarization of macrophages and myeloid-derived suppressor cells (MDSCs) by promoting translation of the transcription factor CREB-2/activating transcription factor 4 (ATF4). Therefore, they blocked the GCN2 signaling by targeting Atf4 with small interfering RNA knockdown, and found that tumor growth was reduced as a consequence. This finding demonstrates blocking GCN2 signaling can promote anti-tumor immunity (68).

PD-1/PD-L1 Signaling Blocking

One study treated immunocompromised mice with either a PD-L1 blocker (HAC, an engineered small protein which can block human PD-L1) or a PD-1 blocker (anti-mouse PD-1 antibody). The results show that both murine and human TAMs express high levels of PD-1, and the level of PD-1 increases gradually with the development of tumors. After PD-1/PD-L1 suppression by inhibitors, the phagocytosis function of TAMs improves, killing tumor cells. In addition, it is likely that PD-1/PD-L1 therapies interact with anti-CD47 in the context of macrophage-mediated immunotherapy, and the combination therapy trends toward increasing the survival rate more than monotherapy (43). According to the PD-1/PD-L1 recognition mechanism, many PD-1 monoclonal antibodies, such as BMS-936558, have been approved by the FDA for use in clinic and have achieved great efficacy in the treatment of certain advanced malignant tumors, although PD-1 inhibitors have a curative effect only on a small proportion of cancer patients (69).

Monoclonal Antibodies and Inhibitors

Immune escape is one of the most important mechanisms of tumor establishment and diffusion. Currently, the most widely used tumor immunotherapy is monoclonal antibodies. Monoclonal antibodies can block multiple pathways involved in TAMs and tumors recognition, disrupting tumors escape pathways and thus acting as antitumor agents. After discovering the CD47-SIRPα recognition mechanism of tumor cells and macrophages, researchers used an anti-CD47 monoclonal antibody to carry out *in vivo* experiments on tumor-bearing mice, and found the antibody can block the CD47-SIRPα pathway to interdict the signal of anti-phagocytosis (**Figure 3B**). This antibody shows targeting to tumor cells, which increases the macrophage phagocytosis of tumor cells and at the same time, does not affect normal cells (70). The CD47 molecule is also expressed on the surface of normal cells, and the anti-CD47 mAb triggers a strong self-reaction (71–73). Current researches have found that anti-CD47 monoclonal antibodies mainly induce transient anemia and mild neutrophil reduction as well as no other obvious adverse effects or the occurrence of autoimmune diseases (74, 75). However, Hu5F9-G4, an anti-CD47 monoclonal antibody, selectively eliminates malignant cells that express CD47 and not normal cells (76). As is mentioned in a recent study, glutaminyl-peptide cyclotransferase-like protein (QPCTL) is identified as a new target to interfere with the CD47 pathway and promotes the efficacy of antibody therapy of cancer (77). Recently, Arely and colleagues switched to an anti-SIRPα monoclonal antibody in the study and blocked this mechanism to enhance the tumor phagocytosis of macrophages; the effect was better than that in previous experiments (78), and another research team found the anti-human SIRPα antibody, KWAR23, could significantly promote the anti-tumor activity of neutrophils and TAMs when it was in combination with the tumor-opsonizing antibody rituximab (79) (**Figure 3B**).

Though the mechanism of the anti-CD47 antibody is not yet clear, the possible pathways are as follows: preventing the combination of CD47 on tumor cells and SIRPα on macrophages to activate phagocytosis, promoting the cytotoxic effect of antibody dependence and complement dependence based on Fc, directly inducing apoptosis to tumor cells, or stimulating the phagocytosis of dendritic cells to tumor cells. Additionally, it is likely the combined result of several mechanisms mentioned above (45). Because the overexpression of CD47 in myeloid leukemia cells prevents macrophages from clearing tumor cells, the survival rate of tumor cells increases. Taken together, these findings provide a rational basis for targeting the interaction of CD47-SIRPα in cancer, particularly to enhance the efficiency of antibody therapy in cancer. Similarly, drugs of another recognition mechanism, LILRB1/MHC class I, such as the LILRB1 monoclonal antibody GHI/75 are still in the clinical trial stage, and no obvious damage to the human body has been found for the time being (46) (**Figure 3B**). These drugs have clear targets and few adverse reactions, providing a theoretical basis and good prospects for clinical application. In addition, in some studies, macrophage-mediated antibody-dependent cell phagocytosis (ADCP) has been elucidated, which needs more experiments to study its mechanism (80).

Regulation of Macrophages Polarization

In recent years, using molecular targeted drugs to treat hepatocellular carcinoma has led to new breakthroughs with deep researches in the molecular biology of liver cancer. The treatment strategies for macrophages in the microenvironment of hepatocellular carcinoma include promoting M2 macrophages to transform into M1 macrophages (81) and blocking the immunosuppressive effect. Trabectedin is a targeted drug for macrophages and is used to treat soft tissue sarcomas. This drug is a marine bioactive extract that is toxic to macrophages. Other potential drugs, such as the immunomodulator linemode, can block the activity of macrophages in tumor angiogenesis. The CCL2 antibody can reduce the aggregation of macrophages as a potential treatment. C-Fms is a CSF receptor that regulates the function of macrophages. Clinical research is conducted using many

drugs and these drug combinations may affect the interaction of C-Fms with other immune cells, change macrophage phenotypes and change the microenvironment that maintains M2 macrophages.

Combination therapy can also be put in to use in the treatment of cancer. A recent study held by Travers found that DNMTi 5-Azacytidine (AZA) and α-difluoromethylornithine (DFMO) in combination could significantly improve survival, reduce tumor burden, and then they combined therapy in a mouse model of ovarian cancer with normal immune function. The survival rate significantly decreased, more than that the two drugs were used alone. Significant reduction in M2-polarized macrophages and increased number of tumor-killing M1 macrophages in combination therapy suggest that combination therapy can alter macrophage polarization in the tumor microenvironment, recruit M1 macrophages and prolong survival period (82). This type of tumor suppression treatment will have great prospects for clinical application.

In addition, a new study by Ashish Kulkarni and his colleagues have reported that self-assembled dual-inhibitor-loaded nanoparticles (DNTs) target M2 macrophages and make M2 macrophages repolarize to active M1 macrophages. In the meanwhile, this drug simultaneously inhibits CSF1R and SHP-2 signaling pathways. This research provides an idea for anti-tumor therapy of macrophages and DNTs has good perspective potential for individual drug treatments (83).

Engineering Macrophages

Macrophage Gene Modification

In the discovery of a new mechanism for the recognition between macrophages and tumor cells, the MHC class I component β2-microglobulin/LILRB1 protein, researchers used the gene modification of macrophages to knock out the gene for the LILRB1 protein and downregulated its expression on the membrane surface, allowing the macrophages to transform from the state of promoting the growth of tumor cells to eliminating the tumor cells (**Figure 3C**). While inhibiting the receptor with the simultaneous administration of anti-CD47 monoclonal antibodies, the phagocytosis and killing capacity of macrophages on tumor cells is significantly increased (46) (**Figure 3B**). In recent research, researchers have found that CD 24 expressed on tumor cells is a dominant innate immune checkpoint, and can promote the escape of tumors with the interaction of Siglec-10 on TAMs. Ablating the genes of CD 24 or Siglec-10 has been demonstrated an effective way to enhance the phagocytic function of TAMs (47). At present, due to the convenience of clinical application and cost issues, macrophage gene modification is not as frequently used, as it is only at the research stage; however, in future, with the development of the technology, gene therapy, especially the progress of genetic engineering, will have better prospect because of its stability and longevity. When the technology is mature and applied on a large scale, tumor treatment and precision medicine will take a new step.

iSNAPS Smart Protein Molecules

A group of researchers designed a smart protein called the integrated sensing and activating proteins (iSNAPS) protein, which could reprogram white blood cells and ignore the self-defense signaling mechanisms on which tumor cells rely for survival and spreading *in vivo*. The emergence of this protein will present new approaches and ideas for the editing of immune cells. The iSNAPS protein is inserted into macrophages in the study, and it reconnects macrophages, covering the escape signals recognized by the tumor cells and interpreting them as phagocytic signals. In addition, its rapid response and strong lethality can significantly enhance the ability of macrophages to divide, phagocytose, and kill tumor cells rapidly (84).

The design principle of this intelligent protein molecule can also be used to redesign other immune cells for cancer treatment. At present, the team plans to test iSNAPS in mice and may study its application in other areas (84). This protein may influence not only tumor treatment but also other diseases and self-regulation, and further research is needed.

CONCLUSIONS

This review introduces the origin, classification and immune function of macrophages and further explores the mechanisms of the participation of macrophages in tumor microenvironment. We focus on the killing effect and mechanisms of macrophages on tumors, while tumor promoting factors such as IL-6, IL-8, IL-10, TLR4 are briefly introduced as well (29, 37, 40, 85). Based on existing research, we discuss the molecular mechanisms of the interaction between macrophages and tumor cells, not only the chemokines and cytokines but also some recognition mechanisms including. For instance, the promoting pathways of PD-1/PD-L1, SIRPα/CD47, and LILRB1/MHC I (41, 46, 86) and the killing factors such as PrCR (54) are presented. In addition, based on existing researches, we summarize a new pathway by which TAMs can promote the development of tumors through exosomes. The pathway has been found and may exist in certain kinds of tumors, which opens a new door for the study of tumor immunity (51, 52). Moreover, several types of treatments, such as inhibiting M2 macrophages to promote the growth of tumor cells, motivating the transition of M2 macrophages to M1 macrophages, enhancing macrophage phagocytosis of tumors and reinforcing the role of macrophages in preventing tumor growth and metastasis, suggest that macrophages can participate in tumor cells immune regulation through various molecular mechanisms and should be given more attention.

At present, with the development of precision medicine, the therapeutic direction of tumors has gradually turned to targeted therapy because non-discriminatory medicine for the whole body during the treatment of tumors has many disadvantages. With tumor immunity becoming a popular research direction, increasing researches has been conducted to overcome the unresolved issues in traditional tumor treatment, but this area of research has been very limited in terms of adaptive immunity until recent years. As suggested by some studies, macrophages

influence tumor cells through various mechanisms and have become a new research hotspot in immunotherapy research (45, 46, 64), and researchers have found that certain cytokines (56) secreted by macrophages or modified macrophages can be used to kill tumor cells. In recent years, a variety of recognition mechanisms have been discovered, and related targeted therapies, such as the application of antibodies or inhibitors (43, 71), genetic modification (46), and adoptive transfer of immune cells, are under in-depth research. In summary, macrophages are promising in terms of tumor-targeted therapy, as several kinds of therapy has been applied, but the technology is still immature at present, and current researches are limited because cancer still cannot be completely cured. Thus, quite a few unknown molecular mechanisms may play a vitally important role in the regulation of tumor growth and development, and some potential targets need more research and attention. Thus, it is necessary to investigate the communication of macrophages and tumor cells a bit deeper in further studies.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript and JZ designed the figures. ZT and CL collected the related references. SG and YF edited the manuscript. XZ provided guidance and revised this manuscript. All authors approved the final manuscript.

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Expression of Monocarboxylate Transporter 1 in Immunosuppressive Macrophages Is Associated With the Poor Prognosis in Breast Cancer

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Monocarboxylate transporter 1 (MCT1) participates in the transport of lactate to facilitate metabolic reprogramming during tumor progression. Tumor-associated macrophages (TAMs) are also involved in the inflammatory adaptation of the tumor microenvironment (TME). This study aimed to determine the correlation between metabolite changes and the polarization of macrophages in the TME. We demonstrated that the expression of CD163 on macrophages was significantly higher in breast cancer tissues than in normal tissues, especially in the HER2 subtype, although it was not statistically associated with recurrence-free survival (RFS). The presence of $MCT1^+$ and $CD163^+$ macrophages in the invasive margin was significantly correlated with decreased RFS. A significant correlation existed between MCT1 and CD163 expression in the margin, and high infiltration of $MCT1^+CD163^+$ macrophages into the margin predicted rapid progression and poor survival outcomes for breast cancer patients. These data suggested that MCT1 at least partially promoted the alternative polarization of macrophages to inhibit antitumor immunity, and blocking this interaction may be a promising method for breast cancer therapy.

Keywords: breast cancer, tumor-associated macrophage, MCT1, CD163, recurrence-free survival

INTRODUCTION

The tumor microenvironment (TME) is a heterogeneous ecosystem, including infiltrating immune cells, mesenchymal support cells, and matrix components (1). With the metabolic and inflammatory reprogramming of tumor cells during cancer progression, the TME is converted into an advantageous microenvironment with altered generation of metabolites, such as lactate, pyruvate and ketone bodies, and adaptive infiltration of tumor-infiltrating lymphocytes. Macrophages are one of the important immune cells recruited to the TME, which have two subsets, "classically activated" M1 macrophages and "alternatively activated" M2 macrophages (2). Generally, M1 macrophages are thought to be proinflammatory and are characterized by high expression of proinflammatory factors, such as interleukin (IL)-12, nitric oxide synthase 2 (NOS2), and tumor necrosis factor (TNF)-α. However, M2 macrophages are considered to be immunosuppressive and generate high levels of anti-inflammatory cytokines, such as IL-10 and transforming growth factor (TGF)-β, and low levels of proinflammatory cytokines, to facilitate

tumor evasion (2, 3). The pan-macrophage marker CD68 is now generally utilized to identify tumor-associated macrophages (TAMs) in diagnostic biopsy samples, and CD163 and CD206 are used to identify M2 macrophages (3).

Monocarboxylate transporters (MCTs) are proteins located primarily in the plasma membrane that transport monocarboxylates bidirectionally depending on the concentration gradient of their substrates, including lactate, pyruvate, and ketone bodies (4). MCT1 is ubiquitously expressed in normal tissues, such as gut epithelium (5), heart and red skeletal muscle fibers (6), as well as in various cancer types, including breast cancer (4), melanoma (7), and prostate cancer (8). MCT1 can mediate lactate influx as well as efflux, while MCT4 mainly facilitates the efflux of lactate to maintain steady intracellular pH (9). In high-lactate microenvironment, MCT4 is the major exporting transporter of lactate (10), and high expression of MCT1 on macrophages regulates the lactate uptake and induces M2-like polarization of macrophages (11). LPS and TNFα stimulate the expression of MCT1 in macrophages (12). Macrophages increase the uptake of lactate through MCT1, the possible reason is that lactate can be utilized as energy source to generate ATP to meet the need for production and secretion of cytokines. However, the expression of MCT1 on tumor-associated macrophages is still unknown.

Lactate, generated by glycolytic tumor cells and immune cells, such as macrophages, and dendritic cells (13), is involved in almost all of the main processes following carcinogenesis, including immune evasion, angiogenesis, cell metastasis, and metabolism (14). Functionally, a high lactate concentration serves as an immune suppressor. Lactate derived from tumor cells suppressed the proliferation and cytokine generation of cytotoxic T lymphocytes (CTLs) (15). In addition, the lactate concentration in cancerous tissues was increased almost 10-fold compared to that of healthy tissues. Lactate taken up by macrophages can also induce alternative polarization of macrophages through hypoxia-inducible factor 1α (HIF-1α) stabilization and the resulting increased production of vascular endothelial growth factor (VEGF) (16).

Here, we focus on the expression levels of MCT1 and CD163 on macrophages in breast cancer specimens to investigate the correlation between the expression of MCT1 on macrophages, macrophage phenotypes, and survival outcomes to explore the impact of tumor metabolic reprogramming on the remodeling of the immune microenvironment.

MATERIALS AND METHODS

Tissue Specimens

A total of 108 formalin-fixed paraffin-embedded (FFPE) tissue samples of breast cancer were collected from Renmin Hospital of Wuhan University, People's Republic of China, and 12 cases of benign breast disease were used as controls. Clinical information was extracted from medical records and pathology reports, and the detailed clinicopathological characteristics of the patient are shown in **Table 1**. Patients were all followed-up for at least 5 years from the date of first diagnosis. All patients involved in the study have written an informed consent form, and this study was approved by the Institutional Ethics Committee of the Renmin Hospital of Wuhan University (approval no. 2018K-C09). Patients did not receive any financial compensation. All methods were performed in accordance with the relevant guidelines and local regulations.

Immunohistochemistry

A series of 108 paraffin-embedded human breast cancer specimens was characterized by histopathology at Renmin Hospital of Wuhan University from 2011 to 2013. Immunohistochemistry (IHC) staining was performed as follows: deparaffinization, antigen retrieval, blocking (2% bovine serum albumin, 37°C, for 30 min), incubation with the primary antibody (dilution 1:100, 37°C for 2 h), washing, blocking, incubation with the horseradish peroxidase (HRP)-conjugated secondary antibody (dilution 1:500, 37°C for 30 min), washing, and staining with diaminobenzidine (DAB). The specimen incubated without the selective antibody was used as the negative control. And we used the paraffin-embedded human non-small cell lung cancer (CD163) or paraffin-embedded human liver tissue (MCT1) as positive control provided by the antibody companies. The staining results were scored by two independent pathologists based on both the proportion of positively stained tumor cells and the intensity of staining. According to the expression, the protein expression level of CD163 was described according to the numbers of $CD163^+$ macrophages using software Image-Pro plus, while the expression level of MCT1 was described according to the percentage of positive cells calculating by the software ImageJ (17, 18). The proportion of tumor cells was scored as follows: 0 (<10% positive cells), 1 (10–20% positive cells), 2 (21–50% positive cells) or 3 (more than 50% positive cells). The intensity of protein expression was determined as follows: 0 (no staining), 1 (weak staining, light brown), 2 (moderate staining, brown), or 3 (strong staining, dark brown). The protein staining positivity was determined using the following formula: overall score = percentage score × intensity score. In addition, the numbers of $CD163^+$ macrophages were counted in 10 random fields of each breast cancer specimen at 400× magnification. Receiver operating characteristic (ROC) analysis was used to determine the optimal cut-off values of all protein expression levels in regard to survival rate.

Immunofluorescence Imaging

Immunofluorescence (IF) imaging was performed to investigate the localization of MCT1 and CD163 as well as the colocalization of CD68 (a marker of all macrophages) and CD163. Tissue specimens undergoing IF staining were incubated with a Cy3-conjugated secondary antibody or a FITC-conjugated secondary antibody for 1 h at room temperature, followed by counterstaining with DAPI for 5 min. Images were captured using a fluorescence microscope (Olympus BX63; Olympus Corporation).

TABLE 1 | Clinicopathological associations of MCT1&CD163 expression in breast cancer.

Clinicopathological parameters	CD163 margin		CD163 tissue		MCT1		MCT1—CD163 margin		MCT1—CD163 Tissue	
	High expression (%)	*p*	High expression (%)	*p*	High expression (%)	*p*	High expression (%)	*p*	High expression (%)	*p*
Age at diagnosis, y		0.333		0.645		0.298		0.350		0.750
≤50	33 (50)		10 (58.8)		29 (49.2)		22 (46.8)		6 (50.0)	
≥51	33 (50)		7 (41.2)		30 (50.8)		25 (53.2)		6 (50.0)	
Tumor size (cm)		**0.005**		0.239		0.183		**0.025**		0.322
<2	17 (25.8)		4 (23.5)		18 (30.5)		11 (23.4)		3 (25.0)	
≥2	49 (74.2)		13 (76.5)		41 (69.5)		36 (76.6)		9 (75.0)	
Lymph node metastasis		**0.005**		0.283		0.471		0.062		0.191
Negative	24 (36.4)		6 (35.3)		26 (44.1)		16 (34.0)		3 (25.0)	
Positive	42 (63.6)		11 (64.7)		33 (55.9)		31 (66.0)		9 (75.0)	
Vascular invasion		0.545		0.698		0.091		0.469		0.198
Negative	59 (89.4)		15 (88.2)		51 (86.4)		40 (85.1)		10 (83.3)	
Positive	7 (10.6)		2 (11.8)		8 (13.6)		7 (14.9)		2 (16.7)	
ER		0.069		0.053		0.051		**0.038**		0.054
Negative	37 (56.1)		12 (70.6)		34 (57.6)		29 (61.7)		8 (66.7)	
Positive	29 (43.9)		5 (29.4)		25 (42.4)		18 (38.3)		4 (33.3)	
PR		**0.006**		**0.004**		**0.004**		**0.002**		**0.002**
Negative	40 (60.6)		14 (82.4)		37 (62.7)		32 (68.1)		10 (83.3)	
Positive	26 (39.4)		3 (17.6)		22 (37.3)		15 (31.9)		2 (16.7)	
HER2		0.058		0.238		0.086		0.062		0.196
Negative	46 (69.7)		11 (64.7)		41 (69.5)		31 (66.0)		8 (66.7)	
Positive	20 (30.3)		6 (35.3)		18 (30.5)		16 (34.0)		4 (33.3)	
Molecular subtypes		**0.004**		**0.038**		0.088		**0.007**		**0.001**
Luminal A	10 (16.9)		14 (21.2)		1 (5.9)		23 (48.9)		10 (83.3)	
Luminal B	15 (25.4)		15 (22.7)		4 (23.5)		7 (14.9)		1 (8.3)	
HER2	10 (16.9)		10 (15.2)		4 (23.5)		4 (8.5)		0 (0.0)	
Basal-like	24 (40.7)		27 (40.9)		8 (47.1)		13 (27.7)		1 (8.3)	
Ki67		**0.002**		**0.006**		**0.000**		**0.000**		**0.000**
<14%	24 (36.4)		3 (17.6)		18 (30.5)		13 (27.7)		0 (0.0)	
≥14%	42 (63.6)		14 (82.4)		41 (69.5)		34 (72.3)		12 (100.0)	
Recurrence		**0.012**		0.191		**0.001**		**0.001**		**0.006**
No	38 (57.6)		9 (52.9)		31 (52.5)		23 (48.9)		5 (41.7)	
Yes	28 (42.4)		8 (47.1)		28 (47.5)		24 (51.1)		7 (58.3)	

**P-values calculated by Log-rank testing; Bold if statistically significant, P < 0.05. ER, estrogen receptor; PR, progesterone receptor; HER2, human epithelial growth factor receptor-2.*

Analysis of Gene Expression Data

The expression data of breast cancer cases were downloaded from The Cancer Genome Atlas (TCGA) database to analyze the correlation between the mRNA expression of MCT1 (SLC16A1) and CD163 in breast cancer patients. In addition, the association between MCT1 and CD163 mRNA levels and survival outcomes of patients with breast cancer was analyzed.

Statistical Analysis

Statistical analyses were performed and survival probabilities were determined with SPSS 22.0 (IBM Corporation, Armonk, NY, USA). The relationships between MCT1 and CD163 and the clinical characteristics of patients with breast cancer were evaluated by the Chi-square test. Kaplan-Meier analysis was utilized to calculate the patient survival probability, and the log-rank test was used to assess the heterogeneity in the survival data for each prognostic factor. Multivariate Cox proportional hazard regressions were used to obtain hazard ratios (HRs) and their respective 95% confidence intervals to show the strength of the estimated relative risks. Pearson's correlation analysis was used to evaluate the correlation between MCT1 and CD163 expression levels. Significance levels were set at a $p < 0.05$. All tests were two-sided.

RESULTS

Significant Differences Existed in the Expression of CD163 Between the Tumor Invasive Margin and Malignant Tissues

Tumor-associated macrophage (TAM) were phenotypically different between the invasive margin and the core in malignant tumors (19, 20). In colorectal carcinoma, strong infiltration of intraepithelial CD163$^+$ macrophages was correlated with unfavorable clinicopathological features, such as lymph node invasion (21); however, in endometrial cancer, stromal TAMs rather than tumor core TAMs promoted lymph node metastasis (22). Therefore, we investigated whether this discrepancy also existed in breast cancer tissues. Immunohistochemistry staining was utilized to examine the expression level of CD163 in 108 cases of primary breast cancer and 12 cases of benign breast disease. As shown in **Figure 1A**, CD163 protein was positively expressed in both the tumor tissues and the invasive margin near adipose tissues. Of 108 breast cancer specimens, 66 (61.1%) exhibited high expression of CD163 in the margin (CD163Margin), whereas only 2 (16.7%) specimens of benign breast disease showed high expression. However, for the expression of CD163 in the tumor tissues (CD163Tissue), only 17 (15.7%) cases of breast cancer and no (0%) cases of benign breast disease showed high expression. There are significant differences in CD163 protein expression in the margin or tumor tissue between 108 breast cancer specimens and 12 controls (**Supplementary Figure 1A**, $p = 0.0015$, $p = 0.0002$, respectively). The significant difference also existed between the expression level of CD163 in the margin and that in tumor tissues, and the expression of CD163Tissue was much higher than that of CD163Margin ($p < 0.0001$) (**Figure 1B**). In addition, **Table 1** shows the association between CD163 expression and the clinicopathological features of breast cancer patients. Our results demonstrated that compared with low expression of CD163Margin, high expression of CD163Margin was significantly associated with larger tumor size ($p = 0.005$), lymph node metastasis ($p = 0.005$), PR status ($p = 0.006$), and higher Ki67 ($p = 0.002$), which indicated that CD163Margin might be a predictor of prognosis for breast cancer patients. On the other hand, high expression of CD163Tissue was only significantly related to PR status ($p = 0.004$) and higher Ki67 ($p = 0.006$). No correlations were detected between CD163Tissue and other clinicopathological features, including age, tumor size, lymph node metastasis, vascular invasion, ER status, and human epithelial growth factor receptor-2 (HER2) status. Moreover, Kaplan–Meier analysis and the log-rank test showed that high expression of CD163Margin had a significant association with decreased recurrence-free survival (RFS) (**Figure 1C**). Multivariate Cox proportional hazard regressions showed that CD163Margin was an independent prognostic predictor in breast cancer (**Figure 4**, $p = 0.016$; HR = 2.705, 95% CI 1.203–6.083). However, such a relationship was not observed between the expression of CD163Tissue and RFS (**Figures 1D**, **4**).

CD163 Overexpression Was Found in HER2 Breast Cancer Patients

It has been reported that there are significant differences in the types and numbers of tumor-infiltrating lymphocytes (TILs) among different molecular subtypes of breast cancer (23, 24). Therefore, we compared the infiltration level of CD163$^+$ macrophages in different breast cancer subtypes. As for CD163 in the tumor margin, the HER2 subtype had the highest expression level (**Figures 2A,B**), and a similar condition was observed in the expression level of CD163 in the malignant tissue (**Figures 2C,D**).

Increased Infiltration of MCT1$^+$CD163$^+$ Macrophages Was Associated With Poor Prognosis in Breast Cancer

Previous observations have shown that high expression of MCT1 is significantly correlated with poor prognosis in breast cancer (**Supplementary Figure 1D**) (25). Here, we further explored the correlation between the expression of MCT1 and CD163 and whether MCT1 has an impact on the polarization of macrophages to promote the expression of CD163. For this purpose, we performed immunohistochemistry (IHC) staining and immunofluorescence (IF) staining to detect the expression of MCT1 and CD163 in a series of 108 cases of breast cancer. We compared the expression of CD163 in MCT1$^-$ and MCT1$^+$ groups, and the results demonstrated that the margin and tissue expression of CD163 were higher in MCT1$^+$ group than that of MCT1$^-$ group (**Supplementary Figure 1E**, $p = 0.0072$, $p = 0.0166$, respectively).

Pearson's correlation analysis revealed that MCT1 expression was positively correlated with the level of CD163Margin (**Figure 3A**, $r = 0.202$, $p = 0.036$). The IHC and IF results also revealed that MCT1 was frequently present in the margin between tumor tissues and adipose tissues, accompanied by positive expression of CD163 (**Figures 3B,C**). In addition, IF images showed that the expression of MCT1 was almost completely coincident with that of CD163, which meant that macrophages were both MCT1-positive and CD163-positive (**Figure 3B**). Overall, 47 (43.5%) breast cancer specimens exhibited high expression of both MCT1 and CD163Margin, and this combined high expression was significantly correlated with tumor size ($p = 0.025$), ER status ($p = 0.038$), PR status ($p = 0.002$), and increased Ki67 staining ($p < 0.0001$) (**Table 1**). Kaplan-Meier analysis revealed that patients with high infiltration of MCT1$^+$CD163$^+$ macrophages in the margin displayed shorter RFS than patients with negative expression of both markers or positive expression of one marker (**Figure 3D**, $p = 0.0012$). Furthermore, multivariate Cox proportional hazard regressions showed that high expression of both MCT1 and CD163Margin was an independent prognostic factor for poor prognosis in breast cancer (**Figure 4**, $p = 0.002$; HR = 3.145, 95% CI 1.516–6.526; $n = 108$). These observations indicate that high infiltration of MCT1$^+$CD163$^+$ macrophages in the margin can be a useful biomarker for predicting rapid progression.

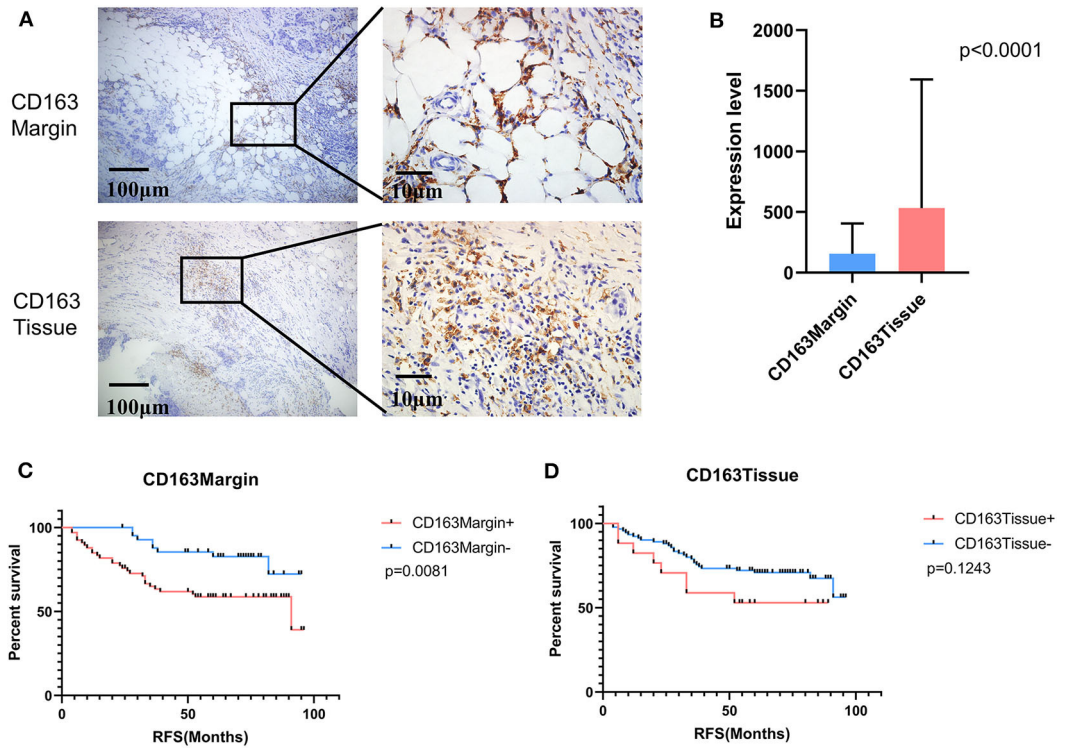

FIGURE 1 | The expression of CD163 in tumor invasive margins and malignant tissues was associated with recurrence-free survival (RFS). **(A)** The positive expression of CD163 in tumor invasive margins and malignant tissues, respectively. **(B)** A comparison of the expression levels of CD163 in tumor invasive margins and malignant tissues. **(C,D)** Kaplan-Meier survival analysis of patients with CD163-positive and -negative IHC staining in the margin or tumor tissues, respectively.

MCT1 expression was also positively associated with the level of CD163Tissue (**Figure 3E**, $r = 0.209$, $p = 0.030$). The IHC and IF results showed similar co-occurrence of MCT1 and CD163 staining in the malignant tissue (**Figures 3F,G**). However, only 12 (11.1%) specimens had high expression of both MCT1 and CD163Tissue, and this combined expression only displayed a significant association with PR status ($p = 0.002$) and increased Ki67 staining ($p < 0.0001$) (**Table 1**). The Kaplan-Meier analysis showed similar results: the RFS of patients with high infiltration of $MCT1^+CD163^+$ macrophages in the tissue was much shorter (**Figure 3H**, $p = 0.0026$) than that of patients with low infiltration of $MCT1^+CD163^+$ macrophages in the tissue. Multivariate Cox proportional hazard regressions indicated that high infiltration of $MCT1^+CD163^+$ macrophages in the tissue might not be an independent predictor of poor RFS (**Figure 4**, $p = 0.081$; HR = 2.165, 95% CI 0.910–5.153; $n = 108$). These findings suggest that high infiltration of $MCT1^+CD163^+$ macrophages in the tissue is not superior to high infiltration of $MCT1^+CD163^+$ macrophages in the margin for the prediction of breast cancer progression.

Validation in the TCGA Database

To explore whether the correlation between MCT1 and CD163 also existed in additional breast cancer cases, we downloaded breast cancer expression files from The Cancer Genome Atlas (TCGA) database. Although mRNA expression of MCT1 or CD163 alone was not significantly associated with overall survival (data not shown) or recurrence-free survival (**Supplementary Figures 2A,B**), there was a significant correlation between MCT1 and CD163 expression (**Supplementary Figure 2C**) and between the high mRNA expression of both MCT1 and CD163 and shorter overall survival (**Supplementary Figure 2D**), which may be a potential prognostic marker for breast cancer.

DISCUSSION

There are increasing studies concentrating on tumor-infiltrating lymphocytes, including T lymphocytes, macrophages and mast cells, as well as the spatial distribution of these cells (21, 22, 26). Tumor-associated macrophages are important cells involved in the tumor microenvironment and participate in tumor progression, immune suppression, metastasis, and tumor angiogenesis through cross-talk with tumor cells and other stromal cells. Here, we showed that the expression of MCT1 and CD163 on macrophages in the infiltration boundary of breast cancer was significantly increased and can be regarded as a useful biomarker for predicting rapid progression. Likewise, overexpression of both MCT1 and CD163 by macrophages in the adjacent tissue may serve as a high-risk factor for poor prognosis in breast cancer patients.

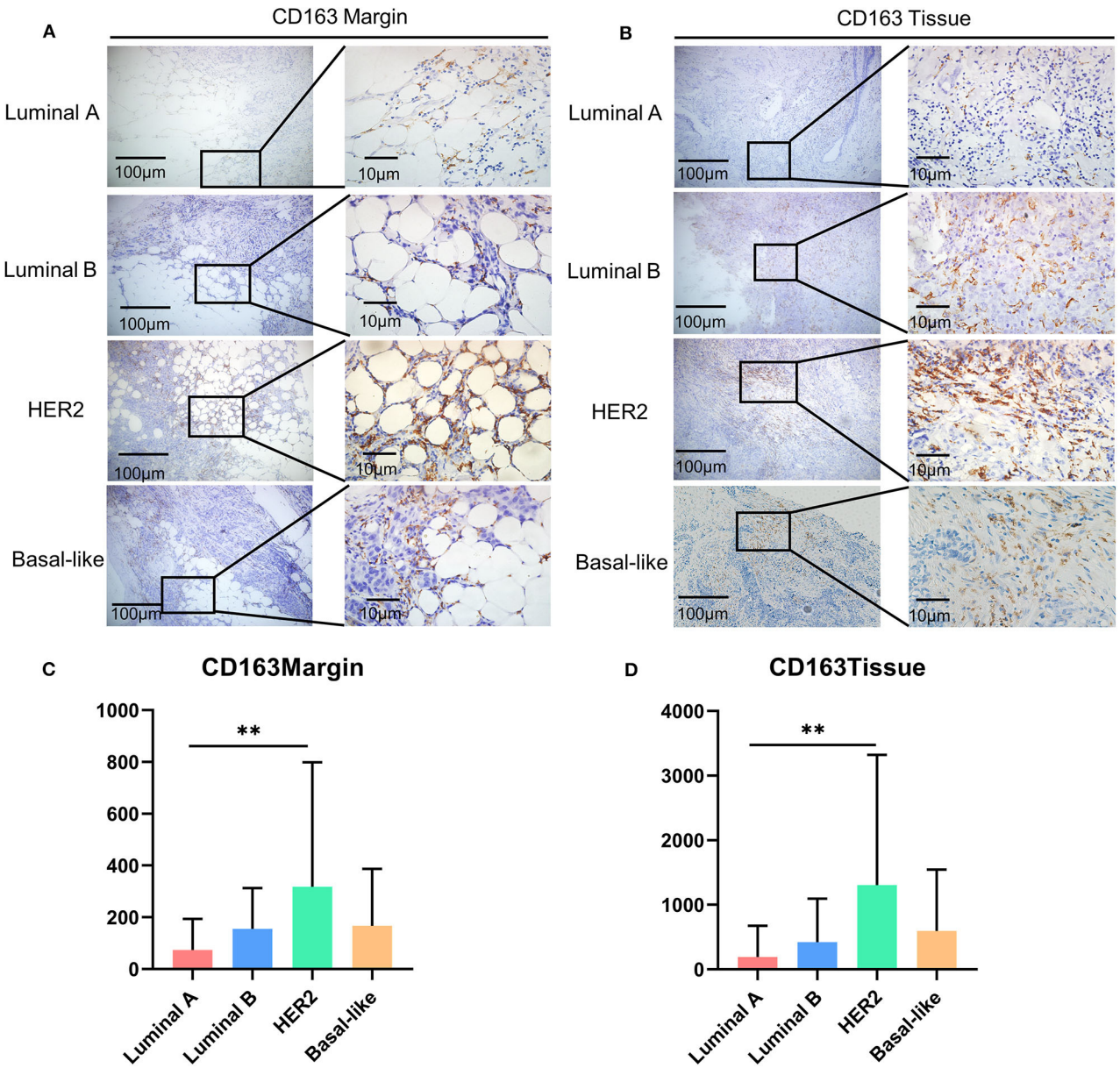

FIGURE 2 | The expression of CD163 among molecular subtypes of breast cancer. **(A)** Representative images of CD163 protein abundance in the margin in different subtypes. **(B)** A comparison of expression levels of CD163 in the margin in different subtypes. **(C)** Representative images of CD163 protein abundance in malignant tissues from different subtypes. **(D)** A comparison of CD163 expression levels in malignant tissues from different subtypes. $^{**}p < 0.01$.

The impacts of TAMs on clinicopathological features and survival outcomes partially depend on their spatial distribution (27, 28), which is consistent with the finding of the present study that high numbers of $CD163^+$ macrophages are an unfavorable clinicopathological feature. In gastric cancer, the number of infiltrating macrophages in the malignant tissues was much higher than that in peritumoral tissues; however, infiltration of TAMs into the tumor core was not correlated with any clinicopathological characteristics, but the presence of TAMs in the invasive front was associated with poor prognosis and unfavorable survival (27, 28). The TAMs exhibited a more M2-like phenotype at the margin, while a significant increase in the proportion of M1-like TAMs was observed in the core (20). Mechanically, some studies have speculated that TAMs in the core of tumor are protective because they secrete signals to kill tumor cells (29, 30). In contrast, TAMs located in the invasive margin are immunosuppressive, promote tumor progression and facilitate tumor evasion. Overexpression of cytokines in the TME, such as chemokine (C–X–C motif) ligand 2 (CCL2) and CCL5 (1), contributes to the progression of breast cancer.

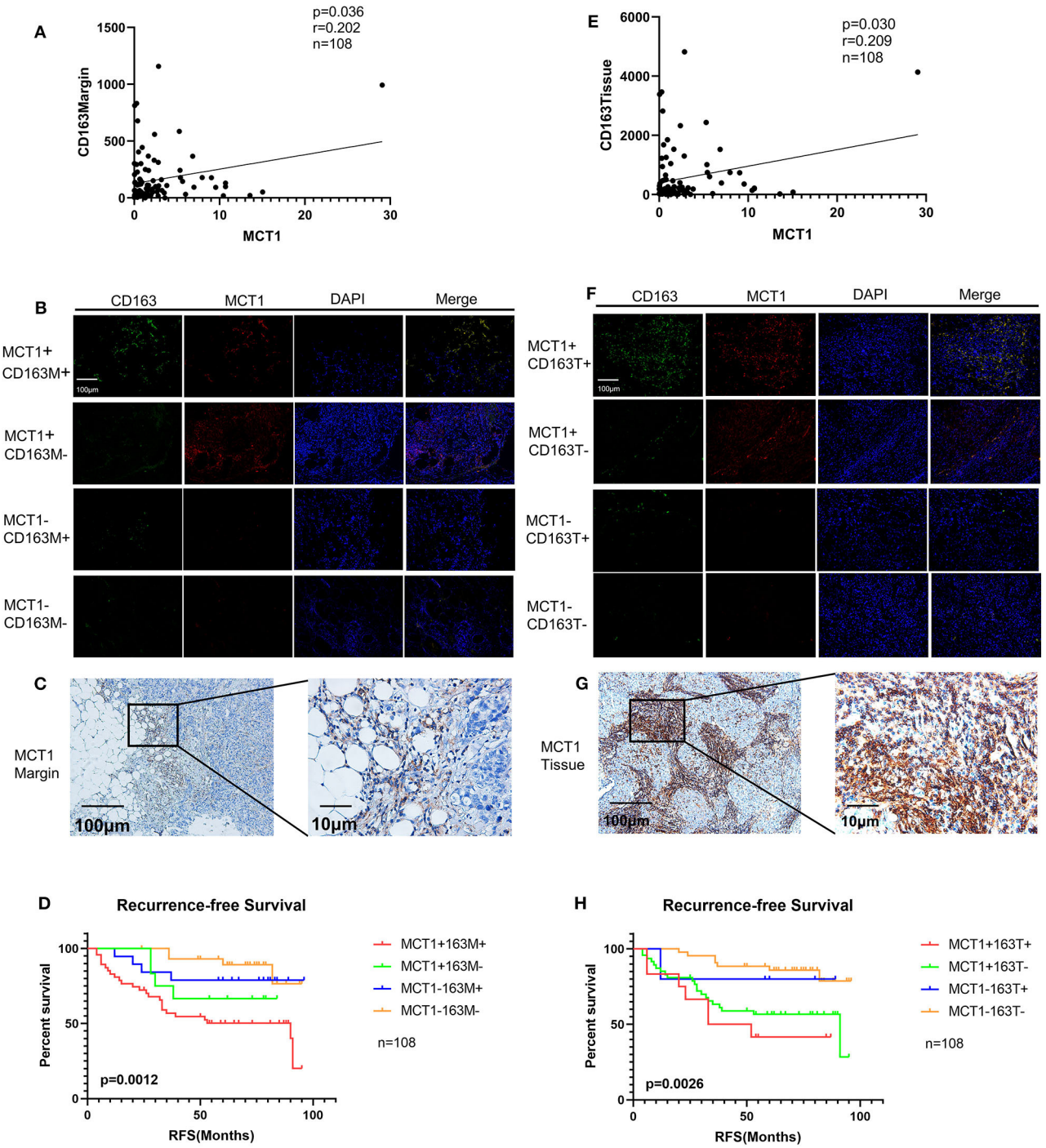

FIGURE 3 | Increased infiltration of MCT1^{+}CD163^{+} macrophages is correlated with poor prognosis. **(A)** Correlation analyses between the protein expression levels of MCT1 and CD163 in the margin. **(B)** Representative IF images of MCT1 and CD163 in the margin (red immunofluorescent signal for MCT1 and green immunofluorescent signal for CD163). **(C)** Representative images of MCT1 in the margin. **(D)** Kaplan-Meier survival analysis of patients with biomarker-positive and -negative IHC staining in the margin. **(E)** Correlation analyses between the protein expression levels of MCT1 and CD163 in malignant tissues. **(F)** Representative IF images of MCT1 and CD163 in malignant tissues. **(G)** Representative image of MCT1 in malignant tissues. **(H)** Kaplan-Meier survival analysis of patients with biomarker-positive and -negative IHC staining in the tissues.

CCL2 recruits more macrophages into the tumor to promote lymphatic metastasis via VEGF-C secretion (31). In addition, elevated CCL2 induces the secretion of chemokine (C–X–C motif) ligand 12 (CXCL12) in macrophages, which acts on blood vessels to enhance angiogenesis (32). Moreover, increased CCL5 binding to CCR5 activates the protein kinase B/mechanistic

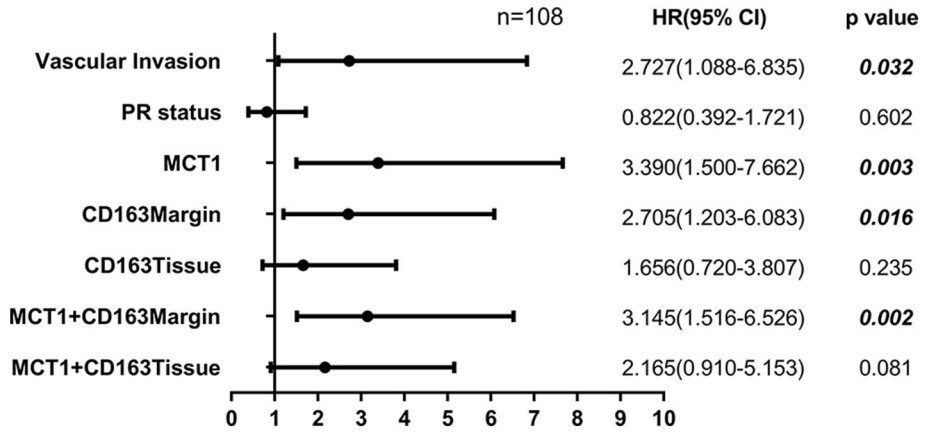

FIGURE 4 | Forest plot showing the results of multivariate Cox proportional hazard regression analyses.

target of rapamycin (AKT/mTOR) signaling pathway to promote tumor cell growth and invasion and induces the production of matrix metalloproteinase (MMPs) by macrophages to decrease adhesion and facilitate migration (33). Our results established that the infiltration of TAMs into the tumor margin rather than into the malignant tissues was significantly associated with poor prognosis in breast cancer patients. Further studies are needed to clarify the potential mechanisms by which TAM spatial distribution influences human solid tumors.

The subtype and number of tumor-infiltrating lymphocytes track with tumor heterogeneity. P53 gene mutation is common in multiple tumors, and inactivating mutations of P53 have been associated with reduced immune infiltration (34). Interestingly, induction of P53 resulted in increased expression of colony-stimulating factor 1 (CSF1), CCL2, CXCL1, and IL-15 as well as of the adhesion molecules intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), which further recruit natural killer (NK) cells to trigger tumor regression, arguing that oncogenic pathways might also influence immune cell types. Previous studies revealed that $CD163^+$ macrophages were positively correlated with lymph node metastasis, hormone receptor negativity, and Ki67 positivity (35–37). The present study validated this relationship between CD163 expression and clinicopathological features by showing that CD163 expression was lower in PR-positive tumors that had a low proliferation level than in highly proliferative PR-negative tumors, and high CD163 expression was associated with poor survival outcome. In addition, this study showed the highest infiltration level of $CD163^+$ macrophages in the HER2 subtype. It has been well-established that in response to the Th2 cytokines interleukin-4 (IL-4) and interleukin-13 (IL-13), macrophages undergo alternative activation, gaining abilities to support tumor growth and inhibit antitumor immunity (38). The expression of IL-4 and IL-13 was similarly correlated with hormone receptor status, and IL-4 was increased in samples with an ER-negative status (39, 40). IL-4 is generated by both tumor cells and stromal cells, and IL-4 neutralization resulted in reduced levels of the chemokines CCL2, CCL11, and CXCL5 in the TME (41). Another study demonstrated that high expression of the plasma membrane receptor for IL-13 (IL-13Rα1) was observed in breast cancer patients with HER2 positivity (42). Therefore, increased levels of IL-4 and IL-13 may partially explain the higher infiltration of $CD163^+$ macrophages in the HER2 subtype. Given the wide range of changes in chemokine production associated with dysregulation of the HER2 pathway, additional studies will be needed to investigate which immune cell types are affected in patients with distinct types of cancer.

MCT1 functions as a transporter of lactate and has been reported to be generally expressed in various human tumors, including prostate, colon, breast, and lung tumors (43). In line with a previous study (25), the present study demonstrated that high expression of MCT1 was significantly associated with poor prognostic clinicopathological parameters, including PR-negative status and proliferation, as MCT1 was correlated with Ki-67 positivity. Therefore, MCT1 contributes to the aggressive features and is an independent prognostic factor for breast cancer. In addition to participating in tumor metabolism, as the IF results showed, MCT1 and CD163 were colocalized on macrophages, and MCT1 may participate in the lactate uptake into $CD163^+$ macrophages in the high-lactate TME. MCT1 played a role in suppressing the phagocytosis of tumor-associated macrophages (44). In glioblastoma, branched-chain ketoacids excreted from tumor cells were taken up by TAMs through MCT1 and were converted to branched-chain amino acids, which attenuated the phagocytosis by TAMs. Furthermore, lactate, another important substrate of MCT1, can induce alternative polarization of macrophages (45). Mechanically, lactate activated the extracellular regulated protein kinase/signal transducer and activator of transcription 3 (ERK/STAT3) signaling pathway to stimulate M2 macrophage polarization to promote proliferation, migration, and angiogenesis in breast cancer, which were abolished with the suppression of ERK/STAT3 signaling (46). On the other hand, lactate activated macrophage G protein-coupled receptor 132 (Gpr132) to promote an alternatively activated macrophage (M2)-like phenotype, which in turn facilitated cancer cell migration and invasion to promote lung metastasis in breast cancer (47). However, MCT4, which facilitates lactate efflux, was highly expressed in the surrounding stromal cells (48).

Therefore, in the tumor invasive margin, macrophages with high expression of MCT1 uptake large amounts of lactate, leading them to have immunosuppressive effects in the TME. However, in the core of tumor tissues, due to an insufficient supply of nutrients, tumor cells preferentially consume lactate, which restricts the uptake of lactate by macrophages, thereby resulting in reduced immunosuppressive effects. The present study revealed that there was a significant correlation between MCT1 positivity and CD163 positivity on macrophages; however, the underlying mechanisms are worthy of further investigation.

This is the first attempt to correlate monocarboxylate transporters with macrophages utilizing immunohistochemistry and immunofluorescence imaging methods. We demonstrated that alternations of metabolic-associated proteins are greatly associated with the infiltration and polarization of macrophages in the TME. Increased infiltration of $MCT1^{+}CD163^{+}$ macrophages in the margin, rather than in the malignant tissues, was associated with poor prognosis for breast cancer patients and was an independent risk factor for predicting rapid progression of breast cancer. This increased infiltration will be a promising therapeutic target to impede breast cancer progression.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of the Renmin Hospital of Wuhan University (approval no. 2018K-C09). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BL, ZX, and SiS are responsible for collecting and collating documents. BL, QY, and ZL performed these experiments. BL and QW are responsible for writing this article, while ShS is responsible for proofreading and submission. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.574787/full#supplementary-material

Supplementary Figure 1 | (A) Comparison of CD163 protein expression data for 108 breast cancer specimens and 12 controls. **(B)** Comparison of CD163Margin and CD163Tissue in 12 benign controls. **(C)** Comparison of the CD163 expression in the margin and tissues without HER2 subtype sample. **(D)** Kaplan-Meier survival analysis of patients with MCT1-positive and MCT1-negative IHC staining. **(E)** Comparison of the CD163 expression in $MCT1^{-}$ and $MCT1^{+}$ groups.

Supplementary Figure 2 | (A) Kaplan-Meier survival analysis of patients with SLC16A1 (MCT1)-positive and -negative mRNA expression from the TCGA database. **(B)** Kaplan-Meier survival analysis of patients with CD163-positive and -negative mRNA expression from the TCGA database. **(C)** Correlation analyses between the mRNA expression levels of SLC16A1 and CD163. **(D)** Kaplan-Meier survival analysis of patients with biomarker-positive and -negative samples.

Supplementary Table 1 | Antibody information.

Supplementary Table 2 | Clinicopathological associations of CD163-Margin expression in breast cancer.

Supplementary Table 3 | Clinicopathological associations of CD163-Tissue expression in breast cancer.

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Cancer-Associated Fibroblasts and Tumor-Associated Macrophages in Cancer and Cancer Immunotherapy

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Our understanding of the tumor microenvironment (TME), including the interplay between tumor cells, stromal cells, immune cells, and extracellular matrix components, is mandatory for the innovation of new therapeutic approaches in cancer. The cell-cell communication within the TME plays a pivotal role in the evolution and progression of cancer. Cancer-associated fibroblasts (CAF) and tumor-associated macrophages (TAM) are major cell populations in the stroma of all solid tumors and often exert protumorigenic functions; however, the origin and precise functions of CAF and TAM are still incompletely understood. CAF and TAM hold significant potential as therapeutic targets to improve outcomes in oncology when combined with existing therapies. The regulation of CAF/TAM communication and/or their differentiation could be of high impact for improving the future targeted treatment strategies. Nevertheless, there is much scope for research and innovation in this field with regards to the development of novel drugs. In this review, we elaborate on the current knowledge on CAF and TAM in cancer and cancer immunotherapy. Additionally, by focusing on their heterogenous functions in different stages and types of cancer, we explore their role as potential therapeutic targets and highlight certain aspects of their functions that need further research.

Keywords: cancer-associated fibroblasts, tumor-associated macrophages, tumor microenvironment, cancer immunotherapy, cancer biology

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INTRODUCTION

Originating from the neighboring healthy tissues and recruited from the circulation, a multitude of proliferating non-neoplastic cells such as fibroblasts, macrophages, immune cells, and endothelial cells contribute to carcinogenesis within the tumor microenvironment (TME) (1). Cancer-associated fibroblasts (CAF) and tumor-associated macrophages (TAM) are the major cell populations within the stroma of all solid tumors in which they often exert protumorigenic functions. Although their precise interactions remain to be elucidated, CAF and TAM strongly modulate disease progression, therapy resistance, and clinical outcomes (2–7) and may function in synergy.

Targeting the cytokines, inhibitory immune checkpoint ligands expressed by CAF and TAM, and antiphagocytic signaling by tumor cells have shown some efficacy in preclinical trials. The results of clinical trials are nonetheless ambiguous. Antibodies, chemokines, and chemokine ligands

that interfere with CAF/TAM interactions, and their combinations hereof, are highly prioritized in experimental clinical regimens that are aimed at modulating the TME (8).

THE FIBROBLAST

Fibroblasts can be clearly identified and characterized by their elongated morphology, the lack of epithelial, endothelial, leukocytic, and malignant-cell markers, and the positivity for mesenchymal markers such as vimentin. Under normal circumstances, fibroblasts are present in abundance in the connective tissues in a dormant state, transiently being activated during periods of tissue remodeling and repair. They are involved in the production of extracellular matrix (ECM) and modulation of inflammation, as well as the proliferation and differentiation of epithelial cells.

Well-established fibroblast-activating signals include inflammatory mediators, transforming growth factor-beta (TGF-β), and lysophosphatidic acid which increase the activity of SMAD transcription factors and drive the expression of alpha-smooth muscle actin (α-SMA) that provides the fibroblast with a highly contractile phenotype (usually known as myofibroblast or α-SMA$^+$ fibroblast). The activated fibroblasts produce chemokines and cytokines to regulate the communication with other mesenchymal, epithelial, and immune cells (9). Importantly, all of these functions are utilized and enhanced in cancer (10, 11).

CANCER-ASSOCIATED FIBROBLASTS

Structure and Functions

Within a tumor, the mesenchymal cells that comply with the aforementioned definitions above, are generally referred to as CAF. Compared with regular fibroblasts, they tend to be slightly larger with darker nuclei and branched cytoplasm. CAF may differentiate from quiescent fibroblasts and bone marrow-derived mesenchymal stem cells or trans-differentiate from epithelial cells, smooth muscle cells, pericytes, and adipocytes (12).

Abbreviations: α-SMA, alpha smooth muscle actin; b-FGF, basic fibroblast growth factor; CAF, cancer-associated fibroblasts; CAFEx, CAF-derived exosomes; CCL, C-C motif ligand; CRC, colorectal cancer; CTC, circulating tumor cell; CXCL, C-X-C motif ligand; CCR, C-C chemokine receptor; CD, cluster of differentiation; CSF1R, colony-stimulating factor 1 receptor; CSM, consensus molecular subtypes; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; FAP, fibroblast activation protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; HCC, hepatocellular carcinoma; IL, interleukin; myCAF, cancer-associated myofibroblast; miR, micro-RNA; MMP, matrix metalloproteinase; OPN, osteopontin; PDAC, pancreatic ductal adenocarcinoma; PD-1, programmed death receptor 1; PD-L1 and 2, programmed death receptor ligand 1 and 2; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth receptor; STAT3, signal transducer and activator of transcription 3; TAM, tumor-associated macrophages; TGF, transforming growth factor; TME, tumor microenvironment; TNF, tumor necrosis factor; TSE, tumor-derived exosomes; TSF, tumor-derived factors; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

CAF are present during all stages of solid malignancies (13) and their functional impact on the biology of cancer is assumed to be similar across all tumor types (14).

CAF are the predominant cell type in the tumor stroma and they contribute to the proliferative, pro-inflammatory, immunosuppressive, angiogenic, pro-invasive, and pro-metastatic TME that is required for the evolution and progression of cancer (15).

Inflammatory mediators such as TGF-β, interleukin (IL)-1, and IL-6 produced by tumor cells and non-malignant stromal cells promote CAF activation and contribute to a pro-inflammatory profile, that directly support carcinogenesis (16). The activation of specific transcriptional programs and the lack of negative feedback mechanisms launch CAF into self-sustaining trajectories (17, 18).

Additionally, CAF drive the epithelial-mesenchymal transition (EMT), whereby cancer cells lose polarity and adhesion molecules and gain the motility necessary for dissemination (19). Despite the overall pro-tumorigenic effects, functional dualities have been observed. A hypothesis is that initially CAF are tumor suppressive but as cancer evolves they transform into pro-tumorigenic cells (20).

Heterogeneity of CAF Subtypes

Within a multi-clonal solid tumor, CAF are differentially exposed to a multitude of tumor secreted factors (TSF) explaining their heterogeneity. However, the essential molecular mechanisms underlying the activation and pro-tumorigenic activities of fibroblasts may be common to various cancers, which present a manifold of targets for innovative CAF-targeted therapies. Signaling cascades mainly involve the Wnt/β-catenin, TGF-β, epidermal growth factor receptor, JAK/STAT, and Hippo pathways.

Several studies have characterized distinct CAF subgroups that differentially express the CAF markers, e.g. α-SMA, fibroblast activation protein (FAP), and platelet-derived growth factor receptor (PDGFR), and show that CAF subpopulations may have various and even opposing functions. Tumor-suppressive CAF populations have been characterized by activated Hedgehog signaling pathways in mouse models of colon, pancreatic, and bladder cancers. However, the full complement of CAF populations remains unclear, and more detailed classifications and functions of CAF subtypes are needed (21–25).

In a mouse model of pancreatic ductal adenocarcinoma (PDAC), the ablation of CAF led to enhanced hypoxia, EMT, increased vascularity, cancer cell proliferation, and disease progression demonstrating that CAF to some extent can restrain tumor growth (26, 27). Similarly, an initial expansion of local fibroblasts circumscribing early or premalignant lesions in response to tissue neoplasia was observed in mouse models and human tissue studies (14, 28, 29).

Thus, the TME comprises a heterogeneous population of CAF subtypes or clusters with different functions associated with immunomodulation, immunosuppression, and immunotherapy resistance (30).

Furthermore, in a mouse model on early and late PDAC stages, fibrosis associated with type I collagen provided a protective response from the host rather than a pro-tumorigenic response (26).

These results demonstrate that at least some stromal constituents may restrain rather than promote tumor progression and illustrate the high degree of temporal differentiation plasticity within the diverse cell populations of tumors. This may also explain the conflicting reports regarding antitumor and pro-tumor functions of CAF.

In a preclinical trial on lung cancer, the depletion of CAF significantly reduced the number of metastases (31, 32). To establish the clinical relevance of primary tumor CAF in the formation of metastasis, this research group examined human brain metastases (since the normal brain is devoid of fibroblasts) from lung, breast, kidney, and endometrium, and found a distribution of activated CAF within these metastases. These findings support the view that the CAF shed from the primary tumor, together with cancer and non-tumor cells from the TME, survive during the blood circulation and proliferate at the metastatic site (31).

With respect to human PDAC specimens, the patients with a higher expression of FAP were found to be associated with shorter disease-free survival and overall survival when compared to those with low FAP expression (33). The immune suppression caused by FAP^+ CAF is mediated by the CXCL12 receptor CXCR4 that excludes T cells from the tumor. Notably, CXCR4 inhibition leads to an elimination of tumor cells by a rapid accumulation of cytotoxic $CD8^+$ T cells (34). Moreover, the deletion of FAP^+ CAF using a FAP-targeted immune-based therapeutic approach or a genetic ablation approach inhibited cancer growth in murine PDAC models (32, 35). Thus, the inhibition of CAF-induced pro-tumorigenic signals is a highly attractive future strategy to improve outcomes in pancreatic cancer.

In human triple-negative breast cancer, a subset of CAF with myofibroblast characteristics (myCAF) (α-SMA^+/FAP^+ or S1 CAF) was identified as a key player in immunosuppression through the attraction of T_{regs} and inhibition of effector T cell proliferation (36) and it was hypothesized that targeting the CAF-S1-mediated immunosuppression could enhance anti-tumor immunity.

In PDAC, CAF are linked to worse overall survival. PDAC is infamous for the abundance of fibrotic ECM with the majority of the tumor volume being composed of α-SMA^+ CAF. Preclinical and clinical trials targeting stromal α-SMA^+ CAF, however, resulted in an apparent, paradoxical acceleration in disease progression and reduction in survival, halting clinical trials and adding further layers of complexity to CAF functions (26, 37).

Another study on murine models of lung carcinoma and PDAC revealed that the deletion of FAP led to a significant reduction in CAF infiltration and tumor tissue necrosis, and an increase in infiltration of $CD8^+$ T cells (38). Moreover, in murine models of breast and colon cancer, the administration of a DNA-based vaccine targeting FAP induced the killing of CAF by $CD8^+$ T cells and lead to a substantial increase in the uptake of chemotherapeutic agents by otherwise multi-drug-resistant cancer cells (39). Further, in immunocompetent mice, the cell transfer of FAP-specific chimeric antigen receptor T cells boosted host immunity and arrested pancreatic tumor growth; however, it also led to significant lethal toxicity and cachexia (40). These examples indicate that specific CAF subsets could be potential targets for improving immunotherapy. Future studies are needed to develop targeted therapies aimed at specific CAF populations (41).

Secreted Factors and Exosomes in CAF-Tumor Cells Interplay

The cytokines and chemokines produced by CAF may have both immunosuppressive and immuno-activating effects on various leukocytes, including $CD8^+$ T cells, immunosuppressive regulatory T cells (T_{regs}), and macrophages (**Figure 1**). However, the consensus is that the overall effects of CAF are immunosuppressive (14). IL-6, CXC-chemokine ligand (CXCL) 9, and TGF-β, which are produced by CAF, have well-established roles in suppressing anti-tumor T cell responses (34). This is also supported by an inverse association between CAF and $CD8^+$ T cell cytotoxicity.

The staining of the inhibitory immune-receptor ligand programmed death-ligand 2 (PD-L2) and tumor necrosis factor-alpha (TNF-α) ligand OX40L in human breast cancer sections revealed T lymphocytes at the surface of CAF. This confirmed that subsets of CAF attract and retain T lymphocytes at the periphery of the tumor through distinct mechanisms involving chemokine signaling (chemokine ligand [CCL]-11, CXCL12–14), cell adhesion molecules, activation of inhibitory immune checkpoints, and $CD8^+$ T cell anergy (36).

In a murine PDAC model, it was demonstrated that CAF, programmed by TGF-β to express a leucine-rich protein (LRRC15), were associated with a poor response to anti–PD-L1 therapy (42). Additionally, CAF are a source of various growth factors including TGF-β, vascular endothelial growth factor (VEGF), fibroblast growth factor 5, growth differentiation factor 15, hepatocyte growth factor and insulin-like growth factor (43, 44). The secretion of pro-stemness paracrine factors such as insulin-like growth factors, inflammatory cytokines (IL-6 and IL-8), and chemokines (CCL2 and CCL5) promotes the conversion of cancer cells into cancer stem cells and reinforce the stemness of existing cancer stem cells (45–47). Moreover, the secretion of IL-6 make CAF an important mediator of EMT in cancer cells (48, 49).

Exosomes are extracellular vesicles released by all cell types and are found in all bodily fluids (50). They contain genetic material, proteins, and lipids and are essential for intercellular communication. The activation, recruitment, and conversion of fibroblasts into activated CAF depend on TSF and tumor-secreted exosomes (TSE) containing various oncogenic molecules such as microRNAs (miRs), fusion gene mRNAs, long non-coding RNAs, mutated DNA fragments, and a manifold of cell-signaling molecules (51). The circulating levels of exosomal miRNA accurately reflect disease progression and could serve as a prognostic tool among various cancers following resection of the primary tumor (52–58).

In addition to TSF, TSE and CAF-derived exosomes (CAFEx) secreted by tumor cells and CAF, respectively, in the primary tumor are critical mediators of cancer cell-immune cell communication and they drive the formation of pre-metastatic niches (PMN) (59). Moreover, CAF may enter the circulation and promote the development of PMN and subsequent metastatic lesions (60, 61).

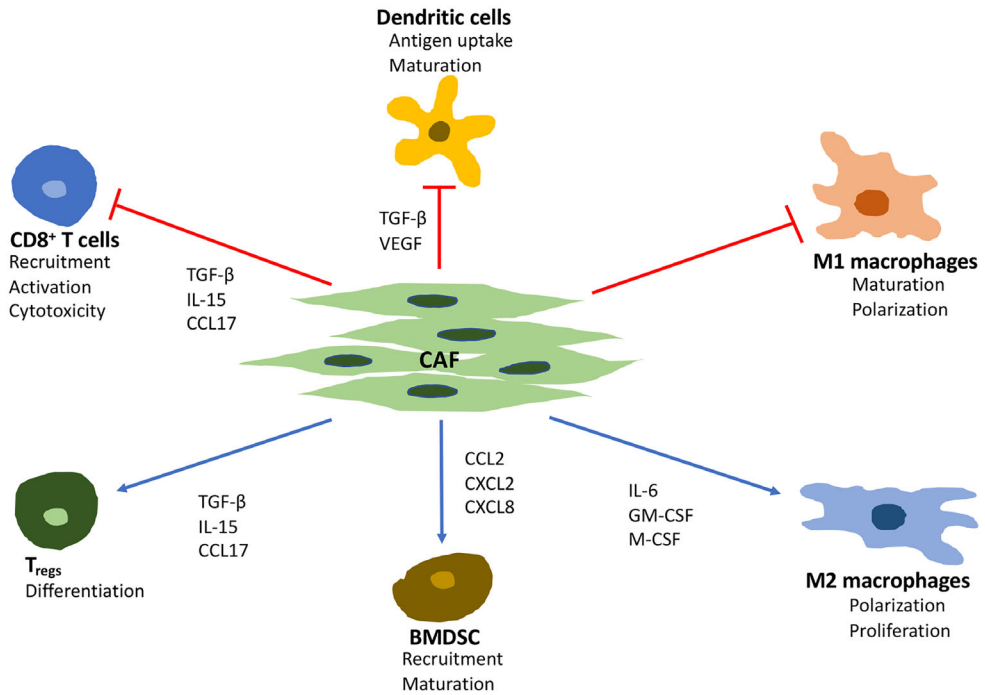

FIGURE 1 | Major effects of CAF on immune cells in the tumor microenvironment. TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor; IL, interleukin 6; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony stimulating factor; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif ligand.

Integrins (ITG) are known to determine tumor cell organotropism. In a mouse model, CAF promoted lung metastasis by the construction of PMN *via* CAFEx. CAFEx-derived ITG$\alpha_2\beta_1$ were found to home to the lung fibroblasts and subsequently activate the TGF-β signaling pathway. To prepare for subsequent colonization of the lung tissue by extravasating circulating tumor cells (CTC), the lung microenvironment is remodeled by the activated lung fibroblasts (58). Surface ITG guide the TSE to organ-specific ECM ligands (collagen, fibronectin, fibrinogen, and E-cadherin) in the target organs, e.g. ITG$\alpha_6\beta_1$ and ITG$\alpha_6\beta_4$ adhere to the epithelial cells and fibroblasts in the lung and ITG$\alpha_v\beta_5$ binds to resident liver macrophages (Kupffer cells) and upregulate the genes for cell migration and S100 protein (62). Organ-specific TSE have been identified for 28 different metastatic cell lines. Furthermore, TSE comprising TGF-β and PDGF mediate the activation, differentiation, and recruitment of CAF through all stages of all solid cancers (13).

In early-stage colorectal cancer (CRC), TSE were found to promote highly proliferative and angiogenic CAF, while those from late-stage metastatic CRC cell lines were observed to induce highly invasive CAF which, through the secretion of ECM-degrading proteases and increased expression of the pro-invasive modulators of membrane protrusion, enabled the penetration of ECM (51).

In addition, TSE alter CAF metabolism and induce the production of CAFEx containing nutrient metabolites (amino acids and tricarboxylic acid cycle intermediates) that fuel the tumor cells and increase their survival (31, 63). A study on breast-cancer cell lines revealed that TSE containing miR-105 could re-program CAF metabolism and enable them to increase glucose metabolism when nutrient levels were sufficient as well as detoxify metabolic wastes into energy-rich metabolites when nutrients were scarce (64).

As shown in PDAC, lactate produced by cancer cells promotes extensive epigenomic reprogramming of CAF (65). In CRC, and during protein deprivation, CAF accumulate fatty acids, phospholipids, and fatty acid synthetase. The uptake of lipid metabolites by the CRC cells secreted by CAF seem to be essential for their migration (66).

Another potent promotor of malignancy is the heat shock factor 1 which is frequently activated in CAF. It drives a program that supports the survival and metastatic potential of cancer cells by inhibiting apoptosis and promoting migration. The activation of heat shock factor 1 has been associated with poor outcomes in CRC, lung-, breast-, and hepatocellular carcinoma (HCC) (67).

Of the important players, the gene that deserves mentioning is the HMG-box 2 (SOX2). It codes for transcription factors controlling the expression of several genes involved in early embryonic development. The upregulated stromal SOX2 drives the reprogramming of colonic fibroblasts that results in enhanced β-Catenin and TGF-β signaling in CRC cells supporting cancer progression. Nonetheless, the precise mechanism remains to be determined (68).

The subset of CAF with myofibroblasts characteristics (myCAF) mediate a chronically deranged wound healing program in tumors and play a key role in the development of a continuously evolving

fibrotic stroma. myCAF are highly responsive to chemokines and metabolically and morphologically distinctive from CAF. When activated, their proliferation rate drops and the production of ECM components increases dramatically. The cytoplasmic microfilaments of myCAF connect to the extracellular fibronectin domains, creating very contractile mechanisms. The following extracellular deposition of collagen reinforces and stiffens the ECM (69).

Not only does it contribute to the increasing stromal density, but the remodeling of the stroma by CAF-produced matrix-enzymes also provides tracks for cancer cell invasion and migration (14). The stromal stiffness results in increased interstitial pressure, abnormal vasculature, collapsed blood vessels, hypoxia, and acidity which lead to inefficient drug delivery and reduced response to therapy. These physical and chemical barriers are hostile to cytotoxic immune cells such as $CD8^+$ T cells and natural killer (NK) cells (70).

CAF and Circulating Tumor Cells (CTC)

The presence of CAF in the circulation of cancer patients and their levels in the peripheral blood correlates with cancer progression and worse prognosis. Notably, the high levels of CAF-CTC aggregates in the blood samples from patients should be considered an important marker of worse clinical outcomes (71). For instance, CTC have higher viability in the blood stream when accompanied by stroma cells that also provide an advantage with respect to early survival and growth of tumor cells at the metastatic site (31). Traveling in clusters with macrophages, immune cells, and platelets, CAF support, shield, and increase the survival of CTC. Adjoining neutrophils may aid in the survival of CTC through the suppression of leukocyte activation (72). Through strong intercellular adhesions, CAF maintained the viability and proliferative capacity of CTC in cellular aggregates in presence of high levels of hemodynamic forces (> 1,000 dyn/cm^2). This protective role was observed in prostate cancer, usually spreading through blood vessels rather than the lymphatic system (61).

Only a minority of CTC travel in clusters; however, in a mouse model, it was estimated that the probability of metastasis formation originating from clusters (and especially those of oligoclonal tumor cell groupings) is fifty times higher compared with that originating from a single CTC (73).

As EMT of tumor cells may proceed within the clusters, the association between neutrophils and CTC drives tumor cell mitosis and expands the metastatic potential of CTC (74). Upon arrival in the PMN, tissue-resident fibroblasts contribute to the mesenchymal-epithelial transition (MET). Thus, CAF are considered key players in promoting the survival of CTC.

Targeting CAF-Associated Pathways

To revert CAF to a quiescent state by targeting the activation pathways is an appealing concept. CAF-secreted Wnt2 accelerates the Wnt/β-catenin signaling pathway which corresponds with the absence of $CD8^+$ T cells. The effects of vitamin D seen in epidemiological studies of PDAC and CRC are partly related to the reduced CAF-related Wnt/β-catenin signaling which was relayed by vitamin D metabolites (**Table 1**) (75).

Alternatively, targeting CAF-derived cytokines and chemokines (e.g. CXCL, IL-6, and TGF-β) could improve anticancer efficiency in combination with immunotherapy. Several IL-6 inhibitors such as sarilumab and tocilizumab that are already approved for autoimmune and myeloproliferative disorders, are being investigated for their role in anticancer therapy either alone or in combination.

Anti-TGF-β in combination with anti-PD-L1 antibodies inhibited TGF-β signaling in CAF and facilitated T cell penetration into solid tumors (76). A summary of RCT examining the effects of targeting IL-6 and TGF-β have been presented in **Table 2**. The complexity and incomplete understanding of CAF functions necessitate further research before anti-CAF targeted therapy can be integrated into clinical practice.

THE MACROPHAGE AND ITS M1 AND M2 SUBTYPES

Representing another major stromal cell population, macrophages are remarkable, heterogenic, and versatile cells. These cells are capable of switching functions and phenotypes, depending on their unique microenvironment (77). They engulf

TABLE 1 | Clinical trials targeting Wnt/β-catenin signaling related to CAF in different types of cancer.

Cancer type	Trial number	Target	Mechanism of action	Treatment/Intervention
CRC	NCT04094688	CAF-related Wnt/β-catenin signaling	Wnt pathway: Vitamin D3 promotes the upregulation of DKK-1 (tumor suppressor) and downregulation of DKK 4 β catenin: Vitamin D3 promotes VDR-dependent inhibition of β-catenin (1)	High dose vitamin D3 + FOLFOX/FOLFIRI + Bevacizumab
PDAC	NCT03520790			Gemcitabine + Nab-paclitaxel + Paricalcitol IV/oral
Melanoma	NCT01748448			Vitamin D
Urothelial cancer	NCT04197089			Vitamin D
Prostate cancer	NCT03103152			High/Low dose Aspirin + Vitamin D
Gynecologic cancers	NCT03192059			Vitamin D + Aspirin + Cyclophosphamide + Lansoprazole + Pembrolizumab + Radiation + Curcumin
Breast cancer	NCT02786875			Low glycemic diet, Physical activity, and Vitamin D

FOLFOX: leucovorin, 5-fluorouracil, and oxaliplatin; FOLFIRI: leucovorin, 5-fluorouracil, and irinotecan

CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; CAF, cancer-associated fibroblasts; VDR, vitamin D receptor; DKK 1, DICKKOPF 1.

(1) Pendás-Franco, Natalia et al. "Vitamin D and Wnt/beta-catenin pathway in colon cancer: role and regulation of DICKKOPF genes." Anticancer research vol. 28,5A (2008): 2613-23.

TABLE 2 | Clinical trials targeting CAF associated pathways involving IL-6 and TGF-β in different cancers.

Cancer type	Trial number	Target	Mechanism of action	Treatment/Intervention
Pancreatic cancer	NCT02767557	IL-6	Anti-IL-6 antibody	Tocilizumab + Nab-paclitaxel + Gemcitabine
Melanoma	NCT03999749	IL-6		Tocilizumab + Nivolumab + Ipilimumab
Prostate cancer	NCT03821246	IL-6		Tocilizumab + Atezolizumab + Etrumadenant
Esophageal cancer	NCT04595149	TGF-β + PD-L1	Bifunctional antibody against 3 isoforms of TGF-β and PD-L1 (1)	Paclitaxel + Carboplatin + Bintrafusp alfa + Radiotherapy
Head and neck cancer	NCT04247282	TGF-β + PD-L1		Bintrafusp alfa alone/+ TriAd vaccine + N-803
HPV-associated cancers	NCT04432597	TGF-β + PD-L1		PRGN-2009 alone/+ Bintrafusp alfa

Bintrafusp alfa, Anti-PD-L1/TGF-Beta Trap; N-803, IL-15 super agonist; TriAd vaccine, novel agent targeting 3 human tumor-associated antigens-CEA, MUC1, and brachyury; PRGN-2009, HPV vaccine.

IL, interleukin; TGF-β, transforming growth factor β; PD-L1, programmed death-ligand 1; HPV, human papillomavirus.

(1) Lind, Hanne et al. "Dual targeting of TGF-β and PD-L1 via a bifunctional anti-PD-L1/TGF-βRII agent: status of preclinical and clinical advances." Journal for immunotherapy of cancer vol. 8,1 (2020): e000433. doi: 10.1136/jitc-2019-000433.

tissue and microbial debris; orchestrate inflammatory processes (78); and contribute to tissue remodeling, angiogenesis, and homeostasis. The conventional binary model distinguishes between the M1 and M2 macrophages.

The M1 subtype consists of classically activated, pro-inflammatory macrophages with bactericidal, tumor-suppressive, and anti-angiogenic functions. They express inducible nitric oxide synthase (CD86 and CD169) and are activated through their pattern recognition receptors upon recognition of damage- or pathogen-associated molecular patterns such as bacterial lipopolysaccharides and DNA damage. They produce inflammatory cytokines (e.g. IL-1β, IL-6, IL-12, IL-23, and TNF-α), proliferate, and self-renew in a macrophage colony-stimulating factor 1 (M-CSF1)- and granulocyte-macrophage (GM)-CSF-dependent manner (79).

The M2 subtype, the alternatively activated macrophages expressing CD163, CD206, and CD204, are commonly known as TAM. They are characterized by the production of anti-inflammatory, immunosuppressive chemokines and cytokines, such as IL-4, IL-6, IL-8, IL-10, IL-13, and TGF-β (80, 81), and are devoid of cytotoxic activity. They produce various growth factors, such as basic fibroblast growth factor (b-FGF), placental growth factor, insulin-like growth factor, epidermal growth factor (EGF), VEGF, and PDGF (82).

It should be emphasized that macrophages are extremely plastic. Many context- and tissue- dependant phenotypes on the spectrum between M1 and M2 exist, depending on multiple factors of stimulation, and these in-between phenotypes are not captured by the classical nomenclature. A more comprehensive classification system that takes the dynamic nature of macrophages into account has been proposed but so far not adopted in the literature (83).

Although their origin is still debated, it is generally believed that macrophages originate *via* common dendritic cell precursors in blood, spleen, and from bone marrow hematopoietic stem cell-derived progenitors with myeloid restricted differentiation. Embryonic precursors may seed tissues already in the fetal period and become tissue-resident macrophages (84). Attracted by chemokines, macrophage progenitors enter the circulation from reservoirs in the bone marrow and spleen. They leave the peripheral blood flow and migrate to tissues where local growth factors and cytokines control their differentiation (85).

TUMOR-ASSOCIATED MACROPHAGES

The level of infiltrating TAM correlates with tumor progression and reduced survival in patients (**Figure 2**). Growth factors and immunosuppressive cytokines produced by TAM can enhance motility, intravasation, and invasion of tumor cells, as well as stimulate angiogenesis and prevent attacks by T cells and NK cells (90, 91) as observed in various tumor types including carcinomas, sarcomas, and lymphomas (92–94). The recruitment of macrophages and their differentiation into TAM are primarily promoted by TSF and CAF-derived factors such as M-CSF1, GM-CSF, CCL2, VEGF, IL-6, and IL-8 (95); and are related to local anoxia, acidity, and inflammation. The infiltration into the TME is determined by CC chemokines such as the C-C motif ligands CCL2, CCL11, CCL16, and CCL21 produced by local lymphatic endothelial cells and stromal cells as demonstrated in breast-, lung-, oesophageal-, ovarian-, and cervical cancers (96, 97). Especially CCL2 exhibits strong chemotactic activity for macrophages. Producing CCL2 themselves, macrophages recruit macrophages in a feed-forward loop.

Homing towards increasing gradients of chemotactic molecules, TAM massively infiltrate hypoxic/necrotic regions of tumors and survive by shifting their metabolism towards glycolysis (98). Hypoxic TAM express the transcription factor hypoxia-inducible factor 1α and secrete VEGF, b-FGF, PDGF, cyclooxygenase-2, prostaglandin E2, and MMPs (99, 100). In response to hypoxia, TAM also overexpress PD-L1, PD-L2, and cytotoxic T-lymphocyte-associated protein 4 ligands that contribute to immune cell dysfunction and limit the effects of checkpoint inhibitors (101, 102). Furthermore, the high levels of IL-10 and TGF-β produced by TAM block T cell proliferation and T cell cytotoxicity, while activating T_{regs} (92, 103, 104).

Exploring TAM in Different Cancer Types

Activated TAM are significant prognostic biomarkers for breast cancer, PDAC (105), non-small-cell lung cancer (106), gastric cancer (107), HCC (108), and stage II colon cancer (109).

In breast cancer, TAM produce metalloproteinases (MMP) and cathepsins which degrade the ECM and release angiogenic factors stored in the ECM. TAM-derived MMP-2 and MMP-9 have been correlated to a worse prognosis (110). Using human metastatic breast cancer cells, it was demonstrated that these cells

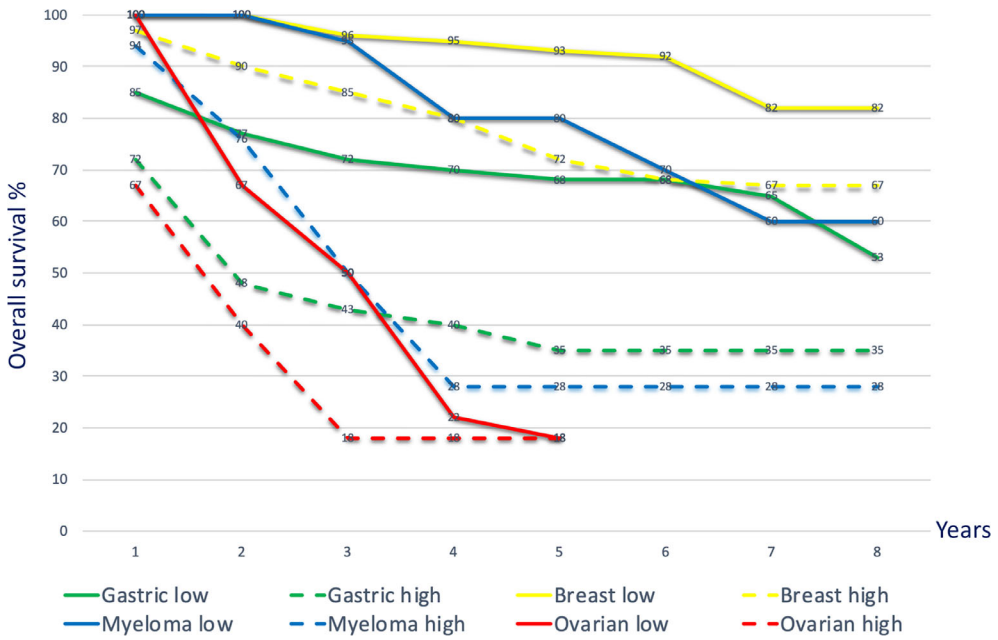

FIGURE 2 | Kaplan–Meier curves depicting overall survival for high and low TAM densities across different cancer types. Overall survival curves, merged data, various cancers. Gastric cancer: Kaplan–Meier overall survival curves for gastric cancer patients with high TAM density (> 671 cells in five 400x microscopic fields; green dotted line) and low density (< 671 cells in five 400x microscopic fields; green solid line). The TAM density in the tumor tissue was negatively associated with overall survival [p=0.0073; (86)]. Breast cancer: Kaplan–Meier curves showing significant correlation (p<0.001) with overall survival according to the numbers of M2 TAM (CD163 high: yellow dotted line; CD163 low: yellow solid line) (87). Multiple myeloma: overall survival outcome based on low and high CD163 TAM (≤ 55 per high power microscopic field; solid blue line) vs. high CD163 TAM (> 55 per high power microscopic field; dotted blue line) showing significant survival difference (p<0.001) (88). Ovarian cancer: Kaplan–Meier survival curves comparing high and low M1 (CD80)/M2 (CD163) ratios in patients with ovarian cancer. Patients with an M1/M2 ratio ≥ 1.4 (solid red line) showed a significantly higher overall survival (p=0.02) than those with an M1/M2 ratio < 1.4 (dotted red line) (89).

stimulate TAM with M-CSF1 and in turn, TAM supply EGF to them. This paracrine feed forward mechanism between tumor cells and TAM facilitates the dissemination, intravasation, and metastatic spread of cancer (111, 112).

In gastric cancer, TAM-derived exosomes that are rich in miRNA, lncRNA, and specific proteins contribute to tumor cell dissemination. Mass spectrometric analysis revealed that these exosomes activated mitogenic signaling through the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in tumor cells, inducing EMT and increasing the metastatic potential (113).

In PDAC, TAM-derived exosomes reportedly contribute to the resistance of tumor cells to gemcitabine. Using a genetic mouse model of PDAC and electron microscopy analyses, it was demonstrated that TAM exosomes are selectively internalized by tumor cells indicating that TAM and tumor cells communicate closely with each other. Furthermore, it was shown that the sensitivity of PDAC to gemcitabine was significantly reduced by the exosomal TAM-derived miR-365 (114).

In non-small-cell lung cancer tissue samples from 104 patients, M1 macrophages and TAM were identified using multiplex immunofluorescence staining. TAM predominated over M1 macrophages in number and proximity to tumor cells, which was linked with tumor cell survival, particularly in the hypoxic regions (109).

In stage II colon cancer, postoperative adjuvant chemotherapy generally has limited effect, with an improved survival rate of less than 5% at 5 years after surgery (115, 116). In a clinical study on human stage II colon cancer, a high density of $CD206^+$ TAM was significantly associated with poor differentiation and worse disease-free survival. A high CD206/CD68 ratio (CD68 being an unspecific marker for the macrophage lineages) was significantly associated with poor differentiation, T4 stage, and lymphatic/vascular/perineural invasion. This ratio was a more reliable prognostic factor than $CD206^+$ TAM density and other traditional clinicopathologic high-risk factors. Notably, the CD206/CD68 ratio identified patients with a low and high risk of tumor recurrence and effectively predicted which patients would benefit from adjuvant chemotherapy (117).

Targeting TAM-Associated Pathways

The field of research exploring the mechanisms by which TAM impact the tumor progression and lower the response to anticancer therapies is very active, and it includes several pharmacological strategies to target TAM. While some strategies revolve around blocking recruitment and depleting TAM through direct inhibition using small molecules and monoclonal antibodies, others focus on reprogramming of TAM.

With respect to TAM recruitment, it has been demonstrated that the blockade of CCR2 suppresses the accumulation of TAM in tumors. CCR2 inhibitors and anti-CCL2 antibodies (CNTO 888) have demonstrated efficacy in reducing tumor growth and metastasis in several pre-clinical murine models (118, 119).

It has been reported across multiple murine tumor- and metastasis models that CCR2 antagonism in combination with anti-PD-1 therapy lead to sensitization and enhanced tumor response over anti-PD-1 monotherapy (120). Additionally, in a clinical trial on PDAC, an objective tumor response was observed in 16 of the 33 patients (49%) receiving a CCR2 inhibitor (PF-04136309) plus FOLFIRINOX, compared to FOLFIRINOX alone (**Table 3**) (121). CCR5 is another receptor which is highly upregulated in metastatic cancers, and a study in mice showed promising response upon treatment with CCR5 antagonist, maraviroc (118, 122).

Furthermore, several trials investigating the effect of dual inhibition of CCR2 and CCR5 in patients with locally advanced pancreatic cancer, CRC, HCC, advanced renal cell carcinoma and non-small-cell lung cancer are underway (**Table 3**).

Another strategy is to deplete TAM by pharmacological blockade of CSF and its receptor CSF-1R, in mono- or combination therapy, preferentially in patients with advanced solid tumors. The depletion of TAM by CSF-1R blockade showed increased infiltration of CD8+ cytotoxic T cells and improved treatment response in murine models of breast, prostate, and cervical tumors (123–125). Inhibition of the CSF-1/CSF-1R axis, using antibodies (AMG 820, IMC-CS4) and small molecule inhibitors like pexidartinib, is presently being explored in phase I/II clinical trials (**Table 4**).

Additionally, the plasticity of macrophages opens up new avenues for reprogramming TAM to switch to an anti-tumor, M1-subtype. While drugs targeting toll-like receptors (imiquimod) are already approved for use, many novel antibodies and fusion proteins targeting CD47/SIRPα axis are under investigation (**Table 5**) (126). Adding to that list, some preclinical trials are currently investigating the use of CAR-T adoptive cell transfer and mRNA tumor vaccines. Theoretically, strategies to reprogram TAM by the delivery of mRNA are attractive, but this research is still in its nascent stages.

To this end, TAM are a promising therapeutic target and further research will benefit in the development of combinational regimens utilizing multifaceted targeting of the cancers.

THE CAF–TAM COLLABORATION

Although CAF and TAM can play both supportive and restrictive roles in carcinogenesis and tumor progression, they are emerging as key players in orchestrating cancer-promoting inflammation and their interactions likely increase the malignancy of tumors (127).

Further to the recruitment of monocytes and M2 polarization, recent data have linked CAF and TAM to a reciprocal interplay with cancer cells. The anti-inflammatory and immunosuppressive M2 phenotype facilitates tumor growth and converts healthy fibroblasts into CAF. Activated CAF secrete factors that promote TAM, cancer cell aggressiveness, EMT, and stemness. In return, cancer cell secrete factors that increase CAF activation and reactivity in a complex that involves various interleukins, chemokines, growth factors and proteinases (128).

As the synergistic interaction between TAM and CAF was only recently identified, only a few studies describe their cell-cell interactions. In CRC and oral squamous cell carcinoma, high levels and combined presence of CAF and TAM within the TME was reported as a negative prognostic factor (7, 129). In high-risk neuroblastoma, pro-inflammatory lipid mediators produced by CAF contributed to tumor growth and were accompanied by a high infiltration of $CD163^+$ TAM (130).

The CAF secretome seems to regulate the composition of tumor-related inflammation, including the presence, phenotypes, and levels of infiltrating TAM (13). In this case, CAF together with tumor cells shape the environment to which monocytes/macrophages are recruited to promote tumor progression (127, 131). To evaluate the effects of CAF on tumor growth and metastasis, monocytes were co-cultured with colon cancer cells and stimulated with colon cancer-activated CAF. The inducible factors that drove monocyte differentiation into pro-invasive TAM were primarily characterized as CAF-derived GM-CSF and IL-6, and are known to regulate the presence of TAM and promote

TABLE 3 | Clinical trials targeting CCR2-CCL2 axis and CCR2/CCR5 in TAM.

Cancer type	Trial number	Target	Mechanism	Treatment/Intervention
Metastatic PDAC	NCT02732938	CCR2	PF-04136309 binds to CCR2 and inhibits	PF-04136309 + Nab-paclitaxel + Gemcitabine
Locally advanced PDAC	NCT01413022		interaction between CCR2 and CCL2	PF-04136309 + FOLFIRINOX
Solid tumors, Bone metastases	NCT01015560	CCR2	Monoclonal antibody	MLN1202
Locally advanced PDAC	NCT03767582	CCR2 +	BMS-813160 is a small-molecule dual	BMS-813160 + SBRT + Nivolumab +/- GVAX
CRC and PDAC	NCT03184870	CCR5	antagonist of CCR2 and CCR5	BMS-813160 alone or combined with: Nivolumab, Gemcitabine, Leucovorin, Irinotecan, Nab-paclitaxel, 5-FU
PDAC	NCT03496662			BMS-813160, Nivolumab, Gemcitabine, Nab-paclitaxel
NSCLC, HCC	NCT04123379			BMS-813160 + BMS-986253 + Nivolumab
Advanced RCC	NCT02996110			Nivolumab + Ipilumab/Relatlimab/BMS-986205/BMS813160
Solid tumors	NCT00537368	CCL2	Anti-CCL2 recombinant monoclonal antibody	CNTO 888 (discontinued)

FOLFIRINOX, 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin; SBRT, stereotactic body radiotherapy; 5-FU, 5-fluorouracil; GVAX, granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected tumor cell vaccine; PDAC, pancreatic ductal adenocarcinoma; CRC, colorectal cancer; HCC, hepatocellular carcinoma; RCC, renal cell carcinoma; NSCLC, non-small-cell lung cancer; CCR2/5, C-C chemokine receptor type 2/5; CCL2, chemokine (C-C motif) ligand 2.

TABLE 4 | Clinical trials targeting TAM through CSF-1 inhibition.

Cancer type	Trial number	Target	Mechanism of action	Treatment/Intervention
PC, CRC, NSCLC	NCT02713529	CSF1R	CSF1R antibody inhibiting binding of	AMG 820 + Pembrolizumab
Solid tumors	NCT01444404		CSF1 and IL34	AMG 820 monotherapy
Advanced solid tumors	NCT02734433	CSF1R, c-KIT,	Multi-targeted receptor tyrosine	Pexidartinib monotherapy
	NCT01525602	FLT3	kinase inhibitor	Pexidartinib + Paclitaxel
Acral and mucosal melanoma	NCT02071940			Pexidartinib monotherapy
PVNS, GCT-TS, TGCT	NCT02371369			Pexidartinib monotherapy
Sarcoma and Malignant Peripheral Nerve Sheath Tumors	NCT02584647			Pexidartinib + Sirolimus (rapamycin)
Metastatic breast cancer	NCT01596751			Pexidartinib + Eribulin
Breast cancer, neoplasms, and angiosarcoma	NCT01042379			Standard/neoadjuvant therapies with novel agents (Pexidartinib in one arm)
Leukemia and solid tumors	NCT02390752			Pexidartinib monotherapy
Prostate cancer	NCT02472275			Pexidartinib + radiation + antiandrogen therapy
Glioblastoma	NCT01790503			Pexidartinib + radiation + Temozolomide
Metastatic/Advanced PC and CRC	NCT02777710			Pexidartinib + Durvalumab
Melanoma, NSCLC, GIST, HNSCC, and ovarian cancer	NCT02452424			Pexidartinib + Pembrolizumab
Advanced solid tumors	NCT01346358	CSF1R	Monoclonal antibody against	IMC-CS4 monotherapy
	NCT02718911		CSF1R	IMC-CS4 + Durvalumab/Tremelimumab
PC	NCT03153410			IMC-CS4 + Cyclophosphamide + Pembrolizumab + GVAX
Breast/Prostate cancer	NCT02265536			IMC-CS4 monotherapy
Metastatic sarcomas	NCT04242238	Switch pocket of	Highly selective kinase inhibitor	DCC-3014 + Avelumab
TGCT and advanced tumors	NCT03069469	CSF1R		DCC-3014

PC, pancreatic cancer; CRC, colorectal cancer; PVNS, Pigmented villonodular synovitis; GCT-TS, Giant cell tumors of the tendon sheath; TGCT, Tenosynovial Giant Cell Tumor; CSF1, colony stimulating factor 1; IL-34, interleukin 34; c-KIT, KIT proto-oncogene receptor tyrosine kinase; CSF1R, CSF1 receptor; FLT-3, FMS like tyrosine kinase 3; NSCLC, non-small-cell lung cancer; GIST, gastrointestinal stromal tumor; HNSCC, head and neck squamous cell carcinoma; GVAX, granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected tumor cell vaccine.

TABLE 5 | Clinical trials investigating reprogramming of TAM in combination with other therapies.

Cancer type	Trial number	Target	Mechanism of action	Treatment/Intervention
Ovarian cancer	NCT03558139	CD47	Monoclonal antibody recognizes CD47 and	Magrolimab + Avelumab
Hodgkin lymphoma	NCT04788043		blocks the "don't eat me" signal on SIRPα	Magrolimab + Pembrolizumab
Urothelial carcinoma	NCT03869190		receptor on TAM	Several treatment combinations including Magrolimab
AML	NCT04435691			Magrolimab + Azacitidine + Venetoclax
AML and myelodysplastic syndrome	NCT03248479			Magrolimab +/- Azacitidine
Solid tumors and advanced CRC	NCT02953782			Magrolimab + Cetuximab
Non-Hodgkin lymphoma	NCT02953509			Magrolimab + Rituximab + Gemcitabine + Oxaliplatin
Hematologic malignancies and solid tumors	NCT02663518	CD47	TTI-621 is SIRPαFc, a recombinant fusion protein blocking CD47:SIRPα axis	TTI-621 alone/+ Rituximab/+ Nivolumab
Lymphoma and myeloma	NCT03530683	CD47	SIRPα-IgG4Fc, a recombinant fusion protein binding to CD47	TTI-622 alone/+ Rituximab/+ Nivolumab/+ Carfilzomib
Hematologic cancers and advanced solid tumors	NCT03512340	CD47	Anti-CD47 antibody	SRF231
PDAC	NCT01456585	CD40	CP-870,893 is a fully human, CD40-specific	CP-870,893 + Gemcitabine
Metastatic melanoma	NCT01103635		agonist monoclonal antibody	CP-870,893 + Tremelimumab
Metastatic CRC	NCT03555149	CD40	Selicrelumab is a human IgG2 agonistic anti-	Several combinations including Selicrelumab
Metastatic PDAC	NCT03193190		CD40 monoclonal antibody	Several combinations including Selicrelumab
Locally advanced and metastatic solid tumors	NCT02304393			Selicrelumab + Atezolizumab

AML, acute myeloid leukemia; CRC, colorectal cancer; PDAC, pancreatic ductal adenocarcinoma; CD47, cluster of differentiation protein-47; TAM, tumor associated macrophages; IgG, immunoglobulin G; SIRPα, signal regulatory protein α.

cancer cell invasion and metastasis. Therefore, in the triple cross-talk between tumor cells, CAF, and TAM, IL-6 and GM-CSF could become important targets for modulating their interaction (95).

In HCC, osteopontin (OPN) was identified as a key molecule involved in cancer-CAF-TAM interactions. OPN is a chemokine-like phosphorylated glycoprotein released by TAM in the TME. The TAM-secreted OPN promotes the secretion of OPN from CAF and leads to increased cancer cell malignancy through upregulation of proliferation, ECM degradation, and migration. Thus, OPN could be a potential new therapeutic target to inhibit cancer-CAF-TAM interactions in HCC (132).

Based on global gene expression profiles in CRC, bioinformatics and immunohistochemistry identified stromal markers that were significantly associated with resistance to therapy, recurrence and

poor prognosis. The predictive power of stromal cell genes was higher than the power of tumor cell genes (4). In accordance with and by investigating the four consensus molecular subtypes (CMS) in CRC, the CMS4 tumors were characterized by heavy infiltration of mesenchymal cells and displayed worse recurrence-free survival and overall survival compared with other CMS subtypes. Moreover, the CMS4 tumors showed a clear upregulation of genes controlling EMT, TGF-β signaling, angiogenesis, matrix remodelling, and inflammation (5).

Compared with TAM, and playing a dominant role in the evolution of TME, a higher density of CAF is usually observed in tumors of the gastrointestinal tract, pancreas, lung, and prostate. It is important to mention that TAM are associated with migration and intravasation of tumor cells, CTC formation, and aiding CTC clusters in the peripheral circulation in the patients (73, 133, 134). Despite them being appealing targets, owing to the lack of selectivity, strategies to attack CAF and TAM have resulted in unwanted side-effects and thereby, limited their clinical use (14, 135).

THE EXTRACELLULAR MATRIX

The ECM mainly consists of proteins and glycosaminoglycans that are constantly remodeled by fibroblasts and macrophages in response to environmental changes.

In preclinical trials on breast and lung cancer, it was reported that CAF-produced collagen and CAF-derived FAP transformed the ECM into an environment facilitating the cancer cell motility through a parallel alignment of the collagen fibers that enhanced the direction and speed of the migrating cells (136).

Regular tissue fibroblasts synthesize and release ECM components such as collagen, elastin, fibronectin, and a variety of proteoglycans that combine to form a web of fibers. This network regulates the homeostasis of cells, tissues, and organs and allows the ECM and tumor cells to resist a wide range of chemical and mechanical stress factors (137).

In a solid tumor, the assembly of ECM fibrils is crucial for the barrier formation and exclusion of immune cells and therapeutics. Further, the collagen network in the stroma is key for the maintenance and exchange of fluids and solutes within the tumor.

Elastin, an abundantly expressed protein in the ECM, is secreted by fibroblasts as a precursor protein, tropoelastin, which assembles in the elastic fibers that are rich in crosslinks. The crosslinks render the elastin insoluble and equip the fibers with the ability to withstand repeated distension. Additionally, the elastin fibers are tightly associated with collagen fibrils which are mediated by the cell surface proteoglycans (138).

Fibronectin, also secreted by fibroblasts, binds to the ECM components such as collagen and fibrin and anchors the fibrils to the cell-surface integrin receptors (139).

The tyrosine kinase inhibitor, Imatinib—specific to ABL1, PDGFR, and c-kit—is used to treat hematological malignancies and gastrointestinal stromal tumors. It is found to increase the flow of fluids through the interstitial compartment of the tumor, improving drug delivery, mainly due to a decreased collagen fibril diameter (140, 141).

CAF and TAM produce various enzymes, including matrix metalloproteinases (MMP), fibrinolysin, and cathepsins that degrade ECM components, accelerate local invasion of tumor cells, and facilitate their dissemination (142, 143). Some ECM degradation fragments may even stimulate angiogenesis and migration (144). MMPs are zinc-dependent ECM-remodelling endopeptidases deeply implicated in almost all steps of metastasis. A high MMP expression in the tumor correlates with poor prognosis and increased risk of recurrence (145). The CAF expression of MMP-11 in CRC, MMP-2 and MMP-9 in breast cancer, and MMP-21 in HCC was significantly related to a high risk of tumor recurrence (146–148).

The presence of hypoxia, acidity, increased interstitial pressure, and aberrant vasculature in the TME confer tumor cells with a survival advantage. The environment inhibits the penetration, navigation, and functionality of cytotoxic immune cells in their quest to kill tumor cells (149, 150).

To prevent intracellular acidity, tumor cells express various proton flux regulators, such as H^+-ATPases, Na^+/H^+ exchangers, monocarboxylate transporters, carbonic anhydrases, and Na^+/HCO_3 transporters. Proton pump inhibitors are currently being used in clinical trials (151, 152), in combination with therapies targeting carbonic anhydrases: Acetazolamide (carbonic anhydrase inhibitor) and radiotherapy for small cell lung cancer (NCT03467360), carbonic anhydrase IX inhibitor and Gemcitabine (antimetabolite) for PDAC (NCT03450018), and Acetazolamide and Temozolomide (alkylating agent) for malignant glioma of the brain (NCT03011671). Additional clinical trials of therapies that aim to target ECM and ECM-associated molecules are on-going; however, as therapeutics, ECM degrading agents must be used with caution as they may have fundamental consequences on cell and tissue functions, which could ease the metastatic spread instead of inhibiting tumor progression (153).

CAF AND TAM IN IMMUNOTHERAPY AND ANTI-ANGIOGENESIS

The introduction of monoclonal antibodies targeting inhibitory receptors on immune cells, known as immune checkpoint inhibitors, has been a great breakthrough in oncology, immensely improving the clinical outcomes of several cancers. This therapeutic strategy enhances the efficacy of anti-tumor immune responses and revitalizes exhausted killer cells such as $CD8^+$ T cells and NK cells (154).

The exclusion of immune cells from solid tumors is not only caused by the physical and chemical barrier of the ECM, but also by the immune checkpoint ligands expressed by cancer cells, CAF, and TAM (155). In line with this, a study on tissue samples from patients with PDAC demonstrated that PD-L1 and PD-L2 (both ligands to PD-1) expressed by CAF were involved in immune cell exclusion and anergy (156).

Adding to the complexity of stromal cell functions, preclinical studies suggest that some CAF, along with normal fibroblasts, have the ability to overrule oncogenic signaling from the surroundings

and act as tumor suppressors (20, 157). Whether these fibroblasts are subtypes of normal fibroblasts resistant to CAF conversion or distinct anti-tumor CAF subpopulations remains unknown. However, the CAF/TAM collaboration do play a vital tumor-promoting role. It fuels the growth of tumors; induces stemness and EMT in cancer cells by the production of cytokines, chemokines, e.g. interleukins, TGF-β, CCL, and CXCL chemokines (158). It supplies the tumors with energy-rich metabolites and upregulate the tumor-cell mitochondrial oxidative phosphorylation (159). Thus, therapeutic regimens targeting the TAM-CAF interaction in combination with immunotherapy could improve anti-tumor therapeutic efficacy (160).

CSF-1R receptors are overexpressed on TAM in many cancers, controlling the production, differentiation, and function of macrophages. In a mouse model, a CSF-1R-inhibitor blocked the production of inflammatory mediators in TAM, inhibited the recruitment of bone marrow-derived suppressor cells (BMDSC), and enhanced T-cell infiltration and $CD8^+$ T cell activity. However, the inhibition of CSF-1R signaling caused CAF to secrete chemokines and chemokine ligands that neutralized the CSF-1R inhibitor. The supplementation of a chemokine receptor antagonist reduced the tumor burden, and tumor growth was completely blocked when an immune checkpoint inhibitor (anti-PD-1) was further added to the combination (161).

There are currently several clinical trials evaluating the effect of CSF1R monoclonal antibodies in combination with immune checkpoint inhibitors in a variety of solid tumors (**Table 4**).

In a human trial on solid tumors, dual antibody blockade (anti-TGF-β and anti-PD-L1) led to a significant increase in the number of cytotoxic $CD8^+$ T cells in the TME. The co-inhibition of TGF-β and PD-L1 converted an immune excluded tumor phenotype to an inflamed phenotype, supporting the fact that TGF-β signaling prevents T-cell invasion. T cell localization was not affected with either antibody as monotherapy (162). Thus, TAM expressing immune checkpoint receptor ligands limit the functions of effector T cells, NK cells, and dendritic cells, and attenuate the effects of immune checkpoint inhibitor therapy (101, 102).

To prevent phagocytosis, upregulated CD47 surface proteins on tumor cells provide a "do not eat me" signal by ligating the inhibitory TAM-receptor signal regulatory protein alpha (SIRPα). As CD47 also promotes the proliferation of cancer cells *via* the PI3K/AKT pathway, the CD47 signaling pathway is considered an important mechanism of therapy resistance. Inhibition of CD47 could be a promising therapeutic strategy, particularly in combination with immune checkpoint inhibitors.

In mouse models of melanoma, colon carcinoma, and lymphoma, dual targeting of CD47 and PD-L1 was found to enhance anti-tumor effects (163–165) and several clinical trials evaluating the efficacy of CD47 or SIRPα monoclonal antibodies as monotherapy or in combination with immune checkpoint inhibitors are underway (**Table 5**; ClinicalTrials.gov).

As VEGF-A is overexpressed in both tumor cells, CAF, and TAM and is associated with cancer progression and dissemination, it represents the main target of anti-angiogenic drugs in cancer therapy. These drugs are widely used in the treatment of various cancers and have resulted in increased overall survival or progression-free survival in gynecologic cancers (166), CRC (167), and gastric cancer (168). However, due to antiangiogenic drug resistance of tumor cells, metastasis and mortality continue to occur during and after cessation of treatment. This resistance comprises the amplification of pro-angiogenic genes, secretion of multiple proangiogenic factors, and recruitment of proangiogenic BMDSC (169). Bevacizumab, a humanized monoclonal antibody that targets all VEGF-A isoforms and the first anti-angiogenic drug approved for clinical application, is efficacious in various malignancies such as CRC and glioblastoma (170). Today, most clinical studies use anti-angiogenetic drugs in combinatory regimens, e.g. lenvatinib (multiple kinase inhibitor) inhibiting both VEGFR 1–3 and PDGFR and Pembrolizumab (anti-PD-1) for the treatment of endometrial cancer (NCT03517449).

The anti-diabetic drug metformin appears to be a promising therapeutic agent in neoadjuvant and adjuvant settings. The metformin-induced antitumor and anti-angiogenic effects are partly related to the skewing of TAM polarization from M2- to M1-like phenotype and significant inhibition of tumor angiogenesis. Currently, there is very little insight into the mechanism through which metformin modulates macrophage function. However, an *in vitro* study on breast cancer cells and TAM polarization revealed that metformin treatment activated AMPK-NF-κB signaling in cancer cells. These molecules participate in the regulation of M1 and M2 inducing cytokines. Metformin was observed to increase macrophage expression of M1-related cytokines IL-12 and TNF-α and attenuate the expression of the M2-related cytokines IL-8, IL-10, and TGF-β. Furthermore, the secretion of important cytokines for the M2 phenotype (e.g. IL-4, IL-10, and IL-13) was inhibited in metformin-treated cancer cells (171).

In cultures of human cholangiocarcinoma cells, and at concentrations corresponding to plasma levels of metformin in diabetic patients, metformin inhibited proliferation and cell migration and induced apoptosis. Expression of vimentin (mesenchymal marker) and EMT genes was downregulated and expression of cytokeratin-19 (epithelial marker) was upregulated (172). The findings from the multiple ongoing trials (173) may convey a deeper understanding of the anti-tumor function of metformin in the near future.

DISCUSSION

In a solid tumor, the balance between growth and differentiation is determined by the TME. TAM and CAF promote cancer evolution through the inflammatory, immunosuppressive, angiogenic, energy-rich environment, and also suppress cancer cells *via* predominantly unknown mechanisms. The presence and precise functions of CAF and TAM in the TME are extremely complex (**Figure 3**) and incompletely understood, and only a few studies describe the interplay between these cells. The general perception is that the TME strongly modulates tumor cells through all phases of disease progression, and as each tumor is comprised of multiple clones with myriads of cell types and signaling molecules, the heterogeneity of each tumor may therefore require unique therapeutic approaches.

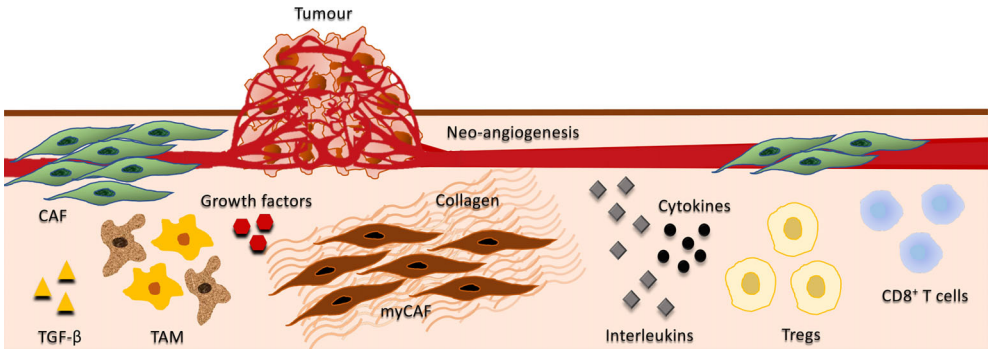

FIGURE 3 | Interplay between tumor, stromal, and immune cells. Depicts the interplay between cancer-associated fibroblasts (CAF), tumor-associated macrophages (TAM), growth factors, cytokines, interleukins, and immune cells in the tumor microenvironment (TME). CAF are the predominant cell type in the tumor stroma, contributing to the proliferative, pro-inflammatory, immunosuppressive, angiogenic, pro-invasive and pro-metastatic TME. They secrete various growth factors including TGF-β, vascular endothelial growth factor (VEGF), fibroblast growth factor 5, growth differentiation factor 15, and hepatocyte growth factor. CAF also produce cytokines and interleukins that may have both immunosuppressive and immuno-activating effects on various leukocytes, including $CD8^+$ T cells, immunosuppressive regulatory T cells (T_{regs}) and macrophages. A subset of CAFs have myofibroblasts characteristics (myCAF) and play a major role in the development of the fibrotic stroma in the TME including the regulation of collagen fibre elongation. Growth factors and immunosuppressive cytokines produced by TAM enhance motility, intravasation, and invasion of tumor cells, while stimulating angiogenesis and suppressing T cell infiltration. Additionally, TGF-β produced by TAMs activate immunosuppressive T_{regs}.

Improving our understanding of the TME including the impact of stromal cells, immune cells, and ECM components, is vital for the innovation of therapeutic strategies. Hence, cell-cell communication within the TME should be integrated into future cancer research. However, the manipulation of the immune system and/or stromal components within the TME during cancer treatment can be unpredictable. The regulation/eradication of α-SMA^+ or FAP^+ CAF have had variable results and currently, targeting CAF or TAM individually does not seem to be an appropriate approach.

A CAF-directed therapy could be designed against specific pro-tumorigenic factors that in turn could prevent CAF activation or CAF functions. The reprogramming of CAF back into a normal resting phenotype would be a desirable option; however, targeting FAP has had a minimal response in human trials (20). Drugs that target CAF may emerge as a complement to immunotherapies in solid tumors, though a major obstacle in the precision strategy of CAF-based therapy is that neither α-SMA nor FAP is exclusively expressed by CAF.

In theory, TAM antagonists could be used to overcome resistance to immunotherapy; nevertheless, the type of approach is yet to be determined. The lack of macrophage selectivity has so far hindered its introduction into the clinic.

Monoclonal antibodies blocking the interaction between CD47 on tumor cells and SIRPα on innate immune cells is another interesting direction for future research. Other potential treatment targets are the MMPs. In the TME, MMPs are expressed by various cell types, and a number of MMP inhibitors have been tested in phase 1, 2, and 3 clinical trials. Unfortunately, all trials across different cancer types and stages have failed to provide any improvements in the clinical outcomes (174). Nonetheless, the field is advancing fast with the development of small-molecule inhibitors and antibodies targeting specific domains of pro-tumorigenic MMPs.

In PDAC, the TME is an important contributor to tumor progression and prognosis. The increasing amount of ECM and fibrosis promote tumor progression and correlate with shorter survival. The aberrant TGF-β signaling in cancer cells leads to an increased epithelial signal transducer and activator of transcription 3 (STAT3) activity, resulting in increased ECM fibrosis (175). Therefore, the concept of reducing tumor aggressiveness by interfering with STAT3 hyperactivity seems intriguing.

Notably, a recent study demonstrated that increased phosphorylation of STAT3 in CAF was associated with reduced overall survival in CRC patients (176). To improve response rates and increase the number of responding cancer types, combination therapies using STAT3 inhibitors and immune checkpoint inhibitors are now being undertaken (177).

In conclusion, combinations of immune-modulating agents are gaining more and more ground in oncology. CAF and TAM hold significant potential to improve targeted therapy and outcomes in cancer treatment when combined with existing therapies. Although in its naive stages, the TME modulating technology is an active field of research that holds immense prospects for researchers, clinicians, and patients.

AUTHOR CONTRIBUTIONS

HR: idea, design, intelectual contents, writing the manuscript, and crreation of ilustration. AO: intelectual contents, writing the manuscript, and creation of illustrations. SG: language editing, intelectual contents, manuscript structure, and creation of tables. IG: overall design, intelectual contents, and editing. All authors contributed to the article and approved the submitted version.

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Significance of macrophage infiltration in the prognosis of lung adenocarcinoma patients evaluated by scRNA and bulkRNA analysis

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Purpose: To investigate the significance of macrophage infiltration to the prognosis of lung adenocarcinoma.

Methods: R language bioinformatics analysis technology, was used to obtain macrophage infiltration-related module genes through WGCNA (Weighted Gene Co-Expression Network Analysis). Marker genes of macrophage subtypes were identified using single-cell sequencing of lung adenocarcinoma tissue. Risk score models were constructed and validated using external data cohorts and clinical samples.

Results: Analysis of cohorts TCGA-LUAD, GSE11969, GSE31210, GSE50081, GSE72094 and GSE8894, revealed a negative correlation between macrophage infiltration and survival. Immunohistochemical analyses of clinical samples were consistent with these data. Based on cell-cluster-markers and TAMs-related-genes, TOP8 genes were obtained (C1QTNF6, CCNB1, FSCN1, HMMR, KPNA2, PRC1, RRM2, and TK1) with a significant association to prognosis. Risk score models including 9 factors (C1QTNF6, FSCN1, KPNA2, GLI2, TYMS, BIRC3, RBBP7, KRT8, GPR65) for prognosis were constructed. The efficacy, stability and generalizability of the risk score models were validated using multiple data cohorts (GSE19188, GSE26939, GSE31210, GSE50081, GSE42127, and GSE72094).

Conclusions: Macrophage infiltration negatively correlates with prognosis in patients with lung adenocarcinoma. Based on cell-cluster-markers and

TAMs-related-genes, both TOP8 genes (C1QTNF6, CCNB1, FSCN1, HMMR, KPNA2, PRC1, RRM2, TK1) and risk score models using C1QTNF6, FSCN1, KPNA2, GLI2, TYMS, BIRC3, RBBP7, KRT8, GPR65 could predict disease prognosis.

KEYWORDS

macrophages, prognosis, lung adenocarcinoma, ScRNA, bulkRNA, infilitration, marker gene, WGCNA (weighted gene co- expression network analyses)

1 Introduction

Lung cancer remains the most common malignancy worldwide and a leading cause of cancer-related death, despite advances in screening and treatment (1, 2). Whether it was for the non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) patients, immunotherapy was the most shining one among many treatment methods, which had changed the landscape of anti-tumor therapy and brought anti-tumor therapy into a new era (3–7). However, there were still many details in the screening of immunotherapy benefit populations and related predictors needed to be further elucidated (8–16). Specific macrophage phenotypes can act as indicators of lung cancer prognosis and the efficacy of immunotherapy (17–24). Sequencing technologies and R language based bioinformatics, formerly reported (25–27), can be used for studies in this area (28–30). Based on our previous studies (28–30), we performed bioinformatics analysis and clinical sample validation to identify specific macrophage signatures that can act as indicators of therapeutic efficacy.

2 Methods

2.1 Data analysis

2.1.1 TCGA data

mRNA expression profiles, clinical information, copy number alterations and mutations of GDC TCGA Lung Adenocarcinoma (LUAD) samples were downloaded from https://xenabrowser.net/datapages/. Tumor samples were screened according to sample name. RNA-seq data for 513 tumor samples and 59 paracancerous samples were obtained.

Abbreviations: UMAP, Uniform Manifold Approximation and Projection; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; LUAD, lung adenocarcinoma; WGCNA, Weighted Gene Co-Expression Network Analysis; TAM, tumor-associated macrophage; GSVA, Gene Set Variation Analysis; FFPE, formalin fixation and paraffin embedding; IHC, immunohistochemical; DAB, diaminobenzidine; DEGs, Differential Expression Genes; LASSO, Least Absolute Shrinkage and Selection Operator.

2.1.2 GEO data

Expression data and sample survival information for GSE11969, GSE19188, GSE26939, GSE31210, GSE42127, GSE50081, GSE72094 and GSE8894 were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo). Survival information of the samples were summarized as follows: (Supplementary Table 1_train_clin.tsv; Supplementary Table 1_GSE11969_clin.txt; Supplementary Table 1_GSE19188_clin.txt; Supplementary Table 1_ GSE26939_clin.txt; Supplementary Table 1_GSE31210_clin.txt; Supplementary Table 1_GSE42127_clin.txt; Supplementary Table 1_GSE50081_clin.txt; Supplementary Table 1_ GSE72094_clin.txt; Supplementary Table 1_ GSE8894_clin.txt). Single-cell sequencing data from GSE131907 were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo). A total of 42,995 cells and 29,634 genes were obtained.

2.2 Immune infiltration analysis

Immune infiltration for each sample was calculated using IOBR of the R package for the training set TCGA expression matrix and GEO data, respectively (method = 'cibersort').

2.3 Survival analysis

For survival assessments, R packages "survminer" and "survival" were analyzed and survival curves were constructed based on survival time and status. Differences in prognosis among the groups were assessed.

2.4 Screening of modules corresponding to macrophages using WGCNA

Hierarchical clustering analysis was performed on the TCGA expression matrix using the R package "hclust", "method=average". Phenotypic information was obtained

using the infiltration ratio of macrophages. A correlation between different modules and macrophages was obtained.

2.5 Clustering analysis of samples

The R package "ConsesusClusterPlus" was used to perform consensus clustering analysis. After clustering on the TCGA and GEO data, the optimal number of categories were determined according to the change of area under the CDF curve. The k value of the cluster category ranged from 2 to 6.

2.6 Analysis of single-cell data

Single-cell data were filtered using the R package "seurat" to remove cells with ≥ 20% mitochondrial expression. Data were analyzed using the "seurat" normalization pipeline. To identify tumor-associated macrophage (TAM) populations, marker genes from published studies were used to identify corresponding clusters. TAM populations were selected for standardization analysis using "Seurat".

2.7 Trajectory analysis of single-cell data

Trajectory analysis was performed on TAM subclasses using the R package "monocle" with default parameters. This resulted in differentiation trajectories and key genes determining these trajectories.

2.8 Gene set variation analysis

To investigate differences in the expression patterns of specific TAM isoforms in biological processes, GSVA enrichment analysis was performed using the R package "GSVA". GSVA is a nonparametric, unsupervised method primarily used to assess alterations in signaling pathways and biological processes in samples.

2.9 Construction of risk scoring model

Univariate cox regression analysis was performed on "cell-cluster-markers" and "TAMs-related-genes", and genes significantly associated with OS survival were screened at the $p<0.05$ level. According to the identified prognosis-related genes, the R package 'glmnet' was used to construct a prognosis model (or classifier model) with a 10-fold cross-validation fold using the cox method. Characteristic factors were then screened. Kaplan-Meier survival analysis and ROC curves were used to evaluate the predictive power of the prognostic model.

2.10 Clinical sample validation (sample collection and immunohistochemistry)

Lung Cancer samples were collected from the First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital from June 2012 to February 2020. Written informed consent was provided by all participants. Tumor tissues were surgically resected, formalin fixed and paraffin embedded (FFPE) for histological evaluation. HE-stained and immunohistochemical (IHC) slides were examined by two independent and experienced pathologists according to the WHO criteria.

Samples were IHC stained with mouse anti-human CD68 monoclonal antibodies (MAB-0863, clone MX075) and mouse anti-human CD163 monoclonal antibodies (MAB-0869, clone MX081). CD68 was used as a general surface marker for macrophages, whilst CD163 was used as a marker for M2 macrophages (31). Double-labeled immunohistochemical staining was performed using alkaline phosphatase and horseradish peroxidase conjugated secondary antibodies. Substrates were fast red (AP-Red) and diaminobenzidine (DAB) (Roche Ltd) stained. Slides were processed using an automated Roche BenchMark XT staining system according to the manufacturer's protocol.

3 Results

3.1 Proportion of immune infiltrating cells and the prognostic efficacy of macrophages

CIBERSORT was used to evaluate the levels of immune-infiltration from different lung adenocarcinoma datasets (TCGA-LUAD, GSE11969, GSE31210, GSE50081, GSE72094, and GSE8894). According to the median macrophage ratio, samples were divided into high- and low-levels of macrophage infiltration. Survival differences between high- and low-groups showed a significant correlation with macrophage infiltration (Figure 1; Supplementary Table 2_train_cibersort.txt; Supplementary Table 2_GSE11969_cibersort.txt; Supplementary Table 2_GSE31210_cibersort.txt; Supplementary Table 2_GSE50081_cibersort.txt; Supplementary Table 2_GSE72094_cibersort.txt; Supplementary Table 2_GSE8894_cibersort.txt).

3.2 Screening of modules corresponding to macrophages

To identify macrophage-related genes related to infiltration, WGCNA module analysis was performed on the training dataset (Supplementary Figure 1, Supplementary Table 3_gene_module.txt).

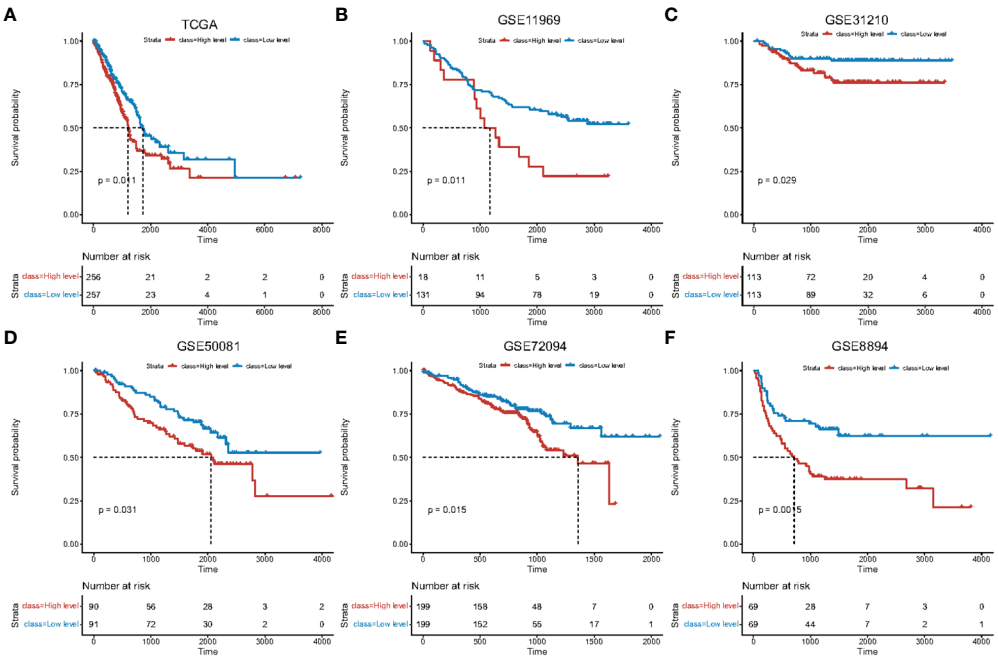

FIGURE 1
Survival curves of high and low macrophage infiltration in lung adenocarcinoma datasets. Horizontal axis: survival time. Vertical axis: survival probability. Color: level of macrophage infiltration. Survival analysis using **(A)** TCGA data, **(B)** GSE11969, **(C)** GSE31210, **(D)** GSE50081, **(E)** GSE72094, **(F)** GSE8894.

Genes corresponding to red modules were named "TAMs-related-genes" for subsequent analysis.

3.3 TAMs-related-gene-based clustering analysis, molecular typing and prognostic assessments

We analyzed the expression profiles of TAMs-related-genes in samples from different lung adenocarcinoma datasets (TCGA-LUAD, GSE13213, GSE31210, GSE72094, and GSE8894) to construct consistent clustering profiles. Based on cumulative distribution functions and incremental area maps, we selected stable clusters of TAMs-related-genes to obtain multiple subtypes (Supplementary Figures 2A–E, Supplementary Table 4_TCGA_consensusClass.csv; Supplementary Table 4_GSE13213_consensusClass.csv; Supplementary Table 4_GSE31210_consensusClass.csv; Supplementary Table 4_GSE72094_consensusClass.csv; Supplementary Table 4_GSE8894_consensusClass.csv).

Survival analysis was performed on cluster subtypes from different datasets, revealing significant survival differences (Supplementary Figures 2F–I). Dimensionality reduction analysis was performed on each dataset, revealing significant differences in sample characteristics between different subtypes (Supplementary Figures 2K–O).

3.4 Preprocessing of single-cell data

To further investigate the role of macrophages in lung adenocarcinoma, published single-cell sequencing data of lung adenocarcinoma patients was analyzed (PMC7210975) (32). Gene distribution and mitochondrial gene expression were screened (Supplementary Figures 3A–C). Cells with mitochondrial expression ≥20% were identified as dead and removed.

3.5 Identification of TAMs in total cells

Markers were used to detect the presence of TAMs in the lung adenocarcinoma single-cell datasets (Supplementary Figures 3D–I). TAMs were then extracted and subtype analysis performed to obtain a TAMs subtype map (Supplementary Figure 3I).

3.6 Screening of differential expression genes among TAMs subsets

To identify marker genes amongst the different TAM subgroups, samples were screened in "Seurat". Dot and violin plots revealed the top5 marker genes for each TAM subtype (Supplementary Figure 4; Supplementary Table 5_TAM_marker_genes.txt).

3.7 Simulation of dynamic changes in macrophages

"Monocle" was used to identify dynamic changes of macrophages in the tumors and cell polarization (Supplementary Figures 5A–C). Cluster 0 could be divided into Cluster 1 and Cluster 2 amongst TAM subtypes. The identified genes were found to regulate differentiation (Supplementary Figure 5D). Gene enrichment analysis on the subtypes of TAM showed that Cluster 2 positively correlated with E2F TARGETS and G2M CHECKPOINT, whilst Cluster 4 negatively correlated with these pathways (Supplementary Figure 5E).

3.8 Screening of prognostic factors based on cell-cluster-markers and TAMS-related-genes using univariate cox regression analysis

Markers of each TAM subtype and TAMs-related-genes were used to identify genes related to the prognosis. Samples were divided into high- and low-expression groups according to the median of gene expression. Univariate Cox analysis was performed and survival curves of the top8 prognostic genes were displayed (Figures 2A–H; Supplementary Table 6_cox_significant.txt).

3.9 Construction of risk score models and evaluation of the prognostic efficacy

Based on the "GLMNET" of the R package, LASSO (Least Absolute Shrinkage and Selection Operator) regression analysis was used to construct a regression model for the expression matrix of prognosis related genes corresponding to "Cell-Cluster-Markers" and "Tams-Related-Genes". By analysis, when the value of the freedom degree was 9, the model was accurate (Figures 2I–O; Supplementary Table 7_forest.univariate_cox.txt, Supplementary Table 7_Signature_Coef.txt). The calculation formula of the risk score model are listed as follows:

Risk Score = 0.0354754835*C1QTNF6 (Expression Value) + 0.0023344103* FSCN1 (Expression Value) + 0.0022298189*GLI2 (Expression Value) + 0.0001616254 * KPNA2 (Expression value) + 0.0005176419*TYMS (Expression Value) + 0.0037498174 *BIRC3 (Expression Value) + 0.0033257017*RBBP7 (Expression Value) + 0.0002465129 *KRT8 (Expression Value) - 0.0263442444 *GPR65 (Expression Value). Kaplan-Meier survival curves indicated a significant difference in survival between high and low risk groups. The ROC curve indicated high performance of the risk score model.

3.10 Validation of risk score prognostic models in external datasets

To further verify the stability of the risk score model, external and independent data GSE19188, GSE26939, GSE31210, GSE50081, GSE42127 and GSE72094 were used to verify predictive efficacy. Through Kaplan-Meier survival analysis, the constructed risk score model performed well for all external data predictions (Figure 3).

3.11 Robust principal component analysis of risk scoring models in clinical factors

To confirm the stability of the risk score model according to clinical characteristics, differences in survival status between high- and low-risk groups in terms of age, gender, radiotherapy, clinical characteristics and Pathlogic M were explored. Significant differences in survival between high- and low-risk groups were observed in those aged ≥ 60 and ≤ 60 years (Figures 4A, B; Supplementary Table 8_clinical_inf.txt). Similar differences were observed between gender subgroups (Figures 4C, D). In the radiotherapy group, differences between high- and low-risk groups were more pronounced (Figures 4E, F). In Pathologic M (Figures 4G, I), significant differences between high- and low-risk groups were observed for M0, indicative of higher stability.

3.12 Differences in risk score models among cancer clinical factors

To investigate the relationship between the risk score model and clinical characteristics, specific features were selected for analysis. The risk score was found to be related to radiation therapy, pathologic T and tumor stage. No significant relationship to age or gender were observed (Supplementary Figure 6).

3.13 Evaluation of risk score models through univariate and multivariate cox regression analysis

To determine whether the risk score model could act as an independent prognostic factor for cancer, the "coxph()" function in the R package "survival" was adopted for univariate and multivariate regression analysis on training and test sets, respectively. We found that in all validation and test sets, the p value of the risk score was ≤ 0.05 (Figure 5; Supplementary Table 9_TCGA_clinical.multivariate_cox.txt; Supplementary Table 9_TCGA_ clinical.univariate_cox.txt; Supplementary

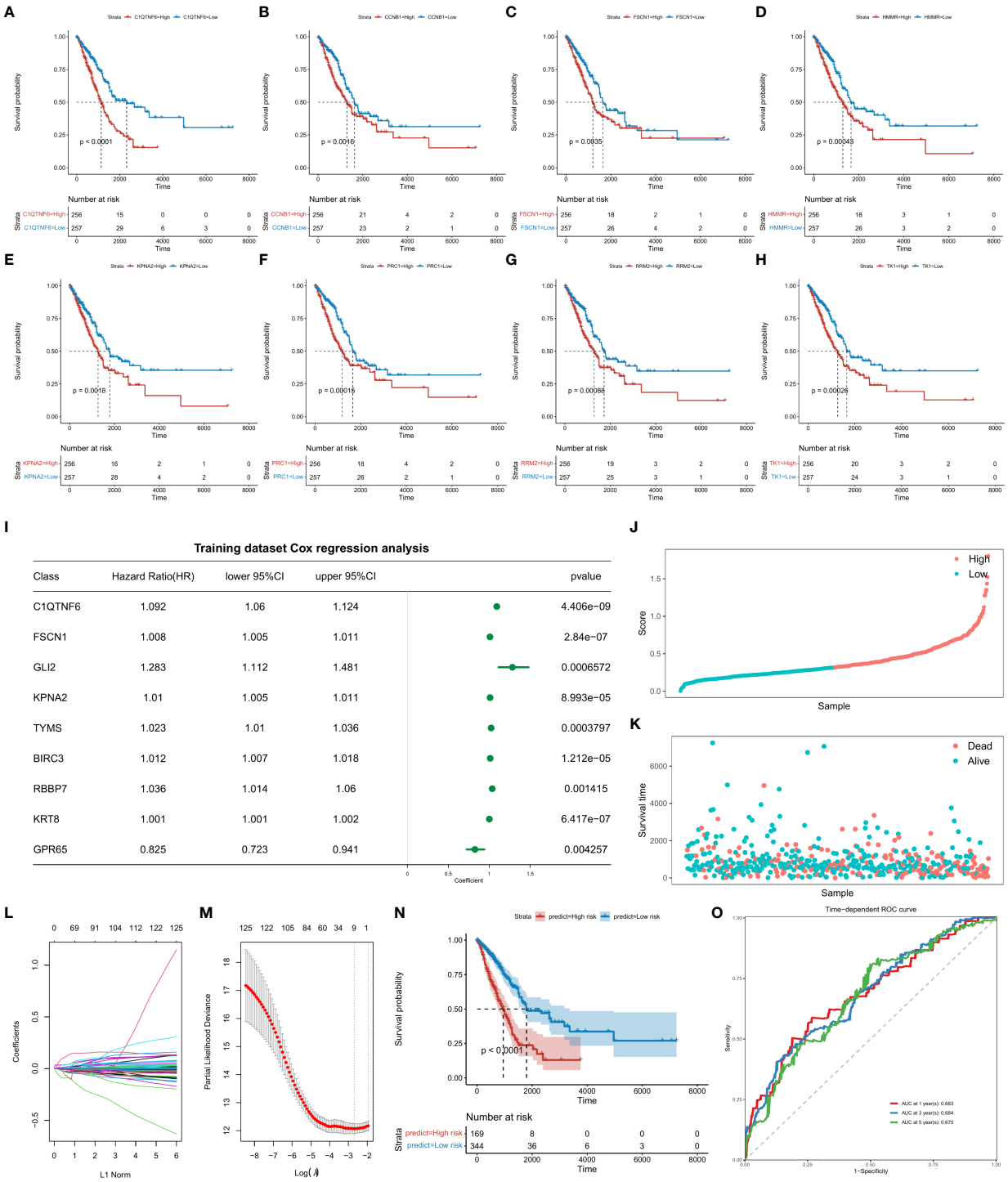

FIGURE 2
Survival analysis of top8 genes is significantly associated with prognosis. Abscissa axis: survival time. Ordinate axis: survival probability. Colors: differential gene expression. Survival analysis of **(A)** C1QTNF6, **(B)** CCNB1, **(C)**, FSCN1, **(D)** HMMR, **(E)** KPNA2, **(F)** PRC1, **(H)** RRM2, and **(I)** TK1. **(J)** Construction of the risk score model and evaluation of its prognostic efficacy. Forest plots of genes included in the risk score model. Right column: 9 genes included in the risk score model. Left column: corresponding forest plot. **(K)** Risk score plot for cancer samples (line graph). **(L)** Risk score plot for cancer samples (scatter plot graph). **(M)** Dynamic process diagram of variables screened by LASSO regression analysis and selection process diagram of the cross-validation parameter λ. **(N)** Survival analysis of the training dataset. Abscissa axis: survival time; ordinate axis: survival probability. **(O)** ROC curve of training datasets. Abscissa axis: specificity; Ordinate axis: sensitivity. Colors represent different years.

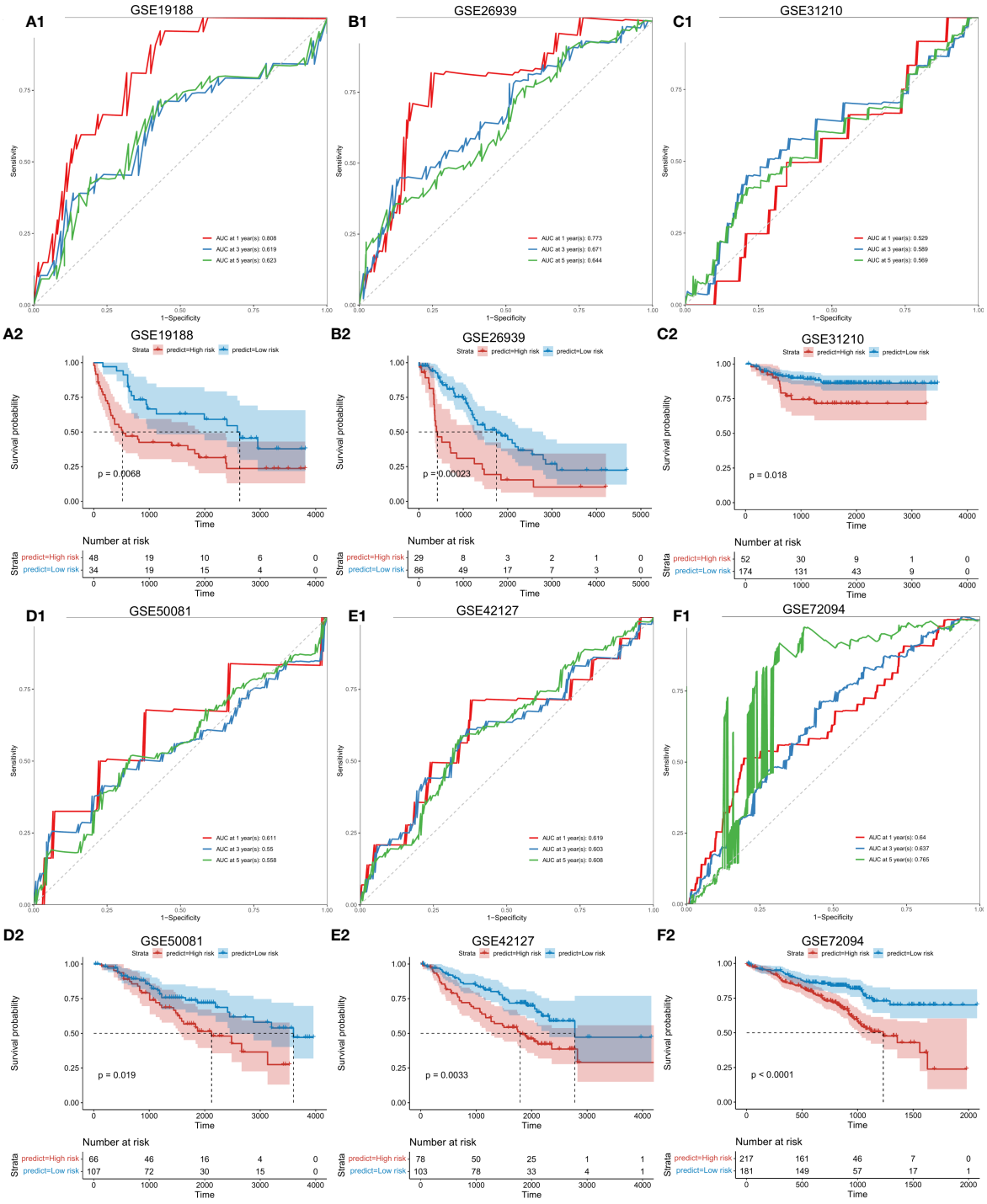

FIGURE 3
Validation of the risk score model with external independent data. A1: Validation (ROC curve) of the risk score model using external independent data GSE19188; A2: survival analysis. B1: Validation (ROC curve) of the risk score model using GSE26939. B2: Survival analysis using GSE26939. C1: Validation (ROC curve) of the risk score model using GSE31210. C2: Validation (survival analysis) using GSE31210. D1: Validation results (ROC curve) of the risk score model using GSE50081. D2: (survival analysis) of the risk score model using GSE50081. E1: Validation results (ROC curve) of the risk score model using GSE42127. E2: survival analysis using E42127. F1: Validation (ROC curve) of the risk score model using GSE72094. F2: Survival analysis using GSE72094. Abscissa axis: survival time; Ordinate axis: survival probability. Colors: different risk groups.

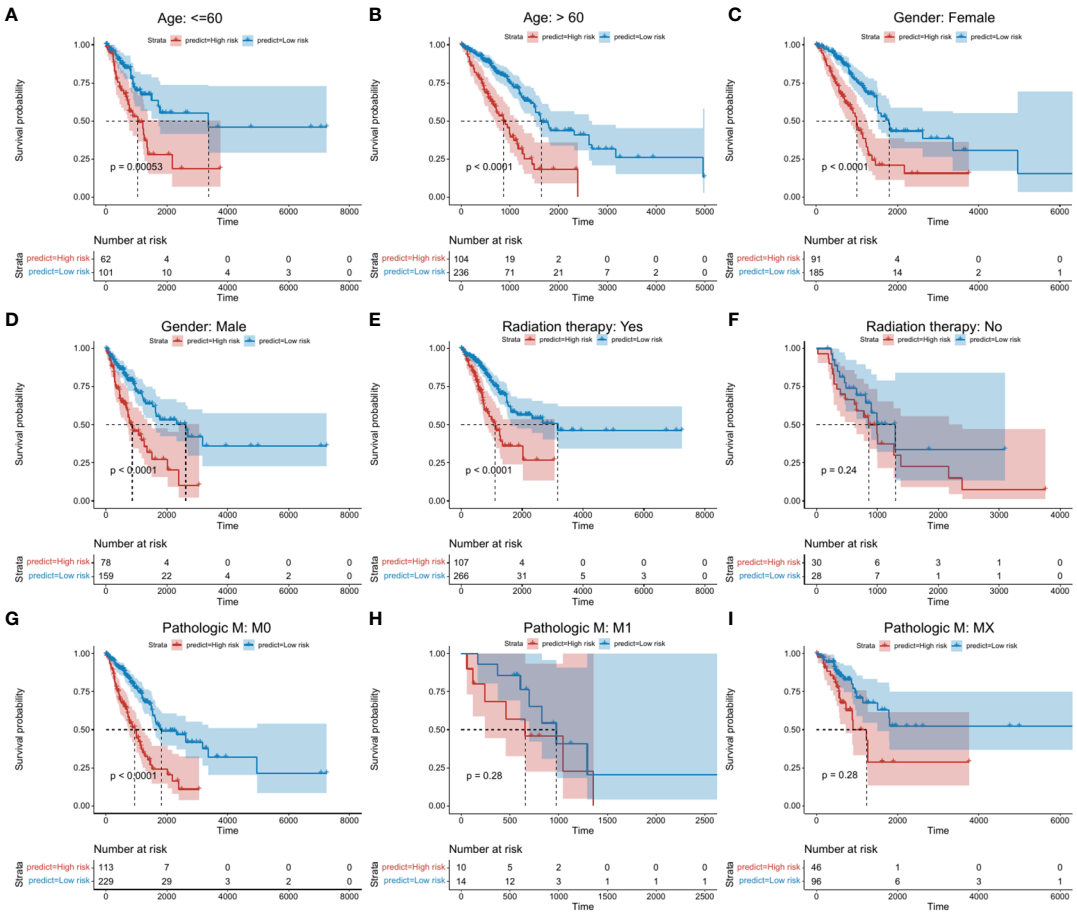

FIGURE 4
Kaplan-Meier survival analysis between high- and low risk groups. Abscissa axis: survival time; Ordinate axis: survival probability. Colors: different risk groups. **(A)** Kaplan-Meier survival analysis between high- and low-risk groups in those aged ≤ 60 years. **(B)** >60 years. **(C)** Female patients. **(D)** Male patients. **(E)** + Radiation therapy. **(F)** - Radiation therapy. **(G)** M:M0. **(H)** M: M1. **(I)** M: Mx.

Table 9_GSE19188.multivariate_cox_result.txt; Supplementary Table 9_GSE19188.univariate_cox_result.txt; Supplementary Table 9_GSE26939. multivariate_cox_result.txt; Supplementary Table 9_GSE26939.univariate_cox_result.txt; Supplementary Table 9_GSE42127.multivariate_cox_result.txt; Supplementary Table 9_GSE42127. univariate_cox_result.txt; Supplementary Table 9_GSE50081. multivariate_cox_result.txt; Supplementary Table 9_GSE50081.univariate_cox_result.txt; Supplementary Table 9_GSE72094. multivariate_cox_result.txt; Supplementary Table 9_GSE72094.univariate_cox_result.txt). This indicated that the risk score model was an accurate independent prognostic factor for cancer.

3.14 Construction of a nomogram model of risk scores and clinical factors to predict cancer progression

We next sought to apply the risk scoring model to the prediction of cancer progression in the clinic. The R package "rms" was adopted to construct a nomogram using a variety of clinical features. Calibration curves were used to calculate 1, 2, 3, and 5-year survival times (Supplementary Figure 6, Supplementary Table 10_nomogram_patient_info_part.txt). All survival calibration curves were near the 45° slope, indicating high accuracy of the nomogram.

FIGURE 5
Univariate and multivariate cox regression analysis of the risk score model in training and validation datasets. **(A)** Univariate cox regression analysis. **(B)** Multivariate cox regression analysis. **(C)** GSE19188 univariate cox regression analysis. **(D)** GSE19188 multivariate cox regression analysis. **(E)** GSE26939 univariate cox regression analysis. **(F)** GSE26939 multivariate cox regression analysis. **(G)** GSE42127 univariate cox regression analysis. **(H)** GSE42127 multivariate cox regression analysis. **(I)** GSE50081 univariate cox regression analysis. **(J)** GSE50081 multivariate cox regression analysis. **(K)** GSE72094 univariate cox regression analysis. **(L)** GSE72094 multivariate cox regression analysis.

3.15 Prediction of immunotherapy efficacy amongst subtypes

We next investigated whether the risk score model could predict the prognosis of immunotherapy. Data were calculated using the risk score model and the K-M survival status between high- and low-risk groups evaluated (Supplementary Figure 7A, Supplementary Table 11_Immune_treatment.xlsx). Upon statistical analysis of the distribution of CR/PR and PD/SD, the proportion of treatment response rates significantly differed between high- and low-risk groups (Supplementary Figure 7B, chi-square test p= 0.004133). No significant differences in the risk scores between the different treatment response groups were observed (Supplementary Figure 7C).

3.16 Overall survival analyses of M1 and M2 macrophage subtypes in patients with lung cancer

A total of 32 patients with lung cancer were evaluated for M1 and M2 macrophage subtypes. Samples were stained using double-labeled IHC. The majority of patients were in pathological Stage II (62.5%) and the dominant histopathological type was adenocarcinoma (68.8%). The clinicopathological characteristics of the lung cancer patients are shown in (Table 1).

To identify M1 and M2 macrophage subtypes, CD68 and CD163 antibodies were used for double-labeled IHC staining. CD68 (brown/yellow) as a surface marker for all macrophages primarily localized to the cytoplasm, whilst CD163 (red) localized to the plasma membrane. M2 macrophages were identified through double staining for CD68 and CD163. M1 macrophages were identified through staining with CD68 alone. Representative IHC images are shown in (Figures 6A, B).

TABLE 1 Basic characteristics of enrolled clinical samples.

Characteristic	levels	Overall
n		32
Age, n (%)	>65	12 (37.5%)
	≤65	20 (62.5%)
Gender, n (%)	Female	16 (50%)
	Male	16 (50%)
T stage, n (%)	T1	5 (15.6%)
	T2	20 (62.5%)
	T3	7 (21.9%)
N stage, n (%)	N0	21 (65.6%)
	N1	8 (25%)
	N2	3 (9.4%)
Pathological Stage, n (%)	I	8 (25%)
	II	20 (62.5%)
	III	4 (12.5%)
Histologic type, n (%)	Adenomcarcinoma	22 (68.8%)
	Mucoepidermoid carcinoma	2 (6.2%)
	Squamous cell carcinoma	8 (25%)

The prognostic value of macrophage infiltration was next evaluated. Total macrophages, M2 to M1, and M2 macrophage infiltration were identified as detrimental to patient survival (Figures 6C, E, F), whilst M1 macrophage infiltration was beneficial to prognosis (Figure 6D). The infiltration of M1 macrophages in adenocarcinoma was significantly higher than that in squamous cell carcinoma of lung cancer. No significant differences in M2 nor total macrophage infiltration were observed between these two histological subtypes (Figure 6G).

4 Discussion

Macrophages with different phenotypes are frequently cited as indicators of the prognosis of lung cancer patients and the efficacy of immunotherapy (17–24). In our preliminary analyses, macrophage infiltration, rarely reported in lung cancer, had a significant detrimental effect on the prognosis of lung cancer patients (Figure 1). These data were consistent across cohorts (Figures 1A–F) and further verified in follow-up immunohistochemical analysis of clinical samples (Figure 6C). Collectively, these data highlight how macrophages not only act as innate immune cells to regulate immunological responses (23), but play an important role in the prognosis of lung cancer. This lays the foundation for subsequent module analysis based on macrophage infiltration (Supplementary Figure 1).

Based on the expression profiles of TAMs-related-genes, a consistent clustering profile was constructed (Supplementary Figures 2A–E). Significant differences in both survival analysis and PCA (Supplementary Figures 2F–O) were observed. These apparent differences were further identified in single-cell data (Supplementary Figure 3) confirming the importance of macrophages to the prognosis of lung cancer patients (17–24, 32). These data also highlight the need for further refinement of relevant factors to more favorably evaluate patient prognosis.

Given the advantages and progress of single-cell sequencing in lung cancer immunity (33–35), the single-cell data was further analyzed (Supplementary Figures 4, 5, and Figure 2) (32). Based on cell-cluster-markers and TAMs-related-genes, TOP 8 genes (C1QTNF6, CCNB1, FSCN1, HMMR, KPNA2, PRC1, RRM2, and TK1) significantly associated with prognosis were obtained (Figure 2). These have obvious benefits to clinicians for the assessment of patient prognosis (36–49). The same data were used to construct a risk score model containing 9 factors (C1QTNF6, FSCN1, KPNA2, GLI2, TYMS, BIRC3, RBBP7, KRT8, and GPR65) for prognostic evaluation (Figure 2) (50–55). The model was validated using external data cohorts (Figure 3) and identified as robust and accurate for prognostic evaluation (Figure 4). Significant differences in the risk scores

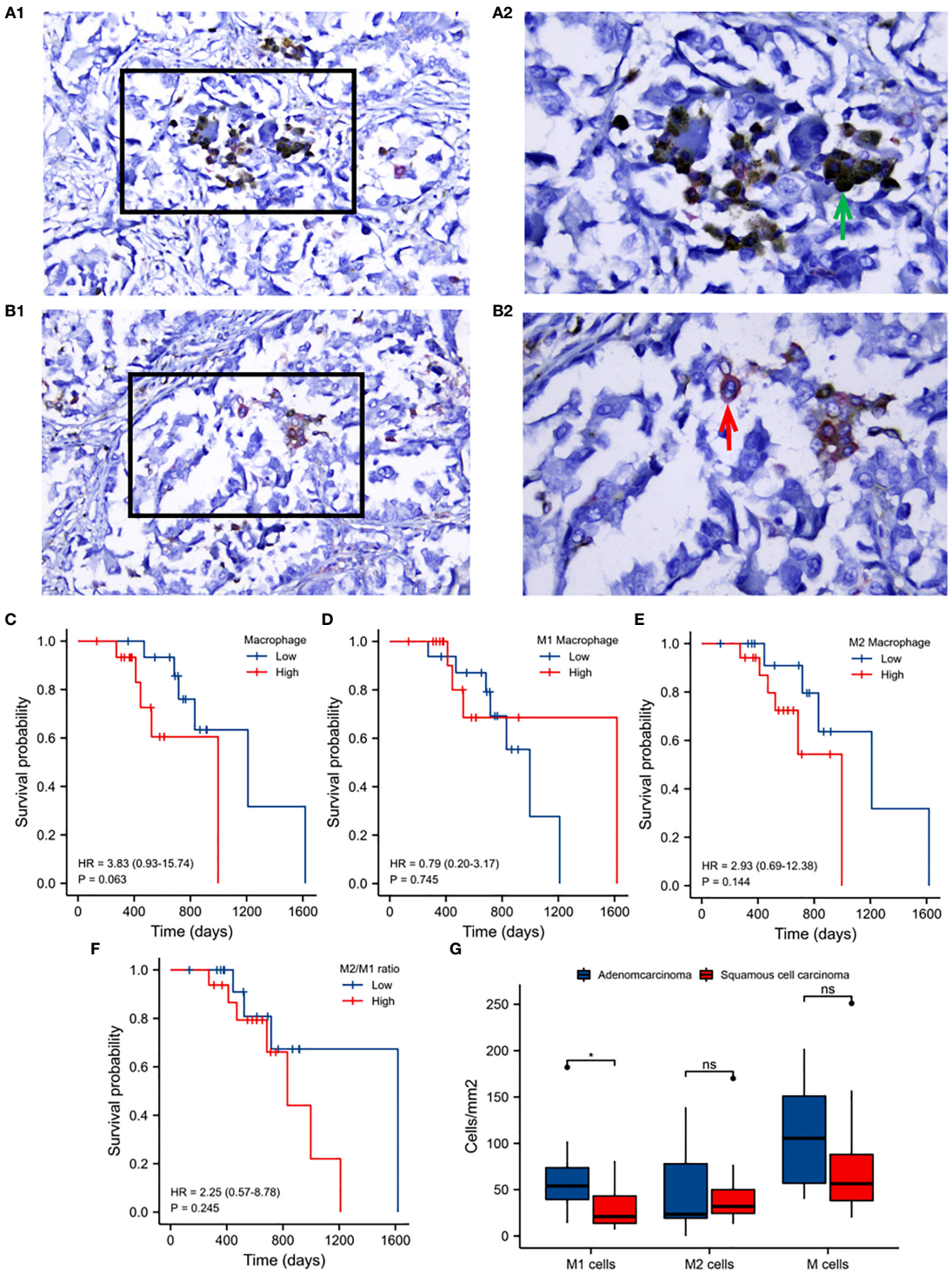

FIGURE 6
Validation analysis of clinical samples. A1: Immunohistochemical staining of M1 macrophages. A2: Enlargement of the boxed regions. B1: Immunohistochemical staining of M2 macrophages. B2: Enlargement of the boxed regions. Images were obtained at 40×10 magnification under a light microscope. **(C)** Kaplan-Meier survival analysis of macrophage infiltration. **(D)** M1 macrophage infiltration. **(E)** M2 macrophage infiltration. **(F)** M2 to M1 macrophage infiltration. **(G)** M1, M2 and total macrophage infiltration between adenocarcinoma and squamous cell carcinoma. Horizontal axis: survival time. Vertical axis: survival probability. Colors: macrophage infiltration.

were observed for clinical characteristics including radiation therapy, pathologic T, and Tumor stage (Supplementary Figures 6A–E). This further highlighted the efficiency of the risk score to predict therapeutic efficacy.

Through univariate and multivariate cox regression analysis, the risk score model held utility as an independent prognostic factor for cancer, further affirming its clinical benefits (Figure 5). Furthermore, cancer progression could be more accurately predicted using nomogram models constructed based on risk scores and clinical factors (Supplementary Figures 6F–J). For prognostic assessments of immunotherapy, the risk score model could also act as an accurate evaluation tool (Supplementary Figure 7). Upon immunohistochemical analysis of clinical tissue samples to verify the correlation between the macrophage phenotype and patient prognosis, similar conclusions were obtained (Table 1; Figures 1, 6). Macrophage infiltration, particularly for the M2 phenotype, were not conducive to the prognosis and survival of patients, consistent with previous studies (20–24, 56, 57).

We used WGCNA to identify macrophage infiltration-related module genes and single-cell sequencing of lung adenocarcinoma tissue to identify marker genes of macrophage subtypes. This permitted the construction of a risk assessment model with high prognostic efficacy. The model performed well on external and independent datasets. Immunohistochemistry analysis of clinical samples were consistent with our data. We therefore infer that the risk score has both high clinical practicability and application.

5 Conclusion

Macrophage infiltration was negatively correlated with prognosis for patients with lung adenocarcinoma. Based on cell-cluster-markers and TAMs-related-genes, both TOP8 genes (C1QTNF6, CCNB1, FSCN1, HMMR, KPNA2, PRC1, RRM2, TK1) and the risk score model containing 9 risk factors (C1QTNF6, FSCN1, KPNA2, GLI2, TYMS, BIRC3, RBBP7, KRT8, GPR65) had a high efficacy for the prediction of prognosis.

Data availability statement

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

Ethics statement

The ethics involved in the clinical samples in this study have been approved by the Ethics Committee of Qianfoshan Hospital of Shandong Province (2022-S527). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Corresponding authors (YT) conceived the idea and designed this study. TY was responsible for the final submission. YT, CZ and HZ were responsible for data collection, partial data analysis and drafting the manuscript. HL performed immunohistochemical staining of the clinical tissue and related data analysis. FK, SK, and FC were reviewed the manuscript and were responsible for data corrections. 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1028440/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Screening of modules corresponding to macrophages using WGCNA (Weighted Gene Co-Expression Network Analysis). **(A)** The cluster dendrogram of modular genes associated with macrophage infiltration.

(B) Heatmap of module-trait relationships associated with macrophage infiltration. **(C)** Module genes relevant heatmap related to macrophage infiltration. **(D)** Scatter plot of correlation between modules and gene features.

SUPPLEMENTARY FIGURE 2
Sample clustering, molecular typing, and prognosis evaluation analyses based on TAMs-related-genes. **(A)** Subtypes of clustering profiles obtained through analysis of the expression profiles of TAMs-related-genes in TCGA-LUAD samples (consensus matrix k=3). **(B)** Subtypes of consistent clustering profiles obtained through analysis of the expression profiles of TAMs-related-genes in GSE13213 samples (consensus matrix k=3). **(C)** Subtypes of the consistent clustering profiles obtained from GSE31210 samples (consensus matrix k=2). **(D)** Clustering profiles from GSE72094 samples (consensus matrix k=2). **(E)** GSE8894 samples (consensus matrix k=3). **(F)** Survival analysis of different cluster subtypes in TCGA-LUAD samples. **(G)** Results of survival analysis of different cluster subtypes in GSE13213 samples; The horizontal axis represents survival time; the vertical axis represents survival probability; Curves with different colors represent different cluster subtypes. **(H)** Results of survival analysis of different cluster subtypes in GSE31210 samples; The horizontal axis represents survival time; the vertical axis represents survival probability; Curves with different colors represent different cluster subtypes. **(I)** Results of survival analysis of different cluster subtypes in GSE72094 samples; The horizontal axis represents survival time; the vertical axis represents survival probability; Curves with different colors represent different cluster subtypes. **(J)** Results of survival analysis of different cluster subtypes in GSE8894 samples; The horizontal axis represents survival time; the vertical axis represents survival probability; Curves with different colors represent different cluster subtypes. **(K)** Results of principal component analysis (PCA) on TCGA-LUAD samples; **(L)** Results of principal component analysis (PCA) on GSE13213 samples; **(M)** Results of principal component analysis (PCA) on GSE31210 samples; **(N)** Results of principal component analysis (PCA) on GSE72094 samples; **(O)** Results of principal component analysis (PCA) on GSE8894 samples.

SUPPLEMENTARY FIGURE 3
Single-cell data. **(A)** Number of genes expressed in cells; **(B)** Total counts. **(C)** Mitochondrial gene expression. **(D–I)** UMAP (Uniform Manifold Approximation and Projection) dimensionality reduction analysis results of TAM subgroups. **(D)** C1QA. **(E)** C1QB. **(F)** APOE. **(G)** C1QC. **(H)** Analysis of TAM clusters. **(I)** Subtypes derived from re-clustering of TAM cell populations.

SUPPLEMENTARY FIGURE 4
Screening of differentially expressed genes amongst tumor macrophage subsets. **(A)** Dotplot of Top5 maker genes of each subtype. Abscissa axis: marker genes. Ordinate axis: top5 of TAM subtypes. Colors: mean expression per-group; Dot sizes represent the fraction of cells in each group (%). **(B–G)** Violin plots of the expression of the top5 marker genes. Abscissa axis: different TAM subtypes. Ordinate axis: gene expression.

SUPPLEMENTARY FIGURE 5
Pseudo-chronological analysis of tumor macrophages for simulation of the dynamic changes of macrophages. **(A–C)** Differential states according to monocle trajectory analysis, distribution of TAMs in trajectories, and pseudo-sequences of differentiation. **(D)** Genes influencing differentiation states in the clusters. Left column: different clusters. Right column: names of genes. **(E)** Pathway enrichment analysis of different TAM subtypes. Color: correlation; Red: positive correlation; Blue: negative correlation. Numerical values: correlation p-value.

SUPPLEMENTARY FIGURE 6
Comparison of risk scores corresponding to the clinical characteristics of the different groups. **(A)** Age, **(B)** Gender, **(C)** Radiation therapy. **(E)** Pathologic T cells; **(E)** Tumor stage. Abscissa axis: Different groups. Ordinate axis: risk scores. **(F)** Nomogram model for risk scores and clinical factors according to the clinical characteristics of prognosis. **(G)** Calibration curve for 1-year survival. **(H)**. 2-year survival. (I) 3-year survival. **(J)** 5-year survival. Abscissa axis: predicted probability of survival. Ordinate axis: actual survival.

SUPPLEMENTARY FIGURE 7
Assessment of immunotherapy prognosis according to the risk score. **(A)** Survival analysis of immunotherapy responses in the training set. Abscissa axis: survival time. Ordinate axis: survival probability. Colors represent different risk groups. **(B)** Comparative analysis of the proportion of treatment response states between high and low risk groups. **(C)** Comparative analysis of risk scores for different treatment response states.

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Tumor-associated macrophages in tumor progression and the role of traditional Chinese medicine in regulating TAMs to enhance antitumor effects

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Purpose: To emphasize the importance of tumor-associated macrophages (TAMs) in tumor immunity and to describe the ways in which extracts from Traditional Chinese Medicine (TCM) achieve tumor therapy by modulating macrophages.

Significance: By summarizing these available data, this review focused on TAMs and TCM and can build the foundation for future research on antitumor therapeutics.

Methods: In this review, we summarized the key functions of TAMs in cancer development and overviewed literature on TCM targeting TAMs together with other immune cells aiming to enhance antitumor immunity.

Conclusions: With an indispensable role in antitumor immunity, TAMs contribute to tumor progression, migration, invasion, angiogenesis, lymphangiogenesis, and immunosuppressive microenvironment. In recent years, TCM has gradually gained attention as a potential antitumor adjunctive therapy in preclinical and clinical trials. TCM is also a regulator of cytokine secretion and cell surface molecule expression in balancing the tumor microenvironment (TME), especially macrophage activation and polarization. Therefore, it is believed that TCM could serve as modifiers with immunomodulatory capability.

KEYWORDS

tumor-associated macrophages, traditional Chinese medicine, cancer, immunotherapy, tumor microenvironment

Introduction

Macrophages are unique components of innate and adaptive immunity to defeat foreign pathogens and tumor cells (1). Tissue-resident macrophages spread through the blood and are usually immobile unless they are induced by stimulations (2). The initial state of tissue macrophages is called M0 macrophages, also known as Mφ macrophages, before being stimulated into the M1(classically activated state) or M2 phenotype (alternatively activated state). The phenotypes can be interchanged in response to various stimuli, (or activation) (3, 4) (Table 1). M1 macrophages are induced by Th1 cytokines, such as lipopolysaccharide (LPS), interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF) and glucocorticoid. They highly express major histocompatibility complex (MHC) molecules and produce nitric oxide (NO), reactive oxygen species (ROS), and pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and IL-12. Above all, M1 macrophages are considered to exert antitumor activity.

Initially, macrophages were simply divided into M1 and M2 two subtypes. With the later research, M2 macrophages could be divided into M2a, M2b, and M2c subtypes according to different activators. M2a macrophages are activated by IL-4 or IL-13, and M2b macrophages are stimulated by immune complexes. M2c macrophages are induced by IL-10 and transforming growth factor (TGF)-β. M2 macrophages secrete anti-inflammatory cytokines and chemokines, including a large amount of IL-10 and little of IL-12 as well as chemokine ligand (CCL)-17, CCL-18, CCL-22, vascular endothelial growth factor (VEGF), TGF-β, and Arginase1 (ARG1). Because of the different cytokines and chemokines they secreted, these three subtypes undertake different functions. M2a macrophages are responsible for Th2 responses, and M2b macrophages could also regulate immune status and therefore lead to the Th2 response. M2c macrophages could suppress immune responses and increase tissue remodeling (6). The dynamic character of phenotype allows macrophages to perform various functions. However, due to the complex internal and external environment, it is difficult to comprehensively summarize the types of macrophages by centralized classification. Therefore, when describing the subtypes of macrophages, it is favorable to choose a series of markers to replace the previous classification methods (3).

Tumor-associated macrophages (TAMs), which are exposed to the tumor microenvironment (TME), undergo M1-like or M2-like activation and then display tumor promoting or suppressing activities (7). When defining the classification of TAMs, we should analyze them in combination with the course of the tumor and the time of cell separation. Choose as many markers as possible, including surface proteins and intracellular proteomics (8), to jointly define the properties of macrophages. TAMs can express VEGF, TGF-β, angiogenesis chemokine CXCL12, and platelet-derived growth factor (PDGF), which promote the formation of partial blood vessels and lymphatic vessels of tumor and even further tumor invasion and migration. During tumor initiation, the infiltrated macrophages display the M1 phenotype, which secretes inflammatory cytokines to defeat tumor cells along with other immune cells (9). However, as cancer advances to the later stages, TAMs convert to the M2 phenotype and create an immunosuppressive microenvironment to support further cancer proliferation, invasion, and metastasis, leading to poor prognoses. However, it is difficult to classify them with specific markers, and a series of markers are typically used for classification (10). The biomarkers of tissue macrophages are complicated because of their distinct locations and functions. F4/80^{hi} cells have been identified as the phenotypic definition of tissue macrophages in mice. Additionally, human macrophages exhibit characteristics that are similar to those of mice macrophages (11).

M1 macrophages express CD68, CD86, CD80, and high MHC class II complex. Scavenger receptor (SR), mannose receptor (MR), low MHC class II complex, and ARG1 are used as M2 phenotype markers (5).

Traditional Chinese Medicine (TCM) has developed for many years and is used as an adjuvant to chemotherapy.

TABLE 1 Classically and alternatively activated macrophages (3, 5).

	M1	M2a	M2b	M2c
Activators	LPS, IFN-γ, TNF-α	IL-4, IL-13	immune complexes, TLRs, or IL-1ra	IL-10, TGF-β, or glucocorticoids
Receptors	CD86, CD80, MHC II	CD163, CD206	CD86	CD163
Cytokines	TNF-α, IL-1, IL-6, IL-12, and IL-23	IL-10, TGF-β	TNF-α, IL-1, IL-6, IL-10	IL-10, TGF-β
Chemokines	CXCL10	CCL17, CCL13	CXCL13, CCL1, CCL20	
Arginase metabolism	L-citrulline and NO	polyamine and urea		
Functions	Th1 responses, tumor resistance	Th2 responses, type II inflammation, allergy	Th2 activation, immunoregulation	Inhibition of immune response, tissue remodeling

[1] Characteristics of classically and alternatively activated macrophages. M1 macrophages are classically polarized macrophages, while M2 macrophages could be divided into M2a, M2b and M2c depending on different activators.

Many antitumor natural products come from TCM. However, little is known about its underlying mechanisms and bioactivities because of the complex components and chemical structure and the difficult extraction and purification processes. In addition to the direct cytotoxic effects on tumors, TCM plays various immunomodulatory roles in TME, including angiogenesis inhibition, cell-cycle arrest or apoptosis induction (12), and immune cell regulators, such as activating antigen-presenting cells (APCs) and enhancing NK cell-mediated killing activity. Overall, TCM presents the ability to inhibit tumor progression, angiogenesis, invasion, and metastasis (13).

In this article, we summarized and discussed the characteristics and functions of TAMs in the TME and the mechanisms of TCM targeting TAMs in cancer biological therapy. The evaluations of TCM and TAMs will guide new opportunities in cancer therapeutic strategies.

TAMs and tumor progression

TAMs play indispensable roles in tumor progression, including initiation, promotion, immune suppression, angiogenesis, invasion, and metastasis (14). In the early stages of the tumor, stromal cells secrete colony-stimulating factor (CSF)-1 and other factors to recruit macrophages, which are primarily antitumor M1-like macrophages. However, in the advanced stages, tumor cells secrete other anti-inflammatory cytokines and chemical factors, such as CCL-2 and epidermal growth factor (EGF), leading to the recruitment and conversion of TAMs from the M1 to the M2 phenotype. The flow cytometry results showed that macrophages from advanced stages of hepatic carcinoma were mostly MHC class II^{low} TAMs, which were alternatively activated (15). It has also been confirmed that macrophages could be induced from M1 to M2 in a direct or indirect contact co-culture system with tumor cells (16, 17).

Cancer-related inflammation (CRI) refers to the relevance between the instability of the genome and inflammatory mediators, which are mainly composed of TAMs and other white blood cells, representing a hallmark of cancer. The fact that inflammation induces tumor progression through endogenous and exogenous pathways, suggests a relationship between the initiation of cancer and chronic inflammation caused by inflammatory cytokines produced by TAMs (18).

DNA damage could destroy the stability of genome stability. Poor DNA repair, apoptosis disorder, and radiotherapy or chemotherapy can lead to tumor initiation (19). Oxygen-free radicals have also been found to be critical in the initiation and progression of tumors (20). TAMs could produce IL-1 and TNF-α, promoting the formation of oxygen free radicals and further stimulating the macrophage response to other agonists (21). Meanwhile, the accumulation of reactive oxygen species (ROS) encourages macrophages to differentiate into a pro-inflammatory state, and therefore participate in the inflammation-induced tumorigenesis (22).

The density of the infiltrated TAMs and the M2/M1 ratio increases as tumors develop, leading to a poor prognosis (23). For example, the high proportion of $CD163^{+}$ tumor infiltrated macrophages is related to the poor clinical prognosis in clear cell renal cell carcinoma (RCC) (24), which is supported by another report where the decrease of macrophages partially inhibited the growth of hepatocellular carcinoma (HCC) (15). Clinical datasets show that the overall survival rate of patients with positive expression of M2 macrophages was significantly lower than patients with negative expression. Tian et al. (25) found that among patients with Wilms' tumor, longer survival time is correlated with a lower density of M2 phenotype macrophages, suggesting that the M2 macrophage index could be a predictor in the pathological examination. CD11c/CD206 signature is associated with macrophage polarization and can be used as an index to predict the prognosis. A $CD11c^{high}/CD206^{low}$ immune profile leads to a favorable outcome (26). Moreover, TAMs with the M2 phenotype could even affect the efficacy of chemotherapy and radiotherapy through the suppression of T cells (27).

TAMs in invasion and metastases

The protease produced or induced by invasive tumor cells can degrade the extracellular matrix (ECM). Thus, the invasion and migration of tumor cells are significantly enhanced compared to those of normal cells. In tumor stroma, TAMs produce enzymes, such as matrix metalloproteinases (MMPs) and urokinase fibrinolytic enzymes (uPA) to promote matrix degradation, and hence the invasion and metastasis of tumor cells (28).

As one of the MMPs, MMP-9 is a paracrine regulator of tumor progression (29) that degrades ECM, destructs the basement membrane, and spreads cancer through the circulatory system (30). The secretion of MMP-9 and VEGF by M2 phenotype TAMs was notably higher than that by M1 macrophages (31). It has been identified that MMPs are involved in the degrading and remodelling process of ECM (32), and induce epithelial-mesenchymal transition (EMT) by decomposing the adhesion molecules (33). EMT, a process that transits immotile cells to motile mesenchymal cells and therefore weakens the tight junction of tumor cells (34). Within these pathways, the transforming growth factor-β(TGF-β) is the primary regulator, which is also the key factor facilitating the proliferation and differentiation of TAMs (35). As demonstrated in gastric carcinoma (36) and hepatocellular carcinoma (37), EMT is related to the high infiltration of TAMs, which produce higher TGF-β1 than macrophages with other phenotypes.

TAMs in angiogenesis and lymphangiogenesis

Inducing angiogenesis and lymphangiogenesis is one of the major characteristics of tumor cells, the symbol of tumor expansion to distant metastasis. TAMs regulate tumor angiogenesis and lymphangiogenesis in two approaches: paracrine and cell autonomous mode (38). As the tumor proliferates, the supply of oxygen becomes insufficient, generating a hypoxia tumor microenvironment. Macrophages are recruited to the regions between tumor and interstitial cells where vascularization is poor (39). After being stimulated by hypoxia-inducible factor (HIF-1α), TAMs release a set of angiogenic cytokines, such as vascular endothelial growth factors (VEGF)-A (40), TGF-β, CXCL12, PDGF, and MMPs (7), which in turn promote tumor angiogenesis (40). At the same time, macrophages deliver more VEGF-receptors (VEGFRs) under hypoxia to combine with VEGF in the TME, which affects downstream pathways and promotes the transformation of TAMs to M2 phenotype. TAMs could also activate endothelial cells in cervical carcinoma, which highly express VEGF-C and VEGF-D, and stimulate existing lymphatic endothelial cells' proliferation (41). Furthermore, the existence of macrophage-derived lymphatic endothelial cell progenitors (M-LECP) has proved the autonomous mode. Under the stimulation of inflammatory factors, M-LECP could differentiate into lymphatic endothelial cells (LEC), contributing to pre-existing lymphatic vessels and subsequent lymphogenesis (38).

TAMs could overexpress HIF-1 and HIF-2, further up-regulating CXCL12. CXCL12 was found to be critical in enhancing the GM-CSF/Heparin-binding epidermal growth factor (HB-EGF) paracrine loop of colon cancer metastases in the liver, advancing tumor anti-apoptosis and the recruitment of TAMs (42). CXCL12 has also been identified to promote monocytes to differentiate into $CD163^+$ macrophages and increase the expressions of VEGF and angiogenic chemokine CCL1 (43). To overcome the hypoxia and immunosuppress of the TME, a biomimetic nano-RBC system (V(Hb)) combined with hemoglobin–poly(ϵ-caprolactone) (Hb–PCL) and doxorubicin (V(Hb)@DOX)) was engineered. V(Hb)@DOX could effectively limit the recruitment of $CD163^+$ M2-type macrophages and improve tumor hypoxia by reducing HIF-1α expression. Furthermore, the alleviation of the immunosuppressive TME decreased the secretion of MMP-9 and VEGF-A in tumors, which in turn inhibited tumor growth and metastasis (44).

TAMs and immunosuppressive microenvironment

The immunosuppressive tumor microenvironment consists of tumor cells, endothelial cells, fibroblasts, ECM, and immune cells et al. Immune cells therein include macrophages, dendritic cells (DCs), T cells, B cells, myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells and regulatory T cells (45). As chronic inflammation is essential in the immunosuppressive microenvironment, immune cells and inflammatory factors highly interacted with each other (46), which are summarized in Figure 1. It has been proposed that IL-10 secreted by MDSCs could down-regulate IL-12 produced by macrophages and thus induce macrophage polarization into the M2 phenotype (47).

TAMs restrain T cell-specific response in various aspects according to the recent findings. Extracellular vesicles (EVs), isolated from M2 phenotype macrophages, crippled $CD8^+$ T cell

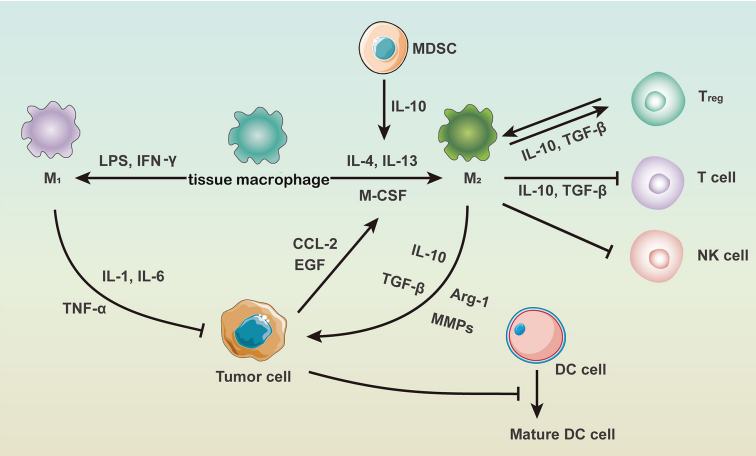

FIGURE 1
The role of macrophages in the immunosuppressive tumor microenvironment.

proliferation, and killing activity, leading to tumor immune evasion in murine hepatocellular carcinoma (48), colon cancer (49) and gastric cancer (50). TAMs depletion with a nanocarrier named $^{BLZ\text{-}945}$SCNs/Pt, which delivers both CSF-1R inhibitor-BLZ-945 and platinum (Pt)-prodrug, can achieve the synergistic antitumor activity of chemoimmunotherapy. The decrease in TAMs significantly reduced the expressions of TAMs-derived VEGF-A and MMP-9 and further less lung metastasis, which demonstrated the therapeutic efficacy of targeting TAMs in tumors (51). Sun (52) reported that Doxorubicin (DOX)-loaded micelles with a hemoglobin crown (Hb-DOXM) have also achieved significant antitumor effects in reprograming the immunosuppressive microenvironment into the immunostimulatory microenvironment by augmenting the release of O_2 and DOX and reducing the recruitment of M2-type macrophages in tumors.

For one thing, TAMs act as T cell activators using their surface MHC I or II molecules also by producing cytokines. For another, TAMs could induce T cell inhibition and exhaustion. Through direct and indirect regulations, TAMs are important in each of three steps to activate T cell response: specific binding of T cell receptors and MHC molecules on TAMs, costimulatory molecule signaling pathways, and environmental cytokines derived from TAMs (53). TAMs could secrete IL-10 to induce the expression of inhibitory receptors by T cells, such as programmed death (PD)-1 and cytotoxic T lymphocyte-associated antigen (CTLA)-4. The bindings of receptors and the corresponding ligands (PD-L1 and CD80/CD86) on the surface of TAMs, lead to negative regulations of T cell immune response, including apoptosis, anergy, and exhaustion (54).

Regulatory T cells can also weaken the immune functions of $CD4^+$ and $CD8^+$ T cells (55). Thymus-derived $CD4^+CD25^+Foxp3^+$ regulatory T cells could increase the percentage of $CD206^+$ and $CD163^+$ macrophages differentiated from monocytes and up-regulate CCL18 and IL-1Ra produced by macrophages (56). Moreover, TAMs and DCs could increase the production of IL-10 and TGF-β, which transform naïve T cells into regulatory T cells (55) and further inhibit the antitumor immunity contributed by NK cells.

In summary, targeting the regulation of immune cell balance and augmenting tumor immunity in the microenvironment has always been the focus of tumor immunotherapy.

TAMs in canine tumors

Similar to human tumors, many studies suggest that TAMs have a relationship with the grading of malignant tumors in veterinary science.

In canine lymphoma, tumor-infiltrating macrophages could be characterized as M1 and M2 according to iNOS, CD204 and CD163. As shown in the immunohistochemical results, the type of macrophages changed from M1 to M2 in the high histological grade. Among the two immunophenotypes of lymphomas, type B and type T, M2 macrophages have a dominant position in T-type lymphoma (57). Unlike human tumors, in many cases, it has been reported that CD204 is a better choice than using CD163 as the marker of the M2 macrophage subtype in canine tumors (58).

In canine mammary gland tumors, another result supported that tumor-infiltrating M2 macrophages have been correlated with the grading of malignant lesions (59). Furthermore, the high density of TAMs in canine mammary tumors has also been considered as a poor prognosis (60). TAMs also have a significant relationship with the expression of VEGF in canine mammary tumors, suggesting that TAMs synergistically promote tumor angiogenesis (61). Above all, TAMs may act as a potential target in the therapy of canine mammary tumors.

TAMs regulated by TCM

Many studies supported that TCM plays an important role in cancer treatment, including promoting immune function, activating immune cells, enhancing the efficacy of antineoplastic, and reducing the side effects of radiotherapy and chemotherapy (62). Some kinds of TCM can directly inhibit the proliferation of tumor cells, while others suppress tumor growth, invasion, and metastasis indirectly by indirectly regulating the immune system (63). Vincristine and paclitaxel are common commercialized chemotherapeutics extracted from TCM in clinical applications. According to National Medical Products Administration, there are more extractions from TCM that are used as adjuvant therapies with radiotherapy and chemotherapy, such as lentinan, krestin et al. Since the 1980s, both China and Japan have approved the use of the mushroom polysaccharide, lentinan, as an adjuvant medicinal medication for the treatment of cancers. Lentinan was mainly used in treating lung (64), gastric, and colorectal cancers as adjuvant therapies and exhibited better efficacy and clinal response rates, as well as improved the quality of life of cancer patients, according to a review that summarized 9474 reported lentinan-associated cancer treatment cases (65). Additionally, polysaccharide-kureha (PSK), also known as Krestin, was authorized for the treatment of various cancers (66). PSK is frequently given orally, either alone or in combination with other drugs. Together with tegafur/uracil (UFT), PSK significantly increased stage II and stage III colorectal cancer patients' 5-year disease-free survival and reduced the risk of recurrence and lung metastases (67).

The common active components from TCM with macrophage regulatory effects are glycosides, alkaloids, and polysaccharides, which could activate MAPKs, MyD88, and NF-κB related pathways by one or more receptors. The downstream phagocytic activity, ROS, NO, and relevant anti-

tumor cytokines of TAMs are further enhanced accounting for the complete antitumor immune regulation (68).

TCM activates antitumor phenotype and inhibits tumor-promoting phenotype of TAMs

TCM regulates macrophages in various ways, including activating anti-tumor macrophages, inhibiting the recruitment and activation of TAMs, transforming the phenotype of TAMs, and indirectly regulating TAMs by altering cytokine secretions in the tumor microenvironment.

It has been proven that acidic polysaccharides from Plantago major leaves could activate J774 macrophages and increase the release of NO and TNF-α (69). The polysaccharide extracts from Plantago depressa have also been shown as an immunomodulatory agent by promoting lymphocyte proliferation and NO production (70). Emodin inhibited the expressions of CCL2 and CSF1, which were involved in the differentiation of macrophages (71). It could also reduce the growth of EO771 and 4T1 breast tumor cells by suppressing macrophage migration and polarization, and inhibiting IRF4 and C/EBPβ signalings (72). Although astragalus polysaccharide (APS) could not inhibit the MCF-7 cell viability directly, it could activate RAW264.7 cells and up-regulate the production of NO and TNF-α, to induce the apoptosis of breast tumor cells (63). Ginseng polysaccharide (GPS) was shown to have similar functions as APS and exerted a cytotoxic effect against mice tumor cells *via* activating the peritoneal macrophages (PMs) rather than direct cytotoxicity (73).

Furthermore, TCM could cooperate with radiotherapy and chemotherapy, to enhance the curative effect of both and meanwhile alleviate the common side effects. A kind of water-extracted polysaccharides from Fuzi was found to promote the phagocytic activity and the release of NO, IL-6, IL-1, and TNF-α in RAW264.7 cells. Also, it had the ability to reverse the spleen index and thymus index in cyclophosphamide-induced immunosuppressed mice, demonstrating its possible application in antitumor therapy as an immunomodulator (74). Interestingly, not all TCM act as anti-tumor agents. Methanol extracts of Xanthium sibiricum roots (MXS) inhibit the NO, IL-6, IL-1β and TNF-α by suppressing IκBα and STAT3 signaling pathways in LPS-induced RAW264.7 macrophages (75).

Not only the monomers of TCM but also some complex TCM formulas have been proved to have macrophage-regulating functions. Bu-Fei Decoction (BFD), a conventional TCM constituted of six herbs, is often used for tonification and alleviating symptoms of lung cancer. In non-small cell lung cancer (NSCLC), BFD decreased the expressions of IL-10, PD-L1, and CD206 in TAMs induced *in vitro* by PMA and IL-4. Besides, BFD exhibited a dose-dependent inhibition of the invasion and migration of NSCLC cells *via* downregulating IL-10 and PD-L1 both *in vivo* and *in vitro* (76).

TCM and macrophage polarization

M1-like TAM is the dominant phenotype suppressing tumor growth in the initial immune microenvironment of the tumor, but the M2 phenotype gradually replaces its leading position by recruiting tumor cells as the tumor advances to the later stages (7). In the tumor microenvironment, TAMs can switch between M1 and M2 states depending on the different signal inductions (13). Hence, finding new approaches to change TAMs from M2 to M1 phenotype could assist the antitumor immunity and prevent tumors from immune escape.

Astragalus polysaccharide (PG2) has been indicated as a modifier of macrophage polarization in NSCLC both *in vivo* and *in vitro*. PG2 enhanced the M1 polarization and reduced the $CD206^+$ M2 cells in a dose-dependent manner. Also, PG2 could inhibit the tumor enhancement (including proliferation, clonogenicity to form tumorspheres, and invasion *via* IL-6/STAT3 signaling suppression) from a stem-cell-like phenotype of NSCLC induced by M2 macrophage. Furthermore, PG2 prominently strengthened the tumor-suppressive effect of cisplatin in NSCLC tumor-bearing mice models, but also alleviated dysuria and weight loss caused by cisplatin (77). Macrophages play an important role in baicalin-mediated inhibition of hepatocellular carcinoma (HCC). They were re-programmed towards the M1 phenotype to prevent tumor cells from immune escape, which is characterized as the descending proliferation and invasiveness of HCC cells. This repolarization was related to the autophagy-associated up-regulation of RelB/p52 (78).

A novel polysaccharide WCCP-N-b isolated from Cantharellus cibarius can induce M2-like bone marrow-derived macrophages (BMDMs), mouse peritoneal macrophages, and RAW264.7, to M1 phenotype. After being treated by WCCP-N-b, macrophages affected melanoma cell viability *via* increasing the production of TNF-α, which was cytotoxic to tumor cells (79). Water extract of ginseng and astragalus (WEGA) is reported to promote macrophages to express M1 markers and down-regulate M2 marker expressions simultaneously. Furthermore, WEGA also promoted immune responses, which were suppressed by cisplatin (62).

Emodin has received much attention due to its inhibiting effect on TAMs and its antitumor activity. It has been found that Emodin could inhibit IRF4, STAT6, and C/EBPβ signaling pathways *in vivo* to suppress macrophage infiltration and M2 polarization accompanied by T-cell activation, and therefore reduce breast cancer growth (72). Moreover, Emodin significantly inhibited breast cancer lung metastasis by inhibiting M2 polarization in metastatic lungs (80). Emodin

inhibited the activation of NF-κB, STAT1, and IRF5 signaling pathways induced by LPS/IFNγ, and the stimulation of STAT6 and IRF4 signaling pathways stimulated by IL4 (81). Taken together, Emodin adopts an inhibitory effect on tumor growth by restoring macrophage homeostasis in the tumor-suppressive immune microenvironment.

TCM and TME regulation

Some TCMs have direct cell killing effects, while most have lower cytotoxicity, but could enhance the bioactivity of immune cells or inhibit immunosuppressive cells for anti-tumor purposes.

Evidence showed that a polysaccharide extracted from the whole plant of *Plantago Asiatica* L. could recruit immune cells (DCs, macrophages, and T cells) in the murine breast tumor model and accelerate the maturation of DCs, which promoted the proliferation and differentiation of T cells. Plantain polysaccharides had no direct cytotoxicity to breast tumor cells. However, it inhibited tumor growth by promoting the autoimmune response in mice (82). Modified citrus pectin (MCP) has been identified to accelerate the activation of the T-lymphocyte subset, B cells, and NK cells (83). Astragalus polysaccharide (PG2) not only regulated the macrophage phenotype but also promoted the maturation of immature DCs and recruitment of $CD8^+$ T cells for anticancer immune response in NSCLC (77).

Besides, many studies showed a close relationship between immunosuppressive cells and TCM. TCM could suppress the recruitment and metastasis of immunosuppressive cells when tumor-promoting immune cells are dominant in quantity and function. Silibinin, extracted from milk thistle, dwindled tumors in 4T1 tumor-bearing mice by decreasing MDSCs infiltration and M2-like polarization of macrophages. However, in an immunodeficient mouse model, similar efficacy was not observed, suggesting the anti-tumor response of Silibinin was based upon the integrity of the immune system (84). Maitake D (MD)-Fraction, a β-glucan extracted from Grifola frondose, inhibited the growth of mammary carcinoma and colonic adenocarcinoma cells and enhanced immune cell infiltration in the tumor microenvironment, including T cells, B cells, DCs and NK cells. DC maturation, specific T cell responses, and the infiltration and anergy of Tregs and MDSCs were induced by orally administered MD-Fraction, suggesting the significance of converting immunosuppressive elements of the TME in tumor immunotherapy (85).

Not only polysaccharides from TCM could enhance the immune system, but other types of natural products, including terpenes, alkaloids, saponins, and flavonoids, also have the ability. Andrographolide, an isoprenoid extracted from Andrographis paniculate, has been reported to exhibit cytotoxicity to nearly all kinds of cancer cells and mediation of the immune system (86). In another study, Andrographolide released a high level of IL-2 and IFN-γ, promoted cytotoxic T lymphocyte (CTL) production and prolonged the survival time of mice bearing lymphoma (87).

TCM delivery system

Considering the instability and low bioavailability of active components from natural TCM, it is quite challenging to apply them directly *in vivo*. In recent years, the rapid development of nano-drug delivery systems has made it possible to deliver TCM or employ TCM as drug carriers for cancer treatment. A growing amount of TCM has been delivered to tumor tissues and their stroma through nano-drug delivery, such as liposome, or precision targeted therapy and immune regulation. Astragalus polysaccharide liposome (APSL) has been demonstrated to enhance the phagocytosis of murine peritoneal macrophages and speed up the DC-mediated immune reactions compared to applying AP alone (88). Moreover, cell-membrane-coated nanoparticles showed high efficiency in passing through the biofilm barrier and slowing down the metabolism of the loaded drugs. A novel macrophage-biomimetic drug delivery system carrying Saikosaponin D was reported to inhibit cell migration of MCF-7 and 4T1 cells *in vitro* and significantly reduced tumor growth and lung and spleen metastasis by promoting dephosphorylation of AKT and Erk in tumor-bearing mice (89).

TCM and canine tumors

The application of TCM in human treatment has gradually increased because of its chemopreventive and chemotherapeutic effects. However, its role in the small animal clinical field should not be underestimated. TCM could also be considered as an approach for clinical therapy to inhibit the growth of canine tumor cells. For example, Paclitaxel has been widely used to treat lung, ovarian, and breast cancer. It was reported to inhibit the migration of canine hemangiosarcoma (HSA) cells with the increase of time and concentration (90). In canine melanoma cells, oral paclitaxel was also tested to decrease the proliferation of tumor cells both *in vivo* and *in vitro* by arresting cell cycle (91). Canine mammary tumors are common among female dogs and the risks of malignancy are relatively high. BmKn-2, a peptide extracted from the venom of scorpions, has been proved to have antitumor activity in both human and canine tumor cells. It inhibited canine mammary gland tumor cell proliferation *via* inducing apoptosis, which was represented by the decrease of Bcl-2 to Bax ratios (92). Besides the direct tumor-killing effect, TCM could contribute to the immunomodulatory effects targeting immune cells and consequently hinder tumor progress. Our team has been focused on the study of TCM in antitumor immunity regulations and proved that although Plantain polysaccharide (PLP) showed no cytotoxicity to canine mammary cells (CIPp), conditioned medium obtained

from PLP to DCs had inhibitory effects on CIPp cells. Moreover, it could promote the maturation of DCs and thus facilitate the proliferation of lymphocytes, which exert the main toxicity effect (82). All in all, TCM is of great significance in regulating animals with poor immune status, especially for the tumor patients.

Conclusions

Overall, TAMs could suppress anti-tumor immunity by promoting cancer proliferation, invasion, metastases, and angiogenesis. Besides, TAMs contribute to the immunosuppressive microenvironment to further advance cancer development. TCM has been proved to be an effective method for reprogramming TAMs and other immune cells and turning the immunosuppressive microenvironment into an antitumor one. In this study, we provide support for further studies on antitumor immunity and immunotherapy.

Author contributions

Writing—original draft preparation, JZ and JC. Writing—review and editing, JL and JG. Working concept and design, YJ and YW. Data collation, DZ. Supervision, JL and DL. 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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Cutting edges and therapeutic opportunities on tumor-associated macrophages in lung cancer

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Lung cancer is a disease with remarkable heterogeneity. A deep understanding of the tumor microenvironment (TME) offers potential therapeutic strategies against this malignant disease. More and more attention has been paid to the roles of macrophages in the TME. This article briefly summarizes the origin of macrophages, the mutual regulation between anti-tumoral immunity and pro-tumoral statuses derived from macrophage polarization, and the therapeutic opportunities targeting alternately activated macrophages (AAM)-type macrophage polarization. Among them, cellular components including T cells, as well as acellular components represented by IL-4 and IL-13 are key regulators driving the polarization of AAM macrophages. Novel treatments targeting macrophage-associated mechanisms are mainly divided into small molecule inhibitors, monoclonal antibodies, and other therapies to re-acclimate AMM macrophages. Finally, we paid special attention to an immunosuppressive subgroup of macrophages with T cell immunoglobulin and mucin domain-3 (TIM-3) expression. Based on cellular interactions with cancer cells, TIM3+ macrophages facilitate the proliferation and progression of cancer cells, yet this process exposes targets blocking the ligand-receptor recognition. To sum up, this is a systematic review on the mechanism of tumor-associated macrophages (TAM) polarization, therapeutic strategies and the biological functions of Tim-3 positive macrophages that aims to provide new insights into the pathogenesis and treatment of lung cancer.

KEYWORDS

lung cancer, tumor microenvironment (TME), macrophages, anti-tumoral immunity, macrophage polarization

Introduction

Lung cancer is the second most common cancer worldwide and the leading cause of death (1). In recent years, the morbidity and mortality of lung cancer have accelerated significantly. Taking the United States as an example, it is estimated that there will be 1,898,160 new cancer cases in 2021, of which lung cancer ranks second in both male and female patients, accounting for 12% and 13%, respectively. And among the 608,570 estimated cancer deaths, lung cancer ranks first in mortality. Lung cancer is the leading cause of cancer death among men in both developed and underdeveloped countries (2). According to the clinical histological characteristics, lung cancer is mainly divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) which make up 85% of all cases. The common subtypes of NSCLC mainly include lung adenocarcinoma, lung squamous cell carcinoma (LUSC) and large cell carcinoma (3, 4). The annual survival rate of lung cancer cases is only 15.9%, and this data has only improved slightly over the past few decades (5).

The TME is the environment surrounding tumor cells. The TME is heterogeneous and consists of immune cells, fibroblasts, endothelial cells and neuronal cells, their extracellular matrix (ECM) proteins, signaling molecules and surrounding blood vessels (6). The TME is closely related with tumorigenesis and cancer progression through multiple mechanisms, including promoting epithelial-to-mesenchymal transition (EMT), facilitating tumor infiltration and contributing to immune suppression (7). The lung cancer microenvironment is characterized with prominent intra-tumoral heterogeneity, which could be caused by the heterogeneity of TME including mechanical properties, acidity conditions, and signaling molecules (8). A full understanding of the TME will facilitate the further development of effective therapies for lung cancer. In this review, we focused on TAMs, a critical component of the TME that plays an important role in the pathogenesis of lung cancer. We discussed the mutual regulation between anti-tumoral immunity and pro-tumoral statuses derived from macrophage polarization, and explore potential therapeutic opportunities targeting alternately activated macrophages (AAM)-type macrophage polarization in lung cancer.

Abbreviations: EMT, Epithelial-to-mesenchymal transition; TME, Tumor microenvironment; TIM-3, T cell immunoglobulin and mucin domain-3; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; LUSC, lung squamous cell carcinoma; ECM, extracellular matrix; NK cells, natural killer cells; DC cells, dendritic cells; MDSC, myeloid-derived suppresser cells regulatory T Treg cells; TAM, tumor-associated macrophages; MPS, mononuclear phagocytic system; CAM, classically activated macrophages; AAM, alternately activated macrophages; MDSCs, myeloid-derived suppressor cells; TLRs, Toll-like receptors; TGF-β, transforming growth factor-β; GCs, glucocorticoids; IgSF, immunoglobulin superfamily; IgV, immunoglobulin domain; CVB3, coxsackievirus B3; Mtb, mycobacterium tuberculosis; MDSC, myeloid-derived suppressor cells.

Macrophages: An important component in the immune microenvironment of lung cancer

Tumors are increasingly seen as complex 'ecosystems' where multiple interactions take place among cancer cells, immune cells as well as various components in the extracellular matrix (ECM) (9). The ECM comprises the majority of non-cellular TME, such as laminin, collagen, and fibronectin, while the cellular components surrounding tumor cells include immune cells (such as lymphocytes, NK cells, macrophages and dendritic cells) and non-immune cells (such as fibroblasts and vascular endothelial cells), collectively determining their roles in tumorigenesis and tumor progression. More and more evidence suggested that instead of driving uncontrollable proliferation and distant metastases on its own, cancer cells interact with the TME cells to re-shape the lesion into an immunosuppressive, chronic inflammatory, and pro-angiogenic microenvironment (10, 11). During the early stage of tumorigenesis, TME cells including the infiltrating inflammatory cells, endothelial progenitor cells, and cancer-associated fibroblasts constituted the infrastructure of cancer niches. With the proliferation of cancer cells, more immune cells infiltrated in. According to its role in carcinogenesis, TME cells could be divided into pro- and anti-tumoral components (12). Anti-tumoral macrophages, lymphocytes, natural killer (NK) cells, and dendritic cells (DC), which originated from the host microenvironment or recruited from the circulating system, were inhibited and acclimated by the immunosuppressive components, represented by myeloid-derived suppresser cells (MDSC), regulatory T (Treg) cells and M2 subtype macrophages (also known as tumor-associated macrophages, TAM) (10). M2-polarized macrophages can secrete interleukins that promote lung cancer tumorigenesis and metastasis. In turn, some interleukins can prime macrophage M2-polarization through stimulating the expression of interleukin receptors (13). Initially, macrophages performed both phagocytosis and antigen-presentation, while TAMs nourished tumor cells through a multitude of signaling pathways, hindered effector cells from attacking cancer cells, and promoted the occurrence, development and metastasis of malignant cancers (14–17).

Origin of macrophages

In view of its tissue of origin, macrophages can be divided into two main subtypes. Belong to the mononuclear phagocytic

system (MPS), some macrophages are differentiated from monocytes that were released from the bone marrow (18). Other tissue macrophages were derived from embryonic progenitor cells, maintained by *in situ* self-renewal without being replenished from the bone marrow (19–21). For example, in the epidermis and central nervous system, the vast majority of macrophages were maintained in a self-renewing manner instead from the recruitment of circulating monocytes (19, 22, 23). However, in the spleen and gut, bone marrow-derived macrophages contributed more (20, 24). Additionally, based on pathways of activation, macrophages were classified into "activated" macrophages involved in Th1-response and "alternatively activated" macrophages involved in Th2-response, and some researchers proposed that antigen-presenting DC in the circulation were also a member of the MPS lineage (14). Under pathological conditions, the monocyte/macrophage distribution was re-arranged. For instance, cytomegalovirus infection resulted in an accumulation of MPS cells in the bone marrow whereas a decrease in the peripheral circulation (17). Another report demonstrated that Th2-type inflammation promoted rapidly *in situ* proliferation of macrophages to avert potential tissue damage caused by universal recruitment of circulating inflammatory cells (25). In addition, extramedullary sites, such as the spleen, can generate bone marrow-derived monocytes and store, expand, and distribute in response to inflammatory signals (26, 27).

Polarization of macrophages

Under pathological circumstances, TAMs played an indispensable role in the initiation and progression of lung tumors (28, 29). Since the discovery in the 1990s that IL-4 induces macrophage gene expression differently from classical gamma-interferon and bacterial lipopolysaccharide activation, this IL-4-inducible macrophage gene has been termed "alternative activated" macrophages (30). Meanwhile, macrophages are phenotypically and functionally heterogeneous, and macrophages can also be divided into two groups based on their phenotypic profile and local microenvironment: the pro-inflammatory "classically activated macrophages (CAM)" and the anti-inflammatory "alternately activated macrophages (AAM)" (31). CAMs perform the functions of immune surveillance and antigen presentation, secrete pro-inflammatory cytokines and chemokines, participate in positive immune responses. On the contrary, AAMs have a much weaker antigen-presenting ability, while playing an important role in immune regulation by secreting inhibitory cytokines such as IL-10 and/or TGF-β, downregulating anti-tumoral immune response. For surface biomarkers, CD14 is a common biomarker of monocyte/macrophages (32, 33), but the two subtypes of macrophages have differentiated expression of CD206, IL-10, and IL-12 (34, 35). CAM-type macrophages express MHC II, CD86, NO, iNOS, showing the characteristics of pro-inflammatory response and anti-tumor, while AAM-type macrophages express IL-10, arg-1, CD206, CD163, TGF-β, showing immunosuppressive and tumor-promoting characteristics (36). The classification of CAM-type macrophages and AAM-type macrophages was originally proposed for tissue macrophages and can also be extended to peripheral circulating blood monocytes (37). In the field of oncology, two macrophage subclusters were investigated, and the polarization of CAM towards AAM was reported to be correlated with poor prognosis and treatment failure (34, 36). Cellular and molecular mechanisms were reported. The cancer-AAM interactions facilitated the invasiveness of cancer cells and destruction of TME matrix in co-culture system (36). AAM also communicated with cancer cells by chemokines. Interleukin-6 secreted by AAM activated STAT3 signaling pathway and promoted proliferation and sphere formation of lung cancer cells (37).

Mutual regulation between TAM and TME

Chronic inflammation and wound healing have a close relationship with carcinogenesis and tumor progression (38). TAMs are the most abundant immune cells in the TME, and have the characteristic of polarizing towards AAM-type macrophages. As a major component of infiltrating immune cells present in tumor tissue, TAMs are closely related to the inflammatory response in the tumor tissue, and aids tumor progression as well as metastasis (15, 16, 38). After being "educated" into TAMs, macrophages nourish the survival of tumor cells through various signaling pathways (15).

TAMs and tumor cells mutually promote each other through paracrine EGF/CSF-1 signaling (39, 40). Cancer cells secret CCL2 and CSF1 to recruit macrophages from circulating monocytes, and simultaneously IL-10 and PGE2 to facilitate immune evasion (41–43). To fuel tumorigenesis in the TME, TAM can secrete pro-angiogenic cytokines in the hypoxic TME including VEGFA, VEGFC and PDGF to facilitate tumor angiogenesis (14, 40). To destruct the tumor stroma, TAM also secrets proteases such as cysteine cathepsin and further promotes the invasion of cancer cells into the neo-vascularization to drive tumor progression (41). In addition to expressing VEGF-A and other angiogenic factors, TAMs also express Tie2 receptors that interact with endothelial cells and pericytes lining the tumoral vascularization to up-regulate angiogenesis (44). TAMs functions as a pivotal cellular component, in that macrophages also interact with other immune cells in the immunosuppressive TME. The PD-L1/PD-1 pair exists between the antigen-presenting TAMs and cytotoxic T cells, thereby inhibiting the antitumor effect of effector T cells (45, 46). Increased numbers of neutrophils are

closely associated with poor prognosis in non-small cell lung cancer (NSCLC), possibly due to their expression of elastase that degrades the stroma in the microenvironment (47, 48). As tumors grow, immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T (Treg) cells enter the circulation in response to activate cytokine axes such as TGF-β and CXCL5-CXCR2 pathways (49). MDSCs and Treg cells infiltrate into the growing tumors, promote tumor angiogenesis and interferes with innate immunity by immune surveillance and antigen presentation, adaptive immunity *via* disrupting lymphocyte proliferation and biological functions, and damaging cytotoxicity of effector cells (50–53). Moreover, accumulated MDSCs can increase the degradation of stroma, thereby attenuating structural resistance for tumor proliferation, metastasis and angiogenesis (54, 55). In conclusion, TAM is the key to the immunosuppressive TME, and the crosstalk between TAM and various immune cells and TME cytokines plays an irreplaceable role. Understanding the main mechanisms by which TAMs are involved in tumor immunosuppression will help us improve clinical considerations and develop potential new strategies to overcome macrophage-related immune tolerance (Figure 1).

Molecular mechanisms of AAM-type macrophage polarization

IL-4, IL-13 signaling promotes polarization towards AAM macrophages

AAM-type macrophages involved in Th2-type polarization can help the body eliminate parasites, suppress inflammation, promote tissue repair, promote tumor growth, and participate in other immune regulations. Compared with the activation of CAM-type macrophages, the activation of AAM-type macrophages is relatively diverse. The polarization of AAM-type macrophages was first reported to result from the action of Th2-type cytokines IL-4 and IL-13 (30, 56, 57). The main receptors of the IL-4 signaling pathway are type I IL-4 receptors (IL-4Rα or IL-4Rγc) or type II IL-4 receptors (IL-4Rα or IL-13Rα1), while IL-13 signals through type II IL-4 receptor (58). The differential expression of type I or type II receptors on different cell types determines their different sensitivities to IL-4 and IL-13. Monocytes and macrophages have type I and type II receptors and are responsive to both cytokines (58, 59). IL-13Rα2, as a component of type II receptors, can act as a decoy for IL-13 and inhibit the selective activation of monocytes (60). The downstream signaling pathway of the IL-4 receptor involves the activation of multiple Janus kinases (56, 57, 61). Stat3 and Stat6 play crucial roles in AAM-type macrophage polarization (56, 62). Phosphorylated Stat is further transferred into the nucleus to regulate targeted genes involved in macrophage polarization (62). IL-10 secreted by Treg cells and B cells acts on IL-10R of macrophages, and then regulates Stat3 to promote the polarization of AAM-type macrophages and play an immunosuppressive role. In recent years, it has been found that Stat3 is highly activated in various tumor tissues (63, 64).

At the same time, other regulatory genes PPAR-γ, IRF4, JMJD3, and p50 are also involved in regulating the expression of AAM macrophage marker genes such as YM1, FIZZ1, Arg1, CCL17, and CCL22. STAT6 also induces the expression of the transcription factor PPAR-γ, which cooperates with STAT6 to regulate macrophage polarization and increase the expression of AAM-type biomarkers in a murine model of obesity (65). At the epigenetic level, the histone demethylase JMJD3 regulates the AAM macrophage-related genes Arg1, Chi3l3 (Ym1) and Retnla through the mutual change of histone H3 Lys4 (H3K4) and histone H3 Lys27 (H3K27) (Fizz1) transcription (66). IL-4 induces upregulation of JMJD3, which in turn reduces the histone methylation and activates transcription on the promoters of polarization driver genes (30, 62). IL-4 also causes activation of the PI3K signaling pathway, and studies have found that the PI3K subunit PI3Kγ promotes the polarization of AAM-type macrophages in pancreatic ductal carcinoma to exacerbate cancer progression (67, 68). The mutual regulatory function of Stat6 and PI3K in the induction of TAMs polarization has not been directly reported, but IL-4 has been shown to be an important mediator of TAMs polarization in some murine tumor models (59).

Notably, molecular interactions of various signaling pathways also promoted the AAM polarization in the TME. For example, studies found that IL-4 induce the IRF4 expression to promote macrophage polarization not only by Stat6 or PI3K signaling pathway, but also by metabolic regulation such as glycolysis (69), and IRF4 has been reported to be a contributing factor of AAM-type polarization (70). To sum, IL-4 and IL-13 mediated macrophage polarization toward the anti-inflammatory and pro-tumoral phenotype, and function as pivotal molecules connecting several mechanisms.

Other elements inducing AAM polarization

According to different activation mechanisms, AAM macrophages can be further divided into three subtypes: M2a, M2b, and M2c (71). M2a macrophages are mainly stimulated by Th2 cytokines represented by IL-4 and IL-14 (72). M2b macrophages are induced by ICs and agonists of Toll-like receptors (TLRs) or IL-1R (15, 71). M2c macrophages are activated by IL-10, transforming growth factor-β (TGF-β), and glucocorticoids (GCs) to antagonize effector cells and induce immune regulation (71, 73).

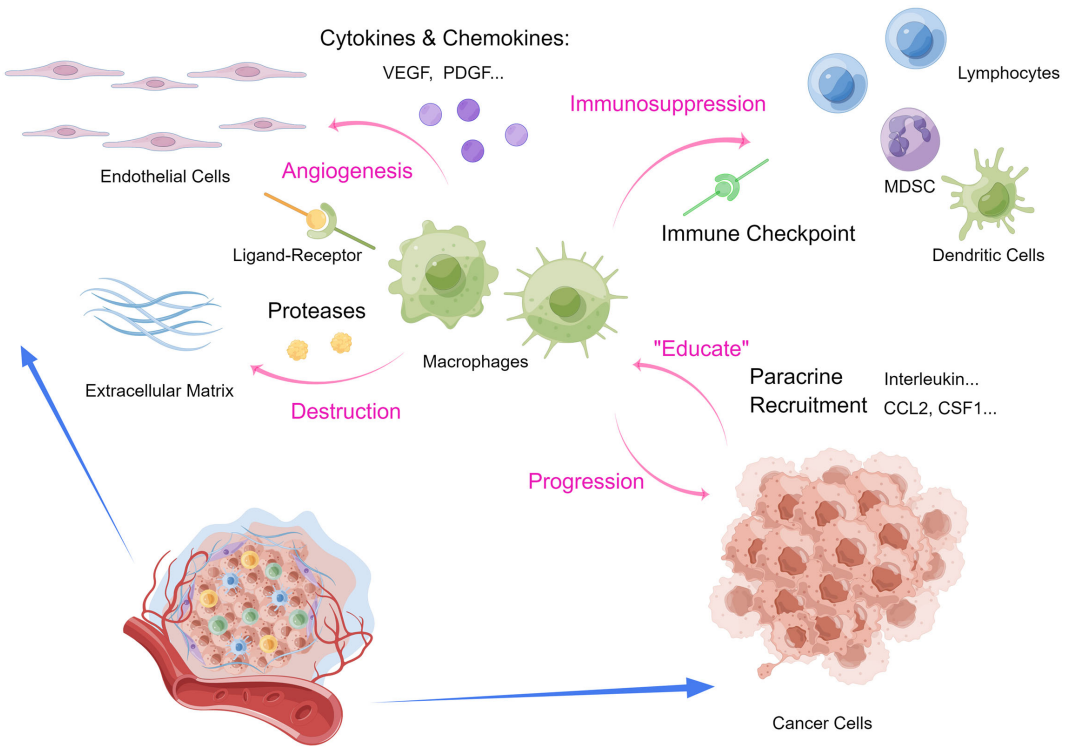

FIGURE 1
Interactions between macrophages and tumor microenvironment. (MDSC, myeloid-derived suppressor cells.) (By Figdraw.).

Immune complexes can promote the polarization of AAM macrophages through FcγR. Binding of immune complexes to activated FcγRs on macrophages triggers a tyrosine kinase Syk-dependent pathway that not only inhibits TLR4 signaling but also inhibits type I interferon through upregulation of IL-10 and negative regulation of A20, ABIN3 and SOCS3 type interferon signal, indicating an increased biological effect of anti-tumoral macrophages (74). Ligation of immune complexes to the inhibitory receptor FcγRIIb on macrophages induces prostaglandin E2 production, thereby inhibiting TLR4-triggered expression of inflammatory cytokines such as IL-6 and TNF7 (75).

Reprogramming metabolism is an emerging hallmark of cancer (76). Cancer cells alter their metabolism to adapt to their microenvironment and facilitate immune evasion. Tumor-derived metabolic factors play key roles in regulating macrophage polarization (77). For instance, lactic acid is highly enriched in the TME due to the intense energy production by glycolysis (78). Lactic acid derived from malignant tumor tissues is found to promote tumor progression by promoting macrophages polarization (79). In addition, lactic acid was shown to drive TAM proliferation during EMT (80). These studies collectively demonstrate a role of lactic acid and glucose metabolic reprogramming in macrophage polarization.

Research on tuberculosis reported that B cells also take part in modulating the phenotype and functions of macrophages (81). In the inflammation milieu, B cells produced type I IFN *via* STING pathway, triggered the preference for M2 polarization and activated the regulatory macrophages (81). In addition, Treg cells also significantly affect the function of macrophages. Human monocytes co-cultured with CD4+CD25+Foxp3+ Treg cells presented high expression of M2 biomarkers (such as CD163, CD206 and CCL18), low expression of inflammatory cytokines such as TNF, IL-1β, IL-6 and CCL3, and were more prone to polarize into AAM-type macrophages (82). Treg cell-driven IL-10 is involved in the suppression of inflammatory cytokines and the expression of CD163 and CCL18 (82, 83). CD4+CD25+ T cells were found to polarize tissue macrophages into AAM-type through arginase, IL-10 and TGF-β pathways (84). In contrast, AAM-type macrophage polarization not only drives the differentiation of CD25+GITR+Foxp3+ Treg cells, but also regulates their recruitment by releasing CCL22 (64, 85). Moreover, research demonstrated that HIV infection up-regulated PD-1 ligation and promoted the recruitment of IL-10-releasing monocytes, and these two molecules synergized to

potentiate AAM polarization *via* ligand-receptor pair and in the milieu (86) (Figure 2).

Therapeutic strategies targeting AAM-type macrophage polarization

As mentioned above, TAMs account for an important proportion of the entire tumor microenvironment, and they are involved in various aspects of tumor progression. Immunotherapy targeting TAMs is gradually becoming a research hotspot. Herein, we discuss potential therapeutic strategies targeting AAM-type macrophage polarization in lung cancer.

CCL2 monoclonal antibody or CSF1R inhibitor

Since chemotaxis is the main contributing factor driving monocyte recruitment and colonization, chemokines regulating chemotaxis become targets to inhibit the subsequent phenotypes and functions of macrophages. The monoclonal antibody CNT0888 (carlumab) targeting CCL2 has been investigated in clinical trials and showed good efficacy and tolerability in patients with advanced malignant tumors (87). Inhibition to CSF1R pathway also attenuated macrophage polarization. There are two ways to inhibit the CSF1/CSF1R signaling pathway: direct inhibition to CSF1R tyrosine kinase, indirect blocking CSF1 from binding to CSF1R. Many inhibitors of the CSF1/CSF1R signaling pathway have been reported, most of which are small-molecule heterocyclic compounds with different scaffold structures. The phosphorylation process of tyrosine residues can achieve the effect of receptor inactivation (88). CSF-1R tyrosine kinase inhibitors that block the CSF-1 signaling pathway have shown good therapeutic effects in preclinical models of various tumors, including acute myeloid leukemia, malignant melanoma, and malignant glioma (88, 89), and CSF-1R inhibitor RG7155 significantly reduced the macrophage infiltration in a case of sarcoma with high CSF-1 expression (90). AZ683 is a potent and highly selective CSF1R inhibitor with good oral bioavailability. *In vivo* experiments show that AZ683 can effectively inhibit TAMs and exert anti-tumor effect (91). However, the latest data show that the therapeutic effect of these antibodies and inhibitors is not durable, and it is easy to relapse and aggravate the disease after treatment is completed. In lung cancer treatment, preclinical study has suggested that CSF1R inhibition by BLZ945, a CSF1R inhibitor, substantially

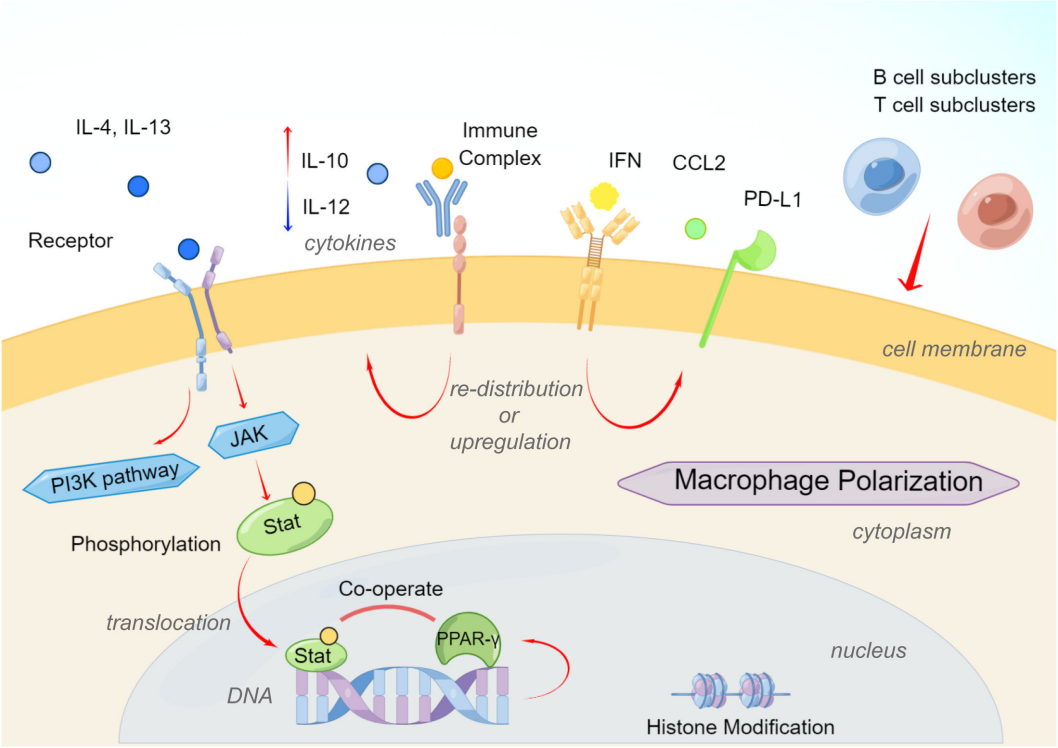

FIGURE 2
Brief mechanisms of macrophage polarization from CAM to AAM. (By Figdraw.).

limits malignant pleural effusion formation induced by lung adenocarcinoma (92). PLX647 is a pyrrolopyridine compound that can bind to the juxtamembrane domain of the kinase to maintain the autoinhibitory state of the protein, thereby inhibiting the phosphorylation of CSF1R with an IC50 of 28 nmol/L. PLX647 improves systematic immunosuppressive state by inhibiting CSF1/CSF1R signaling and has been shown to be effective in the treatment of breast cancer, melanoma and lung cancer (88). The presence of TAMs will also affect the efficacy of chemotherapy drugs. Studies have confirmed that during treatment of docetaxel, CSF-1 monoclonal antibody or CSF-1R blockade will improve the anti-tumoral effects of paclitaxel, and since TAMs secrete the immunosuppressive molecule IL-10, thereby blocking IL-10 combined with docetaxel resulted in better clinical outcomes (93). Therefore, reducing the infiltration of TAMs directly or indirectly will improve the therapeutic effect of malignant tumors.

Re-acclimation of AAM-type macrophages and new strategies

Besides inhibiting TAM infiltration, alternatively, re-educating TAMs by immune checkpoint inhibitors or TAM surface biomarkers reactivate the antitumoral activity of TAMs and relieves their immunosuppressive function. The immune checkpoints on TAMs that have been discovered so far include PDL1, CSF1R, Dectin-1, PI3Kγ, etc., and the corresponding inhibitors and antibodies have achieved good therapeutic effects in clinical practice (94, 95). In the field of immunotherapy, current strategies mainly include blocking immune checkpoints such as PD-1 and CTLA-4 by antibodies or small-molecule inhibitors, thereby "re-firing up" the anti-tumor immune response. PD-1 is mainly expressed in activated T cells and is an important immune checkpoint receptor. After PD-1 binds to its ligands PD-L1 or PD-L2 in tumor cells and tumor microenvironment, it transmits inhibitory signals to T effector cells, hinders T cell survival, and facilitates immune tolerance (96, 97). Chronic exposure to inflammatory cytokines and high levels of antigens can also lead to increased expression of PD-1 and PD-L1, which are hallmarks of T cell exhaustion and dysfunction (98). It was found that PD-1 blocks proximal activation of PI3K/Akt signaling pathway, and the extent of T cell inhibition depends on the signaling of T cell receptors (99). Immune checkpoint antibodies currently in development or clinically approved include the PD-1 antibodies nivolumab and pembrolizumab and the PD-L1 antibodies atezolizumab, durvalumab, and avelumab (100). The latest research has found that combining these immune checkpoint inhibitors and antibodies with chemotherapy or targeted therapy shows synergistic effects.

In recent years, with the rapid development of tumor immunity research, immune checkpoint inhibitors such as CTLA-4 antibody and PD-1/PD-L1 antibody have been successfully applied in a variety of cancers, such as melanoma, non-small cell lung cancer, advanced cervical cancer, hepatocellular carcinoma, skin squamous cell carcinoma, bladder cancer, etc. Immunotherapy has become one of the main treatment options for patients with advanced cancer. Among them, the combination therapy of immune checkpoint inhibitors with precision and multi-pathway targeting has unique advantages in overcoming drug resistance and enhancing the specific recognition and killing of tumor cells by immune cells (101). For example, the combination of nivolumab, a PD-1 inhibitor, and ipilimumab, a CTLA-4 inhibitor, can prolong the progression-free survival of lung cancer patients with good complementarity. Nivolumab combined with LAG-3 inhibitor BMS-986016 in the treatment of advanced melanoma can effectively overcome the resistance of PD-1 monotherapy. The combination of PD-1 inhibitor and TIM-3 inhibitor in the treatment of non-small cell lung cancer can inhibit the resistance to PD-1 inhibitor (102). The combination of CTLA-4 inhibitor and LAG-3 inhibitor can induce immune tolerance through co-inhibiting signaling pathway. The combination with IDO inhibitor can effectively reduce the tumor volume and prolong the survival time of a melanoma murine model. In addition, the emergence of bifunctional antibodies with good targeting property, which can effectively exert synergistic effects through dual-pathway or dual-target blocking, has given new enlightenment to cancer treatment, and may become one of the key therapeutic strategies for human to conquer cancer.

The existing treatment strategies have their own advantages and disadvantages. In order to better improve the tumor treatment effect, new treatment strategies are future-oriented. For example, to improve the "phagocytic ability", in a physiological state, normal cells have a "phagocytic checkpoint", that is, the expression of anti-phagocytic molecules to avoid the self-elimination of phagocytic cells, and tumor cells also rely on this phagocytic checkpoint to carry out immune evasion. Therefore, the identification and intervention of phagocytic checkpoints may provide a new method to re-educate TAMs to restore the phagocytosis against tumor cells. For example, under immunosuppressive conditions, the cancer cell membrane protein CD47 can recognize SIRPα on the surface of macrophages to form the CD47-SIRPα signaling complex, inhibiting the phagocytosis of tumor cells by macrophages and enabling tumor cells to escape immune surveillance for tumor development (103, 104). Therefore, CD47-SIRPα blocking antibody may restore the phagocytosis of macrophages. Furthermore, given that TAMs have the ability to phagocytose nanoparticles, nanoparticles are ideal therapeutic targets. Nanoparticles containing tumor peptides are used to promote the recording of TAMs, and the characteristics of nanoparticles targeting TAMs can be used to promote antitumor immunity.

Biological features of Tim-3 positive TAMs

In recent years, Tim-3 positive macrophages have attracted great attention. The discovery of immune checkpoint molecules and the elucidation of their functions have provided new targets and therapeutic methods for tumor therapy, such as CTLA-4, PD-1, Lag-3 and Tim-3. Tim-3 belongs to the immunoglobulin superfamily (IgSF), which consists of four known domains, including a variable immunoglobulin domain (IgV), a mucin domain, a transmembrane domain, and a cellular inner tail region (105). In the immune system, Tim-3 was initially identified as a specific membrane marker selectively expressed on IFN-γ-producing CD4+ helper T cells (Th1) and CD8+ cytotoxic T cells (Tc1) (105). Later research on tumor microenvironment demonstrated that Tim-3 is expressed by other cell types, such as natural killer cells (NK cells), dendritic cells (DC cells), monocytes, macrophages, and even different types of tumor cells (106, 107). The study of Anderson et al. showed that Tim-3 can be highly expressed on macrophages and promote the inflammatory response of macrophages through the NF-κB pathway (108). Tim-3 expression can be used as an independent prognostic factor in colon cancer patients, and Tim-3 can directly promote tumor growth through STAT3 or STAT3-pSTAT3 pathway. Researchers detected the expression of Tim-3 in tumor-associated macrophages in lung cancer tissues, and in CD68+ tumor-associated macrophages, lung cancer patients with high Tim-3 expression had shorter OS and poorer prognosis (109). The specific mechanism of Tim-3-positive macrophages in lung cancer is still unclear, but its findings in other tumors can provide ideas for our follow-up research. Tim-3 expression on TAMs in hepatocellular carcinoma is induced by tumor-derived signals including TGF-β (110). This further promotes TAM-mediated growth of HCC due to the secretion of soluble factors such as IL-6. Some studies have found that TLR ligand lipopolysaccharide can inhibit the expression of TIM-3 protein in macrophages and restore the immune activity of macrophages (107). This suggests that the expression of TIM-3 may be related to the TLR expression and its downstream signaling pathways. In addition, in HCC, TIM-3 protein regulates the transformation of CAM macrophages towards AAM macrophages, which further inhibits the inflammatory response (110, 111). Secondly, TGF-β-mediated Tim-3 expression in turn regulates the ability of macrophages to secrete cytokines *via* the NF-κB-IL-6 pathway. Researchers detected Tim-3 expression on tumor cells and CD204+ tumor-associated macrophages in clear cell renal cell carcinoma, and found that higher Tim-3 expression levels were associated with shorter PFS in patients, and similar to reports on lung cancer, Tim-3 was found to induce resistance in renal cancer cells to standard treatments as sunitinib and mTOR inhibitors (112). Based on previous findings, we hypothesized that Tim-3 may directly promote tumor growth through the IL-6-STAT3 pathway or the NF-κB-IL-6 pathway, or negatively regulate anti-tumor immunity, thereby facilitating tumor immune escape and promoting tumor cell growth.

In addition, 1 ug/ml LPS treated macrophages for 6 h not only up-regulated TLR4 and MyD88 mRNA expressions, but also significantly up-regulated Tim-3 mRNA expression, indicating that activation of TLR4 signaling pathway can regulate the expression of Tim-3 on the surface of macrophages. Yang et al. used LPS to treat peritoneal macrophages derived from a mouse model of sepsis for 4 hours and found that the expression of Tim-3 mRNA on the cell surface was significantly up-regulated, but they used the same concentration of LPS to treat mouse-derived RAW264.7 cells and found that Tim-3 mRNA expression was down-regulated with the increase of LPS concentration, and decreased to the lowest level at 100ng/ml, suggesting that the regulation of Tim-3 by TLR4/LPS signaling pathway is closely related to the cell origin, and this signaling pathway affects macrophages from different sources (113).

Besides its role in tumor progression, macrophages are also involved in other pathological conditions. Monney et al. established an experimental mouse model of autoimmune encephalomyelitis and showed that Tim-3 can promote the massive activation and proliferation of monocyte-macrophages and promote the inflammatory response (106). The Tim-3-galectin-9 interaction can also transduce reverse signaling, and a murine model of pulmonary infection with Mycobacterium tuberculosis (Mtb) has also shown that the Tim-3 signaling pathway can activate macrophages and activate innate immune responses (114). Tim-3 is essential for the induction of IL-1β and enhanced macrophage anti-mycobacterial activity through a galectin-9-dependent mechanism. When mycobacterium tuberculosis-infected cells were treated with the Tim-3 fusion protein. In the case of macrophages, fewer CFUs were recovered in this case. Tim-3 is essential for induction of IL-1β and enhanced macrophage anti-mycobacterial activity through a galectin 9-dependent mechanism. Zhang et al. showed that blocking or silencing Tim-3 on the surface of macrophages can induce increased secretion of pro-inflammatory factors IL-12 and IL-6, as well as increased secretion of anti-inflammatory factor IL-10. The authors speculate that the regulatory role of Tim-3 on immune inflammation is influenced not only by Tim-3 expression itself, but also by the state of macrophages and the balance between inhibitory and stimulatory molecules involved (115). Tim-3 expression is lower on M1 macrophages that have multiple functions (eg, phagocytosis, antigen presentation, and production of pro-inflammatory cytokines) and are used to eliminate cancer cells. To illustrate the immunosuppressive role of Tim-3 in various cell types and its role in regulating immune cell cross-talk in the tumor environment.

Extensive preclinical data support that blocking the TIM-3 signaling pathway may promote immune cells to mediate anti-tumor responses, and can be combined with other immune

checkpoint receptor blockers to further enhance the anti-tumor effect. Preliminary signs of clinical efficacy have also been observed in patients with solid tumors, including NSCLC, who received sabatolimab and anti-PD-1 antibody spartalizumab, suggesting that blockade of TIM-3 might represent a potential therapeutic strategy in lung cancer (116).

Summary

TAMs are important immune cells in the immune microenvironment of lung cancer with high heterogeneity. The polarization of macrophages and related mechanisms play an important role in the progression of lung cancer. In this review, we aimed to overview the current understanding of TAMs in the context of lung cancer. First, we discussed mutual regulation between TAM and TME, and established the key role of TAM and TME in supporting tumor cell survival: TAM nourishes tumor cell survival through a large number of signals from the TME, and in turn regulate the TME from many aspects. We also described the molecular mechanism of AAM polarization and therapeutic strategies for cancer-promoting AAM macrophages, including CCL2 monoclonal antibodies or CSF1R inhibitors, AAM re-acclimation targeting immune checkpoints, and new strategies to improve the "phagocytic ability" of cells. Finally, we discussed the involvement of TIM-3 positive macrophages in cancer pathogenesis, and explored TIM-3 inhibition as a potential therapeutic strategy for lung cancer. The extensive involvement of TAM in cancer pathogenesis and promising preclinical and early clinical data summarized above have emphasized the opportunities of the development of AAM-targeting therapeutic strategies against lung cancer.

Author contributions

QX, QH, and RW provided the direction and guidance for this manuscript. QH and RW wrote the whole manuscript. ZZ and HM were responsible for the collation of the paper. GW made significant revisions to 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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Macrophage scavenger receptors: Tumor support and tumor inhibition

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Tumor-associated macrophages (TAMs) are a heterogeneous population of myeloid cells that constitute up to 50% of the cell mass of human tumors. TAMs interact with the components of the tumor microenvironment (TME) by using scavenger receptors (SRs), a large superfamily of multifunctional receptors that recognize, internalize and transport to the endosomal/lysosomal pathway apoptotic cells, cytokines, matrix molecules, lipid modified lipoproteins and other unwanted-self ligands. In our review, we summarized state-of-the art for the role of macrophage scavenger receptors in tumor development and their significance as cancer biomarkers. In this review we focused on functional activity of TAM-expressing SRs in animal models and in patients, and summarized the data for different human cancer types about the prognostic significance of TAM-expressed SRs. We discussed the role of SRs in the regulation of cancer cell biology, cell-cell and cell-matrix interaction in TME, immune status in TME, angiogenesis, and intratumoral metabolism. Targeting of tumor-promoting SRs can be a promising therapeutic approach in anti-cancer therapy. In our review we provide evidence for both tumor supporting and tumor inhibiting functions of scavenger receptors expressed on TAMs. We focused on the key differences in the prognostic and functional roles of SRs that are specific for cancer types. We highlighted perspectives for inhibition of tumor-promoting SRs in anti-cancer therapy.

KEYWORDS

tumor-associated macrophage, scavenger receptor, angiogenesis, extracellular matrix, cancer, tumor microenvironment, endocytosis, phagocytosis

1 Introduction

Tumor-associated macrophages (TAMs) are key innate immune cells that control intratumoral inflammation, cancer cell proliferation, migration and metabolism, angiogenesis, and extracellular matrix composition (1–6). Major sources of TAMs are resident tissue macrophages as well as monocyte-derived macrophages, intensively recruited into the growing tumor by chemotactic factors, like CCL2 (4, 7, 8) The are two major vectors of macrophage polarization: M1-type, classically activated, pro-inflammatory, and M2-type, alternatively activated, generally considered as anti-inflammatory or tolerogenic macrophages (4–6). The classification based on the M1/M2 dichotomy is traditionally used as simplified schema to distinguish between two major directions of macrophage activity. M1 macrophages play an important role in the innate immune response, while M2 macrophages are involved in tissue repair, as well as in the progression of many types of cancer (9, 10).

Within tumor tissues, TAMs interact with cancer cells and with other cell types in tumor microenvironment (TME) not only by secreting different cytokines, chemokines and growth factors, but also by clearance of dying cells, soluble mediators and matrix components mediated by scavenger receptors (SRs). SRs can recognize and internalize high range of unwanted-self ligands including cytokines, growth factors, modified lipoproteins, apoptotic cells, as well as non-self ligands including bacteria, viruses and fungi (11–13).

SRs are large superfamily of transmembrane proteins with high structural diversity (13–15). SRs are categorized into classes A-L depending on their structure, cell-type specific expression and recognition of host-derived ligands (13, 16, 17). Functional diversity of SRs are crucial for numerous biological processes such as endocytosis, phagocytosis, cell adhesion, nutrient exchange and waste clearance, as well as immunity processes, e.g. inflammation regulation and antigen presentation (13, 14).

In tumors, SRs can be expressed by both tumor cells (TCs) and by components of the TME including macrophages, monocytes, endothelial cells and dendritic cells (15, 18, 19). Most commonly used SRs for the identification of TAM subpopulation in different types of tumors include CD68, CD163, CD204 and CD206 (8). Both tumoricidal M1 and tumor-promoting M2 macrophages express CD68, while M2 polarization can be identified by CD163, CD204 and CD206 biomarkers (8). However, this nomenclature does not fully reflect all phenotypic diversity of TAMs that can combine M1 and M2 features and functions (3).

In this review, we focus on functional activity of scavenger receptors expressed by TAMs in tumor. We summarize the latest knowledge on the functional activity of TAM-expressing scavenger receptors in the TME. We discuss how expression of scavenger receptors in TAMs can be used for the evaluation of prognostic value in numerous cancers.

2 Scavenger receptors expressed by TAMs

Several SRs play essential role in the regulation of TME where they can be expressed by TAMs, NK cells, dendritic cells, neutrophils, B cells, endothelial cells, epithelial cells and cancer cells (13). TAM-expressing scavenger receptors are structurally heterogeneous proteins that consist out of diverse structural domains including collagenouse domain, C-type lectin-like domain, fibronectin domain, EGF-like and others (Figure 1). SRs expressed by TAMs are involved in diverse signaling pathway and have a predictive value for tumor progression (Table 1) (23, 25–27, 29, 30, 48).

2.1 Class A scavenger receptors

Scavenger receptors of class A (SR-A) are transmembrane proteins containing a collagen-like domain with collagen-binding activity (15). SR-A family comprises five members: SR-A1 (CD204), SR-A3, SR-A4, SR-A5, and SR-A6 (MARCO), which recognize a variety of ligands such as LPS, LTA and integrins. SR-A are implicated in several pathologies including atherosclerosis, infectious diseases and cancer (13, 70, 71). In most of studies the question about tumor-specific ligand of CD204 was not addressed experimentally. The expression of SR-A was found on monocytes, macrophages, dendritic cells (DCs), mast cells and endothelial cells (71, 72). Out of five SR-A family members, only CD204 and MARCO have been found to be expressed by TAMs (21, 22, 25).

2.1.1 SR-A1/CD204

SR-A1 (also known as CD204, or MSR1) is a pattern recognition receptor expressed primarily on macrophages, and is involved in the inflammatory responses and tumorigenesis (21, 25, 73). CD204 has a dual role in cancer progression (21, 22, 26, 27, 74).

The mechanisms of CD204 anti-tumor activity in TME include inhibition of macrophage infiltration, inhibition of tumor cell migration and invasion, as well as suppression of tumor angiogenesis (21, 22) (Figure 2). In a mouse model of LLC, bone marrow-derived cells transplanted from CD204 KO (knock-out) mice into WT mice enhanced tumor growth and angiogenesis through elevated COX-2, SDF1, VEGF and MMP9 expression in tumor (21). CD204 deficiency activated recruitment of CD68+ and F4/80+ macrophages into tumor mass by upregulation of MCP-1 in the CD204 KO bone marrow transplantation model (22). Peritoneal macrophages isolated out of CD204 KO mice significantly enhanced the migration and invasion of lung cancer cells *in vitro* (22). Moreover, CD204 suppresses tumor development through the upregulation of serum amyloid A1 (SAA1) expression in TAMs *via* JNK/ERK/IκB/NFκB

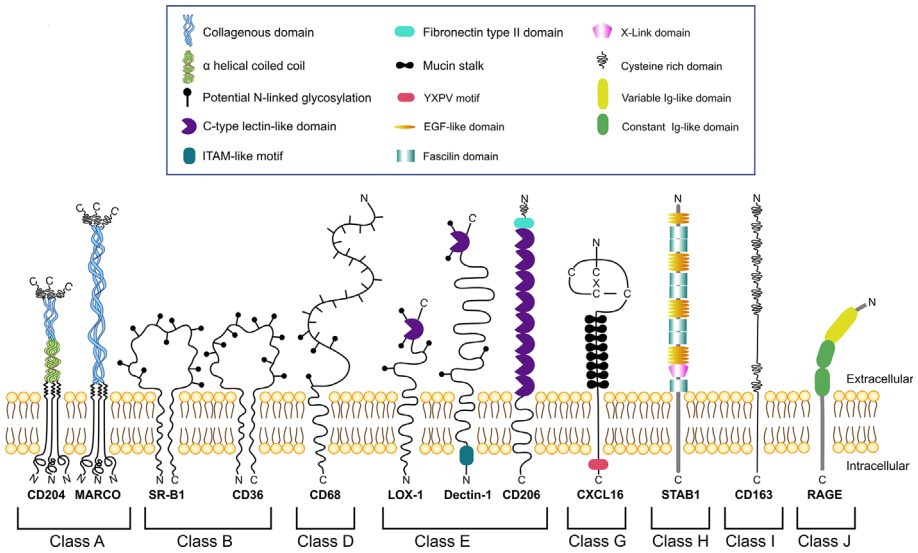

FIGURE 1
Schematic representation of different classes of scavenger receptors expressed by TAMs.

signaling pathways (22). CD204 deficiency promoted tumor growth, angiogenesis and TAM infiltration *via* skewing TAM phenotype toward M2 in murine glioma model (25). Tumor volume as well as the expression of angiogenic factors CD31, CD34, IB4 and VEGF were significantly elevated in CD204−/− mice in comparison with CD204+/+ mice in glioma model (25). In CD204−/− glioma, the number of VCAM1+ TAMs and CCR2 + TAM precursor cells was significantly elevated compared to CD204+/+ glioma (25). *In vitro*, CD204 deficiency resulted in the increased expression of M2-like markers (*MMP2, TGFβ, MRC2, MGL1, FIZZ1*), but no M1-like marker (TNFα) in the presence of GL261 glioma cells (25). Meta-analysis of patients with prostate cancer showed that increased expression of CD204 significantly correlated with better recurrence-free survival (RFS) (23). Immunohistochemical (IHC) analysis demonstrated that high expression of CD204 correlated with better survival rate and less recurrence than those with less CD204 expression in patients with glioma (25).

However, controversial data also exist (Figure 3). Several studies demonstrated that CD204+TAMs promote tumor development and correlate with worse prognoses in prostate cancer, lung cancer, colorectal cancer, cervical cancer, breast cancers and oral squamous cell carcinoma patients (24, 26, 27, 74–76). Tumor-supporting function of CD204+ TAMs was demonstrated for lung cancer and glioma, however anti-tumor function was shown for breast cancer, ovarian cancer and pancreatic cancer (21, 24, 25, 27, 74–76). CIBERSORT analysis of CD204 mRNA expression obtained from TCGA database demonstrated that high CD204 expression correlated to high proportions of M2 macrophages and the expression of immunosuppressive molecules, including HIF1A, FAP, IL-10, and TGFB1 in breast cancer (27). CD204 KO macrophages reduced tumor cell invasion *via* TLR-dependent pathways upon co-culture with ID8 (ovarian cancer cell line) and Panc02 (pancreatic adenocarcinoma cell line) (26). Macrophage-specific loss of CD204 significantly reduced lung metastasis in a mouse model of pancreatic adenocarcinoma (26). *In vitro* CD204+ TAMs promoted proliferation, migration and invasion of MCF7, T47D, SKBR3 and MDA-MB-231 breast cancer cell lines (27). High CD204 expression in TAMs correlates with short overall survival (OS), disease-free survival (DFS) and RFS in colorectal cancer, cervical cancer, breast cancers and oral squamous cell carcinoma (OSCC) (24, 74–76). CD204 expression is associated with T stage, nodal involvement, lymphovascular invasion and tumor relapse after surgery in lung adenocarcinoma (25, 77). In prostate cancer, high CD204 protein expression in the main tumor area predicted a worse prognosis, while CD204 expression in seminal vesicle invasion area was positively associated with the biochemical recurrence (78).

In summary, majority of reports show that CD204 correlates with good prognosis in prostate cancer and glioma, and with worse prognosis in colorectal cancer, cervical cancer, breast cancers, oral squamous cell carcinoma, lung cancer and prostate cancers. Murine experimental systems demonstrated both tumor-promoting and tumor-inhibiting role of CD204+ TAMs (Figures 2, 3).

2.1.2 SR-A1/CD204

SR-A6 (also known as macrophage receptor with collagenous structure, MARCO) is another member of the SR-A family that is also expressed by macrophages and is involved in clearance of cancer cells, in regulation of epithelial-mesenchymal transition (EMT), in the interferon-alpha response, and in antigen presentation (28, 79).

TABLE 1 The function of TAM-expressing scavenger receptors in the TME.

Scavenging receptors	Ligands	Function/mechanism	Correlation with clinical parameters
Class A			
SR-A1 (CD204)	Lipopolysaccharide (LPS), lipoteichoic acid (LTA) and bacterial CpG DNA	Anti-tumor: Suppression of tumor growth and angiogenesis *via* inhibition of COX-2, SDF1, VEGF, and MMP9 expression and down-regulation of JNK/ERK/IκB/NFκB signaling pathway in LLC tumors, as well as inhibition of macrophage polarization (20) and monocyte recruitment (21, 22). Pro-tumor: Promotion of proliferation, migration and invasion of MCF7, T47D, SKBR3, MDA-MB-231, ID8 cell lines *in vitro* (26, 27). Induction of lung metastasis in a mouse model of pancreatic adenocarcinoma (26).	Correlation with better RFS in prostate cancer (23). Negative correlation with human lung cancer progression (21). Positive correlation with short OS and RFS in colorectal cancer (24). Correlation with better survival rate and less recurrence in glioma (25).
SR-A6 (MARCO)	Oxidized lipids, unopsonized particles, bacteria, integrins	Anti-tumor: Clearance of colon carcinoma cells *via* the SYK-PI3K-Rac1 signaling pathway (28). Pro-tumor: Association with high expression of tumor-supporting genes in NSCLC and glioblastoma (30, 32). Activates immunosuppressive phenotype of TAMs (33).	Positive correlation of the number of MARCO+ TAMs with DFS and OS in pancreatic cancer and squamous cell carcinoma (29, 30). Increase amount of MARCO+TAMs associates with prolonged OS in human HCC (31).
Class B			
SR-B3 (CD36)	Thrombospondin-1, long-chain free fatty acids, ox-LDL, advanced glycation endproducts (AGE), collagens I and IV	Anti-tumor: N/A Pro-tumor: Promotion of tumor growth *via* up-regulation of pro-tumor genes, M2-signature genes in TAMs and enhancing TAM infiltration in lymphoma (34). Supporting tumor development through activation of S100A4-PPAR-γ pathway in TAMs in breast cancer and fibrosarcoma (35). Increasing of tumor growth *via* promotion of TAM infiltration in tumor in breast cancer (36).	N/A
Class D			
SR-D1 (CD68)	oxLDL, phosphatidylserine, apoptotic cells, malaria sporozoite	Anti-tumor: N/A Pro-tumor: promotion of angiogenesis in LSCC (37).	Correlation with to worse prognosis in glioblastoma, kidney renal clear cell carcinoma, lower-grade glioma, hepatocellular carcinoma, lung squamous cell carcinoma, thyroid carcinoma, thymoma and a favorable prognosis in chromophobe renal cell carcinoma, LSCC, breast cancer (37, 38). Correlation with recurrence in cutaneous melanoma (39). Positive correlation with favorable neoadjuvant chemotherapy responses in osteosarcoma (40). Correlation with anti-tumor TAM phenotype in melanoma (41).
Class E			
SR-E1 (LOX-1)	Ox-LDL, apoptotic cells, gram-positive and gram-negative bacteria, acute phase C-reactive proteins, HSP	Anti-tumor: N/A Pro-tumor: Promotion of M2 TAM polarization *via* PI3K/Akt/mTOR signaling in HNSC (43).	Decreased amount of LOX-1+ TAMs is associated with poor OS in colorectal cancer (42).
SR-E2 (Dectin-1)	β-1,3-glucan, galectin-9, annexins, vimentin, N-glycan	Anti-tumor: Supporting of TAM tumoricidal activity against lymphoma and ovarian adenocarcinoma (44). Pro-tumor: Promoting pancreatic cancer progression *via* increasing TAM infiltration,	Correlation with shorter patient recurrence free survival and overall survival in cell renal cell carcinoma (45).

(Continued)

TABLE 1 Continued

Scavenging receptors	Ligands	Function/mechanism	Correlation with clinical parameters
		reprogramming TAMs toward M2 phenotype and reduced T-cell infiltration (46).	
SR-E3 (CD206)	Collagens, N-acetylgalactosamine (GalNAc)	Anti-tumor: Suppression melanoma growth *via* activation of tumoricidal T cells (47). Pro-tumor: Promoting proliferation and invasion of OSCC cells by producing EGF (49).	Positive correlation with tumor relapse and metastasis after chemotherapy in breast cancer (48) and correlation with worse clinical prognosis in OSCC (49). Increased amount of CD206+TAMs associated with improved overall survival in cutaneous melanoma (47).
Class G			
SR-G1 (CXCL16)	oxLDL	Anti-tumor: overexpression in colorectal cancer cells causes TNFα-mediated apoptosis (50). Pro-tumor: enhancing tumor cell migration, invasion, proliferation and promoting M2 TAM polarization (51–54).	Associated with aggressive pathologic phenotypes, the higher TNM staging and lymph node metastasis in papillary thyroid cancer (51). Decrease of the overall survival due to *CXCR6* overexpression, receptor of CXCL16 (52).
Class H			
SR-H1 (STAB1)	ac-LDL, placental lactogen, SPARC, advanced glycation end products, apoptotic cells, microparticles from gram-positive and negative bacteria	Anti-tumor: N/A Pro-tumor: Supporting breast cancer progression through activation of PKCβ expression in TAMs resulted in SPARC uptake from TME by TAMs (57).	Positive correlation with long DSS and favorable prognosis in early stage I CRC patients (55). Correlation with poor OS, RFS, tumor stage and histological grade in urothelial carcinoma of the bladder and rectal cancer (55, 56).
Class I			
SR-I (CD163)	Haptoglobin-hemoglobin complex	Anti-tumor: N/A Pro-tumor: Promoting TAM polarization toward tumor-supporting TAM phenotype in cholangiocarcinoma (63). Induction of tumor progression *via* production of IL-6 and CXCL2 and activation of STAT3 in fibrosarcoma (64).	Correlation with tumor grade in breast cancer (58). Positive correlation with severe prognosis of myeloma, gastroesophageal adenocarcinoma, triple negative breast cancer (59–61). Correlation with lymph node metastasis poor prognosis in breast cancer (62). Negative correlation with recurrence and poor overall survival in primary melanoma (39).
Class J			
RAGE	AGEs, HMGB1, S100 proteins, amyloid-beta peptide, dsDNA and dsRNA	Anti-tumor: AGEs and HMGB1 promote M1 polarization in macrophages and TAMs, respectively (65),[93,94]. HMGB1 exposure to M1 macrophages abrogates invasion of gastric tumor cells and growth of endothelial cells (66). Pro-tumor: HMGB1 activation of RAGE in M2 macrophages promotes invasion of gastric tumor cells (66), production of VEGF (66, 67, 69) and angiogenesis (66, 67). Mediates $KRAS^{G12D}$ uptake, which promotes M2 polarization of TAMs (68).	HMGB1 and CD163 positive macrophages were found as detrimental prognostic factors for OS in laryngeal squamous cell carcinoma (67). High $KRAS^{G12D}$ expression in $CD68^{+}$ cells in PDAC patients correlated with worse OS rates (68).

In vitro, TAMs suppress tumor development utilizing MARCO to phagocytose cancer cells (Figure 2) (28). MARCO overexpression in peritoneal murine macrophages led to the increased expression of SYK, PI3K and Rac-1, and facilitated macrophage-mediated phagocytosis of SL4 (colon carcinoma cell line) *via* binding to integrin β5 on cancer cells and activation of SYK-PI3K-Rac1 signaling pathway in TAMs in the co-culture system (28) (Figure 4).

Several studies on clinical material revealed tumor-supporting phenotype of MARCO-expressing TAMs (30, 32, 33). Single-cell transcriptomic analysis of glioblastoma demonstrated that a cluster of MARCO+ TAMs coincides with high expression of genes involved in epithelial-mesenchymal transition, angiogenesis, glycolysis, hypoxia and low expression of genes associated with interferon-alpha response, interferon-gamma response, allograft rejection, and TNFα signaling (32). MARCO+ TAMs support tumorigenesis by activating immunosuppression in the TME. Transcriptomic analysis of non-small cell lung cancer (NSCLC) samples showed that MARCO expression significantly correlated with gene

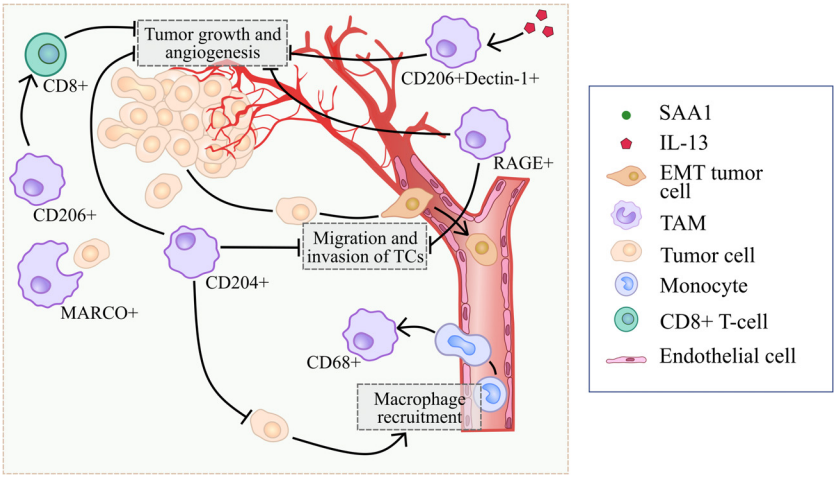

FIGURE 2
Summary of processes that are regulated by scavenger receptors in tumor-suppressing microenvironment.

expression of immunosuppressive TAM-related genes (CD163, MSR1, IL4R, CHIA, TGFB1, and IL10), genes involved in T-cell regulation (FOXP3, TGFB1, IL10, EBI3, PDCD1, and CTLA4) and genes encoding immune checkpoint molecules (PD-L1, PD-L1, VISTA, PD-1, and CTLA4) (30). High infiltration of MARCO + TAMs in tumor was associated with worse OS and DFS in patients with squamous cell carcinoma (30) and pancreatic cancer (29).

MARCO-expressing TAMs suppressed tumoricidal activity of T cells and NK cells through skewing TAM phenotype toward anti-inflammatory one (Figure 3). *In vitro* MARCO-expressing TAMs suppressed activation, proliferation and IFNγ production in T cells, resulted in inhibition of T-cell killing activity towards NSCLC tumor cells (33). Moreover, human PBMC-derived MARCO+ TAMs inhibited migration, degranulation, proliferation and IFNγ production in NK cells (33). MARCO+ macrophages cultured with lung cancer cell lines displayed decreased expression of pro-inflammatory cytokines (TNFa, IL1B and IL12B) and increased expression of anti-inflammatory molecules (IL10, MRC1, COX2, TIMP1, and FIZZ1) (33). High expression of MARCO in TAMs from the tumor tissues was associated with increased OS in patients with hepatocellular carcinoma (HCC) (31).

Thus, role of SR-A family members depends on the tumor context. Despite strong tumor-supporting activity identified for several members of SR-A family expressed by TAMs (26, 27, 30, 32, 33), solid body of evidence is available for SR-A1 and SR-A6 demonstrating their anti-tumor action that depends primarily on the cancer types (20–22, 28) (Figure 3, Table 1).

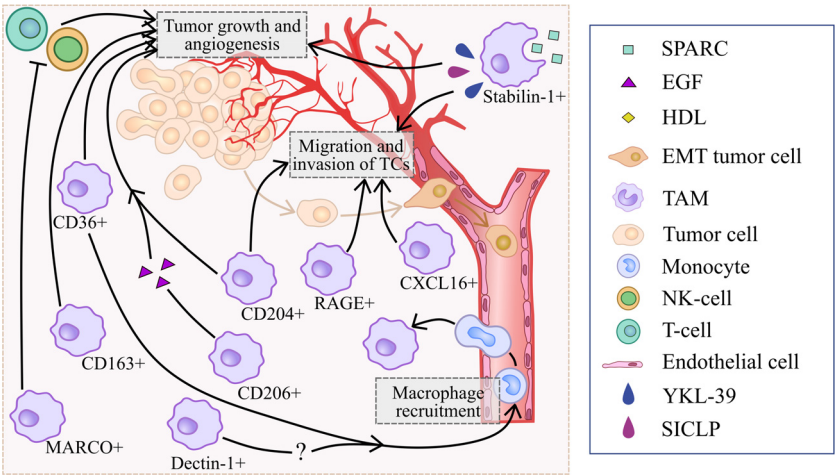

FIGURE 3
Summary of processes that are regulated by scavenger receptors in tumor-supporting microenvironment.

2.2 B scavenger receptors

Class B scavenger receptors includes the following members: SR-B1, SR-B2 and SR-B3. Structurally Class B scavenger receptors are constructed out of two transmembrane domains flanking an extracellular loop, with both the N- and C-termini located within the cytoplasm (80). Class B scavenger receptors mediate transport of cholesterol and lipids, and are involved in tumor development (34, 35, 81). The role of TAMs expressing SR-B1 and SR-B3 was demonstrated in several types of cancers, including liposarcoma, nasopharyngeal carcinoma, breast cancer, colon cancer and prostate cancer (35, 36, 82).

2.2.1 SR-B1

Scavenger Receptor Class B Type 1 (SR-B1) is a transmembrane protein that act as a major high-density lipoprotein (HDL) receptor (81, 82). SR-B1 is expressed by endothelial cells, smooth muscle cells, keratinocytes, adipocytes, tumor cells and macrophages (19). In the TME, SR-B1 participates in HDL metabolism and promotes invasion, proliferation and metastasis of tumor cells (82). In macrophages, SR-B1 regulates cholesterol metabolism through selective uptake of HDL-cholesterol and cholesteryl esters (83). In a syngeneic mouse model of prostate cancer, knock out of SR-B1 inhibited HDL-mediated tumor growth and progression (84). In SR-B1−/− mice had lower levels of total cholesterol and HDL-cholesterol. SR-B1−/− mice developed smaller tumor compared to SR-B1+/+ mice, and SR-B1−/− mice showed also the decreased survival (84). Application of HDL-mimetic nanoparticles that interacted with SR-B1 reduced tumor growth in a mouse xenograft model for human nasopharyngeal carcinoma (82). Thus, the selective uptake of HDL-cholesterol by SR-B1 in macrophages is a promising pathway for pharmacological inhibition of pro-tumor TAM actions. SR-B1 activity in macrophages is mediated by Src/PI3K/Akt/Rac1 and PPARγ/LXRα signaling pathways (85, 86). The data about the role of TAM-expressing SR-B1 in cancer are limited, but SR-B1 expression was found in head and neck cancer, lung cancer, prostate cancer and breast cancer, where it positively correlates with the tumor aggressiveness and poor prognosis (82, 87, 88).

2.2.2 SR-B3/CD36

SR-B3 (also known as CD36) is expressed on monocytes, macrophages, platelets, endothelial cells, adipocytes (89, 90). CD36 mediates lipid uptake, ligand, clearance of apoptotic cells and cell-cell adhesion (90–92). CD36-expressing macrophages facilitate tumor progression, pro-tumor TAM polarization and mediate fatty acid uptake from TME (Figure 3) (35, 36, 93).

CD36 regulates polarization of TAMs towards pro-tumor phenotype and promotes tumor growth *via* regulation of fatty acid (FA) metabolism (94, 95). CD36 was demonstrated as a major SR on macrophages involved in the lipid uptake and accumulation, FA oxidation and oxidative phosphorylation (94, 95). In TME extracellular free fatty acids, including palmitic acid, oxLDL or oleic acid, are transported into cells *via* membrane-associated CD36 and promoted tumor growth and metastasis (94–97). Essential feature of CD36 is that its endocytic function is linked to the inflammatory pathways. In macrophages CD36 is involved in diverse signaling cascade including NF-κB pathway, TLR1/2 signaling, TLR4 signaling and NOD-, LRR-, and pyrin domain-containing protein inflammasome pathways (98).

In co-culture of human PBMCs and tumor cells CD36 regulates macrophage response by enhanced lipid uptake and increased expression of pro-tumor genes in modeled TAMs

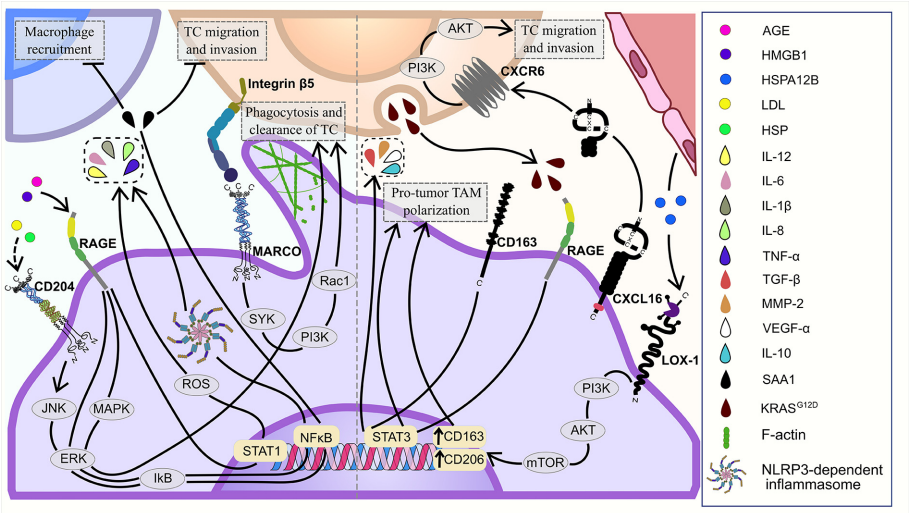

FIGURE 4
Major signaling pathways of TAM-expressing scavenger receptors in the TME: tumor-supporting and tumor-inhibiting.

(Arg1, Ccl2) (34). Subcutaneously injection of CD36-KO TAMs in a mouse model of lymphoma decreased tumor volume, impaired TAMs infiltration into tumor site, increased expression of M1-signature genes and decreased expression of M2-signature genes (34). CD36 in TAMs mediates FA uptake through S100A4-PPAR-γ axis that promotes tumor growth in a mouse models of breast cancer and fibrosarcoma (35). In the mouse model of breast cancer CD36 regulates TME *via* clearance of tumor-derived miR-375, a prominent tumor suppressor (36, 93). In co-culture system of MCF-7 cells and human PBMCs, apoptotic tumor cell-derived miR-375 binds to LDL and is scavenged by TAMs *via* CD36 receptor resulting in increased macrophage migration and infiltration into tumor (36).

Association of CD36 with worse prognosis was demonstrated in several human cancers including bladder cancer, glioblastoma, oral carcinoma and gastric cancer (97, 99). However, currently prognostic significance of CD36 expressed specifically on TAMs is still unclear.

2.3 Class D scavenger receptors

Scavenger receptor SR-D1 (also known as CD68) is the only known class D scavenger receptor that is highly specifically expressed on macrophages and other mononuclear phagocytes but not on other cell types, even of myeloid origin. CD68 is the major biomarker for the quantification of total TAM amounts. CD68 is also a well-established pan-macrophage marker used as a cancer-associated diagnostic and prognostic marker (8, 13).

CD68+TAM infiltration and accumulation in tumor results in tumor progression and adverse prognosis in numerous cancers (8, 37, 38). In our recent review we have summarized data from large number of studies on patients with 5 types of cancer: breast, colorectal, lung, ovarian and prostate (9). Number of studies on patients representing diverse genetics, life style and geographical localizations indicate that amount of intramural CD68+TAMs positively correlates with negative prognosis, distant hematogenous and local lymphatic metastasis in breast, lung, ovarian and prostate cancers. However, amount of intramural CD68+TAMs showed negative correlation with the bad outcome in patients with colorectal cancer. Recent analysis of Genotype-Tissue Expression datasets (TCGA) and immunohistology have demonstrated that high expression of CD68 was correlated with worse prognosis in glioblastoma, renal clear cell carcinoma, lower-grade glioma, HCC, lung squamous cell carcinoma, thyroid carcinoma, and thymoma, but with favorable prognosis in chromophobe renal cell carcinoma (38). In laryngeal squamous cell carcinoma (LSCC), CD68+ cells were involved in angiogenesis and correlated with worse prognosis (37). High expression of CD68 was associated with CD34+ cells in tumor and low 5-year DFS in 45 patients with LSCC from China (37). High amounts of CD68+ TAMs in tumor nest correlated with recurrence in 184 cutaneous melanoma patients from Finland (39). Number of CD68+ TAMs in tumor stroma were positively correlated with tumor size in breast cancer both in 144 patients from Sweden and in 60 patients from Egypt (100, 101). Controversially, in melanoma, CD68+ TAMs characterized by M1 phenotype, however, not statistically significant correlations were found for the total amount of CD68+TAMs and clinical parameters of melanoma progression in patients (41). RNA-seq and IHC analysis of 57 human melanoma samples showed that CD68+ TAMs were associated with increased iNOS and arginase expression (41). In human osteosarcoma, elevated expression levels of macrophage and CD4 T-cell markers (defined as CD4/IFNGR2/CD68/CSF1R signature) was associated with favorable neoadjuvant chemotherapy responses (40).

In several independent studies amount of intratumoral CD68+ TAMs were indicative for reduced tumor growth and better prognosis (8). CD68+ macrophage infiltrates correlated with better RFS in 468 patients with ER-negative tumors from Scotland (102). In a Norway study of 553 primary NSCLCs CD68+ expression correlated with favorable NSCLC-specific survival (103). Correlation of high expression of CD68 with favorable prognosis was also demonstrated in colorectal cancer (104, 105). Thus, total amount of CD68+ TAMs is a potential prognostic biomarker that can predict negative scenario in progression for majority of human cancer types, however opposite correlations were identified for specific cancer types, in particular colorectal cancer, raising urgent question about intrinsic anti-tumor activates of TAMs that cannot be converted by growing tumor. However, functional role of CD68 in inflammation and carcinogenesis is not sufficiently understood despite its routine application as an immunochemical marker of TAMs.

2.4 Class E scavenger receptors

The class E of scavenger receptors comprises SR-E1, SR-E2, SR-E3 and SR-E4 members (13). The class E SRs belong to a subfamily of NK cell C-type lectin-like (CLEC) receptor family that plays role in diverse biological processes such as immune response, antigen presentation and phagocytosis (13). Several members of the Class E SR family are expressed in TAMs and involved in tumor progression (42, 43).

2.4.1 SR-E1/LOX-1

SR-E1 (also known as LOX-1) is mainly expressed by endothelial cells, but is also found on smooth muscle cells, cardiomiocytes, adipocytes, platelets and on TAMs (106). LOX-1 participates in multiple physiological and pathological processes, including lipid metabolism, cholesterol biosynthesis and tumorigenesis (107, 108). In tumors, LOX-1 regulates macrophage polarization (43). Correlation analysis of TCGA

data, single-cell RNA-seq data and *in vitro* models showed, that TAMs increased the uptake of heat shock protein HSPA12B by LOX-1 that resulted in the activation of PI3K/Akt/mTOR signaling and enhanced M2-type marker expression (CD163 and CD206) in TAMs in head and neck squamous cell carcinoma (HNSC) (43) (Figure 4). At the moment, the detailed mechanism of LOX-1+ TAM activity in the TME is not well defined, but the prognostic value of LOX-1+ TAMs was found in colorectal cancer (42). IHC analysis demonstrated that low expression on TAMs was associated with poor OS in patients with colorectal cancer (42).

2.4.2 SR-E2/Dectin-1

SR-E2 (also known as Dectin-1) is a C-type lectin receptor that is involved in large number of biological processes such as phagocytosis, activation of signaling pathways, generation of reactive oxygen species (ROS) and production of cytokines (109). Dectin-1 is encoded by Clec7a gene and primary expressed on the surface of the myeloid-monocytic lineage cells including macrophages, but can be also found on neutrophils, dendritic cells, and on a minor subpopulation of splenic T cells (46, 110). Dectin-1 is an innate immune receptor playing role in anti-fungal immune response. In cancer, dectin-1 regulates immune microenvironment and has an ambiguous function in tumor progression (44, 46, 111).

IL-13-activated macrophages expressing both dectin-1 and mannose receptor (MR) inhibited T-cell lymphoma and ovarian adenocarcinoma progression *via* binding to tumoral sialic acid (44). Dectin-1 and MR interaction of with sialic acid enhanced antitumor effect of IL-13- activated macrophages *in vitro* (44). Depletion of dectin-1 and MR in IL-13-activated macrophages resulted in inhibition of TAM tumoricidal activity and decrease in death of Jurkat (human T-cell leukemia cell line) and EL4 (murine T-lymphoma cell line) tumor cells (44).

Dectin-1 promotes tumor progression *via* the regulation of immune microenvironment of human OSCC (111). Dectin-1 deficiency decreased the amount of IL-1β+ cells, Tregs, MDSC cells and PD-1 induction in CD8+ T cells resulted in slower dysplasia progression and lower number and size of tumors in mouse model of OSCC (111). Dectin-1 promotes pancreatic ductal adenocarcinoma (PDA) progression by enhanced TAM infiltration and by reprogramming TAMs towards M2 phenotype (46). In a mouse model of PDA, Clec7a deletion significantly reduced the infiltration of PDA with F4/80+, CD206+ and Arg1+ TAMs, as well as upregulated MHCII, TNF-α and iNOS expression in tumor (46). Moreover, depletion of Clec7a in macrophages *in vivo* elevated infiltration by CD4+ and CD8+ T cells selectively in wt hosts, but not in Clec7a−/− hosts, indicating that dectin-1-expressing macrophages drive T cell suppression in PDA (46). In renal cell carcinoma, high expression of tumor cell-derived but not TAM-derived dectin-1 was associated with shorter RFS and OS (45).

2.4.3 SR-E3/CD206

Scavenger receptor SR-E3 (also known as CD206) is a C-type lectin that mediates antigen presentation, endocytosis, phagocytosis and immune homeostasis (112, 113). It is commonly accepted that CD206 is a marker of tumor-supporting M2 phenotype of TAMs, but recent studies demonstrated controversial activity of CD206+ TAMs in tumor (47–49).

CD206+ TAMs produced EGF to promote OSCC progression *in vitro* (49). Proliferation and invasion of OSCC cells cultured with conditioned medium of CD206+ TAMs were strongly enhanced by EGF (49). CD206 mediated breast cancer post-chemotherapy progression (48). In mouse model of breast cancer high expression of CD206+F4/80+ TAMs was associated with tumor relapse and lymph node metastasis after cyclophosphamide treatment (48). Number of CD206+ TAMs positively correlated with worse clinical prognosis in OSCC, CRC, lung cancer (49, 114, 115).

In contrast, CD206+TAMs were shown to program T cells to attack melanoma tumor cells (47). Antigen cross-presentation in tumor remains to be a challenging issue for development of anti-cancer therapy. Primary human as well as mouse CD206+ macrophages were recently shown to be efficient in functional cross-presentation of soluble self-Ag and non-self-Ag, including tumor-associated Ag (TAA) (47). CD11b+CD206+ TAM were found to express a unique cell surface repertoire, promoting antigen cross-presentation and antigen-specific activation of CD8+ T cells. In murine tumor models, the levels of cross-presenting CD206+ TAMs correlated with reduced tumor burden (47). CD206+ TAMs also correlated with improved overall survival of cutaneous melanoma patients. It is an intriguing question to be addressed in future, which self-antigens can be presented to the adaptive immunity in different types of solid cancers by CD206 TAMs, and what is the impact of this process in overall role of CD206 in cancer (47).

2.5 Class G scavenger receptors

2.5.1 SR-G/CXCL16

CXCL16 (also known as SR-G1, or SR-PSOX) is a scavenger receptor mediating endocytosis of oxidized low-density lipoproteins (OxLDL). CXCL16 is primarily expressed on macrophages and dendritic cells. CXCL16 exists in both transmembrane and soluble forms. The soluble form acts as a chemokine specifically binding to CXCR6, and the transmembrane SR-G1 represents an adhesion molecule for CXCR6-expressing cells (116, 117).

CXCL16 has been shown to have a pro-tumoral function in papillary thyroid cancer (PTC) (53). In co-culture of PTC cells with primary monocytes or macrophage-like THP1 celles, high levels of CXCL16 were detected compared to separate PTC cell

culture. Treatment of PTC cells with CXCL16 or co-culture with macrophages enhanced their migration potential. In turn, co-culture up-regulated the expression of M2-markers in macrophages, e.g., CD163, IL-10 and CD206, that was abrogated by an anti-CXCL16 antibody (53). An analysis of the TCGA PTC revealed an association of CXCL16 with M2 macrophage- and angiogenesis-related genes. High CXCL16 expression was associated with aggressive pathologic phenotypes, the higher TNM staging and lymph node metastasis in 77 patients with papillary thyroid cancers, in 25 patients with thyroid follicular adenomas, and 81 - with normal thyroid tissues from the SNUH cohort (51).

CXCL16 pro-tumoral activity was suggested for glioblastoma (GBM) patient's (52). CXCL16 expression in GBM tissues was upregulated, compared to normal brain tissues. However, isolated tumor cells, even if cultured for 1-3 passages, had a substantial reduction in the CXCL16 expression levels. Treatment of mouse glioblastoma microglia with both recombinant and glioma-released CXCL16 increased the expression of anti-inflammatory genes ARG1, CHIL3, RETNLA and CD163 that was impaired by anti-CXCL16 antibodies. Microglia from glioma-bearing CXCR6-ko mice had lower expression levels of anti-inflammatory genes, compared to glioma-bearing wt mice that suggested CXCL16/CXCR6 axis involvement in the anti-inflammatory programming of microglia (Figure 4). Patient-derived GBM cells significantly increased cell chemotactic index, invasion and proliferation under CXCL16 exposure. Use of TCGA data with GBM patients revealed a significant increase in patient's survival associated with CXCR6 deletion and a significant decrease in the survival associated with CXCR6 mRNA overexpression (52).

Human ovarian cancer tissue significantly increased expression of CXCL16 in comparison with both corresponding adjacent and para-cancerous tissues (54). The correlation analysis indicated a positive association of CXCL16 expression with an activation of macrophages in ovarian cancer (54). Macrophage-derived CXCL16 promoted migration and invasion of ovarian cancer SCOV3 cells by enhancing the activity of the PI3K/Akt pathway (54). Silencing of CXCR6 by shRNA in SCOV3 cells diminished above-mentioned effects of CXCL16 (54). In the co-culture of AIF1-overexpressed macrophages with hepatocellular carcinoma Hepa1-6 cells, CXCL16 secreted by macrophages enhanced proliferation and migration of cancer cells, and this effect was abrogated by a neutralizing antibody against CXCL16 (118).

We were able to find only one report describing CXCL16-mediating tumor-inhibiting function in colorectal cancer (CRC) (50). In co-culture of colorectal cancer SL4 cells with RAW 264.7 cells, CXCL16 induced tumor cell apoptosis mediated by TNFα-expressing macrophages. A susceptibility of CXCL16-overexpressing CRC cells to apoptosis was attenuated by neutralization of TNFα with a corresponding antibody (50).

In summary, CXCL16 tend to have predominantly a pro-tumoral role through promoting an anti-inflammatory phenotype of TAMs, and by activating proliferative and invasive potentials of cancer cells. Nevertheless, there is an evidence of CXCL16 anti-tumoral role too through sensitizing of CRC cells to apoptosis.

2.6 Class J scavenger receptors

2.6.1 SR-J1/RAGE

SR-J1 (AGER, or RAGE) is a cell surface receptor from the immunoglobulin superfamily that specifically binds advanced glycation end products (AGEs) (119). RAGE is the only member of class J scavenger receptors and capable of binding multiple ligands (72). Except for AGEs, SR-J1 recognizes HMGB1 (120), members of the S100 protein family (121), amyloid-beta peptide (122) and binds dsDNA and dsRNA directly (123). RAGE is expressed by diverse cell types, including macrophages, monocytes, endothelial cells, fibroblasts and smooth muscle cells (124).

Multiple evidences indicate a pro-inflammatory role of RAGE activation, in particular, the HMGB1-induced activation of RAGE in inflammation-related context (125–127). RAGE is involved in ROS production and M1 polarization of macrophages under AGE exposure (65, 128). In macrophages, AGEs significantly elevated the expression of IL-6, IL-12, TNFα and TLR4, as well as the phosphorylation levels of STAT1 in cytoplasm in RAGE/ROS dependent manner (Figure 4). TLR4 inhibition by siRNA diminished effect of the AGE-dependent RAGE activation, while both RAGE expression and ROS production remained unchanged. This evidence suggests TLR4 as a downstream regulator of RAGE activation and further ROS production (65).

RAGE was studied in human GBM treated with temozolomide (TMZ) (129). TMZ treatment caused HMGB1 release from GBM cells in tumor tissue of patients. Affinity examination showed that RAGE is the main receptor binding extracellular HMGB1. Immunofluorescent analysis of patients` GBM samples indicated co-localization of RAGE and HMGB1 on TAMs. *In vitro* stimulation of THP-1 macrophages with recombinant HMGB1 promoted release of pro-inflammatory cytokines through NLRP3-dependent inflammasomes that was diminished by RAGE inhibition (129). The mechanism of RAGE activation by HMGB1 was related to phosphorylation of ERK1/2 and IKB resulting in NFκB activation. In patients with GBM, HMGB1 expression is associated with improved OS. These results indicate that RAGE interaction with HMGB1 can be favorable factor in GBM treatment response (129). Irreversible electroporation caused the release of nucleus HMGB1 out of PDAC cells followed by binding of HMGB1 to RAGE in THP1-derived macrophages, that skewed macrophages toward pro-inflammatory phenotype *via* MAPK-ERK activation (130).

Macrophages enhanced phagocytosis of dying electroporated PDAC cells. This effect was neutralized by RAGE inhibition. MAPK-ERK inhibition significantly decreased the RAGE expression and the release of autocrine HMGB1 by macrophages (130).

In vitro RAGE is equally expressed in both M1- and M2-polarized macrophages, but has distinct effects on the cancer cells that depends on a polarization state of macrophages (69). In contrast to M1 macrophages, HMGB1-dependent stimulation of RAGE facilitated pro-tumor activity in M2 macrophages. RAGE activation by HMGB1 enhanced invasion of gastric tumor cells (MKN45) in co-culture with M2-polarized THP1 macrophages and vice versa with M1 macrophages (66). RAGE induced VEGF production in M2 macrophages. The conditioned medium of M2 macrophages treated with HMGB1 stimulated the growth of endothelial cells *in vitro*; this effect was opposite for M1 macrophages. In contrast to M1 polarization, the RAGE activation in M2 macrophages did not lead to NFkB activation. Two negative regulators of the NFkB activation, SOCS1 and SHIP-1, were significantly upregulated under the HMGB1 exposure in M2 macrophages (66). HMGB1-mediated RAGE activation in THP1-derived M2 macrophages also stimulated lymphangiogenesis by increasing both proliferation and migration of lymphatic endothelial cells as well as VEGF-C production in M2, but not in M0 macrophages (67). RAGE inhibition significantly reduced M2-dependent lymphangiogenesis (67). HMGB1+CD163+ M2 macrophages were found as detrimental prognostic factors for OS in laryngeal squamous cell carcinoma patients (67). RAGE mediates chemotaxis of THP1-differentiated macrophages upon stimulation with a conditioned medium of S100A7-overexpressing breast cancer MDA-MB-231 cells. This effect was significantly abrogated by RAGE blockage (131).

In vivo RAGE-depleted mouse models of GBM indicated RAGE as a significant TAM-specific factor participating in inflammation and angiogenesis in the TME (69). Survival analysis of tumor-bearing mice revealed that RAGE ablation significantly prolonged survival of mice in comparison with wild type (wt) mice. RAGE-depleted tumor exhibited lower expression of pro-inflammatory cytokines, and RAGE-depleted TAMs expressed significantly lower levels of IL-6 and VEGF-A. RAGE expression in tumor microglia or bone marrow-derived macrophages stimulated angiogenesis in GBM. Patient's GBM samples had abundance of CD163+ TAMs with high RAGE expression (69).

RAGE was shown to mediate uptake of an oncogenic mutant KRASG12D protein by peripheral blood mononuclear cell-derived macrophages during autophagy-dependent ferroptosis of PDAC cells (68). Under oxidative stress conditions, tumor cells released KRASG12D protein *via* exosomes secretion. Exosomes were engulfed by macrophages in a RAGE-dependent manner that was confirmed by the knockdown of RAGE by shRNAs in macrophages. KRASG12D promoted M2 polarization *via* STAT3-dependent fatty acid oxidation (FAO). Inhibition of FAO reduced mRNA expression of IL10, ARG1, and TGFB1 in the macrophages. The knockdown of RAGE and ablation of STAT3 by shRNA abrogated the FAO and the M2 polarization. In PDAC patients, high KRASG12D expression in CD68+ cells correlated with worse OS rates. The KRASG12D uptake by macrophages may significantly contribute to the human PDAC progression (68).

In summary, RAGE activation of TAMs has controversial impact on TME and tumor cells. Evidence indicates that pro-tumor and anti-tumor RAGE role through distinct TAMs activation depends on TME context and RAGE ligands. Is of great interest to identify in future the spectrum of tumor-specific sets of RAGE ligands, and to examine how cooperation of M1 or M2-specific receptors with RAGE can a decide about pro-and anti-tumor programming of TAMs.

2.7 Class H scavenger receptors

The class H scavenger receptors are transmembrane protein receptors containing in their extracellular part fasciclin, EGF-like and lamin-type domains. Class H scavenger receptors has two members: SR-H1 (also known as Stabilin-1, or Clever-1) and SR-H2 (known as stabilin-2, or HARE) (132, 133). Despite high similarity in domain organization and endocytic functions, stabilin-1, but not stabilin-2 is expressed on TAMs and plays an essential role in tumor development.

2.7.1 SR-H1/Stabilin-1

SR-H1, originally identified as stabilin-1 (134) and as CLEVER-1 (135) is multifunctional scavenger and intracellular sorting receptor with adhesive activities expressed by immunosuppressive monocytes and macrophages, sinusoidal endothelial cells and lymphatic endothelial cells (134, 136–138). Stabilin-1 performs endocytosis, phagocytosis, intracellular sorting of newly synthetized proteins and transcytosis of growth hormone family member placental lactogen (9, 139–144). Large body of evidence demonstrated that stabilin-1/CLEVER-1 can mediate cell-matrix and cell-cell interactions during primary tumor growth and in metastatic state (135, 138, 145–147).

Stabilin-1 is abundantly expressed on TAMs in number of solid cancers in patients and in murine models (8, 57, 132, 148). TAM-expressed stabilin-1 mediates clearance of tumor growth-inhibiting factor SPARC in a mouse model of breast cancer, and germinal knock-out of stabilin-1 results in the statistically significant reduction of primary tumor growth in this model (57). In orthotopic mouse models of lung cancer, breast cancer and lymphoma, genetic deficiency of macrophage stabilin-1 significantly reduced tumor growth (149). In stabilin-1 KO mice TME was shifted towards inflammatory program and was enriched in the activated endogenous CD8+ T cells. Immunotherapeutic blockade of stabilin-1 had similar

consequences, and had synergistic effect with anti-PD-1 checkpoint inhibition (149).

Strong association of stabilin-1+ TAMs with worse prognosis was shown in several human cancers (55, 56, 150). Stabilin-1 expression in TAMs was associated with poor OS, RFS, tumor stage and histological grade in patients with urothelial carcinoma (56). High intratumoral expression of stabilin-1 on CD68+ TAMs was associated with poor DSS in stage I–IV rectal cancer (55). In contrast, high number of CD68+ stabilin-1+ TAMs correlated with longer DSS and predicted a favorable prognosis in early stage I colorectal cancer (CRC) patients (55).

Contribution of intracellular sorting function of stabilin-1 to tumor progression is linked to the ability of the extracellular domains of stabilin-1 to interact with at least two human chitinase-like proteins, SI-CLP and YKL-39, while the interaction with true chitinases CHIT1 and AMCase and with YKL-40 was not studied to date (6, 141, 151, 152).

We have demonstrated that stabilin-1 mediates intracellular delivery of newly synthetized SI-CLP, stabilin-1interacting chitinase-like protein, that interacted with a fasciclin domain of stabilin-1 in the yeast two-hybrid screening and in the affinity chromatography assay (141, 152). In a murine model for breast adenocarcinoma we demonstrated that SI-CLP being ectopically expressed in subcutaneously injected TS-A cells significantly reduced tumor growth and reduced infiltration of TAMs (153). Recently, we have also found that stabilin-1 is able to interact with YKL-39 (CHI3L2), that for a long-time was known as highly specific biomarker of rheumatoid arthritis, and later has been found to be overexpressed in glioblastoma affecting biology of transformed cells (6, 154). In patients with glioma high levels of CHI3L2 expressed in cancer cells and on microglia cells correlated with poor prognosis. Mechanistically the authors found that CHI3L2 induces the apoptosis of CD8+ T cells (155). We found that YKL-39 has two functions that can promote tumor growth: it stimulates monocyte migration, and it stimulates as well angiogenic activity of endothelial cells *in vitro* (6). In patients with breast cancer YKL-39 was exclusively expressed in TAMs in tumor mass, and elevated levels of YKL-39 in primary tumors significantly correlated with metastatic relapse after therapy onset (6). However, whether similar mechanisms can act in other types of cancer has to be studied, while application of purified SI-CLP and blocking agents for YKL-39 is a promising strategy to reprogram tumor-promoting microenvironment.

In summary, stabilin-1 has a highly complex function in cancer. Its deficiency is cancer-inhibiting, at least due to the reduction of SPARC clearance. Its ability to modulate concentrations of tumor-promoting YKL-39 and tumor-inhibiting SI-CLP can contribute to tumor growth and metastasis in a cancer-specific way, since not necessarily both proteins are present at the same time in TME. In particular, the role of stabilin-1 in CRC is of interest, while total amount of TAMs in this cancer type, in contrast to majority of other types, correlates with reduced tumor growth and good prognosis (8).

2.8 Class I scavenger receptors

SR-I (also known as CD163) is a hemoglobin-haptoglobin complex scavenger receptor that is mostly expressed in monocytes and macrophages (13, 156). In tumors, CD163 promotes tumor development and is associated with worth prognosis in breast cancer, head and neck cancer, lymphoma and melanoma (39, 59–62, 64, 100). CD163, mediates clearance of hemoglobin-haptoglobin complexes out of circulation is a silent way, however, in hyperglyceamic conditions this scavenging process leads to inflammatory macrophage responses (157).

CD163 is commonly defined as a marker for tumor-supporting TAM phenotype. In human breast cancer, CD163+ TAMs accumulation was inhibited by tumor suppressor TAp73 (58). Amount of CD163+ TAMs negatively correlated with Tap73 expression and positively correlated with tumor grade (58). High amount of CD68+ and CD163+ TAMs was associated with lymph node metastasis, high Ki67 expression and poor prognosis in 1579 breast cancer patients from Zhejiang Provincial People's Hospital and Zhejiang Tiantai People's Hospital (62, 100). Elevated levels of CD163+ TAMs in tumor stroma and tumor nest correlated with poor prognosis in 107 patients with triple negative breast cancer operated on at Dokkyo Medical University Hospital (59). CD163 was identified as a good predictor of pre-metastatic status of colorectal cancer (158). High levels of CD163+ cells were associated with tumor node metastasis stage, depth of infiltration, and lymphatic metastasis in 197 patients with colorectal cancer from China (158). Using multispectral immunofluorescence it was demonstrated that CD163+ cells have immunosuppressive phenotype in 17 patients with colorectal cancer who underwent resection of primary and liver metastases (159). High number of CD163+ cells was found in peritumoral region of tumor and in liver metastases (159).

Several studies confirmed that tumor-supporting effect of CD163+ TAMs is mediated by the activation of STAT3 signaling pathway (60, 63). Tumor-mediated activation of STAT3 in CD163+ TAMs resulted in pro-tumor TAM polarization (63). *In vitro*, conditioned medium from cholangiocarcinoma cell lines (HuCCT1, RBE and MEC) induced activation of STAT3 in modeled TAMs and enhanced production of IL-10, VEGF-α, TGF-β and MMP-2 in CD163+ TAMs (63) (Figure 4). CD163+ TAMs produced tumor-supporting cytokines (IL-6 and CXCL2)

activated STAT3 in tumor cells and supported tumor progression. CD163-KO TAMs had decreased production of IL-6 and CXCL2 in comparison to WT TAMs in co-culture with MCA205 (mouse fibrosarcoma) cells (64). Conditioned medium of CD163-KO TAMs significantly impaired activation of STAT3 in MCA205 cells (64). CD163-expressing TAMs displayed elevated levels of pSTAT3 and correlated to poor prognosis in 77 patients with myeloma from STAT3 is over-activated within CD163pos bone marrow macrophages in both Multiple Myeloma and the benign pre-condition MGUS (60). Increased infiltration of both CD68+ and CD163+ TAMs in tumor mass correlated with decreased survival of 174 patients with gastroesophageal adenocarcinoma from Sweden (61). CD68 and CD163 overexpression was indicative for worse prognosis in 105 HCC patients from Japan (160). Increased levels of CD163+ TAMs correlated with decreased OS and higher histological grade in human sarcoma (64). Oppositely, in human primary melanoma low amount of CD163-expressing TAMs in tumor stroma was associated with recurrence and poor OS (39).

Overall, at least two molecular mechanism for tumor-supporting function of CD163+ TAMs were identified to date: inhibition of tumor suppressor TAp73 in breast cancer and activation of STAT3 signaling in TAMs and in r fibrosarcoma cells (58, 63, 64). Moreover, high expression of CD163+ TAMs was related to poor prognosis in breast cancer, gastroesophageal adenocarcinoma, HCC, human sarcoma, but not in melanoma patients (39, 59, 61, 62, 64).

3 Genetics of scavenger receptors

Deleterious germline mutations cause a broad range of distinct pathological conditions including cancer (161) There is limited information describing association of SR gene mutations with tumor progression, especially in non-malignant cells, e.g., macrophages. The only reliable evidence for such association are germline mutations in MSR1 coding scavenger receptor CD204 (162, 163). Genetic analysis of hereditary prostate cancer revealed significant co-segregation of prostate cancer with the nonsense mutation R293X in man of European descent and the missense mutation D174Y in man of African American descent (163). The truncating mutation R293X resulted in deletion of most of the collagen-like domain of MSR1 gene, including the ligand-binding region and the cysteine-rich domain. The missense mutation D174Y can affect proper polymerization of three MSR1 polypeptide chains. Both mutations disrupted MSR1 function that affected MSR1 ability to bind oxLDL involved in the oxidative stress. MSR1 is predominantly expressed by macrophages in both benign and cancerous prostate tissues, emphasizing the role of macrophage-derived mutated MSR1 in prostate cancer development (163).

MSR1 mutations are also involved in Barrett esophagus (BE) and esophageal adenocarcinoma (EAC) development (162). The nonsense R293X and missense L254V mutations contributed to BE/EAC risk, or were required for BE/EAC predisposition. The L254V mutation was found within the conserved coiled-coil domain of MSR1, so both R293X and L254V led to MSR1 function disruption. MSR1 mutation caused overexpression of key nuclear cell cycle molecule Cyclin D1 (CCND1) in BE and EAC tissue samples that was impaired by overexpression of wild-type MSR1 in HEK293 cells (162).

Association of MSR1 mutations with progression of prostate cancer and esophageal adenocarcinoma confirmed the involvement of this scavenger receptor to carcionogenesis.

4 Conclusions

Macrophage SRs have dual role in tumor development. Tumor-supporting activity mediated by macrophage SRs includes regulation of tumor invasion, proliferation and migration (for CD204, CD206, CXCL16, Stabilin-1, and RAGE), as well as M2-like TAM polarization (for CD36, LOX-1, CXCL16, CD 163, and RAGE) and tumor angiogenesis (for CD68, Dectin-1, RAGE). The anti-tumor functions of TAM-expressing SRs include suppression of tumor angiogenesis (for CD204), tumor invasion (for RAGE), inducing tumor cells clearance (for MARCO) and M1-like TAM polarization (for CD204 and RAGE). In cancer patients, number of TAM-expressed SRs (CD204, MARCO, CD68, LOX-1, Dectin-1, CD206, CXCL16, Stabilin-1, CD163, and RAGE) associates with negative and more sever prognosis. Targeting of tumor-promoting SRs can be a promising approach in cancer immunotherapy. Accumulating clinical data demonstrate that SRs can serve as potential prognostic biomarkers for patients with cancer (29, 84, 164). For example, in mouse model of triple negative breast cancer specific targeting of CD206+ TAMs inhibited tumorigenesis and metastatic dissemination of tumor cells (165). Application of antibodies against MARCO resulted in the reduction of tumor growth and inhibition of metastasis in murine models for melanoma and breast cancer (166). There are multiple studies describing effects of SR targeting in various cellular *in vitro* and pre-clinical *in vivo* models (145, 165, 167–169). Drug targeting of CD36 demonstrated promising results for patients with advanced soft tissue sarcoma in the initial clinical trials (167, 170). However, targeting CD36 failed in phase 2 clinical trials because of ineffective performance and severe adverse events. The complications can be explained by the expression of majority of various SRs on resident macrophages and on other cell types in healthy organs and tissues. Moreover, we still have very limited information about cancer-

specific ligands of SRs, in particular for the ability of SRs to internalize and target for degradation cytokines and growth factors.

The investigation of the mechanisms of tumor development and progression mediated by SRs is a foreground goal for the developing immunotherapeutic approaches that can help to suppress tumor cell invasion, proliferation and migration, to inhibit macrophage recruitment and pro-tumor macrophage polarization as well as to enhance clearance of tumor cells by TAMs. Moreover, the ability of SRs to internalize both particles and molecular complexes still remains to be explored for the design of targeted drug delivery for macrophage re-programming in tumor microenvironment.

Author contributions

EK performed literature analysis and drafted the manuscript. EK designed Figures. PI and IL contributed to literature analysis and wrote manuscript chapters. JK developed the concept of the manuscript, designed tables, wrote manuscript chapters, edited final text. All authors contributed to manuscript revision, read 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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Human macrophage-engineered vesicles for utilization in ovarian cancer treatment

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Background: Ovarian cancer is a deadly female malignancy with a high rate of recurrent and chemotherapy-resistant disease. Tumor-associated macrophages (TAMs) are a significant component of the tumor microenvironment and include high levels of M2-protumor macrophages that promote chemoresistance and metastatic spread. M2 macrophages can be converted to M1 anti-tumor macrophages, representing a novel therapeutic approach. Vesicles engineered from M1 macrophages (MEVs) are a novel method for converting M2 macrophages to M1 phenotype-like macrophages.

Methods: Macrophages were isolated and cultured from human peripheral blood mononuclear cells. Macrophages were stimulated to M1 or M2 phenotypes utilizing LPS/IFN-γ and IL-4/IL-13, respectively. M1 MEVs were generated with nitrogen cavitation and ultracentrifugation. Co-culture of ovarian cancer cells with macrophages and M1 MEVs was followed by cytokine, PCR, and cell viability analysis. Murine macrophage cell line, RAW264.7 cells were cultured and used to generate M1 MEVs for use in ovarian cancer xenograft models.

Results: M1 MEVs can effectively convert M2 macrophages to an M1-like state both in isolation and when co-cultured with ovarian cancer cells *in vitro*, resulting in a reduced ovarian cancer cell viability. Additionally, RAW264.7 M1 MEVs can localize to ovarian cancer tumor xenografts in mice.

Conclusion: Human M1 MEVs can repolarize M2 macrophages to a M1 state and have anti-cancer activity against ovarian cancer cell lines. RAW264.7 M1 MEVs localize to tumor xenografts *in vivo* murine models.

KEYWORDS

ovarian cancer, tumor-associated macrophage (TAMs), M1 macrophage, M2 macrophage, vesicle, immunotherapy

1 Introduction

Ovarian cancer is the leading cause of death in gynecological cancers. The American Cancer Society estimates that in 2022 there will be 19,880 new cases of ovarian cancer and 12,810 deaths (1). Most patients are diagnosed at an advanced stage, with a 5-year survival rate of less than 50% (2). Patients with advanced-stage ovarian cancer are treated with combination platinum and taxane chemotherapy in the front-line setting. While many patients initially show a response to chemotherapy, the majority will ultimately relapse (2, 3). Unlike other solid tumors, immunotherapy has been largely ineffective in ovarian cancer (4, 5), emphasizing the need for novel immunotherapies to treat this disease.

Recent research suggests that tumor-supportive tumor-associated macrophages (TAMs) promote tumor vascularization and metastasis and are predominantly anti-inflammatory, M2-like macrophages (6, 7). In contrast, pro-inflammatory, M1-like macrophages can clear cancer cells and are associated with a better prognosis (8–10). A recent meta-analysis demonstrated that high numbers of TAMs are negatively associated with overall survival in multiple solid tumor types, including ovarian cancer (11). As macrophages are highly plastic, an area of growing interest is the repolarization of anti-inflammatory TAMs to pro-inflammatory TAMs as a potential mechanism of increasing the sensitivity of cancer cells to multiple therapies, including immunotherapy. Approaches to initiate macrophage repolarization include small molecule inhibitors, *in vitro*-transcribed mRNA, toll-like receptor (TLR) agonists, and siRNAs delivered *via* nanoparticles, all of which have demonstrated repolarization of M2-like TAMs to a M1 phenotype, resulting in downregulation of pro-tumor markers, such as vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β), and upregulation of pro-inflammatory markers, including tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ). However, the aforementioned approaches are limited because they fail to localize to tumor associated cells, and therefore heighten the potential for off-target side effects (7, 12–14). Additional approaches include increasing the antibody-dependent cell-mediated cytotoxicity (ADCC) of TAMs utilizing low-fucosylated antibodies, such as humanized glyco-engineered anti-AMHRII monoclonal antibody murlentamab, holds potential promise, *via* stimulating an antitumor adaptive immune response *via* TAM repolarization (15). Interest in using vesicles as potential therapeutics has grown significantly in recent years (16). Vesicles are structures of varying sizes that are created endogenously by cells and they can also be bioengineered by several techniques. In biological systems, vesicles enable cell-to-cell communication, *via* the transfer of proteins, lipids, and nucleic acids (17, 18). As a therapeutic modality, vesicles can encapsulate various therapeutic agents, while minimizing immunogenicity and can efficiently target the same cell type as the donor cell (16, 19, 20). This targeting property has led to the investigation of endogenous vesicles, exosomes, isolated from cancer cells to target comparable primary malignant cells (21, 22). Currently, there is limited data on the role of cancer cell exosomes to specifically target ovarian cancer. One study examined exosomes from SKOV3 ovarian cancer cells, subsequently loaded with triptolide, an antineoplastic agent, and demonstrated anti-tumor efficacy in ovarian cancer models (23). Yet, it should be noted there are significant theoretical and practical concerns with the utilization of exosomes derived from cancer cells as prior studies have suggested that tumor cell exosomes may enhance tumor progression and metastasis (17, 21, 22, 24–31).

Another approach is the utilization of vesicles derived from macrophages to target the macrophage-abundant tumor microenvironment seen in ovarian cancer (32). M1-type exosomes from RAW 264.7 cells, a murine macrophage line, can polarize unstimulated RAW 264.7 macrophages to the M1 phenotype (33). However, exosomes are limited in their therapeutic use due to low production yields and limitations in loading drug cargo. An alternative approach that has recently shown promise is bioengineering vesicles from macrophage cell membranes. These macrophages engineered vesicles (MEVs) can be formed by rupturing the cell membrane into fragments *via* nitrogen cavitation and allowing them to reconstitute into smaller distinct vesicle units. Engineered vesicles derived from the mouse RAW 264.7 cell line show similar properties as macrophage exosomes and can be loaded with a broad range of cargo, including therapeutics (34, 35).

MEVs derived from M1 macrophages can serve dual purposes; they can be used as a novel delivery vector for chemotherapeutics and can immunomodulate TAMs (35–37). Prior studies have demonstrated that mouse-derived M1 MEVs can repolarize mouse M2 macrophages back to an M1 state *in vitro* (35, 36). In addition, mouse M1 MEVs can be loaded with platinum-chemotherapeutics and have *in vitro* anti-cancer activity (36). Additional studies have shown that macrophage-derived vesicles loaded with paclitaxel have anti-cancer effects against multidrug-resistant cancer cell lines and murine breast cancer models (38, 39).

Here we describe the generation of MEVs from human peripheral blood mononuclear cells (PBMCs) that have been differentiated into macrophages. This is an advancement in our prior work by utilizing primary non-tumor human cells from fresh primary isolations (35, 36). We show that human M1 MEVs localize to both human macrophages and cancer cells and can repolarize M2 macrophages to an M1 phenotype. Human M1 MEVs display anticancer effects in co-culture with ovarian cancer cells. Additionally, using ovarian xenografts in mice, we demonstrate localization of RAW264.7 M1 MEVs to ovarian tumors *in vivo*.

2 Materials and methods

2.1 Cell lines

The ovarian adenocarcinoma cell lines: Caov-3, OVCAR3, and SKOV3 along with the murine macrophage line: RAW264.7, were obtained from ATCC. Caov-3 cells and RAW264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, ATCC), supplemented with 10% fetal bovine serum (FBS, Sigma). OVCAR3 cells were maintained in RPMI-1640 medium with glutamine and glucose (ATCC), supplemented with 10mg/mL insulin from bovine pancreas (Sigma) and 20% fetal bovine serum (FBS, Sigma). SKOV3 cells were maintained in McCoy's 5a Medium Modified (ATCC), supplemented with 10% fetal bovine serum (FBS, Sigma). Cells were maintained at 37˚C with 5% CO_2.

2.2 Human PBMC isolation and differentiation

Human PBMCs were isolated from buffy coats from 4-5 healthy donors (Kentucky Blood Center, Lexington, KY) by density gradient centrifugation (Ficoll-Paque Premium, GE Healthcare, Sweden) for each preparation of MEVs. Monocytes were isolated from PBMCs by immunomagnetic negative selection (EasySep Human Monocyte Enrichment Kit, Stemcell Technologies, Cambridge, MA). Human PBMC-derived monocytes were cultured in RPMI-1640 (ATCC) with 10% heat-inactivated Fetal Bovine Serum (Sigma-Aldrich, St. Louis, MO), 1% penicillin-streptomycin (Gibco), and recombinant human macrophage colony-stimulating factor (M-CSF, 50ng/mL, PeproTech, Rocky Hill, NJ) for 5-6 days. Media was replaced every 48 hours. M0 macrophages were stimulated for 24 hours with lipopolysaccharide (LPS, 20ng/mL, *In vivo*gen) plus recombinant human interferon-γ (IFN-γ, 20ng/mL, PeproTech) for M1 macrophages or with recombinant human interleukin-4 (IL-4, 20ng/mL, PeproTech) plus recombinant human interleukin-13 (IL-13, 20ng/mL, PeproTech) for M2 macrophages. Cells were maintained at 37° C with 5% CO_2.

2.3 Vesicle generation and characterization

M1 MEVs were generated from human M1 macrophages using nitrogen (N_2) cavitation. Cells were washed to remove any remaining cytokines, manually disrupted from cell flasks using a cell scraper, and then resuspended in phosphate-buffered saline (VWR) plus protease inhibitor (Thermo Scientific). N_2 cavitation was performed by maintaining cells in a pre-chilled pressurized chamber (Parr Instruments Company, IL, USA) at 250 psi for 5 minutes at 4 ˚C. Vesicles were purified from cellular debris by centrifugation at 4 ˚C for 20 minutes at 4,000 x *g* then 10,000 x *g*. The supernatant was then withdrawn and ultracentrifuged at 100,000 x *g* for 1 hour at 4 ˚C. The subsequent pellet was washed five times with PBS and resuspended in PBS. Fluorescein-loaded human M1 MEVs were generated as described above, with the addition that the N_2 cavitation step was performed in a 1mM solution of fluorescein in PBS. For the complete removal of free dye, a diluted vesicle suspension was subjected to an additional ultracentrifugation step at 100,000 x *g* for 60 minutes at 4°C. The mean diameter, concentration, and zeta potential values of MEVs were obtained *via* particle tracking analysis using a Zeta View PMX-120 using MEVs generated from $3.1x10^7$ human M1 macrophages. Nanoparticle tracking analysis was performed on human M1 MEVs generated from $2.8x10^7$ human M1 macrophages to determine the vesicle size distribution and concentration (NanoSight 300, Malvern Panalytical, United Kingdom).

2.4 Vesicle electron microscopy

The suspended sample of MEVs was fixed with 4% paraformaldehyde for 1 hour and rinsed with 1X PBS. The sample was serially dehydrated with different concentrations of ethanol from 30%, 50%, 70%, 75%, 80%, 90%, 95%, and 100% for 10 minutes. A droplet of the sample was pipetted and deposited onto a glass cover slip previously treated with 0.1% solution of poly-L-lysine[1] to promote adhesion. Before the sample could fully dry, it was briefly immersed in ethanol (200 proof) and transferred into a critical point dryer (EM CPD 300, Leica Microsystems, Wetzlar, Germany) system. After drying, the surface of the sample was metallized by sputter coating 5 nm of platinum (EM ACE 600, Leica Microsystems, Wetzlar, Germany) to enhance surface electrical conductivity and subsequently imaged using a field-emission scanning electron microscope (SEM, Quanta 250 FEG, ThermoFisher Scientific, formerly FEI, Hillsboro, OR, USA).

SKOV3 cells were incubated with M1 vesicles for 24 hours. After incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 40 minutes at room temperature (RT). The cells were then processed for immunogold labeled silver enhancement stain (IGSS). Cells were blocked with 3% BSA for 2 hours and then incubated with monoclonal rabbit anti-human CD86 (1:250 dilution) overnight at 4^0C. Cells were then incubated with secondary anti-rabbit IgG Alexa Fluor® 647 Fluoro Nanogold (Nanoprobes) at 1:100 dilution for 2 hours at RT. Silver enhancement was performed using HQ silver enhancement kit (Nanoprobes) for 5 minutes at RT. The cells were then washed three times with deionized water and further

incubated with 0.2% osmium tetraoxide in PBS at 4C for 1 hour. Cell samples were exposed with 0.25% uranyl acetate for 1 hour at 4^0C. Samples were then dehydrated using serial concentrations of ethanol: 50%, 70%, 90% and 100% (three times). Samples were then embedded with 100% resin. Samples were washed with resin twice, with the second wash added to samples and incubated for 45-60 minutes in a 60^0C oven. A final resin polymerization was performed for 48 hours at 60^0 C. Cultured cells were then separated from plates and a 100nm section was cut with a microtome and mounted on FCF-200-Cu grids. Images were acquired using a Thermo Scientific™ Talos™ F200X TEM (40).

2.5 Imaging of fluorescently-labeled vesicles

Fluorescein-labeled vesicles were generated as discussed previously and fixed onto a glass-bottom dish before imaging using a fluorescence microscope. Fluorescein-loaded vesicles were imaged using a 488 nm laser of 0.8 mW power and an exposure time of 200 ms.

2.6 Cytokine analysis

Human PBMC-derived monocytes were plated in 24-well plates at 1 x 10^6 cells/well and cultured with M-CSF (50 ng/mL) for six days. Cells were stimulated in duplicate to M1 or M2 macrophages as previously described. M1 macrophages from the same PBMC isolation plated on separate plates were used to generate MEVs. Vesicles were washed to remove any remaining cytokines, then plated with M2 macrophages. Media supernatants were collected following a 24-hour incubation period and were assayed in duplicate using a human TNF-α Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN). Optical density was measured using a microplate reader (Varioskan LUX, Thermo Scientific, Finland). Experiments were performed in triplicate.

2.7 Real-time PCR of macrophage biomarkers

Human peripheral blood monocytes were isolated, plated, and cultured for five days into differentiated M0 macrophages. M0 macrophages plated in a 6-well plate at a concentration of 5.0 x 10^5 per well, after which macrophages were polarized to either an M1 or M2 state using LPS/IFNγ or IL4/IL13, respectively. M1 MEVs were prepared from additional M1 macrophages as previously described and were then used to treat M2 macrophages. Following an additional 24-hour incubation, RNA was purified from human macrophages (M0, M1, M2, MEV-treated M2) with RNeasy Plus Universal Mini Kit (Qiagen), and 500 ng of each sample was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) with random primers. Real-time semi-quantitative PCR measured gene expression using TaqMan Advanced Master Mix with TaqMan Gene Expression Assays (ThermoFisher Scientific). Expression of human CXCL8 (assay ID Hs00174103_m1), CXCL10 (assay ID Hs00171042_m1), relative to endogenous control GAPDH (assay ID Hs02758991_g1) was measured in triplicate using a QuantStudio 3 Real-Time PCR instrument (Applied Biosystems). Relative expression was evaluated across samples with QuantStudio Software (Applied Biosystems) using the Comparative C_T ($\Delta\Delta C_T$) method.

2.8 Co-culture of human M2 macrophages and cancer cells

For co-culture imaging experiments, human M0 macrophages were plated at 5 x 10^4 cells/well in a 96-well clear-bottom, black-walled plate. M0 cells were stimulated to M1 or M2 for 24 hours. Caov-3 ovarian adenocarcinoma cells were then plated at 5000 cells/well with M1 or M2 macrophages. Human M1 MEVs were generated and labeled with a lipophilic dialkylcarbocyanine fluorescent dye, DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, Molecular Probes Inc., Invitrogen, Eugene, OR). DiI labeled vesicles were obtained by incubating MEV-resuspension with 5 μM DiI for 30 minutes at 37°C. The free dye molecules were separated from the fluorescently-labeled vesicles using a size exclusion spin column (PD MidiTrap column) following the manufacturer's protocol. Human M1 DiI-labeled MEVs at a 10% dilution were added to Caov-3 cells, M2 macrophages, or Caov-3 plus M2 macrophage co-culture. After a 24-hour incubation period, cells were imaged at 40x with confocal microscopy (CellInsight CX7 High-Content Screening Platform). Cells were incubated with Hoescht (1:2000) for 30 minutes before imaging to label nuclei.

For cell viability experiments, human M0 macrophages were plated at 2.5-5 x 10^4 cells/well in a 96-well plate. M0 cells were stimulated to M1 or M2 for 24 hours. Supernatant was then removed and Caov-3 ovarian adenocarcinoma cells (ATCC) were then plated at 5000 cells/well with M1 or M2 macrophages. M0, M1, and M2 macrophages and Caov-3 controls were each plated in at least duplicate. Supernatants were collected after 24 hours. A 20% or 10% dilution of human M1 MEVs was added to Caov-3 cells only and Caov-3 plus M2 cells in duplicate. Supernatants were collected after 24-hour incubation with MEVs, and wells were replaced with complete media. A cell viability assay was performed after 96 hours following the addition of MEVs according to the manufacturer's instructions (CellTiter-Glo 2.0, Promega).

Luminescence was measured with a microplate reader (Varioskan LUX). This process was repeated in the same manner with OVCAR3 cells. Experiments for both cell lines were performed in triplicate. The collected supernatants were assayed in duplicate using a human TNF-α Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN).

2.9 RAW264.7 MEV generation and mouse localization experiments

RAW264.7 cells were maintained at 37°C with 5% CO_2. RAW264.7 cells were stimulated to an M1 state using LPS/IFNγ at a concentration of 20 ng/ml for 24 hours. Cells were then manually collected using a cell scraper, and vesicles were generated in the same manner as described above. The vesicle pellet was resuspended in 2 ml of sucrose buffer (10 mM HEPES, 250 mM Sucrose pH 7.5). DiR ($DiIC_{18}(7)$; 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide) (ThermoFisher Scientific) was utilized as a lipophilic fluorescent dye, with 5 µl of 2 mM added to the vesicle solution and then incubated for 30 minutes at 37°C. The vesicle solution was then layered with a 50% and 10% OptiPrep™ density gradient medium. The combined solution was then ultracentrifuged at 112,000 x g for 60 minutes at 4°C. A peristaltic pump was then used to collect DiR labeled vesicles between the gradients. The collected vesicles were purified using size exclusion PD Miditrap columns (Cytiva) to remove any free dye.

Under the University of Kentucky Institutional Animal Care & Use Committee (IACUC) protocol #2017-2674, we did a transperitoneal injection of 5-week-old female BALB/c SCID mice (Jackson Lab) with 5 x 10^6 Caov-3 cells in 100 µl of sterile PBS. After visible tumor progression, 100-200ul of labeled RAW264.7 MEVs were injected *via* lateral tail veins of *via* intraperitoneal injection in the right lower quadrant. Athymic nude homozygous 5-week-old female (Jackson Lab) were subcutaneously injected with 2.5-5.0 x 10^6 SKOV3 cells in 100ul of sterile PBS in the dorsal shoulder region. Mice were imaged 72 hours post-injection using a LagoX Small Animal Optical Imager (Spectral Instruments) at a fluorescent excitation wavelength of 710 nm and emission of 770 nm for 10 seconds. Images were processed with Aura Imaging software (Spectral Instruments). After euthanasia, necropsy performed with tumor and organs of interest isolated and imaged independently.

3 Results

3.1 Characterization of human MEVs

MEVs are formed *via* mechanical disruption of macrophage cell membranes with nitrogen cavitation (35). The generated cellular fragments subsequently reform into vesicles in a pressurized chamber. To determine the ability of human MEVs to encapsulate cargo, human MEVs were generated in the presence of fluorescein, a fluorescent dye. MEVs were imaged using a fluorescence microscope using a 488 nm laser of 0.8 mW power with a gain of 990 and an exposure time of 200 ms. MEVs were visible as bright punctate regions (Figure 1A). This illustrates that human MEVs can encapsulate cargo during vesicle generation, similar to MEVs generated from RAW 264.7 cells (35). To characterize the vesicle size distribution within an individual preparation of MEVs, we quantified the vesicle diameter and concentration using multiple particle tracking using a Zeta View PMX-120 (Figure 1B) and Nanosight 300 (Figures 1C, D). We generated vesicles from 3.1 x 10^7 human M1 macrophages with a cavitation pressure of 250 psi, which yielded 6.6 x 10^{10} vesicles with a mean diameter of 125.1 nm (SD ± 60.2 nm). Additionally, we measured the Zeta potential at -127mV; a large negative value is an indicator of stability in an aqueous solution. Additional characterization performed with Nanosight 300 (Figures 1C–E) using 2.8 x 10^7 human M1 macrophages yielding 6.45x10^{11} with a mean diameter of 165.1nm (SD ± 66.4nm).

Scanning electron microscopy (SEM) was performed in order to determine the shape and morphology of the generated MEVs. MEVs were fixed and serially dehydrated prior to SEM. Examination confirmed the round smooth-edged morphology with diameter of a single MEV of~200nm (Figure 2A). The dense MEV spherical morphology suggests a tendency to encapsulate the cargo drug with firm stability. Utilizing transmission electron microscopy (TEM), M1 vesicles were then identified using CD86 monoclonal antibody. CD86 is a known glycoprotein found in the membrane of the antigen presenting cells, such as blood monocytes and macrophages. Figure 2B shows positive immunogold staining of M1 MEVs (positive for CD86) as seen as dark black silver particles within a SKOV3 cell. The SKOV3 cell membrane and nucleus containing chromatin were also visible.

3.2 M1 MEVs are taken up by M2 macrophages and cancer cells

Next, we examined if M1 MEVs can localize to M2 macrophages and ovarian carcinoma cells. We generated M1 MEVs labeled with DiI, a lipophilic fluorescent dye that is loaded in the membrane. MEVs were incubated with M2 macrophages, Caov-3 cells, and co-culture of M2 macrophages plus Caov-3 cells. Confocal imaging with a CellInsight CX7 High-Content Screening Platform demonstrated that both human M2 macrophages and Caov-3 cells uptake MEVs in co-culture (Figure 3A). Caov-3 cells and macrophages demonstrated different nuclear sizes when cocultured alone, with Caov-3 nuclei significantly larger (Figure 3B). While Caov-3 cells showed a low level of punctate MEVs co-localizing to the cells,

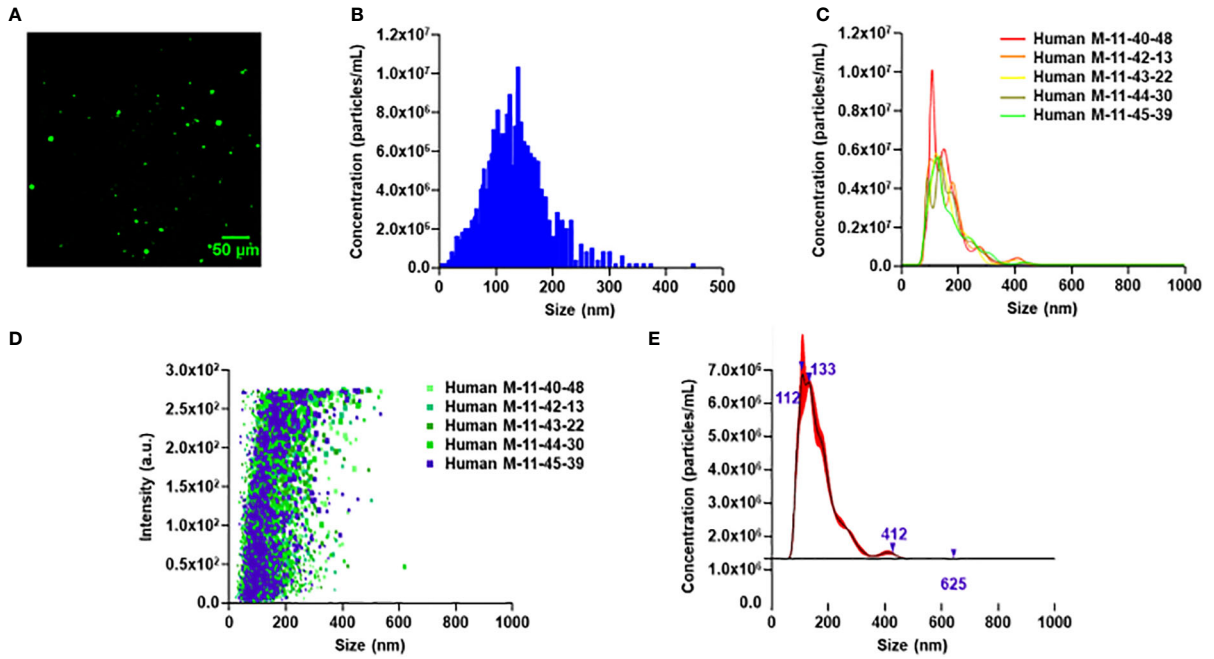

FIGURE 1
Characterization of human PBMC-derived M1 vesicles. **(A)** Wide field image of human M1 MEVs loaded with fluorescein. **(B)** Particle tracking analysis for human M1 MEVs. 2.4 x 10^7 human M1 macrophages generated 2 x 10^{10} vesicles. **(C)** Finite track length adjustment (FTLA)/size graph obtained *via* nanoparticle tracking analysis (NTA) with five separate experimental replicates. **(D)** Intensity/size graph obtained *via* NTA with five separate experimental replicates. **(E)** Composite FTLA concentrations/size obtained *via* NTA with a mean of 165.1nm (SD: 66.4nm).

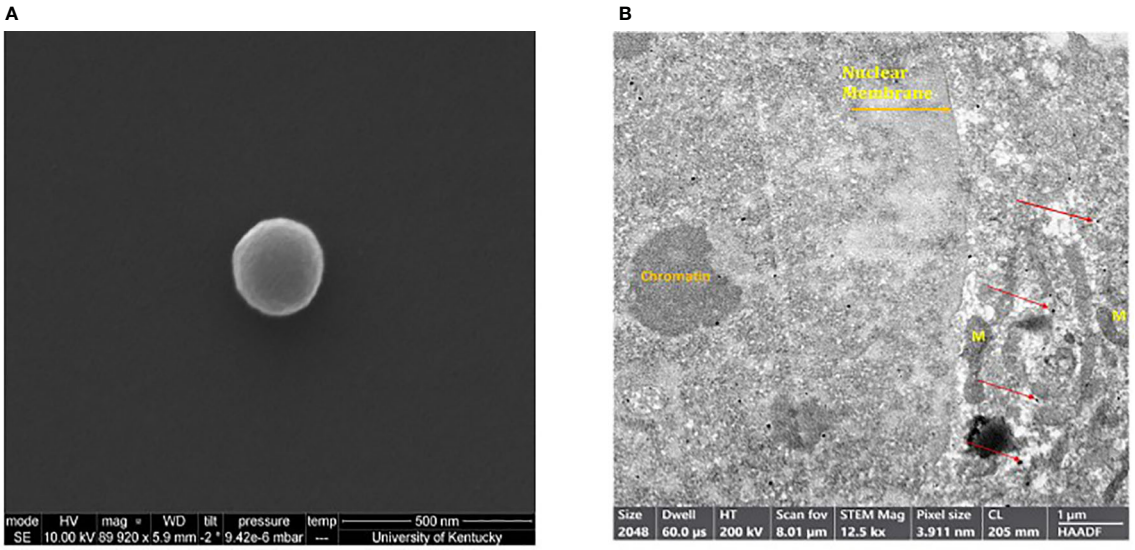

FIGURE 2
(A) Scanning electron microscope image of a single human M1 MEV. Sample was imaged using a field-emission scanning electron microscope. **(B)** Transmission electron microscopy of SKOV3 ovarian cell with intracellular MEVs as identified with positive silver staining for CD86. MEVs (black dots) are identified within the cell (red arrows). The nuclear membrane, chromatin, and Mitochondria (M) are also visible.

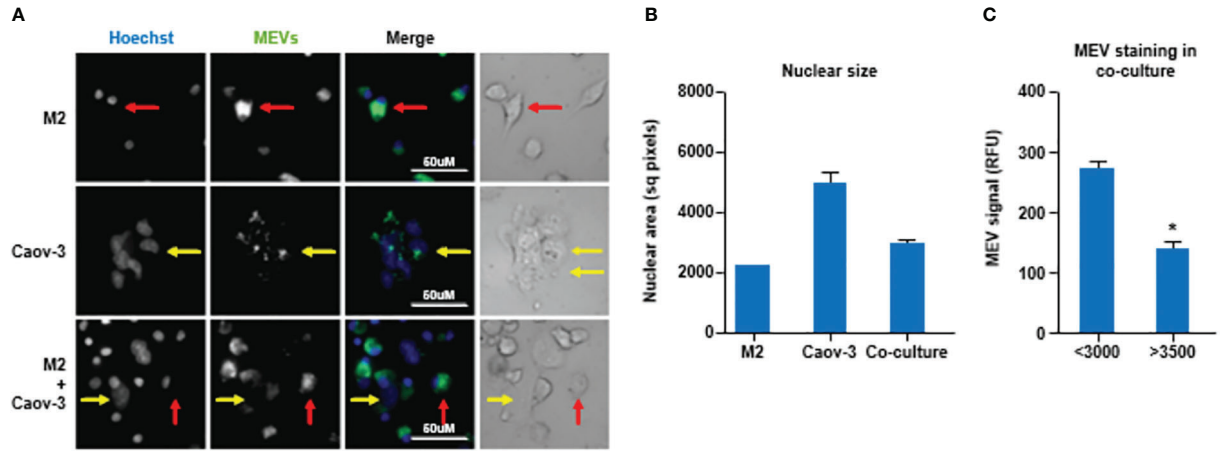

FIGURE 3
(A) Human macrophages display a higher uptake of human M1 MEVs compared to ovarian cancer cells. Confocal imaging of human M2 macrophages alone, Caov-3 cells alone, and co-cultured human M2 macrophages plus Caov-3 cells following a 24-hour incubation with M1 MEVs. Brightfield of co-cultured human M2 macrophages plus Caov-3 cells. Nuclei were labeled with Hoescht (1:2000, blue) and M1 MEVs were DiI-labeled (green). Representative Caov-3 cells (yellow arrows) and human macrophages (red arrows) are indicated. Scale bars indicate 50 µm. Imaging was performed at 40X magnification using a CellInsight CX7 High-Content Screening Platform. **(B)** Graph of the nuclear size mean +/- SEM showing significantly different nuclear area of the M2 cells compared to the Caov3 cells, with coculture mean between the two cell types. Greater than 100 cells were analyzed per cell type. ($P<0.001$ all comparisons by One-way ANOVA with Newman Keuls Multiple Comparison Test.) **(C)** MEV staining in cells with nuclei <3000 sq. pixels (M2) and >3500 sq. pixels (Caov3) from the cocultured wells only, demonstrated significantly less MEV staining in the large nuclei (Caov3) cells in the co-cultured well then the small nuclei (M2) cells as determined by unpaired two-tailed t-test ($p<0.0001$). * indicates a $p<0.05$.

most macrophages, indicated by smaller nuclei, display a distinctly higher number of MEVs (Figure 3C). These results show that human MEVs are capable of localizing to both human macrophages and human ovarian cancer cells *in vitro*.

3.3 M1 MEVs repolarize M2 macrophages

Next, we tested if human M1 MEVs can repolarize M2 macrophages to an M1-like, pro-inflammatory phenotype. We compared the production of the pro-inflammatory cytokine TNF-α in M1 macrophages, M2 macrophages, and M2 macrophages incubated with M1 MEVs. We observed high levels of TNF-α, measured *via* ELISA, in the M1 macrophages and significantly lower TNF-α in the M2 culture and in controls (Mean ± SD pg/ml: M1 vs. M2: 2021 ± 383.8 vs. 259.9 ± 133.7, $p<0.001$, M1 MEVs+M2 vs. M2: 787.5 ± 298.3 vs. 259.9 ± 133.7 $p<0.05$) (Figure 4A). In contrast, we observed an increase in TNF-α in M2 macrophages that were incubated with M1 MEVs, indicating that M1 MEVs can repolarize M2 macrophages towards a pro-inflammatory, M1-like macrophage phenotype. Figure 4B demonstrates the difference in TNF- α levels of M1+M1 MEVs vs M1 cells alone is not significant. However, M2+ M1 MEVs vs M2 cells is statistically significant. From this data we've concluded that the MEVs alone are not the sole driver of the experimental increased TNF-α levels, but rather the interaction with the M2 cells *via* repolarization. The comparatively lower TNF-α levels seen in the M1 between Figures 4A, B is likely secondary to the difference in analyzed time points (24 vs 48 hrs) and experimental methodology. We subsequently sought to validate M1 MEV repolarization of M2 macrophages *via* real-time PCR of mRNA expression of CXCL8 and CXCL10 proteins. Figure 4C shows significant differences in the relative expression of CXCL8 in M2 cells alone compared to M2 cells treated with M1 MEVs ($p<0.0001$). This finding was not demonstrated in relative mRNA expression of CXCL10 (Figure 4D). CXCL8 expression is marker for M1 macrophages (41–43). Therefore, based on CXCL8 mRNA expression, M1 MEVs can repolarize M2 to an M1 state. Taken together, M1 MEVs can repolarize M2 macrophages into an M1-like phenotype based on both cytokine secretion and mRNA expression profiles.

3.4 Human M1 MEVs repolarize M2 macrophages in co-culture

To test if M1 MEVs can convert M2 TAMs to a pro-inflammatory phenotype, we cultured human M2 macrophages with the Caov-3 or OVCAR3 ovarian cancer cell lines and treated the co-cultured cells with M1 MEVs. Co-cultured cells treated with M1 MEVs show an increase in the pro-inflammatory cytokine, TNF-α (Mean ± SD pg/ml: M2+Caov-3+M1 MEVs vs M2+Caov-3; 383.6 ± 120.4 vs. 0.1389 ± 20.03, $p<0.05$, M2+OVCAR3+M1 MEVs vs M2+OVCAR3: 207.1 ± 170.2 vs -45.65 ± 55.35 $p=0.18$)

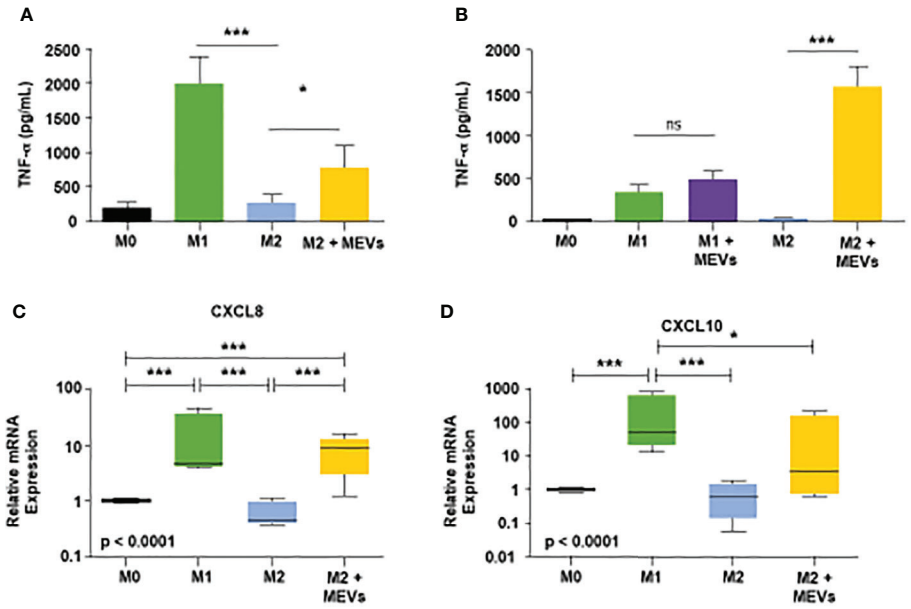

FIGURE 4
(A) Human M1 vesicles repolarize M2 macrophages. Human PBMC-derived monocytes were cultured with M-CSF (50ng/mL) for six days. Cells were stimulated, and supernatants were assayed for human TNF-α after 24 hours. From left to right on the graph: M0 macrophages (black), M1 cells polarized with LPS plus IFN-γ (20ng/mL each, green, M2 polarized with IL-4 plus IL-13 (20ng/mL each, blue, and M2 cells treated with M1 vesicles (yellow). Statistical analysis performed with Statistical analysis performed with one-way ANOVA with *post-hoc* Tukey's Multiple Comparison Test (*p < 0.05; ***p < 0.001). Error bars indicate SD. **(B)** Human PBMC-derived monocytes were cultured, plated, and stimulated to respective states as described above. After 24 hours, supernatant was removed and M1 vesicles were added to M1 and M2 cells. After an additional 24 hours supernatants were collected and subsequently assayed for human TNF-α. Statistical analysis performed with one-way ANOVA with *post-hoc* Tukey's Multiple Comparison Test (*p < 0.05; ***p < 0.001). Error bars indicate SD. **(C, D)** CXCL8 and CXCL10 mRNA expression as biomarkers of human macrophage polarization. Total RNA was purified from human M0, M1, M2 macrophages, and M2 macrophages treated with M1 MEVs (M2 + MEVs) and analyzed by real-time PCR. Relative expression (versus M0 macrophages) of CXCL8 and CXCL10 was measured in 4 independent experiments and summarized in box and whisker plots (median, interquartile range, 5th-95th percentile). Statistical analyses were performed with Kruskal-Wallis tests followed by Dunn's Multiple Comparison tests (*p < 0.05; ***p < 0.001). ns, not significant.

(Figures 5A, D), suggesting that M1 MEVs convert M2 TAMs to an M1 phenotype. The comparatively lower TNF-α levels seen in the M1 plus cancer cells (Figures 5A, D) compared to the high levels of TNF-α in the M1 macrophages alone (Figure 4) is likely secondary to the difference in time points (24 vs 48 hrs) and experimental methodology.

We then tested if M1 MEVs are capable of inhibiting cell viability. M1 MEVs at high concentrations has an inhibitory effect in both Caov-3 (Mean ± SD 100.0 ± 8.232 vs 82.27± 2.853, p<0.0001) and OVCAR3 cell lines (Mean ± SD 100.0 ± 5.710 vs 87.69± 11.62, p<0.05) (Figures 5B, E), with continued significant decreases appreciated at a lower dose (10%) in Caov-3 (Mean ± SD: 100.0 ± 8.232 vs 87.95± 6.069, p<0.0001). Interestingly, in Caov-3 this inhibition appears to be dose-dependent and is significantly higher in the co-cultured cells as compared to cancer cells alone (Mean ± SD 100.0 ± 2.930 vs. 70.54 ± 9.955, p<0.0001) (Figure 5C), indicating that MEVs are more effective in the presence of pro-inflammatory macrophages. The inhibition seen in OVCAR3 cells co-cultured with M2 macrophages is more modest but still significant at a high MEV dose (Mean ± SD 100.0± 6.821 vs 93.61 ± 5.558, p < 0.01) (Figure 5F).

3.5 RAW264.7-derived M1 MEVs localize to ovarian xenografts *in vivo*

As part of a pilot experiment, we sought to demonstrate the localization of M1 MEVs to human tumor xenografts. A BALB/c SCID mouse was injected transperitoneally with Caov-3 ovarian cancer cells and developed a visible tumor xenograft in the abdominal right lower quadrant approximately seven months post-injection. Fluorescent DiR-labeled M1 MEVs were created from RAW264.7 cells and were injected *via* lateral tail vein. Importantly, RAW164.7 are a mouse macrophages cell line. The mouse was imaged 72hrs post-injection (Figure 6) using appropriate corresponding emission and excitation wavelengths for DiR. An additional mouse (left) without a tumor xenograft was not injected was imaged for baseline null comparison purposes. The dye-labeled MEVs demonstrate

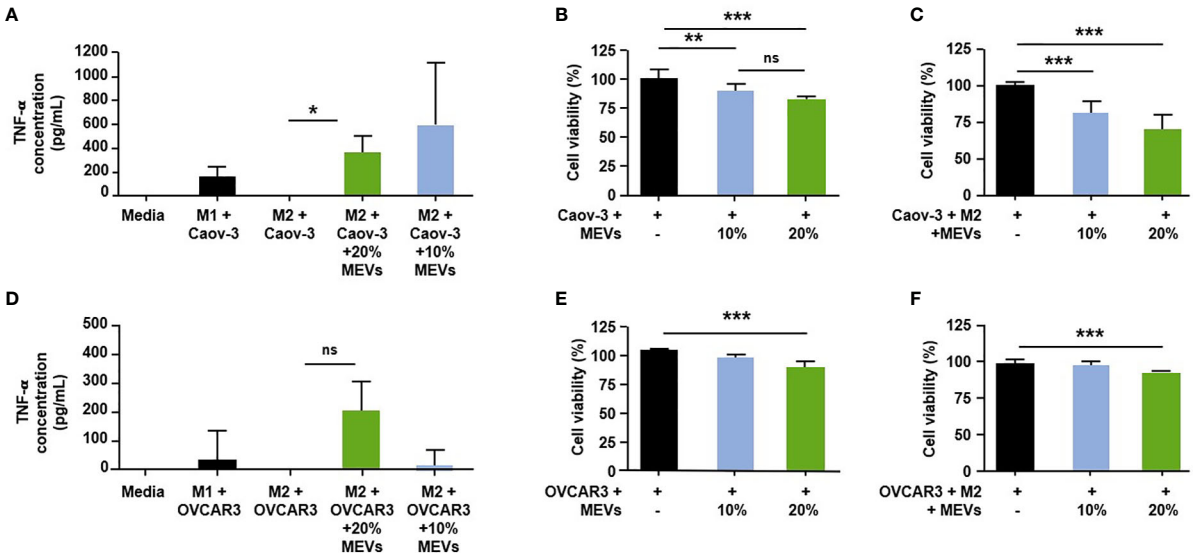

FIGURE 5
Human M1 MEVs shift co-cultured M2 macrophages to M1 phenotype. **(A, D)**. Supernatants were collected 24 hours after the addition of M1 MEVs (48 hours after macrophage plating). Supernatants were assayed in duplicate using a human TNF-α Quantikine ELISA kit (R&D Systems, Inc.). Significance was assessed with a two-tailed paired t-test **(A, D)** ; p = 0.0259 & p=0.18, respectively). Human M1 MEVs show dose-dependent inhibition of cell viability in co-cultured cells. Graphs indicate the percent cell viability of the **(B)** Caov-3 cancer cells alone and **(E)** OVCAR3 cancer cells alone treated with M1 MEVs or **(C)** Caov-3 and **(F)** OVCAR3 co-cultured cancer cells plus M2 macrophages treated with M1 MEVs. Cell viability was measured at 96 hours (CellTiter-Glo 2.0). % of MEVs refers to the relative percentage of supernatant with MEVs added. The percent cell viability was calculated by comparing cells treated with M1 MEVs to the respective untreated control. Statistical analyses were performed using Kruskal-Wallis with Dunn's Multiple Comparison posthoc test (*p < 0.05; **p < 0.01; ***p < 0.001). Experiments were performed in triplicate. Bars correspond to SEM. * indicates a p<0.05.

precise localization to the tumor (Figures 6B, C). Additional pilot experiments were performed with athymic nude mice injected subcutaneously with SKOV3 ovarian cancer cells xenografts in the mouse scapular region. Fluorescent DiR-labeled M1 MEVs were created from RAW264.7 cells and were injected *via* lateral tail vein (Figure 7) or intraperitoneally (Figure 8). Post-necropsy images demonstrate localization of M1 MEVs to tumor (Figure 7E). Intermittent fluorescent signalling demonstrated in the murine cranium at 24 hours is noted, but desists at 72hours. This is suggestive of a transient circulatory

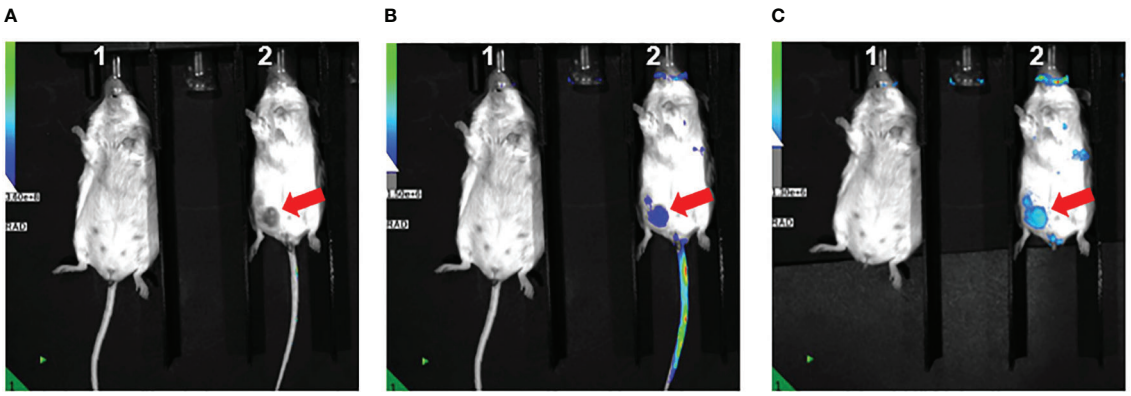

FIGURE 6
RAW.264.7 M1 polarized vesicles localize to Caov-3 tumor xenografts *in vivo*. **(A)** Two BALB/c-SCID mice displayed – one without tumor (left) and one with visible tumor (right) marked by the arrow. The mouse on the right was injected with 100 µl of fluorescent dye-labeled vesicles and imaged 72 hours post-injection. The fluorescent overlay was reduced to display visible tumor for comparison **(B)** Same mice shown in A with clear fluorescent uptake seen in the vicinity of the tumor in the right lower quadrant displayed in Image **(A, C)** Tail veins covered to reduce emission background, displaying accentuated M1 MEV localization to the tumor.

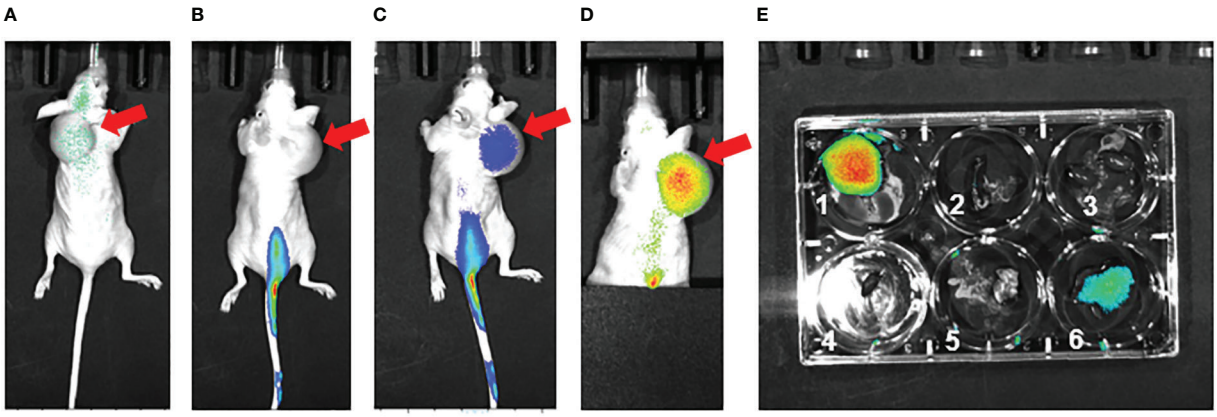

FIGURE 7
RAW.264.7 M1 polarized vesicles localize to SKOV3 tumor xenografts *in vivo via* an intravenous route. **(A)** Preinjection (0 hr – immediately prior to injection) of single athymic nude mouse with SKOV3 tumor xenograft in dorsal shoulder region (red arrow). The mouse was injected with 100 µl of fluorescent dye-labeled vesicles and imaged **(B)** 24 hours post-injection and **(C)** 72 hours post-injection. There is clear fluorescent uptake seen in the tumor. **(D)** 72 hours post-injection Tail vein covered to reduce emission background, displaying accentuated vesicle localization to the tumor 72 hours post injection. **(E)** Post-necropsy with 1=tumor, 2=spleen, 3=kidneys, 4=heart, 5=lungs, 6=liver; there is accentuated localization to the tumor. The size of the subcutaneous lesion resulted sagittal instability and displacement to the right over the time series.

phenomenon or may reflect additional M2 macrophage target populations.

4 Discussion

While there have been several recent advances in immunotherapy for other gynecological malignancies [cervical (25) and uterine (26)], success in ovarian cancer has been limited (27). This lack of activity in ovarian cancer is thought to be related to infiltration of TAMs, which render cancer "cold" and thus immunotherapy ineffective (34, 44). Therefore, strategies to repolarize M2 macrophages to the M1 phenotype may promote anti-cancer activity. Our study, the first to use MEVs derived from human blood monocytes, effectively demonstrates that M1 MEVs can localize primarily to M2 macrophages when co-cultured with ovarian cancer cells and treatment with M1 MEVs repolarizes M2 macrophages to an anti-tumor M1 state with subsequent anti-cancer activity. This effect was demonstrated both in cancer cells alone and with macrophages co-cultured in the presence of cancer cells. Since ovarian cancer cells themselves are significant drivers for macrophage polarization to an M2 state (45), repolarization within co-culture is particularly salient as it suggests the capacity of MEVs to overcome an innate preferential differentiation towards the protumor M2 state.

Macrophages are the most abundant immune system cells within the tumor microenvironment and compose up to 50% of a tumor's volume (46–48). A major benefit of exosome formulations from macrophages is the inherent targeting properties exhibited by their origin cell (18). Exosomes derived from human cells are non-immunogenic compared to liposomal formulation (18). Therefore, the use of exosome-like MEVs derived from human blood cells has the potential to avoid off-target immunogenic effects while honing in on macrophage-laden tissue (e.g., tumors). Additionally, engineered macrophage vesicles carry a higher yield potential than other endogenous sources while avoiding a cancer-derived source that could impact tumorigenesis (27, 49, 50).

One of the main strengths of this study is the exclusive use of non-carcinoma human-derived cells. This eliminates any future translational risk of reintroducing tumor-derived cells into the patient. Another major strength is the immunological and therapeutic potential of M1 MEVs that is demonstrated using several ovarian cancer cell lines. Caov-3 and OVCAR3 are both BRCA wild-type, however, Caov-3 is platinum-sensitive whereas OVCAR3 are platinum resistant. In murine models, SKOV3 is an aggressive platinum resistant cell line that displays rapid xenograft growth. Additionally, pilot animal data demonstrate precise localization of dye-labeled mouse M1 MEVs to ovarian cancer tumor xenografts in mice. This is an intriguing finding and provides further evidence for the tumor precision of MEVs. Localization was seen in both intravenous and intraperitoneal administration routes. This is of compelling interest as ovarian cancer is a peritoneal disease and intraperitoneal chemotherapy has a long-studied role in the treatment of the disease (51, 52). Limitations include a lack of *in vivo* modeling to demonstrate sustained macrophage repolarization. In terms of generalization of *in vivo* models, SCID and nude mice are particularly immunosuppressed, future modeling using syngeic murine models may more accurately reflect physiologic conditions and reveal the interplay of circulating MEVs with the immune system targets. Additionally, there was high variability and size heterogeneity seen with the vesicle preparation that may be

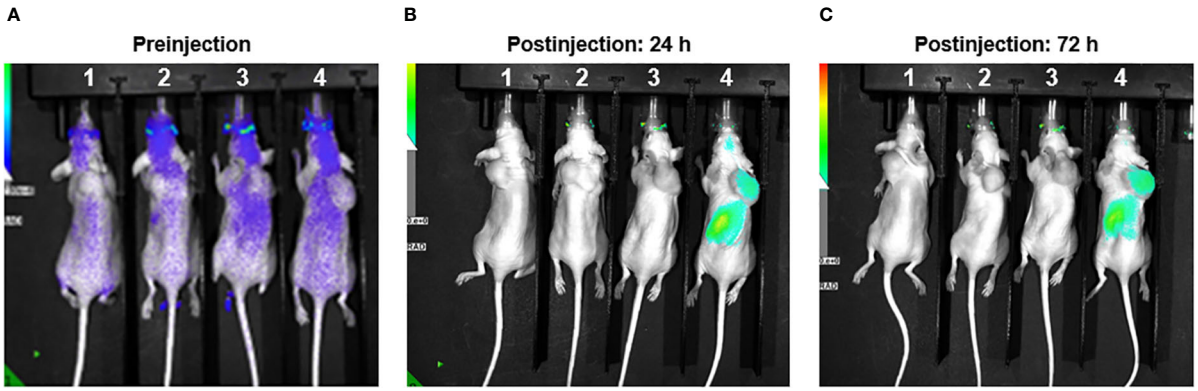

FIGURE 8
RAW.264.7 M1 polarized vesicles localize to SKOV3 tumor xenografts *in vivo via* an intraperitoneal route. **(A)** Four athymic nude mice displayed – each with SKOV3 tumor xenograft in the dorsal shoulder region. Image taken preinjection for comparison purposes. **(B)** Mice 1 & 2 injected with 200ul of sterile PBS. The two mice on the right were injected with 50ul (Mouse 3) and 100ul (Mouse 4) of DiR labeled vesicles and imaged 24 hours post-injection. There is clear localization of fluorescent uptake in the vicinity of the tumor in the far-right mouse. The fluorescent overlay was reduced to display visible tumor for comparison. **(C)** Same mice shown with clear and persistent fluorescent uptake seen in far-right mouse's tumor.

ameliorated in future studies with further filtration methods. Additional characterization methods of the vesicles *via* transmission electron microscopy is warranted. While promising as a therapeutic avenue, significant obstacles remain prior to transition from a preclinical to clinical approach, including standardization of MEV characterization, dosing, precision of imaging localization, and delineation of off-target effects. Future research will be needed to evaluate the role of drug-loaded MEVs as another therapeutic approach and evaluate *in vivo* efficacy in terms of distribution, toxicity, and tumor response.

5 Conclusions

The studies described are the first to demonstrate that human-derived M1 MEVs can serve as immunomodulatory agents by repolarizing M2 macrophages to an M1-like state. This effect was seen in M2 macrophages when cultured alone and in co-culture with ovarian cancer cells. Overall, human-derived M1 MEVs effectively repolarize M2 macrophages. Initial pilot data demonstrates that M1 MEVs target ovarian tumor xenografts. Future *in vivo* studies are warranted.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by University of Kentucky Institutional Animal Care & Use Committee (IACUC).

Author contributions

Conceptualization, AA, DS, CR, and JK; methodology, DS, AA, AN, KN, and NA; validation, DS, AA, NA, AN, KN, BH, and JRM; formal analysis, DS, AA, AN, NA, JRM, KN, KH, and BH; data curation, AA and DS; writing—original draft preparation, AA and DS; writing—review and editing, DS, AA, JRM, KH, CR, FU, BH, KN, AN, and JK; visualization, AA, KH, DS, and KN; supervision, JK; funding acquisition, DS and JK. All authors have read and agreed to the published version of the 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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Pleiotropic effects of the COX-2/PGE2 axis in the glioblastoma tumor microenvironment

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Glioblastoma (GBM) is the most common and aggressive form of malignant glioma. The GBM tumor microenvironment (TME) is a complex ecosystem of heterogeneous cells and signaling factors. Glioma associated macrophages and microglia (GAMs) constitute a significant portion of the TME, suggesting that their functional attributes play a crucial role in cancer homeostasis. In GBM, an elevated GAM population is associated with poor prognosis and therapeutic resistance. Neoplastic cells recruit these myeloid populations through release of chemoattractant factors and dysregulate their induction of inflammatory programs. GAMs become protumoral advocates through production a variety of cytokines, inflammatory mediators, and growth factors that can drive cancer proliferation, invasion, immune evasion, and angiogenesis. Among these inflammatory factors, cyclooxygenase-2 (COX-2) and its downstream product, prostaglandin E2 (PGE2), are highly enriched in GBM and their overexpression is positively correlated with poor prognosis in patients. Both tumor cells and GAMs have the ability to signal through the COX-2 PGE2 axis and respond in an autocrine/paracrine manner. In the GBM TME, enhanced signaling through the COX-2/PGE2 axis leads to pleotropic effects that impact GAM dynamics and drive tumor progression.

KEYWORDS

glioblastoma, COX-2, PGE2, microglia, macrophage, inflammation, tumor microenvironment, cancer

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive form of central nervous system (CNS) tumor. GBM accounts for 48.3% of all malignant brain tumors. GBM patients have a median survival rate of only 14-17 months with standard treatment including surgical resection, chemotherapy, and radiotherapy, and a median survival of less than 6 months without therapeutic intervention (1–3). Poor prognosis in GBM

patients is linked to high intra-and inter-tumor heterogeneity, chemoresistance, and an immunosuppressive environment (4). The GBM tumor microenvironment (TME) plays a crucial role in development and progression of the disease. The TME is a dynamic cellular and molecular ecosystem of tumor cells, glioblastoma stem cells (GSCs), stromal cells (fibroblasts, endothelial), and immune cells (microglia, macrophages, T-cells, B-Cells) actively responding to their surrounding cells, tissues, and molecular cues (5, 6). This highly complex network communicates through production of cytokines, chemokines, bioactive lipids, and extracellular matrix components. Together they dictate diverse pro-inflammatory and anti-inflammatory responses that shape their environment through communication and interaction (7).

The most abundant and multifaceted members of the GBM-TME are the glioma associated microglia and peripheral macrophages (GAMs). They constitute up to 30-50% of tumor associated cells, and thus have a strong influence on the GBM-TME (8, 9). The degree of GAM integration in the GBM-TME is positively correlated with tumor grade and inversely correlated with patient survival (7, 10). The presence of these inflammatory cells in the TME leads to dysregulated inflammation and plays a key role in the immunosuppressive nature of GBM, consistent with the well-established association between cancer and inflammation (11). In contrast to their phagocytic and cytotoxic capabilities against infection, GAMs produce inflammatory mediators that promote tumor growth, immunosuppression, and angiogenesis. GAMs produce an array of cytokines, growth factors, and bioactive lipids that aid in a pro-tumoral shift, such as Interleukin (IL)-1β, IL-6, transforming growth factor-β (TGF-β), epidermal growth factor (EGF), and the prostaglandin E2 (PGE2) (12, 13). PGE2 is highly enriched in the GBM-TME and has a substantial impact on proliferation, migration, immunosuppression, and angiogenesis. Similarly, cyclooxygenase 2 (COX-2), the enzyme responsible for PGE2 production, is also highly upregulated in GBM and is associated with tumor growth (Shono et al., no date; 14–16). Here, we review the current understanding of the COX-2/PGE2 signaling axis in GAMs, its regulation of the tumor microenvironment, and its impact on GBM tumor progression.

Inflammation is well established as a robust driver of cancer and is now considered to be one of the hallmarks of cancer (11). In natural inflammatory responses, infections and cell damage are cleared by immune cells that launch an acute proinflammatory response to neutralize the threat. Once the threat has been neutralized, immune cells launch an anti-inflammatory response to resolve inflammation. In aberrant situations, such as chronic inflammation and cancer, the threat may not be neutralized, causing dysregulation of the inflammatory program. The relationship between glioma and inflammation is characterized by multiple key steps: first, the recruitment and infiltration of immune cells to the site of the tumor; second, complex signaling crosstalk between the tumor cells and multiple types of immune cells mediated through small molecule release and activation of receptors on neighboring cells; third, tumor cell responses including proliferation, transcriptional regulation, migration, and differentiation; and finally, tumor progression driven by immune evasion, neovascularization, and tissue remodeling. Activation of GAMs induces the release of cytokines, growth factors, and other inflammatory mediators that promote tumor growth, angiogenesis, and an immunosuppressed state.

Microglia and macrophages in the CNS

Microglia and brain infiltrating macrophages serve an essential role as immune sentinels, responding to infection and injury in the central nervous system (CNS) to maintain brain homeostasis (5, 17). Bone marrow derived macrophages (BMDMs) originate in the bone marrow as peripheral hematopoietic progenitors, and they become circulating monocytes in the blood stream. BMDMs are highly motile as they locate to target tissue, but motility lowers as they approach a tumor and eventually take residence in the tumor tissue. Microglia, found throughout the brain, represent a distinct myeloid population, and are considered the resident macrophages of the CNS. Microglia are primarily derived from erythro-myeloid progenitor cells in the yolk sac during early embryogenesis and are long lived, relying on self-renewal in the CNS (18, 19). They maintain homeostatic conditions by supporting neurogenesis, synaptic pruning, and phagocytotic clearing of apoptotic cells and debris (20, 21). Microglia exhibit diverse morphologies and phenotypes in response to various stimuli. Surveilling microglia are highly ramified to efficiently respond to environmental stimuli (22). Once activated, they rapidly change to an amoeboid morphology (23, 24). Microglia activation leads to production of IL-1β, which plays an important role in modulating the blood brain barrier (BBB) and promotes a leaky state that allows entrance of bone marrow derived immune cells to enter the brain (25). BMDMs have remarkably similar morphology to the ameboid shaped microglia making it challenging to distinguish between the two histologically (19). Thus, both BMDM-derived brain infiltrating macrophages and resident microglia are present in the brain and in the GBM-TME, and these cells can be functionally and phenotypically difficult to distinguish. Compared to BMDMs, microglia have limited migratory capacity and instead use their processes to extend and retract, constantly surveilling their surroundings (26). These migratory differences lead to the differential distributions between macrophages and microglia in the GBM-TME. Single-cell RNAseq analysis of GBM revealed that highly motile infiltrating macrophages were primarily located in the central regions of the tumor while microglia tend to surround the outer edge of the tumor (27). Additionally, GBM tumors typically display necrotic cores and

microvascular hyperplasia due to the hypoxic environment. GAMs accumulate in these hypoxic/necrotic areas of tumors where they support tumor proliferation and angiogenesis (28, 29).

GAMS in the GBM microenvironment

In glioma, macrophages and microglia are recruited to the tumor site by glioma-derived chemoattractant factors such as colony stimulating factor 1 (CSF1), C-C motif chemokine ligand 2 (CCL2; also known as monocyte chemoattractant protein 1, MCP-1), fractalkine (CX3CL1), and vascular endothelial growth factor (VEGF) (30–33). Following recruitment, GAMs secrete inflammatory mediators that regulate angiogenesis, proliferation, and immunosuppression in the GBM-TME. PGE2 is emerging as a key mediator of these effects, and both PGE2 and its upstream biosynthetic enzyme COX-2 are overexpressed in the GBM-TME, are associated with poor prognosis, and mediate pleiotropic effects that support glioma proliferation, angiogenesis, and immunosuppression (16, 34).

GBM tumors are highly vascular and rely on neovascularization for tumor growth. Microglia and macrophages play a supporting role in this process through the production of angiogenic factors and degradation of the extracellular matrix (ECM) (35). Depletion of microglia and macrophages in an animal model of GBM resulted in reduced micro-vessel density (MVD), proliferation, and overall tumor volume (36). Additionally, selective depletion of only microglia led to a comparable attenuation of MVD to that of total GAM depletion, suggesting that microglia are particularly important immune facilitators of angiogenesis in glioma (36). GAMs release multiple angiogenic factors that promote angiogenesis and invasiveness, including transforming growth factor β (TGF-β), IL-6, and vascular endothelial growth factor (VEGF) (37). VEGF expression is upregulated in hypoxic regions where it acts as a robust chemoattractant to recruit GAMs, which in turn promote angiogenesis (29). In the presence of glioma cells, microglia produce significant amounts of TGF-β, which in turn induces production of matrix metalloproteinase 9 (MMP9) and MMP2, leading to degradation of ECM and supporting glioma stem cell invasion (38). GSCs are treatment resistant, multipotent, self-renewing cells with high heterogeneity (39, 40). GAMs and GSCs are often functionally interconnected and co-localized. Mapping of cellular distribution in human GBM revealed that striking numbers of GAMs were located around GSC clusters and, as observed with GAMS, the density of GSCs positively correlated to tumor grade (40). GAMs accumulate in perivascular regions where they produce proangiogenic factors such as VEGF and CXCL2, due to chemoattractant release from GSCs (36). Taken together, these observations suggest a complex signaling interplay between tumor cells, stem cells, and GAMs to regulate angiogenesis and invasion. Growing evidence suggests that COX-2 and PGE2 are key mediators of the effect of GAMs on angiogenesis. COX-2 and PGE2 are produced by microglia and macrophages, and PGE2 accumulation is particularly high in hypoxic/necrotic regions of the TME (16, 41). PGE2 in the TME is linked to increased expression of glioma-derived monocyte chemoattractant CCL2/MCP-1, leading to active recruitment of GAMs (31, 42). In response, GAMs induce IL-6 production, which increases GBM invasiveness (31). COX-2 and PGE2 regulate expression of VEGF and trigger increased MVD, suggesting that this pathway is critical to the signaling networks that regulate angiogenesis in the GBM-TME (15).

Growing evidence suggests that GAMs also play a key role in establishing the immunosuppressant microenvironment that is characteristic of GBM. Specifically, GAMs regulate the ability of GBM tumor cells to evade clearance by the immune system by down regulation of antigen presentation and subsequent T-cell activation (43). Importantly, elevated levels of PGE2 in the GBM-TME were demonstrated to downregulate major histocompatibility complex class II (MHC class II), responsible for antigen presentation, in microglia (44). In patients, expression of MHC class II is downregulated in GAMs isolated from patients with GBM, leading to ineffective T-cell activation and immunosuppression (45). Induction of COX-2/PGE2 leads to robust production of immunosuppressive mediators such as IL-6, IL-10, and GM-CSF that lead to induction of regulatory T cells, further exacerbating immunosuppression (44, 46). Microglial mTOR/STAT3 signaling is also upregulated in GBM, triggering immunosuppression through induced expression of IL-6 and IL-10 and inactivation of microglial mTOR (43).

Advances in the genomic landscape of the GBM TME has demonstrated the significant roles that GAMs play in tumor progression, but there is still much to be elucidated concerning GAM heterogeneity, plasticity, and classification. It has become apparent that these myeloid populations are highly dynamic, represent spatial diversity, and need to be evaluated multidimensionally. This complexity is in poorly represented by simplified M1/M2 framework that is commonly used to describe macrophage phenotypes. Classically, macrophages and microglia have been categorized through the dualistic lens of M1 (pro-inflammatory) and M2 (anti-inflammatory) activation states. In context of GBM, M1 represents an anti-tumor phenotype, while M2 is described as pro-tumor (47). Microglia being the resident brain macrophages, adopted this nomenclature as well without regard to the distinct differences between them. As research in the field advanced, it became clear that a significant amount of *in vitro* data that supported the M1/M2 framework could not be recapitulated *in vivo* (48–50). Additionally, single cell analysis revealed distinct phenotypic and spatial differences between GAMs in human GBM samples and that both M1 and M2 markers were expressed concurrently in microglia (51). The dichotomous M1/M2 system fails to reflect heterogeneity, spatial landscape, ontogeny, or disease states (52, 53). A recent review has elegantly demonstrated this new concept by presenting GAMs in spatial association to

primary brain tumor type, identified potential markers that differentiate macrophages from microglia, and outlined factors that may support microglia heterogeneity in the TME (22).

COX-2: Activity and expression

COX-1/2, also known as Prostaglandin G/H synthase 1/2 (PTGS1/2) respectively, are key rate limiting enzymes that covert arachidonic acid (AA) into prostaglandin G2 (PGG2) and PGH2 which can then be metabolized by prostaglandin E synthase (PGES) downstream to form 5 bioactive lipids known as prostanoids (16, 54). These five prostanoids are PGE2, PGI2, PGD2, PGF2a, and thromboxane A2 (TXA2). Induction of COX activity and its downstream products are linked to classic inflammatory states such as fever, acute pain, local tissue injury, and arthritis, and as such it is targeted by classic non-steroidal anti-inflammatory drugs in treating these conditions (55). While COX-1 is expressed constitutively throughout most tissues and acts a homeostatic inflammatory mediator for requisite physiological tasks, COX-2 has very low constitutive expression in most tissues, but its expression is rapidly inducible in response to pathological insults and inflammatory stimuli such as cytokines, growth factors, and various tumor promoters (16, 56). COX-2 gene expression is regulated by regulatory cis-elements in its promoter. The two most well characterized critical elements for regulation are the cAMP response element (CRE), which is recognized and activated by dimeric transcription factor activator protein 1 (AP1) and CRE binding protein (CREB), and two nuclear factor kappa B (NF-κB) consensus binding sites, which bind p65 NFκB. Additional sites include a CCAAT/enhancer, which is activated by and the CCAAT/enhancer binding protein (C/EBP). Together, these transcription factors recruit transcriptional co-activator p300 to the AP1/CREB/NFκB/C/EBP regulatory complex, and this complex is essential for proper COX-2 transcription initiation (57). Therefore, COX-2 expression is induced by multiple interacting transcription factors and their associated binding partners (57).

Diverse extracellular stimuli induce the expression of COX-2 through activation of cell surface receptors that initiate signaling cascades which culminate in the regulation of these transcription factors. Classically, lipopolysaccharide (LPS) stimulates toll-like receptor 4 (TLR4) to engage the adapter molecule myeloid differentiation factor 88 (MyD88), which then signals through Mitogen Activate Protein (MAP) kinase cascades to induce AP1 activation and association with the COX-2 promoter. The IL-1 receptor induces COX-2 expression through similar MyD88-dependent MAP kinase activation upon activation by its ligand, IL-1β (58). C/EBP is also activated downstream of MAP kinase activation. LPS/TLR4 activation also triggers MyD88-dependent activation of tumor progression locus 2 (Tpl2), which leads to nuclear translocation and activation of both NFκB and CREB. In addition to receptor-stimulated regulation, COX-2 expression can be upregulated by hypoxia, which triggers NF-κB interaction with the NF-κB regulatory element and recruitment of Hypoxia Inducible Factor 1α (HIF-1α) to the COX-2 promoter (59, 60). Finally, Nitric oxide (NO), a small molecule converted from L-arginine by inducible nitric oxide synthase (iNOS), can enhance COX-2 expression through activation of CREB (61). Therefore, COX-2 transcriptional regulation reflects convergent, integrated regulation by multiple stimuli.

PGE2/EP2 signaling

The diversity of effects of COX-2/PGE2 on angiogenesis, tumor-promoting inflammation, invasion, and immunosuppression in GBM reflects the diversity of signaling pathways regulated by these mediators (Figure 1). PGE2 binds and signals through the EP family of receptors (EP1-4). Due to the functional variability of these receptors, PGE2 initiates pleiotropic downstream effects. EP receptors are all G-protein coupled receptors (GPCRs) with distinct downstream effects depending on their G-protein coupling. Activation of Gq-coupled EP-1 leads to activation of phospholipase C (PLC), which increases intracellular Ca^{2+} and activates protein kinase C (PKC). The EP-3 receptor is primarily Gi-coupled, resulting in inhibition of the adenylate cyclase/cAMP signaling and activation of Gβγ dependent signaling. EP-2 and EP-4 are both Gs-coupled receptors that activate cAMP formation through adenylate cyclase which leads to activation of the protein kinase A (PKA) pathway. EP2/4 activation by PGE2 leads to β-arrestin recruitment, activation of proto-oncogene tyrosine-protein kinase (c-Src), and subsequent transactivation of epidermal growth factor receptor (EGFR), initiating downstream phosphoinositide 3-kinase (PI3K)–Akt, MAPKinase, Ras/Raf, and c-Jun N-terminal kinase (JNK) pathway signaling, all known to increase cell proliferation, migration, and differentiation (62–65). A distinct difference between EP-2/4 is that, upon PGE2 activation, the EP-4 receptor becomes rapidly internalized and desensitized, while EP-2 rarely internalizes and sustains persistent receptor signaling at the cell surface (66).

PGE2 is the predominant downstream product of COX-2 and is implicated in tumor growth and progression in multiple solid malignancies such as breast (67), colorectal (68), lung (69), skin (70), pancreatic (71), prostate (69) and CNS tumors (16). In gliomas COX-2/PGE2 expression is correlated with an increase in glioma grade and poor prognosis. A study of 66 patient glioma samples revealed that 71% of GBM tumor samples had higher than 50% COX-2 positive cells (3% had less than 25% COX-2

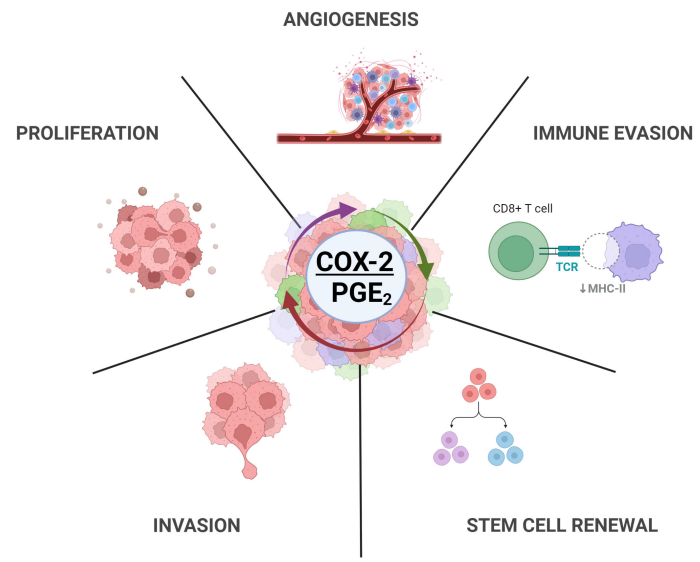

FIGURE 1
Pleiotropic effects of COX2/PGE2 axis in GBM. COX-2 dependent production of PGE2 leads to multiple tumor promoting effects through activation of EP1-4 receptors. These include angiogenesis, immune evasion, glioma stem cell renewal, invasion and ECM remodeling, and enhance proliferation. Created with BioRender.com

positive cells) compared to 30% COX-2 positive cells of low-grade gliomas (40% had less than 25% COX-2 positive cells) (72). COX-2 production of PGE2 is induced upon the treatment of GBM patients with both radiation and chemotherapy leading to a steep increase of immunosuppressive cytokines (16). Elevated COX-2/PGE2 has been shown to correlate with decreased survival and earlier recurrence following radiaotherapy (14, 41). Additionally, levels of circulating PGE2 in patients were shown to decrease significantly following surgical resection of malignant tumors (73). Spatial expression of COX-2 in GBM shows that the majority of COX-2 expression is localized to the core of the tumor, dissipating in the periphery, and is negligible in adjacent tissues. This pattern of expression is consistent with the fact that GBM characteristically maintains a hypoxic microenvironment particularly in the central regions of tumor and hypoxia facilitates COX-2 upregulation in a HIF-1α dependent manner (60, 74).

Multiple feed-back regulatory loops exist between COX-2 production of PGE2 and PGE2 regulation of COX-2 expression, amplifying the pro-tumor, immunosuppressive influences of COX-2/PGE2 on the TME (Figure 2A) (75–77). PGE2 stimulation of the EP2 and EP4 receptors activates nuclear translocation of CREB and binding to the COX-2 promoter, COX-2 expression, and production of more PGE2 (78). In the presence of glioma-derived soluble factors, microglia produce significant amounts PGE2, establishing a paracrine mechanism as well (12). This feedback loop may give context to the high correlation of COX-2/PGE2, as well as the percentage of infiltrating GAMs with high grade gliomas and poor prognosis. Additionally, PGE2 induces VEGF through HIF-1α activation, and VEGF can stimulate COX-2/PGE2 production, suggesting that these mediators are also co-regulated in a feed-forward, amplifying mechanism (76, 79). VEGF overexpression in the GBM-TME is associated with poor prognosis, and this PGE2/VEGF axis may contribute to the prevalence of angiogenesis and invasiveness of GBM. Macrophages, microglia, and tumor cells sustain the ability to produce and respond to COX-2/PGE2 through autocrine/paracrine signals creating a cyclical storm of inflammatory mediators (Figure 2B).

Therapeutic implications

GBM is notoriously resistant to conventional therapies, driving a need for additional targets and approaches. COX-2's multifaceted role in cancer progression suggests it may be a potential target for therapy. Inhibition of COX-2 by nonsteroidal anti-inflammatory drugs (NSAIDs) is a common treatment of cancers and it has increased patient survival in some cancers (80). However, NSAIDs are not selective for COX-2; they also target COX-1 and the related side-effects, including upper gastrointestinal (GI) stress, limit their use (81). The

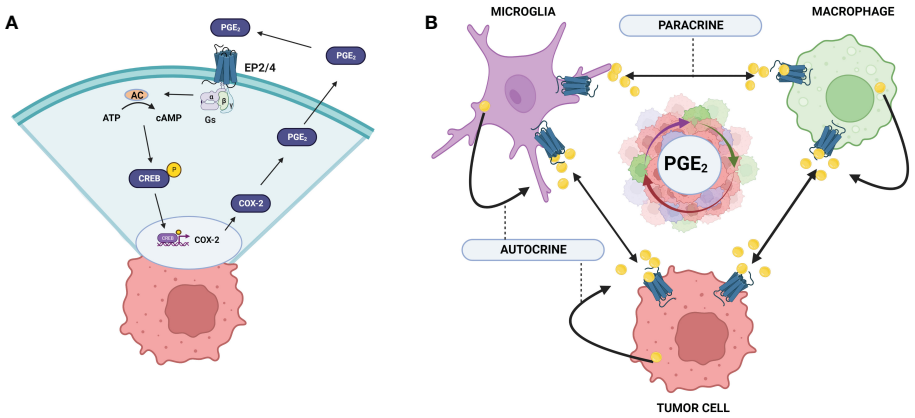

FIGURE 2
COX-2/PGE2 autocrine and paracrine feedback loops in the GBM TME. **(A)** COX-2 expression is induced through the activation of EP-2/4 by PGE2. Activation of CREB leads to association with the COX-2 promoter region and upregulation of COX-2 expression. **(B)** Tumor cells and GAMs upregulate COX-2 expression upon PGE2 activation through autocrine and paracrine mechanisms. Exacerbation of this cycle enhances robust upregulation of COX2/PGE2 in the GBM TME leading to tumor promoting effects and poor prognosis. Created with BioRender.com

development of COX-2 selective inhibitors (COXIBs) in the late 1990s was met with major enthusiasm and great expectations for safer COX-2 inhibition. However, while these drugs do indeed display lower GI stress, the initial enthusiasm for their use has been dampened by significant cardio- and cerebro-vascular toxicities (82). COX-2 selective inhibitors have shown some efficacy in clinical trials as an adjuvant to chemotherapy and radiotherapy (16, 81). Therefore, even though there is clear evidence that COX-2 function is a plausible target in the treatment of GBM, direct inhibition of the enzyme with selective inhibitors may not be an effective strategy.

The multifaceted physiological roles of COX-2 limit its potential as a direct target for long-term therapeutic use. However, therapeutic intervention targeting the cyclical upregulation of COX-2/PGE_2 in the TME can be achieved without direct COX-2 inhibition, and these indirect strategies may provide safety and efficacy advantages. A promising approach is targeting of downstream mediators of COX-2, especially PGE2, and their receptors. The pleiotropic effects of autocrine and paracrine signaling through the COX-2/PGE2 axis in the tumor microenvironment need to be further delineated to target specific paths that lead to malignant progression. Isolating the specific effects of COX-2/PGE_2 for individual EP receptors and how they each shape GBM TME in a spatial and temporal manner will inform future therapeutic avenues. For example, PGE_2/EP_2 signals through a G protein-dependent pathway (cAMP/CREB) and PGE_2 stimulates VEGF production through multiple mechanisms (HIF-1α activation, cAMP signaling, and EGFR transactivation) promoting angiogenesis (76). The essential role of the EP_2 receptor in the autocrine/paracrine signaling establish it as an attractive target for intervention. In recent years, multiple EP_2 small molecule inhibitors have been identified and tested, including butaprost, CAY10399, ONO-AE1–259, and TG6-10-1 (83). The brain-permeable, small molecule EP_2 antagonist TG6-10-1 has shown early promise as a possible therapeutic. In a recent study, inhibition of the PGE_2/EP_2 signal cascade by TG6-10-1 demonstrated significantly reduced GBM tumor growth in both subcutaneous and intercranial *in vivo* models (84).

While there has been extensive research into COX- in inflammation and cancer, the specifics of its dynamic regulation within and among the diverse cell types in the TME has yet to be fully elucidated. Understanding how induction of COX-2 expression is regulated in the context of GBM-TME may reveal therapeutic targets and strategies that are more selective than global COX-2 inhibition. For example, RGS10, a small G-protein regulator, has been shown to be a robust regulator of COX-2/PGE2 in both macrophages and microglia. RGS10 strongly suppresses COX-2 following activation by diverse upstream activators, including LPS, TNFα, and interferon gamma (85, 86). RGS10 does not completely abrogate COX-2, but attenuates the stimulated induction of COX-2 expression in stimulated cells (87). Therefore, RGS10 represents a potential target to break the cycle of COX-2 expression and PGE-2 production in GBM (86).

Concluding remarks

The GBM microenvironment is a dynamic system, and its high heterogeneity leads to an immunosuppressive environment. Tumor cells recruit immune cells which aid in this immunosuppression through production of inflammatory mediators. Infiltration of GAMs leads to dysregulated

inflammatory states that promote tumor progression. COX-2 and PGE2 are increased in GBM, and their pleiotropic signals impact proliferation, angiogenesis, immune evasion, stem cell renewal, and invasion. GBM lacks an effective treatment strategy. Harnessing the COX-2/PGE2 axis and understanding GBM microenvironment dynamics are important steps to revealing potential targets and informing new therapeutics.

Author contributions

SH and PD were responsible for the design, writing, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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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Macrophage – tumor cell interaction beyond cytokines

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Tumor cells communication with tumor associated macrophages is a highly important factor of tumor malignant potential development. For a long time, studies of this interaction were focused on a cytokine- and other soluble factors -mediated processes. Discovery of exosomes and regulatory RNAs as their cargo opened a broad field of research. Non-coding RNAs (ncRNAs) were demonstrated to contribute significantly to the development of macrophage phenotype, not only by regulating expression of certain genes, but also by providing for feedback loops of macrophage activation. Being a usual cargo of macrophage- or tumor cell-derived exosomes ncRNAs provide an important mechanism of tumor-stromal cell interaction that contributes significantly to the pathogenesis of various types of tumors. Despite the volume of ongoing research there are still many gaps that must be filled before the practical use of ncRNAs will be possible. In this review we discuss the role of regulatory RNAs in the development of macrophage phenotype. Further we review recent studies supporting the hypothesis that macrophages may affect the properties of tumor cells and vice versa tumor cells influence macrophage phenotype by miRNA and lncRNA transported between these cells by exosomes. We suggest that this mechanism of tumor cell – macrophage interaction is highly promising for the development of novel diagnostic and therapeutic strategies, though many problems are still to be solved.

KEYWORDS

macrophage, exosome, cancer, miRNA, lncRNA

Introduction

Macrophages are a heterogeneous cell population consisting of cells of various phenotypes. Within the continuum of macrophage functional states two extremes are designated as classically activated M1 macrophages and alternatively activated M2 macrophages. M1 is characterized by the production of signaling molecules that promote inflammation - TNFα, IL-1beta and others (1). M2 macrophages are characterized by the production of anti-inflammatory cytokines - TGFβ, IL-10 and some

others (2). However, the macrophage dichotomy is rather conditional and macrophage phenotype is highly flexible and can be regulated by various factors.

The molecular basis of macrophage polarization by cytokines is quite well understood. The IRF/STAT signaling pathway activated by IFNγ, and various bacterial products *via* TLRs, leads to the development of M1 polarization, while M2 polarization is induced by IL-4 or IL-13. These processes are reversible both *in vitro* and *in vivo* (3, 4). Interferons and TLRs activate the IRF/STAT cascade through STAT1, and M2-stimulating cytokines through STAT6 (2). Additional cytokines or hormones influence the macrophage phenotype in their specific ways. Physical factors, such as hypoxia, may also influence the macrophage phenotype (5).

In the development of solid tumors, macrophages of different phenotypes can play opposite roles. Thus, pro-inflammatory M1 macrophages can suppress tumor progression, while immunosuppressive M2 stimulate angiogenesis and invasion (4, 6, 7). The M1/M2 ratio of tumor associated macrophage population changes significantly with tumor development and depends on the disease stage. For the early stages, M1 macrophages are the predominant population, with tumor development the ratio shifts towards M2 (7, 8). M1 macrophages are able to prevent tumor development, largely due to the presentation of antigens on their surface and the recruitment of CD8+ T cells and NK cells (9). Although interaction of tumor cells and tumor associated macrophages (TAMs) is usually studied in regard of cytokines and other secreted mediators produced by both types of cells, there are several emerging directions of research including regulatory RNA molecules.

microRNA in defining macrophage phenotype

In addition to cytokines, microRNA plays an important role in macrophage polarization and the performance of the corresponding functions by these cells. MicroRNA is a sequence of ~22 ribonucleotides, their main function is the inhibition of mRNA translation. About 60% of all eukaryotic cell mRNAs contain miRNA complementarity sites, both at the 5'- and 3'-non-coding regions (10). Pre-miRNAs are assembled into a RISC complex, which also includes the RNA-specific endonuclease Dicer and Drosha, which are involved in the processing of pre-miRNA into a mature form, as well as proteins from the Argonaut family (11). Guided by miRNA, the RISC complex is involved in the inhibition of translation of an mRNA (10, 12, 13). RISC can inhibit assembly of the 80S translational complex. The Ago2 protein in the RISC complex competes with the 5' recognition site of the eukaryotic initiation factor 4G (eIF4G). According to other data, translation inhibition is associated with the interaction of RISC with the anti-associating factor eIF6, which also prevents the assembly of the 80S translation complex (10).

MicroRNAs can be encoded within introns, exons, and between different genes (14). The expression of miRNA is under the control of various transcription factors, but may also depend on the level of already expressed miRNA by the feedback principle with its own transcription factors (11). MicroRNAs can be used as a diagnostic markers for various diseases (11).

A number of miRNAs control the macrophage phenotype and function. Here we provide just several examples of those. In the case of increased expression miR-720 inhibits GATA3 protein, an important regulator of the M2 polarization of macrophages, suppresses the manifestation of the M2 phenotype and shifts it towards M1, and reduces the phagocytic activity of tumor-associated macrophages. Normally, the expression of this miRNA is significantly reduced in M2 macrophages in comparison with M0 and practically does not change when the M1 phenotype is induced. At the same time, stimulation of GATA3 expression in macrophages overexpressing miR-720 contributed to the restoration of the M2 phenotype, which indicates a close relationship between this microRNA and the macrophage phenotype (15).

Another interesting example is miR-127 that was shown to inhibit the B-cell lymphoma receptor Bcl6 and Dusp1 phosphatase, which promotes JNK activation and development of M1 macrophages. The authors demonstrated that overexpression of miR-127 in macrophages significantly increases the expression of pro-inflammatory markers such as IL-6, IL-1β, tumor necrosis factor alpha, and inducible NO synthase (iNOs), typical for M1 polarization (16).

Both miR-720 and miR-127 are expressed in macrophage upon their stimulation with pro-inflammatory stimuli (15, 16), so they can be considered a part of the intracellular machinery, necessary for the macrophage phenotype development. This contribution can be modulated by transfecting macrophages with corresponding miRNA inhibitors.

Various miRNA-mediated patterns have also been shown to be associated with M2 polarization (17). For instance, the miR-23a/27a/24-2 are overexpressed upon macrophage stimulation with M2-associated cytokines and down regulated by M1-associated stimuli. At the same time forced expression of these miRNAs led to M1 phenotype development *via* different mechanisms. Amplification of miR-23a expression enhances activation of the NF-κB pathway by binding to one of the NF-κB suppressors A20 and simultaneously stimulates the expression of M1 cytokines (18). Therefore, these miRNAs can be considered as a part of a negative feedback loop of M2 phenotype development.

MiR-301a was demonstrated to attenuate macrophage migration and phagocytosis in a mouse KO model. This study was done without induction of any specific macrophage phenotype demonstrating that miRNA affects the basic function of macrophages (19).

There are more studies of miRNAs involved in modulation of macrophage phenotype, reviewed elsewhere (20, 21) though our knowledge of the biological significance of observed effects remains limited due to the absence of unified experimental systems (Table 1). One of the common shortcomings of many studies on microRNA role in macrophage activation is the absence of time course experiments. Especially important this can be for the induction of M1 phenotype that is in many cases a very rapid event.

TABLE 1 ncRNA in macrophage polarization and TAM-tumor cells interaction.

ncRNA	Source	Effect	Experimental system	Reference
miR-720	macrophages, upon inflammatory stimulation	M1 polarization	Human cell lines	(15)
miR-127	macrophages upon stimulation	M1 polarization	Mouse cell lines	(16)
miR-23a/27a/24-2	macrophages upon IL-4 stimulation	M1 polarization	Mouse cell lines, mouse BMDM	(18)
miR-301a	macrophages	decrease of migration and phagocytosis	Mouse cell lines, mouse BMDM	(19)
lncRNA MM2P	macrophages upon IL-4 stimulation	M2 polarization	Mouse cell lines, mouse BMDM	(22)
lncRNA RPPH1	CRC cells exosomes	M2 polarization	Human peripheral blood monocytes	(23)
miR-155, miR-181, miR-451	M1 macrophages	M1 polarization	Mouse BMDM	(24)
miR-146a, miR-125a, miR-145-5p	M2 macrophages	M2 polarization	Mouse BMDM	(24)
miR-511-3p	M2 macrophages	M2 polarization	Mouse BMDM, mouse TAMs	(25)
miR-193a-5p	TAMs	Renal cell carcinoma progression	Human cell lines	(26)
miR-501-3p	TAMs	Pancreatic cancer progression	Human cell lines	(27)
miR-223	TAMs	Breast cancer progression	Human peripheral blood monocytes derived macrophages	(28)
miR-155-5p and miR-21-5p	TAMs	Colorectal cancer progression	Human TAMs	(29)
miR-181a	Tumor-associated fibroblasts educated TAMs	Breast cancer progression	Human peripheral blood monocytes derived macrophages, human cell lines	(30)

Long noncoding RNA

In addition to miRNAs, long noncoding RNAs (lncRNAs) can also be involved in macrophage phenotype development (31). Long non-coding RNAs are sequences of more than 200 nucleotides and are not used as templates for protein synthesis, while carrying exclusively regulatory functions (32). It is noteworthy that various tumors are characterized by impaired expression of lncRNAs associated with tumor progression (33, 34). As miRNAs macrophage phenotype modulating lncRNAs can be expressed by macrophages themselves or delivered to macrophages by exosomes or artificial delivery systems.

For instance, MM2P lncRNA is overexpressed in macrophages upon their stimulation with IL-4 and suppressed by LPS stimulation. Further it was demonstrated that transfection of macrophages with MM2P lncRNA enhance M2 polarization of macrophages induced by IL-4 or IL13 (22). The authors also established that MM2P knockdown leads to a decrease in the concentration of phosphorylated STAT6 in macrophages and by this way prevent their M2 polarization (22).

Not only the lncRNAs are expressed in macrophages upon their stimulation with pro- or anti-inflammatory stimuli. LncRNA RPPH1 is expressed in colorectal cancer (CRC) cells and may be transported to macrophages inside exosomes. In macrophages lncRNA RPPH1 triggers M2 development contributing to tumor aggressiveness (23).

Exosomes

Transfer of molecules by extracellular vesicles (EVs) has been studied as a mechanism of intercellular communication since about 2 decades. EVs is a group of membrane-enclosed vesicles that are naturally released by almost all cell types. EVs are the most important carriers that transport “cargo” from parent cells to target cells, regulating physiological or pathological processes in recipient cells. By origin and size, EVs were originally divided into exosomes (30–200 nm), microvesicles (200–1000 nm), and apoptotic bodies (1–5 μm), but not so long ago, with increasing interest in Other EV subpopulations have also been identified, such as exomers (<50 nm) and large oncosomes (1–10 μm) (35). It has been shown that exosomes carry complex and highly cell-specific cargoes, including DNA, RNA, lipids, metabolites, cytosolic and surface proteins (36). They can be selectively captured by neighboring cells, or cells far from the place of release, and reprogram recipient cells with the help of biologically active molecules contained inside. It is generally accepted

that their content can vary greatly depending on the types of cells, their secretion and their current physiological state. Thus, exosomes represent a mechanism of intercellular communication that plays an important role in many cellular processes, including the immune response (37, 38). Exosomes and the molecules they contain may be of prognostic value in chronic inflammation, cardiovascular and renal diseases, lipid metabolism disorders, and cancer (39, 40). Thus, through exosomes, tumor cells influence their microenvironment, which leads to adaptation of the tumor stroma with subsequent stimulation of tumor growth. On the other hand, exosomes secreted by cells of the tumor microenvironment, in particular tumor-associated macrophages (TAMs), may affect tumor growth.

TAM exosomes

The functions of macrophage exosomes have been widely studied, and the data obtained indicate their key role in disease progression. It should be noted that in recent studies, macrophage extracellular vesicles (EV) are considered to be one of the most important mediators of inflammatory diseases and cancer. As well macrophage EV are thought to be mediators of a positive effect on immunoregulation, tumor therapy, protection against infections, and tissue repair (41).

The content of macrophage exosomes may differ depending on the macrophage phenotype or the composition of their microenvironment. Since macrophages can form a complex mixed phenotype in various diseases or even at different stages of the same disease *in vivo*, it is quite difficult to identify the composition of their exosomes. Proteome analysis revealed different proteins, including cathepsins, 20S proteasome subunits, ribosomal proteins, and heterogeneous nuclear ribonucleoproteins in exosomes released from TAMs, indicating that macrophages may release exosome proteins with increased proteolytic activity and reduced RNA binding capacity (42).

Among the most important molecules contained in macrophage exosomes are various types of RNA molecules. Being protected from ribonuclease degradation within exosomes, ncRNAs can be secreted into various body fluids. MicroRNAs appear to be the most abundant regulatory RNAs in exosomes. In a study by Zhang et al. 109 microRNAs were identified that are differentially expressed in M1- and M2-polarized human and mouse macrophages, including miR-155, miR-181, miR-451 in M1 macrophages and miR-146a, miR-125a, miR-145-5p in M2 macrophages (24). Several miRNAs, miR-146 and miR-155, affect the activation of pathways associated with immune control and the consequences of inflammation (43). Other miRNAs highly expressed in M2 macrophages are miR-511-3p, miR-223 and let-7c, all of which promote M2 polarization (20). MiR-511-3p, which is highly expressed in TAM, targets ROCK2 (Rho-associated helical coil containing protein kinase 2) and maintains the expression of genes associated with M2 polarization (25). TAM-secreted exosomes downregulate TIMP2 expression in RCC cells, promoting vasculogenic mimicry and invasion by miR-193a-5p transfer, which ultimately promotes metastasis (26).

miR-501-3p miRNA isolated from exosomes secreted by tumor-associated M2 macrophages promotes tumor growth and progression of pancreatic cancer. This microRNA inhibits the expression of the TGFBR3 gene, which is an important tumor suppressor, which stimulates an increase in the rate of cell migration and metastasis (27). A decrease in TGFBR3 expression is observed in a number of tumors, which indicates the importance of this cascade in the context of tumor development (44, 45).

The transmission of various microRNAs from macrophages to tumor cells was demonstrated in a study by Mei Yang et al. IL-4 polarized M2 macrophages secrete exosomes containing miR-223. As a result of cocultivation of macrophages with breast cancer cells, it was possible to detect the appearance of this miRNA in tumor cells (28). Data on the differential expression of miR-223 in normal and tumor cells indicate that this miRNA can contribute to the progression of tumors of various types, including renal cell carcinoma and bladder cancer (46, 47).

M2 macrophages are also able to stimulate tumor invasion and angiogenesis through exosomal miRNAs. According to a study by Jingqin Lan et al., in the case of colorectal carcinoma, miR-155-5p and miR-21-5p are transported from M2 macrophages to tumor cells *via* exosomes. In turn, the target of these miRNAs is the BRG1 sequence: this gene is recognized as one of the important suppressors of metastasis in colorectal carcinoma. When miR-155-5p or miR-21-5p interact, a significant drop in the level of BRG1 expression is observed, which may be associated with an acceleration of tumor progression and invasion (29).

Not only tumor cells can modulate TAM phenotype in a way that these cells produce miRNA supporting tumor growth. It was demonstrated that cancer associated fibroblasts stimulate TAMs to express high levels of miR-181a. These TAMs produce miR-181 containing exosomes that activate AKT signaling in breast cancer cells and increase the aggressiveness of the tumor (30).

Recent studies have shown that TAM exosomes also contain various long non-coding RNAs (lncRNAs). The interactions of lncRNA with RNA, DNA, and proteins allow them to regulate gene expression at several levels, so roles in gene regulation are usually divided into epigenetic, transcriptional, and post-transcriptional levels. LncRNAs reside either in the cytoplasm or in the nucleus, where they can interact with miRNAs, mRNAs, RNA-binding proteins (RBPs), transcription factors, and chromatins and act as enhancer-like RNAs (48). Accumulated data have shown that cytoplasmic lncRNAs can be involved in gene regulation at the post-transcriptional level, including acting as ceRNAs and protecting target mRNAs from repression (49).

Accumulated data show that lncRNAs are actively involved in the regulation of many fundamental biological processes of development. At the moment, their participation in epigenetic regulation (gene dosage compensation, genomic imprinting), cell differentiation, and organogenesis has already been shown (50). Some lncRNAs—MALAT1, HOTAIR, and ANRIL—are associated with various pathologies, including cancer (51). Extracellular vesicular transmission of myeloid-derived HIF-1α-stabilizing long non-coding RNA (HISLA) is positively correlated with poor overall survival in breast cancer patients. It has also been shown that HISLA within TAM-derived exosomes can promote aerobic

glycolysis, apoptosis resistance, and chemoresistance in breast cancer cells (52).

Tumor cell-derived exosomes

Studies have shown that tumor cells produce much more exosomes than normal cells. Due to the presence of adhesion receptors and ligands specific for various types of cells and tissues on their membranes, these exosomes "target" certain types of cells, delivering the widest spectrum of biological molecules. Exosomes secreted by tumor cells carry various proinflammatory and immunosuppressive factors, such as macrophage migration inhibitory factor (MIF) and PD-L1, which act in nearby or distant tissues or organs to induce vascular permeability, inflammatory infiltration, extracellular matrix remodeling, and downregulation. immune response. Activated stromal cells can release a variety of cytokines and chemoattractants *via* exosomes, such as IL-6, IL-8 and S100A9, which promote tumor cell proliferation and invasion, as well as the acquisition of chemoresistance and stem cell phenotype (53).

In addition to proteins, miRNAs contained in tumor cell-derived exosomes may affect macrophage polarization. Exosomal miR-301a-3p stimulates macrophage polarization to the M2 phenotype *via* the PTEN/PI3Kγ signaling pathway. Circulating miR-301a-3p levels are positively correlated with later tumor stage, TNM grade; an increase in the level of circulating this microRNA is associated with a worse prognosis of survival in case of pancreatic cancer (54). Tumor cell derived exosomal miR-138-5p inhibits KDM3B expression, thereby promoting the M2 phenotype and blocking M1 polarization. In the case of breast cancer, an increase in the content of exosomal miR-138-5p was associated with a worse prognosis (55). Considering the plasticity of macrophage phenotype it would be important to investigate the stability of macrophage phenotype change induced by tumor cell-derived miRNA.

Conclusions

The investigation of exosome-mediated intercellular communication between tumor cells and tumor-associated macrophages (TAMs) has provided valuable insights into the potential for identifying new targets for anticancer therapy, particularly regulatory RNAs. The results of this research suggest that the inhibitory effects mediated by M1-like macrophages can be a promising approach for cancer therapy. Macrophage reprogramming towards the M1 phenotype, through the modification of exosomal cargoes, may serve as a strategy for suppressing tumor growth.

However, despite the extensive research in this field, there are still many gaps in our understanding of the complex exosome-mediated communication process between tumor cells and macrophages. One of the main challenges is the lack of comparability between different experimental systems, particularly with regards to non-coding RNAs in macrophages and the limited comparability between mouse and human macrophage cell lines (Table 1). Additionally, there is a need for a more nuanced approach to the selection of macrophage phenotype markers, rather than relying solely on the M1/M2 dichotomy. Further research in this area should consider the dynamic changes in macrophage phenotype that can occur in response to different stimuli, and the use of multiple markers to accurately characterize macrophage phenotype.

In order to fully understand the impact of non-coding RNAs on macrophages and tumor cells, extensive kinetics studies are crucial. Studies have been performed to assess the kinetics of LPS-induced TNF production (56) and the cytokine production induced by IFN-γ or IL-4 in macrophages (57), revealing the complexity of macrophage behavior over time. Similar studies of ncRNAs can provide insight into the regulatory networks controlling macrophage biology and identify key hubs that can be targeted for therapeutic intervention. Additionally, further investigation into the role of exosomes in tumor progression and the cross-talk between different cell types within the tumor microenvironment will provide a more comprehensive understanding of the complex interplay between tumor cells, macrophages, and the surrounding microenvironment. This knowledge can be leveraged to design more effective, targeted therapeutic strategies for cancer treatment.

Author contributions

OK wrote the manuscript. MS wrote the manuscript. AE wrote the manuscript. AP wrote and proofread the manuscript. KS wrote and proofread the manuscript. AG designed the concept, proofread the manuscipt. 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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