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Exploring macrophage roles in cancer progression and therapeutic targeting

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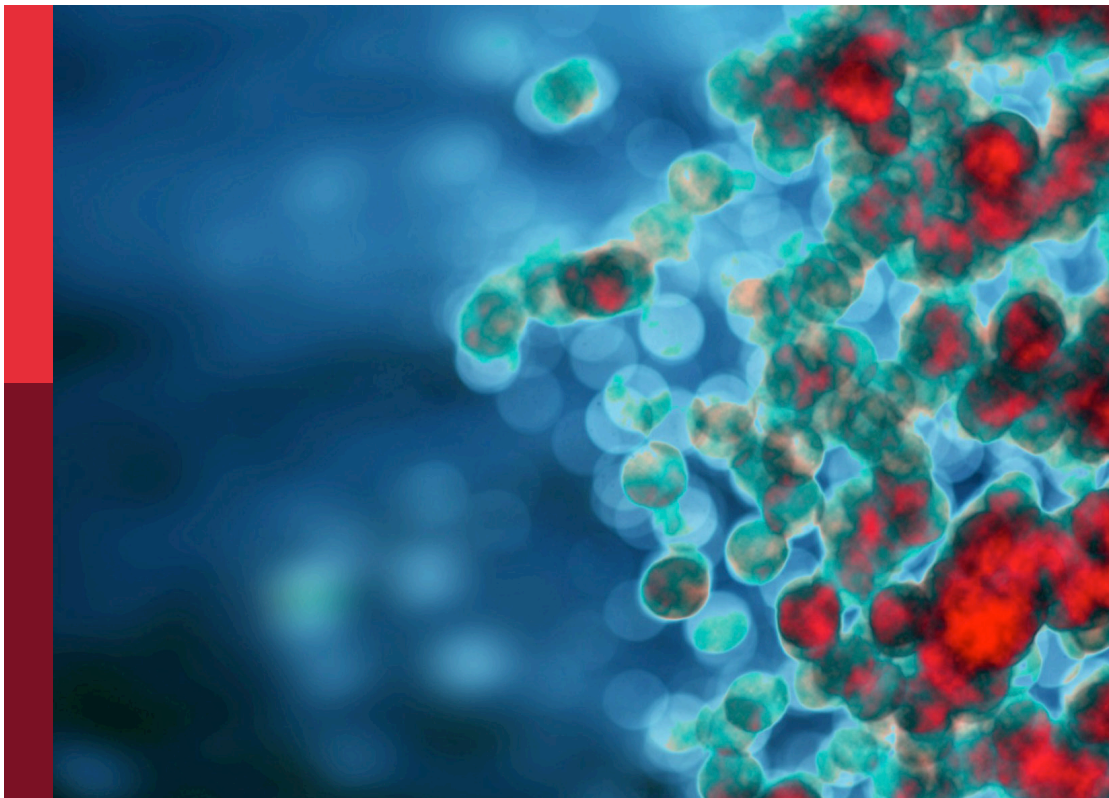

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Exploring macrophage roles in cancer progression and therapeutic targeting

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Editorial: Exploring macrophage roles in cancer progression and therapeutic targeting

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Editorial on the Research Topic
Exploring macrophage roles in cancer progression and therapeutic targeting

Introduction

Macrophages are versatile cells which play pivotal roles in development, tissue repair, and immune response. Lying at the intersection between the innate and adaptive immune systems, these cells serve as sentinels, surveilling tissues for damage, infection, or oncogenic transformation (1, 2). Within cancer microenvironments, these versatile cells transform into tumor-associated macrophages (TAMs) or metastasis-associated macrophages (MAMs), and exhibit a dual nature capable of both promoting antitumor immunity and facilitating cancer progression (3).

Within the primary tumor microenvironment, TAMs are known to facilitate tumor malignancy through various mechanisms: promoting angiogenesis, suppressing antitumor immunity, and enhancing tumor cell invasion and metastatic dissemination. Meanwhile, at secondary sites, MAMs also play a crucial role in the metastatic process, aiding in tumor cell extravasation, survival, and growth. Understanding the complexity of macrophage biology, and how to modulate this biology to inhibit tumor growth and metastasis, is a key focus of current research efforts.

Despite recent advances in cancer immunotherapy (e.g. immune checkpoint inhibition), refractory cancers remain a significant challenge. This persistence highlights the need for novel therapeutic approaches. Targeting macrophages and their associated molecules has emerged as a potential strategy for both anticancer and antimetastatic therapies. However, a comprehensive understanding of macrophage biology in cancer initiation and metastasis is still lacking, necessitating further investigation into their roles and therapeutic potential.

This Research Topic highlights key advances in our understanding of macrophage biology in cancer, focusing on their heterogeneity, molecular mechanisms, and therapeutic potential. Several contributions to this Research Topic showcase recent studies using single-cell and spatial technologies that reveal diverse macrophage subpopulations with context-dependent roles in tumor progression. Additionally, papers included in this Research Topic investigate signaling pathways, metabolism, and intercellular communication, shedding light on how macrophages influence cancer dynamics. Finally, several papers propose innovative strategies to reprogram or target tumor-associated macrophages, offering promising avenues for cancer therapy. Together, this Research Topic will be valuable to researchers and clinicians seeking to develop macrophage-targeted treatments and better understand the immune landscape of tumors.

Contributions to the Research Topic

Heterogeneity and plasticity of macrophages within the tumor microenvironment

The tumor microenvironment is a complex ecosystem where macrophages exhibit remarkable diversity and adaptability. Several contributions include a major focus on the critical role of macrophage heterogeneity and plasticity in cancer progression and immunotherapy, underscoring the need for a nuanced understanding of their function.

The review by Stavrou et al. summarizes recent findings on distinct TAM subsets in the tumor microenvironment and their involvement in breast cancer progression, emphasizing the constant interplay between TAMs and breast cancer cells as a major contributor to disease progression. This interaction involves the polarization of macrophages toward a tumor-promoting phenotype, induction of epithelial-to-mesenchymal transition in cancer cells, and enhancement of cancer stem cell properties. The authors discuss the clinical relevance of these findings, focusing on how a better understanding of TAM involvement in breast cancer metastasis could lead to more effective treatment options. They suggest that a thorough characterization of individual patients' TAM signatures could facilitate the design of personalized treatment strategies and improve the prediction of treatment responses.

The paper by Zhou et al. provides an overview of the role of macrophages in cancer immunotherapy, highlighting recent advances in understanding their complex functions within the tumor microenvironment. Macrophages are a major component of the immune infiltrate in many solid tumors and can exhibit both pro- and anti-tumor activities depending on their phenotype and the signals they receive. The review discusses how TAMs often adopt an immunosuppressive, pro-tumor phenotype that promotes cancer progression and metastasis. However, emerging research has revealed strategies to reprogram these TAMs or harness their anti-tumor potential for cancer therapy. The authors describe several approaches being explored to target macrophages in cancer immunotherapy. These include blocking macrophage recruitment to tumors, depleting TAMs, repolarizing TAMs from a pro-tumor M2-like phenotype to an anti-tumor M1-like phenotype, and enhancing macrophage phagocytosis of cancer cells. The paper also discusses combining macrophage-targeted therapies with other immunotherapies like immune checkpoint inhibitors. Additionally, it highlights new technologies like single-cell RNA sequencing that are providing deeper insights into macrophage heterogeneity and function in the tumor microenvironment. Overall, the review emphasizes that macrophages represent a promising but complex target for improving cancer immunotherapy, with ongoing research aimed at better understanding and manipulating their diverse roles in tumors.

Elucidation of molecular mechanisms by which macrophages contribute to cancer progression

Beyond understanding the broad role of macrophage heterogeneity, many contributions have focused on elucidating the specific molecular mechanisms by which macrophages contribute to cancer progression. This includes investigating the intricate signaling pathways, interactions, and molecules that govern macrophage behavior within the tumor microenvironment.

In the review article by Baig et al., the authors explore the role of adaptor proteins in regulating inflammation in macrophages. Adaptor proteins are non-catalytic proteins that act as molecular bridges between cell surface receptors and intracellular effector molecules, mediating protein-protein interactions and modulating immune cell signaling. These proteins play critical roles in organizing signaling complexes, regulating protein localization, and modulating the intensity/duration of cellular responses. Some adaptor proteins can function to activate signaling pathways, while others inhibit them. This dichotomy offers an opportunity to affect and alter macrophage function.

As such, this article comprehensively reviews 20 adaptor molecules that actively dampen inflammatory signaling pathways in macrophages. The authors discuss how these adaptor proteins regulate signal transduction processes, driving macrophages from pro-inflammatory M1-like states to anti-inflammatory M2-like phenotypes. By mapping the specific functions and structural domains of these molecules, the review illuminates their complex interplay in immune regulation. This work focuses on our current understanding of adaptor dynamics but also paves the way for therapeutic strategies targeting chronic inflammatory conditions, offering new investigative avenues for clinical applications in diseases marked by persistent inflammation.

Another way that TAMs are able to impact tumor cells is by imparting in them aggressive phenotypes (such as increased motility, invasiveness, and epithelial-mesenchymal transition (EMT)). The article by DeLuca et al. discusses the various molecular mechanisms by which TAMs facilitate tumor cell migration and invasion. These include the secretion of proteolytic

enzymes that degrade the extracellular matrix, the production of growth factors and cytokines that stimulate tumor cell motility, and direct interactions with tumor cells that guide their movement. Recent evidence indicates that these factors and interactions may be amplified by traditional anti-tumoral therapies, potentially leading to the emergence of prometastatic phenotypes in tumor cells. The authors continue on to describe how host factors such as diet, race, and obesity, can influence macrophages and their ability to support or counter tumor development.

The review by Murrey et al. views macrophages with a different lens, focusing on the critical role of macrophage motility and migration in various physiological and pathological processes. Macrophages, as tissue-resident immune cells, are highly motile and continuously patrol their environment to maintain tissue homeostasis, respond to injury or infection, and participate in development. The authors discuss the diverse mechanisms that regulate macrophage migration, including chemotactic signals, adhesion molecules, and cytoskeletal dynamics. They highlight how these mechanisms are tightly controlled to ensure proper macrophage recruitment and function in different contexts.

This review also explores the role of macrophage migration in tumor invasion and metastasis. TAMs can promote cancer cell migration and invasion by secreting growth factors, matrix-degrading enzymes, and chemokines. The authors discuss how TAMs are recruited to the tumor microenvironment and how their migratory behavior contributes to tumor progression. They also highlight potential therapeutic strategies for targeting macrophage migration to inhibit tumor invasion and metastasis.

The study by Xie et al. investigates the characteristics and potential antitumor functions of immortalized bone marrow-derived macrophages (iBMDMs) compared to primary bone marrow-derived macrophages (BMDMs) and the RAW264.7 cell line. The researchers found that iBMDMs exhibit similar macrophage biomarkers and polarization responses to BMDMs and RAW264.7 cells, with the ability to polarize into M1 and M2 phenotypes upon appropriate stimulation. iBMDMs demonstrated rapid proliferation and long-term survival both *in vitro* and *in vivo*, while maintaining biosafety in mouse tissues. Importantly, iBMDMs showed strong phagocytic capacity against tumor cells, especially after M1 polarization.

The study also revealed that iBMDMs have potent antitumor effects through various mechanisms. The supernatant from M1-polarized iBMDMs significantly inhibited tumor cell proliferation and promoted apoptosis of tumor cells. Additionally, iBMDMs, particularly M1-polarized ones, demonstrated a remarkable ability to inhibit tumor cell migration by suppressing EMT. *In vivo* experiments showed that M1-polarized iBMDMs could maintain their anti-tumor phenotypes and influence recruited macrophages in recipient mice, leading to improved tumor immune microenvironments and repressed tumor growth. These findings suggest that iBMDMs can serve as a valuable tool for studying macrophage functions and mechanisms, as well as a potential source for macrophage-based immunotherapy in cancer treatment.

The study by Yang et al. investigates the influence of sex disparities on macrophage proliferation and accumulation in hepatocellular carcinoma (HCC). The researchers found higher levels of macrophage density and proliferation in tumor tissues from male HCC patients compared to females. They discovered that the expression of G protein-coupled estrogen receptor 1 (GPER1), a non-classical estrogen receptor, was significantly decreased in proliferating macrophages and inversely correlated with macrophage proliferation in HCC tumors. Activation of GPER1 signaling with a selective agonists, G1, suppressed macrophage proliferation by downregulating the MEK/ERK pathway.

Furthermore, G-1 treatment reduced PD-L1 expression on macrophages and delayed tumor growth in mice. The study also found that patients with a higher percentage of GPER1+ macrophages exhibited longer overall survival and recurrence-free survival compared to those with lower levels. These findings reveal a novel role of GPER1 signaling in regulating macrophage proliferation and function in HCC tumors. The research suggests that understanding sex-related disparities in patients may offer potential strategies for designing more effective therapies for HCC.

Development of novel therapeutic strategies targeting macrophages in cancer treatment

Recognizing the significant impact of macrophages on cancer progression, researchers are actively exploring novel therapeutic strategies to target these cells within the tumor microenvironment. The following studies highlight diverse approaches aimed at manipulating macrophage number and function to improve cancer treatment outcomes.

The review by Cao et al. delves into the major signaling pathways through which TAMs can either promote or suppress tumor progression, and the multifaceted strategies of targeting them for cancer treatment. These immunotherapeutic approaches attempt to alter TAMs from a pro-tumorigenic (M2-like) to an anti-tumorigenic (M1-like) phenotype by blocking M2 macrophage recruitment, depleting them, or modulating their functions to enhance the efficacy of cancer therapies. The authors discuss various mechanisms by which TAM-targeted immunotherapies attempt to exert their effects, including altering cytokine production, enhancing antigen presentation, and promoting cytotoxic T cell infiltration. They also address the challenges associated with TAM-targeted approaches, such as the heterogeneity of TAMs, their plasticity, and the potential for off-target effects. Finally, the review emphasizes the importance of mechanistic studies to better understand the complex interactions between TAMs and cancer cells, as well as the rational design of more effective and selective TAM-targeted immunotherapies.

The paper Lin et al. continues the discussion of how macrophages can play a dual role in cancer progression, but with a particular focus on head and neck squamous cell carcinoma (HNSCC). The authors discuss several modes of tumor cell-macrophage interaction, including phagocytosis and the secretion of cytokines and exosomes. They discuss the potential of macrophages as both diagnostic and therapeutic targets in

HNSCC and the various strategies for targeting TAMs in this carcinoma. These strategies include reprogramming macrophages towards an anti-tumor phenotype, inhibiting macrophage recruitment, and combining macrophage-targeted therapies with conventional treatments. The authors also highlight the use of macrophage-related markers for prognostic and diagnostic purposes in HNSCC. Overall, the paper underscores the importance of understanding macrophage biology in the context of HNSCC to develop more effective treatment strategies and improve patient outcomes.

The primary research article by Schultze-Rhonhof et al. investigates the effects of Plasma-activated liquids (PALs) on human tissue-resident peritoneal macrophages. PALs are an emerging technology with promising applications in medicine and biomedical research. PALs are created by exposing liquids like water or growth media to atmospheric plasma discharges. This process generates long-lived reactive species such as hydrogen peroxide, nitrites, and nitrates, as well as short-lived species like hydroxyl radicals and peroxynitrite. PALs have demonstrated significant potential in wound healing, cancer treatment, and antimicrobial applications due to their ability to induce oxidative stress in target cells while minimizing damage to healthy tissues.

The researchers isolated primary human macrophages from the peritoneum and exposed them to PALs. Using various methods including flow cytometry, Raman microspectroscopy, and DigiWest protein analysis, the study found that macrophages demonstrated a pronounced resistance to PALs, characterized by an upregulation of proliferation and anti-oxidative pathways to counter PAL-derived oxidative stress-induced cell death.

The findings revealed that PAL treatment led to changes in the macrophages' lipid composition and a moderate increase in pro-inflammatory cytokine release. However, the macrophages maintained high viability and showed minimal levels of apoptosis and necrosis. The researchers suggest that these cellular effects of PAL on human tissue-resident peritoneal macrophages could potentially lead to immunomodulatory effects within the human peritoneal cavity. This study contributes to understanding the interaction between PALs and macrophages, highlighting promising prospects for PALs in the adjuvant treatment of peritoneal cancer.

The paper by Wei et al. reviews the potential of natural plant-derived polysaccharides as modulators of macrophage polarization for cancer immunotherapy. Plant polysaccharides have shown promise in regulating macrophage polarization, particularly in promoting the M1 phenotype and inhibiting the M2 phenotype. The review covers the classification, sources, and mechanisms of action of these polysaccharides, including their effects on cytokine production, NO and ROS generation, and activation of signaling pathways such as TLR4, MAPK, and NF-κB.

The paper also explores the clinical translation and application of plant polysaccharides, focusing on compounds like Astragalus polysaccharide and Belapectin. These substances have shown potential in enhancing the efficacy of chemotherapy and immunotherapy in various cancer types. However, the authors note that challenges remain in the clinical translation of plant polysaccharides, including the need for more defined extracts and further research into optimal dosing and potential side effects. The review concludes by highlighting the promise of plant polysaccharides as immunomodulators in cancer therapy while acknowledging the need for further investigation into their specific molecular mechanisms and direct targets.

Finally, the paper by Zhang et al. reviews the role of TAMs in HCC. The authors highlight that TAMs are a major component of the tumor microenvironment in HCC and play a crucial role in tumor progression, metastasis, and therapeutic resistance. TAMs in HCC are primarily derived from circulating monocytes and are polarized towards an M2-like phenotype that promotes tumor growth. The review summarizes how TAMs contribute to HCC development through various mechanisms, including promoting angiogenesis, suppressing anti-tumor immunity, enhancing tumor cell proliferation and invasion, and facilitating metastasis.

The paper also discusses potential therapeutic strategies targeting TAMs in HCC. These include inhibiting TAM recruitment, reprogramming TAMs from a pro-tumor to an anti-tumor phenotype, and depleting TAMs from the tumor microenvironment. The authors review several promising approaches being investigated, such as CSF1R inhibitors, CCR2 antagonists, and CD47 blockade. They emphasize that combining TAM-targeted therapies with other treatments like immune checkpoint inhibitors or anti-angiogenic drugs may be particularly effective for treating HCC. Overall, the review underscores the importance of TAMs as both key drivers of HCC progression and promising therapeutic targets.

Conclusion

The contributions to this Research Topic collectively advance our understanding of macrophage biology in cancer initiation and metastasis. They align closely with the theme of exploring macrophages as potential therapeutic targets and provide valuable insights into the complex roles these cells play in cancer progression.

These studies highlight the heterogeneity and plasticity of macrophages in the tumor microenvironment, the importance of macrophage-derived factors in promoting metastasis, and the potential of targeting macrophages to overcome therapy resistance. The development of new tools, such as iBMDMs hold the potential to facilitate further research in this field.

As we continue to unravel the intricacies of macrophage biology in cancer, studies of novel therapeutics will pave the way towards improved outcomes for patients with refractory cancers. Future research building on these contributions will be crucial in translating our growing understanding of macrophages in cancer into effective clinical interventions.

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Conflict of interest

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Adaptor molecules mediate negative regulation of macrophage inflammatory pathways: a closer look

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Macrophages play a central role in initiating, maintaining, and terminating inflammation. For that, macrophages respond to various external stimuli in changing environments through signaling pathways that are tightly regulated and interconnected. This process involves, among others, autoregulatory loops that activate and deactivate macrophages through various cytokines, stimulants, and other chemical mediators. Adaptor proteins play an indispensable role in facilitating various inflammatory signals. These proteins are dynamic and flexible modulators of immune cell signaling and act as molecular bridges between cell surface receptors and intracellular effector molecules. They are involved in regulating physiological inflammation and also contribute significantly to the development of chronic inflammatory processes. This is at least partly due to their involvement in the activation and deactivation of macrophages, leading to changes in the macrophages' activation/phenotype. This review provides a comprehensive overview of the 20 adaptor molecules and proteins that act as negative regulators of inflammation in macrophages and effectively suppress inflammatory signaling pathways. We emphasize the functional role of adaptors in signal transduction in macrophages and their influence on the phenotypic transition of macrophages from pro-inflammatory M1-like states to anti-inflammatory M2-like phenotypes. This endeavor mainly aims at highlighting and orchestrating the intricate dynamics of adaptor molecules by elucidating the associated key roles along with respective domains and opening avenues for therapeutic and investigative purposes in clinical practice.

KEYWORDS

adaptor proteins, alternative activation, macrophage, inflammation, signaling mechanism

1 Introduction

Macrophages play a crucial role in the development and physiology of an organism as well as in the pathogenesis of various degenerative, infectious, and immunological diseases (1). Macrophages are a type of white blood cells that play an important role in the innate immune system. They are an important component of the first line of defense against pathogens and tumor cells by performing various functions, including ingesting and eliminating microorganisms, clearing debris and dead cells, and secreting pro-inflammatory and antimicrobial messengers. Macrophages are derived from hematopoietic stem cell-derived monocytes and embryonic yolk sac macrophages (2). They exhibit a remarkable diversity of phenotypes in different tissue environments, which is due to localized interactions with other cellular and molecular components. Macrophages actively contribute to physiological and tissue balance through a variety of cell surfaces and secreted molecules (3). To allow a functional classification of macrophage activation/phenotype, Mills and colleagues (4) introduced the terminology of M1 and M2 phenotypes, in analogy to T helper 1- and T helper 2-related inflammation, to distinguish between macrophages with pro-inflammatory and anti-inflammatory properties, respectively. M1 macrophages are often referred to as classically activated macrophages, whereas M2 macrophages are alternatively activated macrophages (5). However, we now understand that changing tissue environments provide molecular clues that lead to the emergence of a variety of macrophage phenotypes, of which the two distinct M1 and M2 subtypes appear as two possible extreme states (6, 7). In this review, we summarize 20 of the macrophage adaptor proteins that inhibit or suppress the immune response by inducing M2-like macrophages and promoting the production of anti-inflammatory cytokines.

Alternatively activated macrophages (such as M2) are usually activated by a series of stimuli (e.g., IL-4 or IL-13) and are typically observed under conditions of parasite infection and also during tissue healing and in the resolution phase of inflammation, when the burden of pathogenic infection is reduced or absent. They are characterized by their secretion of cytokines with anti-inflammatory properties. Moreover, anti-inflammatory macrophages actively support tissue remodeling and repair, e.g., by promoting angiogenesis and participating in debris clearance (8, 9). Malignant tumors attract circulating monocytes/macrophages, maturing them into tumor-associated macrophages (TAMs) with predominantly M2-like phenotypes associated with tissue remodeling and repair (10–14). Hereby, significant amounts of immunosuppressive cytokines are secreted by the anti-inflammatory TAMs, which facilitate metastasis and promote tumor growth (15).

Accurate regulation of macrophage populations is critical for proper immune function at both steady state and during disease. Deviation from this balance may result in immune pathway dysregulation (16). In the context of cellular signaling pathways, adaptor proteins exert a critical influence on the modulation of signal transduction. Despite their lack of inherent enzymatic activity, adaptor proteins are able to transmit signals to desired targets *via* other molecules using their characteristic domain structures (17). Adaptor proteins are equipped with a variety of functional domains that enable specific interactions between proteins and between proteins and lipids (18–23). The modular structure of adaptor proteins, which includes one or more specific domains that enable their interaction with various other proteins, is a characteristic feature shared by all adaptor proteins (17). Adaptor molecules play a key role in the core of various receptor-mediated signaling pathways and act as important mediators bridging the gap between receptors and other molecular components (24).

Recent therapeutic strategies to combat macrophage-mediated inflammation include signal modulation to enable a transition from a pro-inflammatory state to an anti-inflammatory state (25). Hereby, adapter proteins can critically influence the outcome of an external signal, either activating or inhibiting receptor-induced signal transduction (17). Macrophages receive a plethora of microenvironmental stimuli (cytokines, chemokines, and growth factors) that bind on surface receptors and initiate intracellular signaling and that need to be integrated by, among others, adaptor proteins (26). Activating adaptor proteins include myeloid differentiation 88 (MyD88), Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor-inducing interferon-β (TRIF), TNF receptor-associated factor 6 (TRAF6), growth factor receptor-bound protein 2 (Grb2), and caspase recruitment domain-containing protein 9 (CARD9). They regulate the cellular response to a stimulus by inducing the formation of the appropriate signaling complex, spatiotemporal regulation of signaling, activation of binding components, kinase regulation, and sequestration of specific proteins. The amplification of signaling and cell activation is determined by the recruited proteins, complex localization, and signal duration. Also, the binding of the adaptor protein to its target can be sufficient for its activation (27). Signaling pathways that are regulated by the activating adaptor proteins include the NF-κB pathway, AP-1, MAPK, IRFs, JAK/STAT, and PI3K/Akt (28, 29). The final effect of the activation of the aforementioned signaling pathways in macrophages is the production of pro-inflammatory cytokines, cytoskeleton rearrangement, regulation of apoptosis, and proliferation (30).

In contrast to activating adaptor proteins, inhibitory adaptor proteins serve the vital role of negatively regulating signal transduction. Based on the domains that constitute their structure (TIR, ligase domain, SH2, SH3, and IRF association domain), they exhibit different mechanisms of signal inhibition. For example, they can directly or indirectly induce the ubiquitination and subsequent degradation of Toll-like receptors (TLRs) or the activating adaptor proteins, resulting in the inhibition of signal transduction (31, 32). By these means, they may be instrumental in switching macrophage phenotypes from a pro-inflammatory state toward an anti-inflammatory state. In this review, we describe 20 of these inhibitory adaptor proteins such as Sterile α- and Armadillo motif-containing protein (SARM), Toll-interacting protein (TOLLIP), Src-like adaptor protein (SLAP), DNAX-activating protein of 12 kDa (DAP12), *Astragalus* polysaccharide (APS), LnK, β-arrestin, suppressor of cytokine signaling-1 (SOCS-1), interleukin-1 receptor associated kinase-M (IRAK-M),

downstream of kinase 3 (DOK-3), interferon regulatory factor 4 (IRF4), interferon regulatory factor 7 (IRF7), G protein-coupled receptor 108 (GPR108), NOD-like receptor family caspase recruitment domain family domain containing 5 (NLRC5), Disabled-2 (DAB2), Triad-3A, cytoplasmic linker protein 170 (CLIP170), interleukin-1 receptor-associated kinase 1/4 (IRAK-1/4), adaptor protein c-Cbl-associated protein (CAP), and Src kinase-associated phosphoprotein 2 (SKAP2). We explore their role in regulating macrophage activation.

2 Sterile α- and Armadillo motif-containing protein

The adaptor protein SARM is a TLR adaptor protein identified in 2001 by Mink et al. The 690 amino acid long SARM adaptor protein is encoded by the SARM gene located on chromosome 17q11 (33) and is highly conserved in *Caenorhabditis elegans*, mice, and *Drosophila* (34). SARM consists of three domains: an Armadillo repeat motif (ARM) at the N-terminus, two sterile alpha motifs (SAMs), and a TIR domain at the C-terminus (35). The SAM domain is involved in protein–protein interactions through homo- and heterotypic oligomerization to an octamer (36). The 40 amino acid ARM domain mediates autoinhibition as well as interaction with other proteins and beta-catenin with its ligands (35). The TIR domain is responsible for interaction with TLRs and mediates the innate immune response (37). Of the five TIR domain adaptor proteins of TLR, SARM has a unique function by negatively regulating the immune response (35). SARM inhibits the signaling pathway mediated by TLR3 and TLR4 and thus the downstream activation of NF-κB, IRF3, and activator protein-1 (AP-1) (38) through direct TIR–TIR interaction with TRIF and MyD88 (39) (Figure 1). The glycine residue (G601) in the BB loop of the SARM-TIR domain is essential for interaction with the MyD88 adaptor protein (39). Moreover, in rheumatoid arthritis, there is a negative correlation between SARM and TLR2-induced IL-1β expression, and higher SARM levels result in an enhanced response to anti-TNF-α therapy (40). SARM is not exclusively a negative regulator of inflammation and has also been shown to selectively promote TLR4- and TLR7-induced CCL5 expression in macrophages (41). However, SARM regulates TLRs, and TLRs can also control the expression of SARM. For example, treatment of RAW264.7 macrophages with TLR2 ligands increases SARM expression, an effect that requires TLR9 (42). SARM is involved in numerous cellular processes and pathologies, including neuropathy (43), apoptosis, antiviral immune responses (44), mitophagy, and neuronal death (38). SARM's regulatory role in macrophages can be ambiguous at times. Functioning as messengers within cells, these molecules facilitate communication among various proteins. The complexity and specificity of these molecules contribute to the uncertainty in their roles, as they may have diverse functions depending on the cellular context and the molecules they interact with. Additionally, SARM molecules can act as both facilitators and inhibitors in controlling macrophage behavior. Their functionality is dynamic, adapting to ongoing cellular events. Consequently, the apparent ambiguity in their role in macrophage regulation arises from these intricate and context-dependent interactions.

3 DNAX-activating protein of 12 kDa

The signaling adaptor molecule DAP12, also known as killer cell activating receptor-associated protein (KARAP) or tyrosine kinase binding protein (TYROBP), was originally discovered for natural killer (NK) cells (45–47), and later studies showed that it also plays a role in macrophages, dendritic cells, and monocytes (48–50). DAP12 consists of an extracellular domain, a transmembrane domain, and an intracellular domain that specifically includes an immunoreceptor tyrosine-based activation motif (ITAM) (51). The interaction of DAP12 with the receptor present on the cell surface is due to the presence of an aspartic acid residue in its transmembrane domain that forms an electrostatic association (51, 52). The binding of the ligand to the DAP12-bound receptor activates SRC family kinases and, in turn, leads to phosphorylation and activation of the ITAM tyrosine of DAP12 (53). The phosphorylated tyrosine of ITAM serves as a docking site for several tyrosine kinases, namely, ZAP70 and Syk, which further downstream signal transduction (54). Blocking the binding of DAP12 to Syk mediated by Ocilrp2 reduces lipopolysaccharide (LPS)-induced IL-6 production (55, 56). Signaling pathways such as FcεRIγ and CD3ζ, which are considered to be carriers of downstream DAP12 signaling, have been extensively studied (57, 58). One study showed that the knockdown of DAP12 gene in microglial BV2 cells resulted in an increase in mRNA levels of pro-inflammatory cytokines in response to LPS by stabilizing TREM2 (59). TREM2–DAP12 interaction inhibits the activation of Ras and ERK through the recruitment of the proteins Dok-3, Grb2, and Sos1, therefore inhibiting the TLR4-induced pro-inflammatory cytokine production (60) (Figure 1). DAP12/TREM2 signaling was also found to inhibit macrophage activation against non-glycosylated mycolic acid mycobacteria (61). Knockdown of DAP12 has been shown to increase the production of pro-inflammatory cytokines in alveolar macrophages during porcine reproductive and respiratory syndrome virus (PRRSV) arterivirus infection (62). When DAP12 was reintroduced into DAP12−/− macrophages, production of the pro-inflammatory cytokine TNF was significantly reduced (63). IL-4 is thought to be present in alternatively activated macrophages (M2), and thus, its regulation in response to DAP12 is a topic of interest. RNA silencing targeting DAP12 in human monocyte-derived macrophages significantly decreased IL-4-induced macrophage fusion (64). Macrophage fusion refers to the formation of multinucleated giant cells, which play a role in the immune response in granulomatous diseases. It has been suggested that signaling through a receptor such as TLRs or FcγRIII, which tend to activate the ERK pathway, may be inhibited by DAP12 (63). Therefore, further studies focusing on DAP12 are needed to investigate its precise mechanistic role in macrophage polarization.

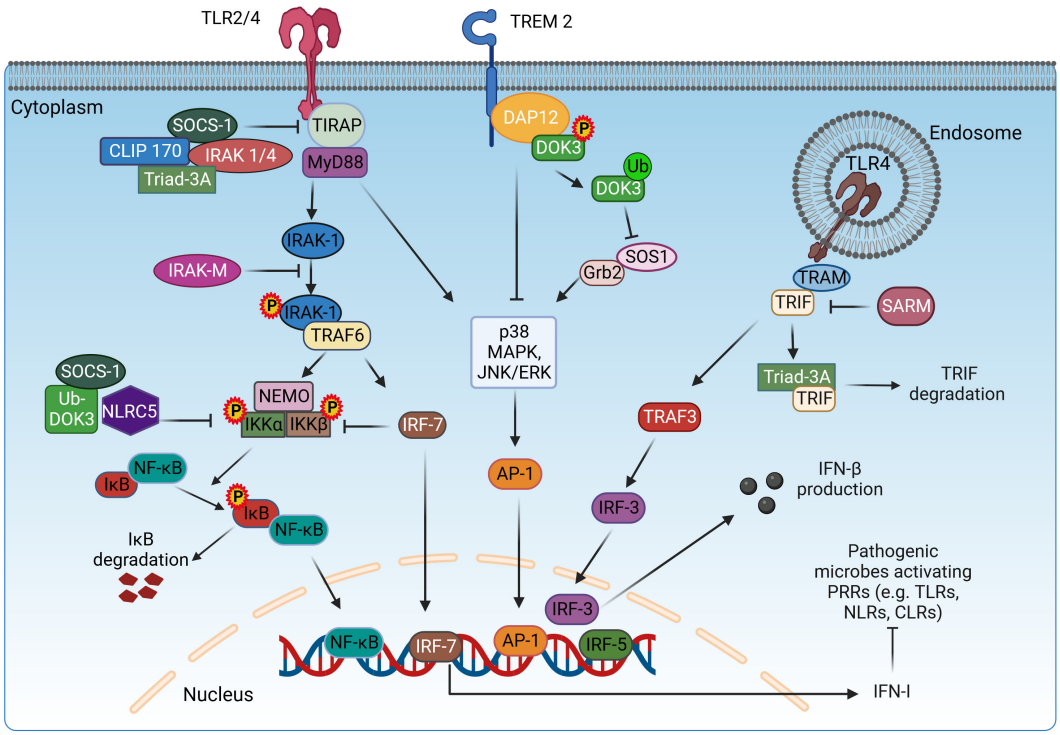

FIGURE 1
Adaptor proteins mediating the signaling of TLR receptors. TLR signaling is mediated *via* two pathways: MyD88-dependent and TRIF-dependent pathway. In the MyD88-dependent pathway, TIRAP mediates the interaction of MyD88 with TLR2/4 for the initiation of the signaling cascade. The adaptor proteins SOCS-1, IRAK1/4, CLIP170, and Triad3A inhibit the function of TIRAP. MyD88 recruits IRAK-1, which will be phosphorylated and bind to TRAF6. Once activated, TRAF6 acts as an E3 ubiquitin ligase, ubiquitinates, and activates NEMO, IKKα, and IKKβ to induce the phosphorylation of IκB and subsequent dissociation from NF-κB, resulting in the nuclear translocation of NF-κB. SOCS-1, Ub-DOK-3, NLRC5, and IRF7 inhibit the activation of IKKα/β, downregulating the NF-κB pathway. In parallel, MyD88 induces the p38, MAPK, and JNK/ERK signaling for the activation and translocation of AP-1 in the nucleus. In the TRIF-dependent pathway, TRIF indirectly interacts with TLR4 *via* TRAM and activates IRF3 *via* TRAF3, which translocates in the nucleus and induces the expression of IFN-β. The role of TRIF is inhibited by SARM. DAP12 associates with TREM2 upon stimulation and induces the phosphorylation and translocation of DOK-3 on the cell membrane, resulting in the inhibition of MAPK and ERK pathways. Meanwhile, ubiquitin-mediated degradation of DOK-3 leads to SOS1 degradation and inhibition of ERK pathway. TLR2/4, Toll-like receptor 2/4; TIRAP, TIR domain-containing adaptor protein; MyD88, myeloid differentiation 88; IRAK-1/4/M, interleukin-1 receptor-associated kinase 1/4/M; TRAF3/6, TNF receptor associated factor 3/6; NEMO, nuclear factor-κB essential modulator; IKKα/β, inhibitory kappa B kinase α/β; NF-κB, nuclear factor-κB; SOCS-1, suppressor of cytokine signaling-1; CLIP170, cytoplasmic linker protein 170; NLRC5, NOD-like receptor family caspase recruitment domain family domain containing 5; DOK-3, downstream of kinase 3; DAP12, DNAX-activating protein of 12 kDa; Grb2, growth factor receptor-bound protein 2; SOS1, Son of sevenless homolog 1; TRIF, TIR domain-containing adaptor-inducing interferon-β; TRAM, TRIF-related adaptor molecule; SARM, Sterile α and Armadillo motif-containing protein; DAB2, Disabled-2; IRF3/5/7, interferon regulatory factor 3/5/7; AP-1, activator protein 1; IFN-β, interferon-β; NLRs, nucleotide-binding oligomerization domain (NOD)–leucine-rich repeat (LRR)-containing receptors; CLRs, C-type lectin receptors. Created with BioRender.com.

4 Suppressor of cytokine signaling-1

SOCS-1 was discovered in 1997 (65) and referred to by several terms: SOCS-1, STAT-induced signal transducer and activator of transcription (STAT) inhibitor-1 (SSI1), structure and function of a new STAT-induced STAT inhibitor, and JAK binding protein (JAB) (66). In humans, SOCS-1 protein consists of 211 amino acids, while in rats and mice, it has 212 amino acids. The SOCS-1 protein consists of a central SH2 domain flanked by an amino terminal with a 12 amino acid kinase inhibitory domain/region and a carboxy terminal with an approximately 40 amino acid SOCS box. The KIR domain is critical for inhibition of the JAK2 kinase domain, the SH2 domain binds to phosphorylated tyrosine regions, and the SOCS box mediates ubiquitin-based proteasomal degradation through the formation of a functional E3 ligase enzyme (67). Originally, SOCS-1 was identified as a negative regulator of cytokine (68) and interferon signaling (69) by interfering with JAK/STAT signaling. SOCS-1 inhibits the JAK/STAT signaling pathway in three ways: the SH2 domain inhibits the kinase activity of JAK by binding to the kinase domain of JAK, binding of the phosphotyrosine residues of the cytokine receptor, and ubiquitination and degradation of activated JAK by the elongin-BC complex (67, 70). SOCS-1 interacts with IRAK-1 and NF-κB, promotes their degradation, and negatively regulates the MyD88-dependent TLR signaling pathway (Figure 1). SOCS-1 also regulates the LPS-induced TLR signaling pathway by acting on the MyD88-dependent and MyD88-independent signaling cascade (71) and by interacting with phosphorylated TIRAP, leading to its ubiquitination and further degradation. SOCS-1 inhibits phosphorylation of p65 by TIRAP and further activation of NF-κB (72). TRIF/TRAM mediates the MyD88-

independent pathway leading to the activation of NF-κB and interferon regulatory factor 3 (IRF3). IRF3 also activates interferon (IFN)-β, which activates STAT-1 *via* signaling through the IFN-α/β receptor. This is an indirect mechanism that inhibits TLR signaling *via* inhibition of the JAK/STAT signaling pathway induced by IFN-β (73). Overexpression of SOCS-1 reduces tyrosine phosphorylation, a step critical for activation of STAT-1 (74, 75).

In activated M2 macrophages, there is increased expression of SOCS-1 but not SOCS-3, and SOCS-1 polarizes macrophages toward an M2 phenotype (76, 77). Expression of SOCS-1 in macrophages inhibits palmitic acid- and LPS-induced signaling and protects mice from insulin resistance and systemic inflammation (78). Survival in IFN-γ−/− SOCS-1−/− or IFN-γ−/− SOCS-1+/− mice was reduced compared to that in wild mice. In SOCS-1−/− macrophages, there was increased production of TNF-α and NO and decreased endotoxin tolerance in response to LPS due to increased phosphorylation of I-κB and p38. NF-κB expression was decreased in macrophages overexpressing SOCS-1 (79). These studies suggest that SOCS-1 regulates LPS-stimulated macrophages independently of IFN-γ (79, 80).

SOCS-1 expression is remarkably high in the early stages of inflammation and decreases in later stages due to methylation of the SOCS-1 promoter region by DNA methyltransferase 1 (DNMT1), which silences SOCS-1 gene expression in LPS-treated crude macrophages. DNMT1 may be a potential target for inhibition of macrophage activation. Apart from inflammation, SOCS-1 also plays a role in cancer (81). Suppression of SOCS-1 can enhance antitumor immunity or promote tumor-promoting inflammation, depending on the cell type (82). Using the CRISPR-Cas9 method, the IRF1–SOCS-1 axis was found to inhibit CXCL9 expression and STAT1 signaling, thereby limiting antitumor immunity (83). However, the knockdown of the SOCS-1 gene in macrophages enhances anticancer inflammation and reduces tumor development (82).

5 Interleukin-1 receptor-associated kinase-M

IRAK-M belongs to the IRAK family but is known to lack kinase activity (84). IRAK-M is also popularly known as IRAK3 and has been studied in the negative regulation of inflammatory states associated with TLRs (Figure 1) (85, 86). IRAK-M is contrarian in function compared to the other members of the family. IRAK-M includes an N-terminal death domain (DD), a kinase domain (KD), and a C-terminal domain (CTD) that contains a conserved motif that helps bind to TRAF6. IRAK-M interacts with other proteins in the family through its DD. The lack of an aspartate residue in its active site has been postulated as the main reason for the lack of kinase activity (85, 87).

Its negative role in inflammatory signaling has been highlighted in several studies. In IRAK-M−/− mouse macrophages, the levels of pro-inflammatory cytokines IL-6, IL-12 p40, and TNF-α were found to increase significantly in response to LPS treatment compared to wild-type (WT) macrophages. Similarly, the effects of IRAK-M deficiency were also evident in the increased signaling *via* NF-κB, JNK, p38, and ERK in LPS-stimulated macrophages (86). TGF-β signaling is known to be involved in the expression of IRAK-M, as evidenced by detailed studies in human peripheral blood mononuclear cells (PBMCs) and mouse macrophage cell lines (88). Downregulation of M2 macrophage surface marker expression was clearly demonstrated under IRAK-M knockdown conditions (89). Regarding the role of IRAK-M in cancer, studies show that tumor cells induce the expression of IRAK-M on human monocytes *via* CD44 and TLR4, resulting in monocyte deactivation and decreased expression of pro-inflammatory cytokines (90). In a lung cancer mouse model, IRAK-M (−/−) mice injected with Lewis lung carcinoma cells exhibited reduced tumor growth compared with WT mice, whereas tumor-associated macrophages isolated from these mice expressed a stronger M1 phenotype. TGF-β signaling was found to promote IRAK-M expression in macrophages during lung tumor growth (88).

6 Downstream of kinase 3

DOK-3 belongs to the Dok family, which includes a total of seven members (Dok-1–7). DOK-3 is known to be involved in the regulation of tyrosine kinase-related signaling (91). Expression of Dok-3 is mainly observed in B cells, plasma cells, neutrophils, macrophages, and dendritic cells (92–95). Structural analysis revealed the presence of a C-terminal domain (190 amino acids), a central domain, and an N-terminal pleckstrin homology (PH) domain. Sequence analysis further revealed that among the three domains, the C-terminal domain varies, while apparent homology with Dok-3 and Dok-2 was found between the central and N-terminal domains (96). Dok-3 plays an important role as a scaffold in inflammatory processes because it lacks enzymatic activity. In neutrophil granulocytes, Dok-3 was found to suppress CARD9 signaling during fungal infection mediated *via* CLR (95). Dok-3 has been shown to regulate TLR4–ERK-mediated inflammatory response in response to LPS mediation and is also involved in DAP12-mediated inhibition of LPS-stimulated inflammatory signaling in macrophages (Figure 1). Dok-3 KO mice had higher mortality and serum TNF-α levels compared with WT mice exposed to LPS (94). Another study reported that NF-κB activation and production of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 were negatively correlated with Dok-3 upon LPS stimulation (97). In gliomas, it was shown that higher expression of DOK-3 strongly correlates with M2 macrophage markers and higher macrophage infiltration (98). Another research showed that vitamin 6 treatment of LPS-stimulated macrophages decreases the expression of pro-inflammatory cytokines, and this effect was abolished in DOK-3 KO macrophages but was enhanced upon overexpression of DOK-3 (99). Also, CpG-mediated ubiquitination and subsequent degradation of DOK-3 *via* interaction with TRAF6 leads to increased production of IL-6 and TNF-α in macrophages (100).

7 Interferon regulatory factor 7

The IRF family of mammals consists of nine members: IRF1–IRF9 (101, 102). IRF7 was originally discovered in the context of Epstein–Barr virus (EBV) infection and has since evolved to become a central controller of type I IFNs in response to pathogenic infections. It is triggered by activating signaling cascades initiated by pathogen recognition receptors (PRRs) that identify pathogenic genetic material. Abnormal formation of type I IFNs has been associated with a variety of diseases, such as malignancies and autoimmune diseases. Therefore, precise control of IRF7 expression and function is critical for the proper production of type I IFNs to maintain normal physiological functions mediated by IFNs. As shown by the process of phosphorylation, which serves as a clear indicator of its activation, post-translational modifications play a crucial role in controlling the activity of IRF7 (103). Activation of IRF7 is a prerequisite for its function as a transcription factor (103). In its resting state, inactive IRF7 is localized in the cytoplasm until activated. In response to pathogenic infection, phosphorylation of IRF7 is triggered, leading to its translocation from the cytoplasm to the nucleus. In the nucleus, IRF7 forms a transcriptional complex with other co-activators that binds to the promoter regions of specific genes and initiates their transcription (102) (Figure 1).

Intriguingly, the IRF family and the NF-κB family have co-evolved and share common evolutionary features. These families are both activated by signaling pathways originating from identical PRRs and IκB kinase (IKK). They also cooperate synergistically in regulating important cytokines such as IFN-β and together serve as central components in innate immune responses (104). The human IRF7 gene is located on chromosome 11p15.5 and is responsible for the production of four unique isoforms: IRF7A, IRF7B, IRF7C, and IRF7D. This discovery highlights the gene's remarkable ability to generate multiple variations, each with its own characteristic features. The human IRF7A (503 amino acids, 55 kDa) differs from its mouse counterpart, IRF7 (457 amino acids, 52 kDa). Notably, IRF7 and its closest relative, IRF3, play a critical role in regulating the type I interferon (IFN-α/β) response (102).

IRF7, a critical transcription factor, has significant regulatory power over the transition from M1 to M2 phenotype, a vital process. Moreover, in pathological interventions, induction of IRF7 expression by IFN-β1 proves to be an effective intervention that effectively attenuates the pro-inflammatory response of microglial cells after injury. By *in vivo* manipulation, activation of IRF7 expression in microglial cells after spinal cord injury resulted in a profound reduction in pro-inflammatory behavior, demonstrating its potent effect in attenuating inflammatory responses (105). IRF7 emerges as a central factor essential for triggering robust induction of type I IFN genes when TLR7 or TLR9 is stimulated. Through phosphorylation, TBK1 modifies IRF7 and converts it to its active form, enabling its participation in important cellular processes and promoting the production of IFN-responsive genes (102). For example, lidocaine inhibits H1N1 virus replication in macrophages by upregulating IFN-a4 *via* TBK1-IRF7 and JNK-AP1 signaling pathways (106). When primary macrophages were transduced with the active form of IRF7, the production of type I IFNs and their tumor-killing effect were increased (107). The potential explanation for IRF7's ability to exhibit different functions is linked to the specific contexts in which macrophages operate (tissue microenvironment), whether they involve the activation or inhibition of specific inflammatory pathways, which provides special power to IRF7 to exhibit transition (switching mechanism) from the profound M1 to the distinguished M2 phenotypic expression, making it the most critical transcription factor used in therapeutics. The roles may be ambiguous and influenced by the particular *in vivo* conditions, and the specific downstream targets of IRF7 can influence the overall macrophage phenotype.

8 NOD-like receptor family caspase recruitment domain family domain containing 5

NLRC5 belongs to the family of intracellular PRRs and NOD-like receptors (NLRs) (108). Immune cells such as monocytes, macrophages, and lymphocytes are known to express NLRC5 at high levels (109). These proteins contain a nucleotide-binding site (NBS) consisting of leucine-rich repeats (LRRs). Pathogens are recognized by the C-terminal domain of these protein molecules. Several domains such as the caspase recruitment domain (CARD), a pyrin domain (PYD), and the baculovirus inhibitor repeat domain (BIRD) together form the N-terminus (109, 110). Oligomerization and activation of NLRs depend on the central nucleotide-binding oligomerization domain. The localization of NLRC5 depends on its expression level; i.e., at elevated expression, it is located in the cytoplasm, whereas at physiological expression levels, it is present in the nucleus (109).

The protective role of NLRC5 in angiogenesis and intimal hyperplasia has been well studied (111, 112). It was observed that NLRC5 was able to downregulate NF-κB signaling in macrophages in coordination with heat shock protein 8 (HSPA8). In the absence of NLRC5, levels of IL-6 were found to increase in macrophages, leading to activation of cardiac fibroblasts (113). The effect of NLRC5 on the production of pro-inflammatory cytokines was also demonstrated in another study, in which overexpression of NLRC5 on macrophages downregulated the expression of IL-6 and TNF-α, whereas its suppression had the opposite effect. The JAK2/STAT3 pathway was shown to control the expression levels of NLRC5 (114). Several studies have shown that IFN-γ regulates the gene expression of NLRC5 upon infection (115, 116), but how NLCRC5 affects RIG-I and IFN response is still controversial. Priya et al. reported that through interaction domain mapping, NLRC5 interacts with RIG-I *via* its N-terminal DD and that NLRC5 enhances antiviral activity in a leucine-rich repeat domain-independent manner. This finding identifies a novel role for NLRC5 in RIG-I-mediated antiviral host responses against influenza virus infection, distinguished from the role of NLRC5 in MHC class I gene regulation (117). It was also shown that NLRC5 acts as a mediator of the IFN-mediated antiviral signaling pathway and that overexpression of NLRC5 activates the IFN-specific response and upregulates antiviral genes (116). In contrast,

another study showed that NLRC5 inhibits the NF-kB signaling pathway and negatively regulates the type I IFN signaling *via* interaction with the RIG-I and MDA5 (118). However, despite its effect on type I IFN signaling and RIG-I, the antiviral effect of NLRC5 can be supported by its role in promoting the activation of NLRP3 inflammasome. In addition to that, NLRC5 overexpression leads to increased activation of caspase-1, which converts pro-1L-1β to the active 1L-1β (119). Moreover, a recently published study demonstrates a novel NLRC5-mediated antiviral pathway for the inhibition of dengue virus infection. In this pathway, the antiviral effect of NLRC5 is exerted *via* the interaction of NLRC5 with the viral non-structural protein 3 (NS3) protease domain, followed by the ubiquitination of NS3 protease domain and degradation of NS3 through a ubiquitin-dependent selective macroautophagy/autophagy pathway (120). Overall, different pathways can mediate the role of NLRC5 in antiviral immune response, which still needs further investigation. In *Helicobacter*-modulated gastric inflammation and lymphoid formation, NLRC5 has been shown to function as a negative regulator. It was observed that significantly higher levels of cytokines and chemokines were produced in NLRC5−/− THP-1 macrophages under the influence of *Helicobacter pylori* than in WT THP-1 macrophages (121).

9 Disabled-2

DAB2 is a clathrin- and cargo-binding endocytic adaptor protein known for its multiple functions in signaling pathways regulating cellular migration, tumor suppression, and other important homeostatic biological activities. DAB2 may help promote immunological tolerance and reduce inflammatory responses (122). This can be validated by several studies. In myeloid cells, the absence of Dab2 promotes an inflammatory phenotype. Systemic inflammation was increased in Dab2-deficient bone marrow, as evidenced by higher serum levels of IL-6 and expression of inflammatory cytokines in the liver (123). Dab2 expression was found to be increased in M2 macrophages but decreased in M1 macrophages, and genetic deletion of Dab2 caused macrophages to develop a pro-inflammatory M1 phenotype (124). Deletion of Dab2 increased activation of TRIF-dependent interferon regulatory factor 3 and production of interferon-inducible genes and subsets of inflammatory cytokines (125) such as IL-12 and IL-6 (126). Dab2 acts as a negative immunological regulator of TLR4 endocytosis and signaling, suggesting a unique role for a Dab2-associated regulatory circuit in modulating macrophage inflammatory responses (125). In contrast, Dab2 has been shown to be involved in the activation of macrophages to the M1 phenotype during central nervous system (CNS) inflammation. It is associated with early activation of macrophages and astrogliosis during CNS inflammation (127). Dab2 promotes central nervous system inflammation by possibly increasing the expression of reactive oxygen species (ROS) in macrophages and microglia (128). One possible reason for Dab2's diverse roles could be that it interacts with various partners in distinct signaling pathways at different body regions. For example, in the CNS, it promotes M1 polarization, but in the liver, it promotes M2 polarization by reducing inflammatory cytokines. Thus, understanding the molecular signaling of Dab2 can be used for therapeutic purposes in various types of diseases.

10 Triad-3A

Triad3A is an E3 ubiquitin-protein ligase of the RING finger type that regulates macrophage activity *via* the mediation of TLR signals (129). Activation of macrophages toward the regulatory M2 type and prevention of their conversion to the M1 phenotype are its key roles (130). Triad3A has been shown to bind to and degrade the TLR4 adaptor proteins TIRAP, TRIF, and RIP1, limiting the release of pro-inflammatory cytokines (Figure 1). It was also discovered that Triad3A triggers K48-linked ubiquitination and degradation of TLR4 and TLR9, preventing the production of pro-inflammatory cytokines (129). The effect of Triad3A-mediated degradation of TLR4 and TLR9 by ubiquitination has also been studied in heart disease, where it was found to play a protective role in the development of cardiac hypertrophy and may improve cardiac function (131). In contrast, K48-linked ubiquitination and degradation of TLR4 triggered by Triad3A have a negative effect on mitochondrial bioenergetics and disease pathology in a model of diabetic cardiomyopathy (132).

In addition, it was discovered that inflammatory cytokine synthesis and necroptosis are limited by Triad3A-dependent necrosomal degradation (133). Through ubiquitin-mediated degradation of the tumor necrosis factor receptor-associated factor 3 (TRAF3) adaptor, Triad3A also has a negative effect on the RIG-I RNA-sensing pathway. Triad3A expression also inhibited the expression of type 1 interferon and antiviral genes by phosphorylating IRF3 and blocking its activation, thereby reducing inflammation (134). In addition to inflammation, Triad3A was also discovered to play a role in autophagy. In RAW264.7 and bone marrow-derived macrophages activated with LPS, it was also discovered that Triad3A interacts with and degrades Beclin-1 through ubiquitination, blocking TLR4-mediated autophagy (135). Thus, we could cure a number of diseases by using it in a variety of ways, such as targeting siRNA to disrupt Triad3A and limiting bacterial growth by triggering autophagy (136).

11 Cytoplasmic linker protein 170

CLIP170, an adaptor protein that controls the dynamics of the growing plus end of microtubules (MTs), consists of two conserved glycine-rich (CAP-Gly) domains of the cytoskeleton-associated protein and two tandem repeats of zinc knuckle motifs. It was found that CLIP-170 is a critical regulator of the stabilization of MT and that stabilized MTs play an important role in cell phagocytosis in activated macrophages (137). Actin polymerization events critical for phagocytosis are controlled by CLIP-170 *via* regulation of the recruitment of the actin core-forming protein form in mDia1 (138). It was discovered to play a role in modulating the anti-inflammatory form of macrophage activation. In one study, TLR4 signaling was found to be negatively regulated by CLIP170 by

targeting TIRAP (Figure 1). Silencing of CLIP170 enhanced LPS-induced production of pro-inflammatory cytokines, while overexpression of CLIP170 in mouse macrophages decreased the expression of pro-inflammatory cytokines, indicating its anti-inflammatory role (139). Pregnenolone hormone was also found to stimulate CLIP170-mediated ubiquitination, leading to increased degradation of TIRAP and TLR4 inhibition (140). In a separate study on the anti-inflammatory properties of SOCS-3, it was discovered that SOCS-3 interacts with the MT plus-end binding proteins CLIP-170 and CLASP2 *via* its N-terminal domain, resulting in the SOCS-3–CLIP-170/CLASP2 complex, which has anti-inflammatory properties (141). Therefore, through various studies, it has been discovered that CLIP170 plays an important role in polarizing macrophages toward the anti-inflammatory type and can be used for therapeutic purposes in a variety of chronic diseases.

12 Interleukin-1 receptor-associated kinase 1/4

IRAK1 and IRAK4 are threonine/serine kinases. The N-terminal death domains of MyD88, IRAK4, and IRAK2 help form a multimeric spiral signaling complex (myddosomes) (142, 143). Asymmetric trans-autophosphorylation of IRAK4 dimers recruited to the myddosome leads to the recruitment of IRAK1, which subsequently participates in extensive autophosphorylation and detaches from the myddosome (142, 144). TLR4/2 signaling is negatively regulated by IRAK1/4, which expresses an auto-active IRAK4 that causes TIRAP degradation (145) (Figure 1). This suggests a regulatory or anti-inflammatory effect of IRAK1/4, which consequently reduces the M1 phenotype. Several studies support its anti-inflammatory role. First, LPS-induced sepsis was alleviated by specific inhibition of IRAK1 (146). In another study, IRAK and Rip2 were found to be deregulated in sarcoidosis (147). Inhibition or silencing of IRAK1/4 reduced Ox-LDL-induced CD36 expression, thereby reducing the development of macrophage foam cells involved in the release of pro-inflammatory cytokines (148). In the same context of macrophage-derived foam cells, Ox-LDL inhibits LPS-induced expression of IFN-β by activation of IRAK1/4. Activated IRAK1/4 induces the mono-ubiquitination of TANK, which in turn will inhibit the recruitment of TBK1 to TRAF3 and the activation of IRF3 (149). LPS-induced formation of ROS was reduced by inhibiting IRAK1/4, which is located downstream of TLR (150). Thus, depending on the type of disease and condition, IRAK1/4 can be used for therapeutic purposes.

13 *Astragalus* polysaccharide

APS (formerly known as adaptor protein with PH and SH2 domains (SH2B2)) is a member of the Src homology 2 B (SH2B) family, which includes three members (SH2B1, SH2B2, and SH2B3). It exhibits a conserved configuration consisting of an initial dimerization domain (DD), a central pleckstrin homology domain (PH), and a terminal Src homology 2 (SH2) domain, all of which contribute to its structural integrity (151). The three components of the SH2B family were originally identified as signaling substances involved in immune cell stimulation (152–154). APS is a macromolecular substance obtained from *Astragalus membranaceus*, which consists of complex polysaccharides rich in α-(1 4) glycosidic bonds, with key constituents like glucan, glucose, galactose, and arabinose influencing its structure and function (155). APS exhibits a variety of properties, especially immune-enhancing, anti-inflammatory, and antioxidant properties (156–158), and is considered an immunostimulant to enhance human immune response (155, 156).

Studies have revealed that APS acts as a potent bio-immunomodulator, augmenting both non-specific and specific immune responses including a role as a vaccine adjuvant (159). *In vitro* experiments showed that APS prevented the conversion of LPS-stimulated THP-1 macrophages to the M1 phenotype. This effect was accompanied by a marked reduction in the formation of ROS and pro-inflammatory mediators (TNF-α, IL-6, and IL-12) and a phenotypic conversion toward M2 polarization, accompanied by the release of anti-inflammatory factors (IL-4, IL-10, and Arg-1) (160). According to Zhou et al., APS enhances the immunomodulatory properties of RAW 264.7 macrophages by activating the TLR4 and MyD88 signaling pathways (161). Further research suggests that APS functions as an immune receptor that specifically targets TLR4 in both mouse macrophages and B cells (162). APS suppresses TNF-α and IL-1β production by preventing NF-κB activation and attenuating phosphorylation of the ERK and JNK signaling pathways (163).

In addition, APS treatment showed a beneficial effect on endothelial cell proliferation while attenuating apoptosis. These results could be replicated *in vivo*, demonstrating the remarkable potential of APS in alleviating thoracic aortic complications in diabetic rats (160). Moreover, SH2B2 connects with the insulin receptor, promotes robust activation of the insulin signaling pathway, and supports its optimal functionality (164). In cell cultures, SH2B2 shows a compelling ability to bind to JAK2 *via* its SH2 domain, resulting in a strong enhancement of JAK2 activation (165). This mechanism contributes to the regulation of energy balance and body weight, particularly by affecting leptin and growth hormone signaling (165, 166).

14 Toll-interacting protein

TOLLIP is a universally expressed protein discovered in 2000 (167). It plays a prominent role as an adaptation molecule in the innate immune response *via* modulation of IL-1RI- and TLR2/4-mediated signaling pathways (168). The receptors IL-1R and TLRs have homologous cytosolic TIR domains that activate signaling pathways upon stimulation with IL-1 and LPS (169). Tollip was originally recognized as a mediator in IL-1 signaling. It has since been found to interact directly with TLR2 and TLR4 (170) and reduce inflammation (171). Structurally, Tollip has three domains with distinct functions and binding partners: the Tom1-binding domain (TBD) at the N-terminal, which is connected to the coupling of ubiquitin to the ER-degradation domain (CUE) through the conserved 2 domain (C2) consisting of 130 amino

acids (172). Of these three domains, the CUE domain plays a critical role in inhibiting the IL-1RI/TLR4 signaling pathways by interacting with the TIR domains of cell surface receptors and also with IRAK-1 and IRAK-2 proteins, thereby inhibiting autophosphorylation (173). Amino acid sequence 1–53 encodes TBD, which is responsible for interaction with clathrin, ubiquitin, and TOM1 during early endosomal interactions (174). The 130 amino acid C2 domain binds to phospholipids, preferably phosphoinositides, allowing Tollip to localize to cell membranes (175, 176).

In resting cells, Tollip is present in a complex with interleukin-1 receptor-associated kinase 1 (IRAK-1), and upon stimulation with IL-1β, the complex recruits to the cytoplasmic TIR domain of IL-1R *via* its accessory proteins. However, phosphorylation of IRAK by co-recruited adaptor molecules leads to complex breakdown. Phosphorylated IRAK is also associated with TRAF6, leading to activation of the NF-κB pathway (177). However, overexpression of Tollip leads to inhibition of NF-κB-mediated release of pro-inflammatory cytokines by inhibiting phosphorylation of IRAK1 (167). In addition, Tollip decreases IL-1-induced inflammation by causing endolysosomal degradation of ubiquitinated IL-1R1 (31) (Figure 2). Tollip also plays a critical role in the cyclic guanosine monophosphate adenosine monophosphate synthase stimulator of interferon genes (cGAS–STING) and acts as a stabilizer of STING, preventing its activation and thus that of the transcription factors IRF3 and NF-κB (178).

In inflammatory bowel disease (IBD), Tollip overexpression in peritoneal macrophages inhibits LPS-induced production of pro-inflammatory cytokines and increases anti-inflammatory cytokines, demonstrating the role of Tollip in macrophage polarization (179). A resveratrol derivative, RM, which is being studied for the treatment of inflammatory diseases, downregulates LPS-induced pro-inflammatory cytokine production, an effect mediated by increasing Tollip expression (180). In essence, Tollip is a negative regulator of acute inflammation. However, at a low dose of LPS, it translocates from lysosomes to mitochondria, increasing ROS levels and causing chronic inflammation (181). In addition to its important role in inflammation, Tollip is also involved in vacuolar trafficking, autophagy, and nuclear interactions (182).

15 β-Arrestin

β-Arrestins 1 and 2 are proteins that are widely distributed throughout the body and affect the signaling of G protein-coupled receptors (GPCRs). β-Arrestin 2 performs a critical function as a signaling adaptor and scaffold protein in modulating cellular inflammatory responses (183). Studies have linked β-arrestins to the signaling and activation of TLRs and genes (184–186). These adaptor proteins, β-arrestins 1 and 2, play a critical role in modulating the function of heterotrimeric guanine nucleotide-

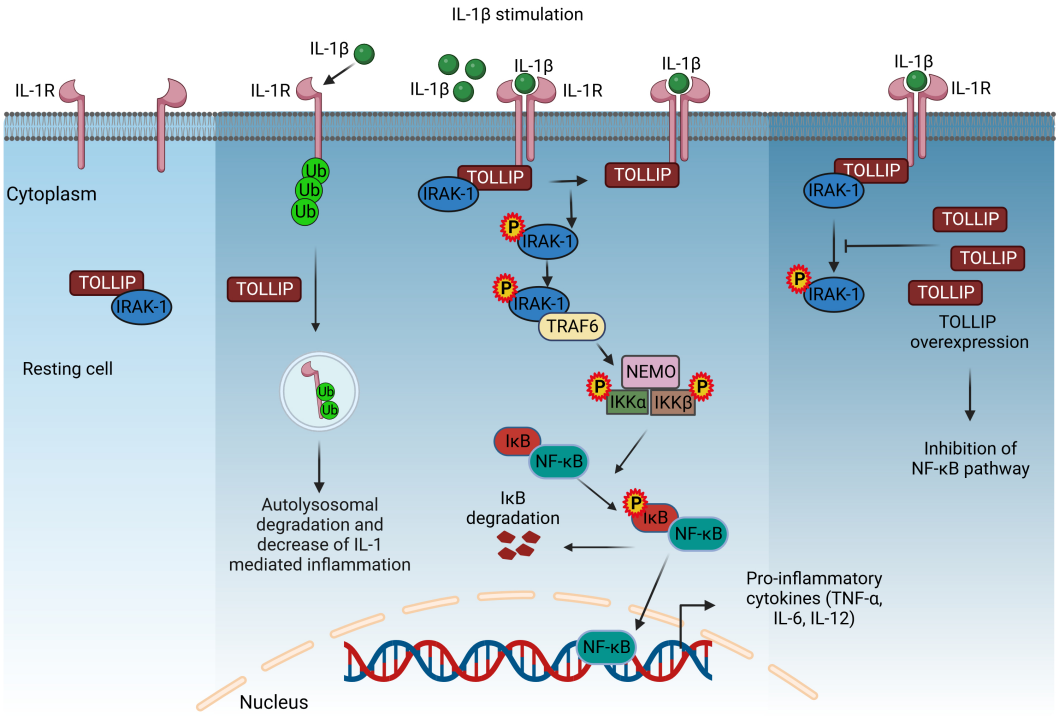

FIGURE 2
TOLLIP-mediated regulation of IL-1R signaling. In resting cells, TOLLIP forms a complex with IRAK-1, and upon stimulation with IL-1β, this complex translocates and binds to the TIR cytoplasmic domain of IL-1R. Co-recruited adaptor proteins induce the phosphorylation of IRAK-1, dissociation from the complex, and binding to TRAF6, which activates the NF-κB signaling, as described in Figure 1. TOLLIP decreases IL-1R-induced inflammation by promoting the endosomal degradation of ubiquitinated IL-1R. Overexpression of TOLLIP inhibits the NF-κB pathway and the expression of pro-inflammatory cytokines by inhibiting the phosphorylation of IRAK-1. IL-1β, interleukin 1β; TOLLIP, Toll-interacting protein; IRAK-1, interleukin-1 receptor-associated kinase 1; TRAF6, TNF receptor-associated factor 6; NEMO, nuclear factor-κB essential modulator; IKKα/β, inhibitory kappa B kinase α/β; NF-κB, nuclear factor-κB. Created with BioRender.com.

binding regulatory (G) proteins by forming complexes with GPCRs (186). Moreover, they are characterized as cytosolic proteins that facilitate the process of desensitization and internalization of activated G protein-coupled receptors (187, 188).

In addition, recent findings have demonstrated the role of β-arrestins 1 and 2 as scaffold/adaptor proteins in the activation of various MAPKs (183, 189). These include extracellular signal-regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinase (JNK), p38 kinases, and Src family kinases in the context of GPCR signaling. β-Arrestin 2 functions as a scaffold for several MAPK components, including JNK and ERK (190, 191). This function promotes phosphorylation, activation, and spatial concentration of MAPKs in specific cellular compartments, leading to their accumulation (191). MAPKs serve as important mediators in the signaling pathways of TLRs and play a central role in the transmission and transduction of TLR signals. MAPKs serve as vital intermediaries in the signaling pathways of TLRs, playing a pivotal role in transmitting and relaying the TLR signals (185). In addition to their involvement in the regulation of MAP kinases, studies have shown that β-arrestins also play a role in modulating the activity of NF-κB (184, 192, 193). Studies have provided evidence for the role of β-arrestin 2 in TLR signaling (183, 194). Specifically, studies have shown that overexpression of β-arrestins 1 and 2 in HEK cells stably expressing TLR4 leads to attenuation of NF-κB activation induced by LPS (186, 194).

In the absence of β-arrestin 2 (β-arrestin 2(−/−) mice), LPS administration resulted in increased expression of pro-inflammatory cytokines in bone marrow-derived macrophages (BMDMs). Moreover, LPS-induced mortality was increased in galactosamine-sensitized mice lacking β-arrestin 2. These comprehensive observations provide compelling evidence that β-arrestins serve as indispensable negative modulators of innate immune activation *via* TLRs (195). Another interesting study demonstrates that the production of IL-6 in polymorphonuclear leukocytes (PMNs) stimulated by LPS was significantly increased in β-arrestin 2 (−/−) mice compared to their wild-type counterparts (+/+). Therefore, β-arrestin 2 serves as an inhibitory regulator of pro-inflammatory mediator production in PMNs (196). Moreover, the absence of β-arrestin 2 negatively affects the formation of IL-10 in response to LPS stimulation (185).

In a remarkable study, researchers demonstrated a direct interaction between β-arrestin and TRAF6 in the activation of TLRs or IL-1 receptors. This interaction serves to prevent autoubiquitination of TRAF6 and thus inhibit subsequent activation of NF-κB and the AP-1 signaling pathway (197). Moreover, β-arrestin 2 shows the ability to directly interact with IκB-α, thereby effectively preventing the phosphorylation and degradation of IκB-α. Consequently, this interaction effectively inhibits the activation of NF-κB (194). In addition to their above functions, both β-arrestin 1 and β-arrestin 2 play a role in cardiac function, as they are expressed in cardiac tissue and have been associated with the regulation of normal cardiac function. In the infarcted heart, a remarkable upregulation of β-arrestin 2 expression was found specifically in infiltrated macrophages, where it exerts a suppressive effect on inflammatory responses. Interestingly, mice lacking arrestin 2 (KO) show a higher mortality rate after myocardial infarction than wild-type mice. Furthermore, the absence of arrestin 2 (KO) in mice resulted in increased production of inflammatory cytokines. These results provide compelling evidence that infiltrated macrophages in β-arrestin 2 KO mice elicit an exaggerated inflammatory response in the infarct region (187).

Upon binding of an agonist to GPCRs, a group of enzymes known as GRKs, comprising seven homologs, phosphorylates intracellular threonine or serine residues of GPCRs. This phosphorylation process enables the recruitment of β-arrestins to agonist-activated GPCRs (198). Due to their ability to prevent G protein activation by steric hindrance and their interaction with clathrin and adaptin, β-arrestins contribute to the desensitization and internalization of GPCRs *via* clathrin-coated pits (199). Apart from their involvement in regulating GPCRs, recent studies have shed light on the ability of β-arrestin to function as a signal transducer (198, 199). As a result, β-arrestins have emerged as promising targets for potential therapeutic interventions in cardiovascular diseases such as heart failure (200, 201).

16 LnK

LnK (SH2B3) belongs to the SH2B family of adaptor proteins, along with SH2B1 and SH2B2 (202). This family shares a common structural framework characterized by proline-rich regions, pleckstrin homology (PH), SH2 domains (202, 203) responsible for binding phosphotyrosine in target proteins, and an N-terminal dimerization domain with a phenylalanine zipper pattern (204). This pattern facilitates the formation of homo- and hetero-dimers among SH2B family members (204). LnK plays a central role as a dynamic adaptor protein in modulating a variety of signaling pathways orchestrated by Janus kinases (JAKs) (Figure 3) and receptor tyrosine kinases (RTKs) (202, 203). The involvement of adaptor proteins that bind to both RTKs and JAKs is of great importance in controlling the intricate network of cytokine signaling pathways (205). Without their own enzymatic activity, these adaptor proteins assume the central role of molecular platforms that skillfully orchestrate and harmonize a variety of signaling events (206). The SH2B family, which includes members of the Src homology 2 proteins, emerges as a dynamic group of adaptor proteins involved in a diverse array of signaling pathways. In mice lacking SH2B family members, the absence of LnK results in the most striking abnormalities in hematopoiesis, highlighting its profound importance in this biological process (205, 206). Another study also indicated the crucial role of LnK (SH2B3) in maintaining normal hematopoiesis as a key negative regulator of cytokine signaling (204).

Lnk forms a direct physical association with c-Fms, effectively attenuating its activity, which includes regulation of macrophage progenitor cell proliferation, macrophage colony-stimulating factor (M-CSF)-induced migration, and production of ROS (Figure 4).

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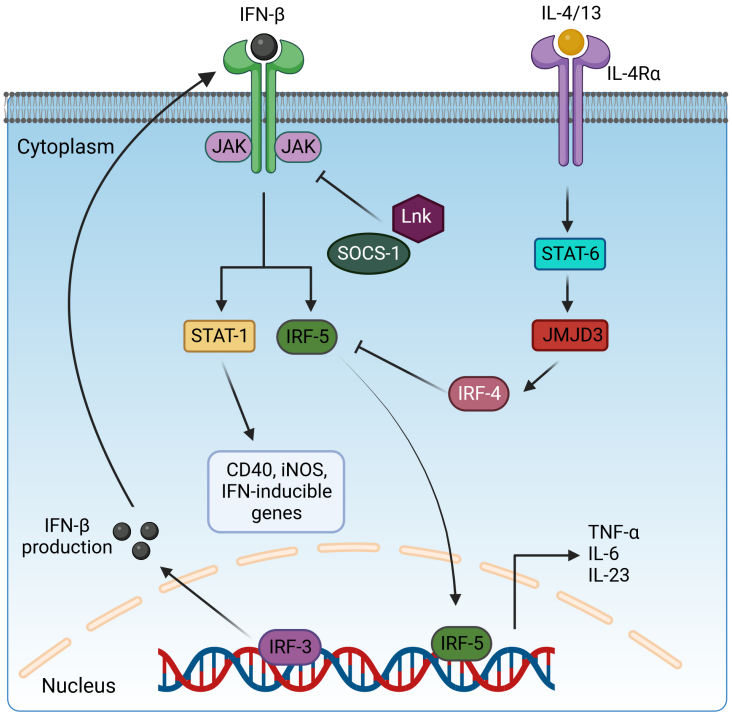

FIGURE 3
Regulation of pro-inflammatory cytokine expression by interleukin receptors *via* STAT and IRF transcription factors. Activated IRF3 translocates in the nucleus and induces the expression IFN-β. IFN-β is secreted and binds to interferon receptors and activates the JAK/STAT1 signaling to drive the expression of CD40, iNOS, and IFN-inducible genes and IRF5 for pro-inflammatory cytokine production. However, this pathway is negatively regulated by the inhibitory adaptor proteins LnK and SOCS-1, which interfere with STAT1 activation, and IRF4, which blocks IRF5 signaling. IRF4 is induced *via* the IL-4/13/STAT6/JMJD3 cascade. IFN-β, interferon β; JAK, Janus kinase; STAT-1/6, signal transducer and activator of transcription-1/6; IL-4/13/6/23, interleukin-4/13/6/23; IRF3/4/5, interferon regulatory factor 3/4/5; SOCS-1, suppressor of cytokine signaling-1; JMJD3, Jumonji domain-containing protein-3; CD40, cluster of differentiation 4; iNOS, inducible nitric oxide synthase. Created with BioRender.com.

The M-CSFR (c-Fms) contributes in complex ways to the control of macrophage proliferation, differentiation, and survival, exerting a critical influence on the regulation of various macrophage functions. Upon interaction with the M-CSF ligand, c-Fms undergoes tyrosine phosphorylation, resulting in docking sites for molecules with SH2 domains. Phosphorylation of Akt was enhanced and sustained in mouse macrophages with LnK knockout in response to M-CSF, whereas phosphorylation of ERK was significantly attenuated. Moreover, Lnk effectively suppressed the migration response of macrophages triggered by M-CSF. After stimulation with zymosan, LnK knockout mouse macrophages showed a remarkable increase in the production of ROS in an M-CSF-dependent manner, highlighting the central role of Lnk in regulating the formation of ROS in response to zymosan-induced activation. Lastly, the absence of LnK led to significant alterations in the cytokine production of macrophages, emphasizing the critical role of Lnk in modulating the immune response and influencing the release of inflammatory cytokines by these vital immune cells (207). LnK plays a crucial role as a negative modulator of TNF signaling, effectively attenuating the pro-inflammatory phenotype (203). Overall, manipulating the expression levels of LnK in macrophages offers a distinctive therapeutic strategy to enhance the innate host defenses, representing a novel approach for potential clinical intervention (207).

Lnk expression levels also correlate with metabolic diseases. Obesity has been shown to lead to a decrease in Lnk expression in immune cells. Deficiency of LnK triggers adipose tissue inflammation and disrupts glucose tolerance, indicating its central role in maintaining the balance of adipose tissue inflammation and ensuring optimal glucose regulation. Through comprehensive qPCR analysis, investigators examined the expression of inflammatory cytokines in adipose tissue and found a significant increase in the levels of IL-1β, IL-12, TNF, and IFN-γ in LnK−/− mice, highlighting the central role of LnK in modulating the inflammatory cytokine profile in adipose tissue (208). Moreover, investigations have demonstrated that Lnk-deficient mice display impaired glucose tolerance and diminished insulin responses, highlighting the critical involvement of LnK in governing these essential metabolic functions (208). The inhibitory effect of the SH2 domain is widely recognized, and its efficacy can be abrogated by the specific point mutation R392E, highlighting the critical importance of this mutation (207). A specific genetic variation in the LnK/SH2B3 gene, known as a missense variant, has been linked to an increased risk of several autoimmune diseases, including diabetes. LnK plays a central role in regulating the ability of dendritic cells (DCs) to modulate the fate of Th1 or regulatory T cells (Treg) in response to stimulation by granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-15, underscoring its

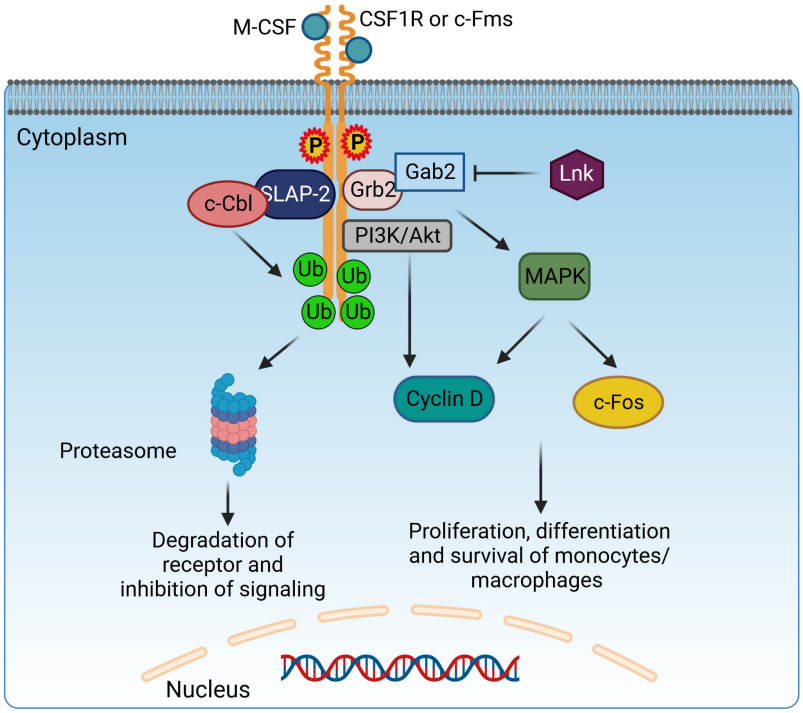

FIGURE 4
Adaptor protein SLAP-2 regulates CSF1R-mediated differentiation and survival of macrophages. Binding of M-CSF to CSF1R (c-Fms) leads to dimerization and tyrosine phosphorylation of CSF1R, generating docking sites to recruit adaptor molecules possessing SH2 domains, like Grb2. Grb2 binds with Gab2 to activate PI3K/Akt and MAPK signaling and induce the proliferation, differentiation, and survival of monocytes/macrophages *via* Cyclin D and c-Fos. Lnk has an inhibitory effect on this pathway. However, SLAP2 can bind both c-Fms and c-Cbl and induce c-Cbl-dependent ubiquitination, internalization, and degradation of the receptor, therefore inhibiting the CSF1R signaling pathway. CSF1R, colony-stimulating factor-1 receptor; c-Fms, colony-stimulating factor-1 receptor; SLAP2, Src-like adaptor protein 2; Grb2, growth factor receptor-bound protein 2; Gab2, GRB2-associated-binding protein 2; MAPK, mitogen-activated protein kinases. Created with BioRender.com.

essential function in orchestrating this complex immune response (209).

17 Interferon regulatory factor 4

IRFs are intracellular proteins that play a critical role in macrophage polarization and immune cell maturation (210, 211). The IRF family consists of nine members, of which IRF1, IRF5, and IRF8 drive the macrophage into the M1 phenotype, and IRF3 and IRF4 switch the macrophage into the anti-inflammatory M2 phenotype (210). IRF4 is a 450 amino acid long transcription factor consisting of a conserved 115 amino acid long DNA-binding domain (DBD) at the N-terminal and IRF association domains (IADs) at the C-terminal (212). The IAD is also associated with an autoinhibitory region at the C-terminus that binds to the DBD and regulates its interaction with DNA (212, 213). The IAD is connected to the DBD *via* a flexible linker. The DBD forms a helix-loop-helix motif with the help of the conserved five tryptophans, which mediates binding to DNA by recognizing GAAA and AANNNGAA sequences (210). The IAD helps interact with other interferons and transcription factors (212).

IRF4 functions as an endogenous antagonist of TLR signaling and competes with IRF5 for binding to the adaptor protein MyD88, suppressing the M1 polarization of macrophages (214, 215). Overexpression of IRF4 increases the release of the anti-inflammatory cytokines IL-4 and IL-10 (216), and in the same way, M2 marker genes (Arg1, Ym1, and Fizz1) are decreased in IRF4 deficiency (217). IRF4-deficient mice are more susceptible to LPS-induced inflammation with increased release of pro-inflammatory cytokines such as TNF-α and IL-6. In IRF4-deficient macrophages, NF-κB and JNK pathway-based cytokine production was increased after stimulation with LPS (218). During helminth infection, the Jumonji domain containing-3 (Jmjd3) methylates IRF4, a step required for its induction (217). Jmjd3 is regulated by STAT6 signaling induced by IL-4. Thus, M2 macrophage polarization is controlled by STAT6-Jmjd3-IRF4 signaling (Figure 3) (219). Another publication indicates that the Jmjd3-IRF4 axis plays a role in M2 polarization induced by IL-13 through the NOTCH4 pathway (220).

18 Src-like adaptor protein

SLAP has a similar structure to Src Homology2 (SH2) and Src Homology3 (SH3) and contains Grb2, Nck, and Crk domains. However, unlike the Src family, SLAP lacks a catalytic tyrosine kinase domain (221). The SH3–SH2 domain facilitates protein–

protein interactions by recognizing phosphorylated tyrosine- or proline-rich sequences. The SH3–SH2 junction sequence is shorter in SLAP/SLAP2 than in Src kinases, resulting in a closer association of Src domains through continuous β-sheet formation (222). The SH2–SH3 domains are flanked on one side by amino terminals and on the other side by unique carboxyl terminals. The myristoylated N-terminus helps associate with membranes, while the non-myristoylated N-terminus isoform is localized in the nucleus (223). The most studied SLAP proteins are SLAP1 and SLAP2, both of which differ in size; they have a difference of 15 amino acid residues. SLAP1 has 276 amino acid residues, while SLAP2 contains 261 amino acids (223, 224). SLAP acts mainly as a negative regulator on intracellular signaling pathways in macrophages as well as in B (222) and T lymphocytes (223, 225, 226). SLAP interacts with cell surface receptors, recruiting a ubiquitin machinery that leads to receptor degradation, thereby inhibiting signal transduction (221). SLAP2 genes are expressed in BMDMs (227). SLAP2 regulates signal transduction of a tyrosine kinase receptor, colony-stimulating factor-1 receptor (CSF-1R) (221, 228). The ligand for CSF-1R is colony-stimulating factor-1 (CSF1), which regulates macrophage growth and differentiation (229). The binding of CSF1 to the tyrosine kinase receptor CSF-1R leads to its dimerization and phosphorylates tyrosine residues, further mediating downstream signaling *via* the recruitment of SH2 adaptor protein and PTB proteins (230). c-Cbl downregulates CSFR-1 signaling. The SH2 domain of c-Cbl binds to an activated tyrosine kinase, and the RING finger domain has ubiquitin ligase activity, leading to ubiquitination and eventual degradation of the receptor (231) (Figure 4). In BMDMs, the association of SLAP2, c-Cblans, and CSF-1R receptor is necessary for the inhibition of downstream signaling. SLAP2 binds with c-Cbl *via* its unique carboxy-terminal tail (227). In FD-Fms cells, the dominant-negative SLAP2 mutant inhibits the binding of c-Cbl to the receptor, thereby inhibiting receptor ubiquitination, internalization, and degradation. SLAP2 plays a role in the recruitment of c-Cbl to activated CSF-1 receptors and the consequent downregulation of CSF-1R signaling by promoting the internalization and degradation of activated receptors (228).

19 G protein-coupled receptor 108

Sophisticated organisms have evolutionarily evolved their intrinsic and acquired defense mechanisms, enabling them to combat foreign substances and pathogens with maximum efficiency. GPR108, a member of the seven-transmembrane protein (7TM) domain family, exerts potent stimulation of NF-κB signaling when overexpressed. Remarkably, its role in a physiological setting proves to be antagonistic to signaling pathways initiated by TLRs (232). Originally identified as lung 7TM receptor-1 and receptor-2 (LUSTR1 and LUSTR2), these genes have since been renamed GPR107 and GPR108, respectively, by the HUGO Gene Nomenclature Committee. The human GPR107 gene with 18 exons is located at locus 9q34.2-3 and covers an area of 86.4 kb. The corresponding cDNA encodes a protein with 552 amino acid residues. In contrast, the mouse GPR108 gene, which is closely related but lacks homology, contains 17 exons and is located in region 17C-D, spanning a shorter length of 12.8 kb (233). The most closely related mammalian ortholog, GPR107, has 49% similarity in the amino acid sequence compared to GPR108 in mice (232).

GPR108 is considered one of the major candidates among candidate genes involved in innate immunity, further highlighting its significant role in this important defense mechanism (234). One possible mechanism is the interplay of GPR108 and TLR4 mediating the interaction with MyD88, modulating the E3 ligase TRAF6 to facilitate ubiquitination of MyD88. Of note, the immunostimulatory function of GPR108 is limited by the expression of TIRAP. According to Donget et al., cells from mice lacking GPR108 show increased secretion of cytokines and enhanced activation of NF-κB and IRF3 signaling pathways. Conversely, GPR108-deficient macrophages, in which GPR108 is restored, show decreased signaling responses. Joint expression of TLRs and GPR108 results in decreased activation of the NF-κB and IFN-β promoter, in contrast to expression of TLRs or GPR108 alone. Upon activation of TLRs, the amount of GPR108 increases, resulting in its interaction with TLRs and their antagonists suppressing the expression of MyD88 and hindering its binding to TLR4 by blocking the ubiquitination of MyD88 and GPR108 negatively regulating MyD88. Of note, the antagonistic effect on GPR108 is exerted by TIRAP, an adaptor protein essential for TLR and MyD88 signaling (232).

20 c-Cbl-associated protein

CAP or c-Cbl-associated protein, also known as sorbin and SH3 domain containing (Sorbs1), belongs to the sorbin homology (SoHo) family of adaptor and scaffold proteins. It is abundantly expressed in immune system cells as well as in cardiac tissue, adipose tissue, and skeletal muscle. CAP plays an important role in regulating cell adhesion, migration, cytoskeletal element reorganization, membrane trafficking, and intracellular signal transduction (235, 236). It also has a protective antiviral function in coxsackievirus virus B3 (CVB3)-induced myocarditis (235). CAP promotes type I IFN production while limiting the release of cytotoxic cytokines, thereby setting a balanced and non-harmful antiviral response. In a recent study by Vdovenko et al., it was shown that the expression of pro-inflammatory cytokines in mouse fibroblasts, cardiomyocytes, and myeloid leukocytes was downregulated after stimulation of TLR. CAP attenuated the expression of pro-inflammatory cytokines, such as IL-6, by limiting the phosphorylation of inhibitor of kappa B (IκB) kinase (Iκκ)-α and Iκκ-β in addition to inhibiting their NF-κB-dependent downstream signaling pathway. The presence of molecular affinity between CAP and Iκκ-α/Iκκ-β was a critical factor in disrupting the NF-κB pathway. CAP is thus an efficient adaptor molecule in inhibiting the NF-κB pathway (237).

21 Src kinase-associated phosphoprotein 2

The Src kinase-associated phosphoprotein (SKAP) proteins consist of SKAP1 [also referred to as SKAP55 (Src kinase-associated phosphoprotein of 55 kDa)] and its homolog SKAP2 [also referred to as SKAP-HOM (SKAP55-homolog) or SKAP-55R [SKAP-55-related]]. Both proteins have the same domain composition as a dimerization domain (DM), a pleckstrin homology domain (PH), and a C-terminal Src homology 3 domain (SH3). At the protein level, they are 44% identical, mainly in their PH and SH3 domains. Human SKAP1 contains three tyrosine-based signaling motifs at amino acid positions 219, 232, and 271 in the inter-domain, while human SKAP2 has only two motifs at amino acids 261 and 298 (238). SKAP2 is abundantly expressed in macrophages (239) as well as in T and B lymphocytes (238). It is a cytosolic adaptor protein found primarily in macrophages and plays an important role in cytoskeletal reorganization, macrophage migration, and chemotaxis.

An interesting study revealed the role of SKAP2 on macrophage podosome formation for the promotion of tumor invasion and metastasis. According to that study, peritoneal macrophages that are derived from SKAP2 null mice have lower invasive ability compared to those from the wild type. Also, injection of lung cancer cells in these mice leads to less lung metastasis, marked by lower macrophage infiltration in the tumor. Furthermore, when SKAP2 null macrophages were injected, the macrophage tumor infiltration and growth were reduced (240). It was observed that deficiency of SKAP2 in mice with colitis resulted in increased LPS-induced inflammation and tumorigenesis. Deletion of SKAP2 in colitis-induced mouse models demonstrated activation of the NF-κB pathway along with upregulation of cytokines such as TNF-α, CXCLs, and interleukins. Thus, consistent expression of SKAP2 is required to reduce inflammatory signal transduction in macrophages triggered by the uptake of exosomes from cancer cells. SKAP2 formed a complex with SHP-1 tyrosine phosphatase *via* association with the Sirpα transmembrane receptor. SKAP2 is also physically associated with the TIR domain of MyD88, TIRAP, and TRAM, adaptors of TLR4. SKAP2-mediated recruitment of the Sirpα/SHP-1 complex to TLR4 attenuated inflammatory responses, whereas direct interaction of SKAP2 with SHP-2 reduced activation of SHP-2. SHP-2 is required for efficient NF-κB activation and suppresses the TRAM/TRIF-IFN-β pathway; therefore, SKAP2-mediated SHP-2 inhibition affected two signaling axes of TLR4. Therefore, TLR4-NF-κB signaling is blocked and TLR4-IFN-β signaling is activated by SHP-1 and SHP-2 of SKAP2, effectively inhibiting inflammation-mediated tumorigenesis (239). In an *in vivo* study of atherosclerosis, SKAP2 was also found to be required for the expression of M2 polarization markers in addition to its athero-protective effects. Because SKAP2 binds to Sirpα, it is possible that Skap2 affects the interaction between CD47 and Sirpα (the "don't eat me" signal) to promote efferocytosis by preventing antiphagocytosis. Although previous studies have found that blocking CD47 signaling to macrophages can increase efferocytosis and attenuate atherosclerosis, the mechanism remains to be explored (241). The connective role of Sirpa with SKAP2 was revealed in one more study, where it was shown that Sirpa is necessary for SKAP2 recruitment to engaged integrins and for regulating the downstream signaling of actin reorganization and cytoskeletal rearrangement, the critical steps for macrophage migration, chemotaxis, and phagocytosis (242).

22 Conclusion

Macrophages are central players in the innate immune response. Macrophage polarization, i.e., the dynamic adaptation of phenotypes, plays an important role in maintaining tissue homeostasis, tissue injury, and repair mechanisms by altering the tissue microenvironment. Adaptor molecules possess a number of domains that play a central role in the recruitment and transmission of inflammatory responses through intricate signaling cascades. They are also responsible for polarizing macrophages into two distinct states: the fierce M1 warriors, which play a pro-inflammatory role, and the calming M2 defenders, which are responsible for fighting inflammation. Several adaptor proteins reprogram macrophages to an anti-inflammatory M2 phenotype and thus control inflammation. Here, we have provided an overview of the role of adaptor molecules in impeding the inflammatory response and the associated signaling pathways, which are summarized in Table 1. Understanding the interactions of adaptor molecules in macrophage polarization is critical for elucidating the signaling pathways associated with inflammatory diseases and cancer and for developing novel therapeutic strategies. Based on the current findings mentioned above about adaptor proteins, we still need to understand more about their molecular mechanisms.

TABLE 1 Function of adaptor proteins in macrophage signaling pathway.

Adaptor protein	Function
SARM	Negative regulator of TLR3 and TLR4 (inhibits TRIF-dependent signaling).
DAP12	DAP12-deficient macrophages produced higher concentrations of inflammatory cytokines in response to a variety of pathogenic stimuli.
SOCS-1	Binds to TIRAP leading to U&PD and blocks the TLR4 and TLR2 signaling mainly.
IRAK-M	Negative regulator of TLR signaling. Prevents dissociation of IRAK-1/4 from MyD88. Hence, no formation of IRAK-TRAF6 complex reduced pro-inflammatory cytokine production.
DOK-3	Blocks LPS signaling in macrophages and reduces NF-κB activation.
IRF7	TIRAP binds with IRF7 and ceases its activation to block specifically IFN-β production.
NLRC5	Negative regulator of TLR signaling through IKK inhibition.
DAB2	Negative regulator of LPS stimulated TLR4 signaling.

(Continued)

TABLE 1 Continued

Adaptor protein	Function
Triad-3A	Promotes downregulation of two TIR domain-containing adapter proteins, TIRAP and TRIF.
CLIP170	Binds to TIRAP.
IRAK-1/4	When TIRAP binds to MYD88, then these act as signal-transducing relay molecules. However, when TIRAP directly interacts with IRAK-1/4, this leads to U&PD and finally leads to downregulation of inflammatory response mediated *via* TIRAP.
APS	Inhibit JAK signaling.
TOLLIP	Inhibitor of MyD88-dependent signaling cascade.
β-Arrestin	The interaction between β-arrestins and IκBα; TRAF6 inhibits NF-κB activity induced by inflammatory cytokines.
LnK	Inhibitor of TNF alpha-dependent pathway.
IRF4	Interacts with MyD88. Acts as negative regulator of TLR signaling. Competes with IRF5 for MyD88 interaction.
SLAP	Negative regulator of TCR signaling.
GPR108	Competes with TLR4 to bind to MyD88; in turn, the expression of GPR108 as an immune activator is restricted by TIRAP.
CAP	Inhibitor of NF-κB pathway.
SKAP2	Interacts with and activates SHP-2, therefore inhibiting NF-κB pathway, but activates TRIF/IFN-β pathway. Interacts with Sirpa and regulates macrophage phagocytosis.

SARM, Sterile α- and Armadillo motif-containing protein; DAP12, DNAX-activating protein of 12 kDa; SOCS-1, suppressor of cytokine signaling-1; IRAK-M, interleukin-1 receptor associated kinase-M; DOK-3, downstream of kinase 3; IRF7, interferon regulatory factor 7; NLRC5, NOD-like receptor family caspase recruitment domain family domain containing 5; DAB2, Disabled-2; CLIP170, cytoplasmic linker protein 170; IRAK-1/4, interleukin-1 receptor-associated kinase 1/4; APS, Astragalus polysaccharide; TOLLIP, Toll-interacting protein; IRF4, interferon regulatory factor 4; SLAP, Src-like adaptor protein; GPR108, G protein-coupled receptor 108; CAP, adaptor protein c-Cbl-associated protein; SKAP2, Src kinase-associated phosphoprotein 2.

Blocking or inhibiting adaptor proteins can shed more light on their role in regulating macrophage activation. There are certain pharmacological agents that act on adaptor molecules and are currently used in clinical trials as agonists of the adaptor molecule, playing an essential role in therapeutic purposes. For example, celastrol is one such drug that enhances the SARM expression and helps in decreasing the effect of incision-associated inflammation (243). Resveratrol is a drug that enhances SOCS-1 expression and helps in the inhibition of microglial activation (244). Pregnenolone is a drug that enhances CLIP170 expression during the degradation of TIRAP and TLR4 suppression (140). Glucocorticoids are drugs that suppress inflammation *via* the upregulation of IRAK-M (245). Similarly, other adaptor molecules can be targeted with pharmacological inhibitors to dampen not only the inflammatory response but also cancers that are closely associated with inflammation. Researchers are exploring the potential role of inflammation in many aspects of cancer, including the spread of the disease within the body and the resistance of tumors to treatment. Moreover, investigating adaptor functions in macrophage crosstalk with other immune cells and tumor cells would provide the tools to better understand cancer progression and design more precise therapeutic interventions.

Author contributions

MB: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. SpB: Writing – review & editing. ShB: Writing – review & editing. AW: Writing – review & editing. NH: Writing – review & editing. RA: Writing – review & editing. RK: Writing – review & editing. RSh: Writing – review & editing. SS: Writing – review & editing. RSa: Writing – review & editing, Conceptualization, Funding acquisition, Writing – original draft.

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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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Human tissue-resident peritoneal macrophages reveal resistance towards oxidative cell stress induced by non-invasive physical plasma

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In the context of multimodal treatments for abdominal cancer, including procedures such as cytoreductive surgery and intraperitoneal chemotherapy, recurrence rates remain high, and long-term survival benefits are uncertain due to post-operative complications. Notably, treatment-limiting side effects often arise from an uncontrolled activation of the immune system, particularly peritoneally localized macrophages, leading to massive cytokine secretion and phenotype changes. Exploring alternatives, an increasing number of studies investigated the potential of plasma-activated liquids (PAL) for adjuvant peritoneal cancer treatment, aiming to mitigate side effects, preserve healthy tissue, and reduce cytotoxicity towards non-cancer cells. To assess the non-toxicity of PAL, we isolated primary human macrophages from the peritoneum and subjected them to PAL exposure. Employing an extensive methodological spectrum, including flow cytometry, Raman microspectroscopy, and DigiWest protein analysis, we observed a pronounced resistance of macrophages towards PAL. This resistance was characterized by an upregulation of proliferation and anti-oxidative pathways, countering PAL-derived oxidative stress-induced cell death. The observed cellular effects of PAL treatment on human tissue-resident peritoneal macrophages unveil a potential avenue for PAL-derived immunomodulatory effects within the human peritoneal cavity. Our findings contribute to understanding the intricate interplay between PAL and macrophages, shedding light on the promising prospects for PAL in the adjuvant treatment of peritoneal cancer.

KEYWORDS

non-invasive physical plasma (NIPP), cold atmospheric plasma (CAP), plasma-activated media (PAM), plasma-treated solutions (PTS), human primary macrophages, immune response, peritoneal cavity, peritoneal cancer

1 Introduction

Non-invasive physical plasma (NIPP), a highly reactive gas at near room temperature, can be applied directly to solids (direct treatment) or transferred from gas to liquid phase (indirect treatment) to propagate plasma-activated liquids (PAL) (1, 2). Biologically active reagents (e.g., reactive oxygen and nitrogen species, RONS) are formed at the interface of plasma discharge, surrounding air and the target (3), inducing dose-dependent anti-proliferative, selective anti-tumoral and wound healing or regenerative effects at a cellular and tissue level (4–7).

Research on human tissue-resident macrophages is scarce due to the increased difficulty of isolation and culture (e.g., surgical procedures, low cell counts) (8). Findings, therefore, largely originate from *in vitro* monocyte-derived or murine macrophages (9), of which fate-mapping studies revealed that in a homeostatic state, the population of tissue-resident macrophages primarily comprises large peritoneal macrophages (LPMs) (10). One-tenth of the population consists of small blood monocyte-derived peritoneal macrophages (SPMs). Differently from SPMs, LPMs stem from yolk-sac progenitors and have self-renewal potential with GATA-binding protein 6 (GATA-6), a transcription factor, responsible for their differentiation and survival (11). Owing to their high plasticity, tissue-resident macrophages can initiate an immune response, regulate wound repair and modulate tumor expansion (12). "Classically" activated (M1) macrophages exert cytotoxic effects, express CD86, a co-stimulatory molecule required for the activation of T cells, and release pro-inflammatory cytokines (e.g., IL-6, IL-17) (13–15). "Alternatively" activated (M2) macrophages can be phenotypically characterized by the scavenger receptor CD163 and have pro-tumoral properties (15–17). The M1/M2 model, however, largely applies to the *in vitro* culture of monocyte-derived macrophages activated with specific factors, whereas *in vivo* macrophages may express a larger spectrum of phenotypes with overlapping properties (12, 18). Polarization of murine macrophages towards an M1-like phenotype demonstrated cytotoxic effects and slowed tumor progression in peritoneal tumor models (19), whereas M2-like macrophages were shown to promote tumor dissemination in gastric cancer via EGFR signaling pathways (20).

Peritoneal macrophages are thus a promising target for PAL-derived immunomodulatory effects. Further research is required for the clinical use of PAL within the human peritoneal cavity for the treatment of cancerous and non-cancerous lesions including inflammatory diseases.

2 Materials and methods

2.1 Isolation and culture of human peritoneal macrophages

Peritoneal lavages were obtained after written informed consent from patients undergoing surgical procedures at the University Women's Hospital in Tübingen. The use of human donor cells was approved by the ethics committee of the medical faculty at the Eberhard Karl's University Tübingen (495/2018BO2). Cells were isolated from these peritoneal lavages as previously reported by Ruiz-Alcaraz et al. (21). 2 - 4 x 10^5 cells were then seeded onto 48-well plates and left to adhere for 2 h at 37 °C and 5% CO_2. Non-adherent cells were aspirated and removed. The plastic-adherent macrophages were washed with warm DPBS and cultured in DMEM Glutamax™ supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, 20 ng/mL macrophage-colony stimulating factor (M-CSF), 2 mM L-glutamine and 10% heat-inactivated FBS (all from ThermoFisher Scientific, OR, USA).

2.2 Generation of PAL and cell treatment

2 mL of Minimal Essential Medium (MEM) without pyruvate (ThermoFisher Scientific, OR, USA, #31095029) supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, 2 mM L-glutamine and 10% heat-inactivated FBS was activated by plasma exposure using an ambient pressure argon plasma jet (kINPen MED, neoplas med, Germany) for 120 s. Following operating conditions were applied: argon gas flow 4.0 L/min, frequency 1 MHz, line voltage 2-3 kV, power 1 W. 2 mL MEM were treated with pure argon gas and used as a control. An argon-treated control, 1:2-diluted and undiluted PAL were performed for experiments (excluding immunostaining and flow cytometric characterization of macrophages). In a 48- well plate, cells were incubated with 200 µL PAL for 4 h at 37 °C and 5% CO_2 before further propagation in culture media for 24 h in total.

2.3 Immunofluorescence microscopy

Macrophages were harvested with Accutase (BioLegend, San Diego, CA, USA, #423201) and reseeded in glass bottom imaging dishes (µ-dish 35 mm, high glass bottom, ibidi, Germany, #81158). Cells were cultured for 24 h prior to fixation with 4% PFA for 10 min. Cells were washed three times with cold DPBS and permeabilized with ice-cold 100% methanol for 20 min at -20 °C. Cells were rinsed with cold DPBS for 5 min and blocked with a blocking buffer (0.5 g BSA + 30 µL Triton + 10 mL DPBS) for 60 min at room temperature (RT) in dark. After the blocking buffer was removed, cells were incubated overnight at 4 °C with a primary antibody diluted in antibody dilution buffer (0.1 g BSA + 30 µL Triton + 10 mL DPBS). The following primary antibody was used: Rabbit (Rb) CD68 (clone D4B9C-specific antibody, Cell Signaling Technology, Netherlands, #76437, 1:800 dilution). Cells were washed three times with DPBS and incubated with diluted secondary antibody for 60 min at RT in dark. The following fluorochrome-conjugated secondary antibody was used: Cy™3 AffiniPure Goat Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch, UK, #111-165-003, 1:500 dilution). Cells were washed three times with DPBS and were incubated with the diluted nuclei-specific dye Hoechst 34580 (ThermoFisher Scientific, OR, USA, #H21486, dilution 1:1000) for 20 min on a plate shaker

covered in aluminum foil. Cells were washed with DPBS prior to image acquisition with a Cell Observer fluorescent microscope (Zeiss, Germany).

2.4 Flow cytometric characterization

Macrophages were harvested with Accutase, washed and resuspended in 500 µL DPBS containing 0.5 µL Zombie NIR, a fixable viability dye, for 20 min at RT in dark. After washing cells twice with FACS buffer (DPBS + 2% FBS + 0.05 mM NaN_3 + 0.1 mM EDTA), cells were resuspended in 50 µL of surface marker antibodies diluted at a 1:50 dilution ratio in FACS buffer supplemented with 10% sterile-filtered, human male AB serum (H2B, France, #21001PM) for 30 min on ice in dark. The following fluorochrome-conjugated antibodies targeted against surface markers were used: CD14-PE (clone HCD14-specific antibody, BioLegend, CA, USA, #325605, dilution 1:50), CD14-FITC (clone HCD14-specific antibody, BioLegend, CA, USA, #325603, dilution 1:50) and CD16-BV605™ (clone 3G8-specific antibody, BioLegend, CA, USA, #302039, dilution 1:50). After washing, cells were resuspended in 100 µL Cytofix/Cytoperm (Fixation/Permeabilization Solution Kit, BD Bioscience, Germany, #554714). Cells were then washed twice with 1 mL 1x Perm/Wash and incubated with 100 µL blocking reagent (10% human male AB serum in 1x Perm/Wash) for 20 min on ice in dark. Intracellular antibodies were added directly to the blocking reagent. Cells were incubated with intracellular antibodies for 30 min on ice in dark. The following fluorochrome-conjugated antibodies targeted against intracellular markers were used: GATA-6-PE (clone D61E4-specific antibody, Cell Signaling Technology, Netherlands, #26452, dilution 1:50) and CD68-PE-eFluor 610 (clone Y1/82A-specific antibody, ThermoFisher Scientific, OR, USA, #61-0689-42, dilution 1:50). Cells were washed once with 1x Perm/Wash and resuspended in 100 µL FACS buffer for data acquisition using LSRFortessa™ Cell Analyzer (BD Biosciences, NJ, USA). Single-color compensation controls were performed with UltraComp eBeads™ (ThermoFisher Scientific, OR, USA, #01-3333-41) for software-based automatic compensation and adjustment of PMT voltages. Data was analyzed with FlowJo™ 10.4.2 software (Tree Star, OR, USA). Gating strategy included the removal of cell debris (FSC vs SSC), doublets (FSC-A vs SSC-A) and dead cells (FSC vs Zombie NIR) to determine positive cell populations (Supplementary Figure S1). FC staining of only surface markers is reported below (section 3.8).

2.5 Raman microspectroscopic analysis

Macrophages were harvested with Accutase and reseeded in glass bottom imaging dishes. 24 h after PAL treatment cells were fixed with 4% PFA for 10 min. Raman imaging was performed using a customized inverted WITec Raman system (alpha 300 R, WiTec GmbH, Ulm, Germany) equipped with a green laser (532 nm) and a charged-coupled device spectrograph with a grating of 600 g/mm. Large area scans were acquired of 9-10 single cells for each argon-treated control, 1:2-diluted and undiluted PAL-treated macrophages with a 63 x apochromat water dipping objective (N.A. 1.4; Olympus, Japan), an integration time of 0.1 s, a pixel resolution of 1 x 1 µm and a laser power of 50 mW. Image analysis was performed with the Project FIVE 5.1 software (WITEC GmbH, Germany), including baseline correction, removal of cosmic rays and cropping of spectra from 300 to 3045 cm^{-1}. True component analysis (TCA) identified prominent spectral components, of which single spectra were extracted using TCA-generated masks from intensity distribution heat maps. Principal component analysis (PCA) was performed as previously reported with the Unscrambler x 14.0 software (Camo Software, AS, Norway) to improve interpretability of the spectral data (22, 23).

2.6 Apoptosis; Apotracker/7-AAD co-staining

Macrophages were harvested with Accutase 24 h after PAL treatment, washed and incubated with 400 nM Apotracker staining solution (BioLegend, CA, USA, #427401) diluted in 100 µL FACS buffer for 20 min at RT in dark. After washing cells twice with FACS buffer, cells were resuspended in 100 µL FACS buffer. Cells were stained with 5 µL of 7-AAD viability dye (BioLegend, CA, USA, #420403) for 10 min at RT in dark, which was added directly to the cell suspension prior to data acquisition with LSRFortessa™ Cell Analyzer. Data was analyzed using FlowJo™ 10.4.2 software. Apotracker/7-AAD co-staining allowed for the discrimination of early and late apoptotic, necrotic and viable cells as a percentage of total cells.

2.7 Protein expression analysis by DigiWest multiplex protein profiling

Macrophages were harvested with Accutase 24 h after PAL treatment. Cell pellets were frozen at -80 °C prior to DigiWest multiplex protein profiling. The high-throughput bead-based Western blot was performed as previously reported by Ruoff et al. (24). Antibody fluorescence intensities were analyzed with the Luminex™ FlexMAP 3D™ Instrument System (Luminex Corporation, TX, USA). An Excel macro-based algorithm identified peaks at the respective molecular weight of the primary antibodies. Streptavidin conjugates were recorded as loading controls to normalize antibody signals.

2.8 FC surface marker expression analysis

Macrophages were harvested with Accutase 24 h after PAL treatment, washed and stained. Following fluorochrome-conjugated specific antibodies targeted against surface markers were used: CD86-PE (clone IT2.2-specific antibody, BioLegend, CA, USA, #305405), HLADR-FITC (clone Tü36-specific antibody, BioLegend, CA, USA, #361603), CD206-BV421™ (clone 15-2-specific antibody, BioLegend, CA, USA, #321125) and CD163-PE/

Cy7 (clone GHI/61-specific antibody, ThermoFisher, OR, USA, #25-1639-42). Antibodies were diluted with FACS buffer supplemented with 10% human male AB serum at a 1:50 dilution ratio for 30 min on ice in dark. After washing, cells were resuspended in 100 µL FACS buffer. 1 µL 7-AAD viability dye was added. Cells were incubated with 7-AAD for 10 min on ice in dark prior to FC analysis. In addition to single-color compensation controls, FMO (fluorescence minus one) controls were performed. Gating strategy included the removal of cell debris (FSC vs SSC), doublets (FSC-A vs SSC-A) and dead cells (FSC vs 7-AAD) to determine MFIs.

2.9 Cell culture supernatant analysis

Cell culture supernatants were collected 24 h after PAL treatment, centrifuged at 3000 x g for 3 min and stored at -80 °C until analysis. Levels of 13 different cytokines and chemokines were determined using the LEGENDplex™ HU Essential Immune Response Panel (BioLegend, San Diego, USA, #740930). The bead-based immunoassay was performed as reported in the manufacturer's instructions. MFIs and absolute concentrations of the cytokines/chemokines were measured as technical replicates (duplicates) using LSRFortessa™ Cell Analyzer and analyzed with the LEGENDplex™ data analysis software.

2.10 Statistical analysis

Statistical comparison was performed with the Student's *t*-test or Mann-Whitney *U* test against the argon-control group (GraphPad Prism 9.2.0. GraphPad Software Inc., San Diego, CA, USA). The data is shown as mean ± standard deviation of a minimum of three independent experimental approaches. *P*-values of < 0.05 were referred to as statistically significant.

3 Results

3.1 Human tissue-resident peritoneal macrophages reveal a heterogenous cellular morphology and co-expression of pro- and anti-inflammatory surface markers

Human peritoneal macrophages were characterized with IF microscopy, FC staining and Raman microspectroscopy. IF microscopy with the intracellular, pan-macrophage marker CD68 demonstrated a heterogenous cellular morphology of the isolated peritoneal macrophages (Figures 1A, B). These became increasingly adherent after isolation, adopting either a round shape or a spindle-shaped elongation. FC staining with CD68 demonstrated that peritoneal macrophages represented the largest population of isolated cells (Figure 1C), of which co-staining with CD14, CD16 and GATA-6 showed that the majority had a high expression of CD14 and CD16 (Figure 1D). GATA-6 was highly expressed, indicating that primarily tissue-resident LPMs were isolated. Simultaneous FC staining of the following surface markers, CD86, HLA-DR (M1), CD206 and CD163 (M2), showed that peritoneal macrophages co-express pro- and anti-inflammatory surface markers in a homeostatic environment (Figure 1E). Peritoneal macrophages showed a higher basal expression of M1 surface markers. 99.9 ± 0.1% of the peritoneal macrophages expressed CD86, while 90.2 ± 5.1% of the cells were positive for HLADR. The basal expression of M2 surface markers was lower with 58.1 ± 18.9% of the cells expressing CD206 and 82.7 ± 11.1% expressing CD163. Label-free Raman microspectroscopy further characterized cellular components of peritoneal macrophages, including nucleic acids, proteins and lipids. Representative Raman images of the false color-coded heat maps are shown in Figure 1F. Nuclei-specific peaks in Figure 1G showed characteristic peaks at 785 cm^{-1} (25), 1458 cm^{-1} (26) and 1655 to 1680 cm^{-1} (25), while protein-specific spectra were identified based on peaks at 1008 cm^{-1} (27), 1308 cm^{-1} (27) and 1667 cm^{-1} (28, 29). Characteristic peaks of lipids in Raman spectra are related to the hydrocarbon chain (e.g., 1250 to 1300 cm^{-1}, 1400 to 1500 cm^{-1}) (30). The C-H stretching, which is found in the bands of the higher wavenumber region, is also distinctive of lipid spectra (30). A detailed molecular assignment of the nuclei-, protein- and lipid-specific peaks is summarized in Table 1.

3.2 PAL-treated peritoneal macrophages maintain resistance towards oxidative cellular death by upregulating anti-oxidative mechanisms

Cellular factors related to apoptosis, necrosis and pro-survival pathways were analyzed in PAL-treated macrophages using FC and DigiWest protein profiling. FC staining of PAL-treated macrophages with Apotracker and 7-AAD demonstrated marginal, non-significant levels of apoptosis and necrosis (Figure 2). Consistent with the low levels of apoptosis and necrosis, PAL-treated macrophages showed a high viability for the 1:2-diluted and undiluted PAL compared to the argon-treated control (argon-treated control: 94.1 ± 4.9%, 1:2-diluted: 92.9 ± 7.3% and undiluted PAL: 91.2 ± 7.8%). Representative dot plots of one donor for the argon-treated control, 1:2-diluted and undiluted PAL are shown in Figures 2C–E. Quadrant 1 (Q1) shows necrotic (Apo-, 7-AAD+), Q2 late apoptotic (Apo+, 7-AAD+), Q3 early apoptotic (Apo+, 7-AAD-) and Q4 viable cells (Apo-, 7-AAD-). Additional apoptosis markers, including the expression of caspases 3 and 9, also showed no significant increase (Figure 3). Signal proteins related to immune response control and proliferation, such as proto-oncogene tyrosine-protein kinase (Src, 1:2-diluted: $p = 0.0981$; undiluted PAL: $p = 0.0661$), S6 ribosomal protein (rpS6, 1:2-diluted: $p = 0.4141$; undiluted PAL: $p = 0.0231$) and phosphatase and tensin homolog (PTEN, 1:2-diluted: $p = 0.3242$; undiluted PAL: $p = 0.0306$) showed an increased expression. The absence of significant spectral changes at a nuclei level in Raman microspectroscopy further supports the PAL-treated macrophages' resistance towards oxidative stress-induced cell death (Supplementary Figure S2). Superoxide dismutase, a redox-

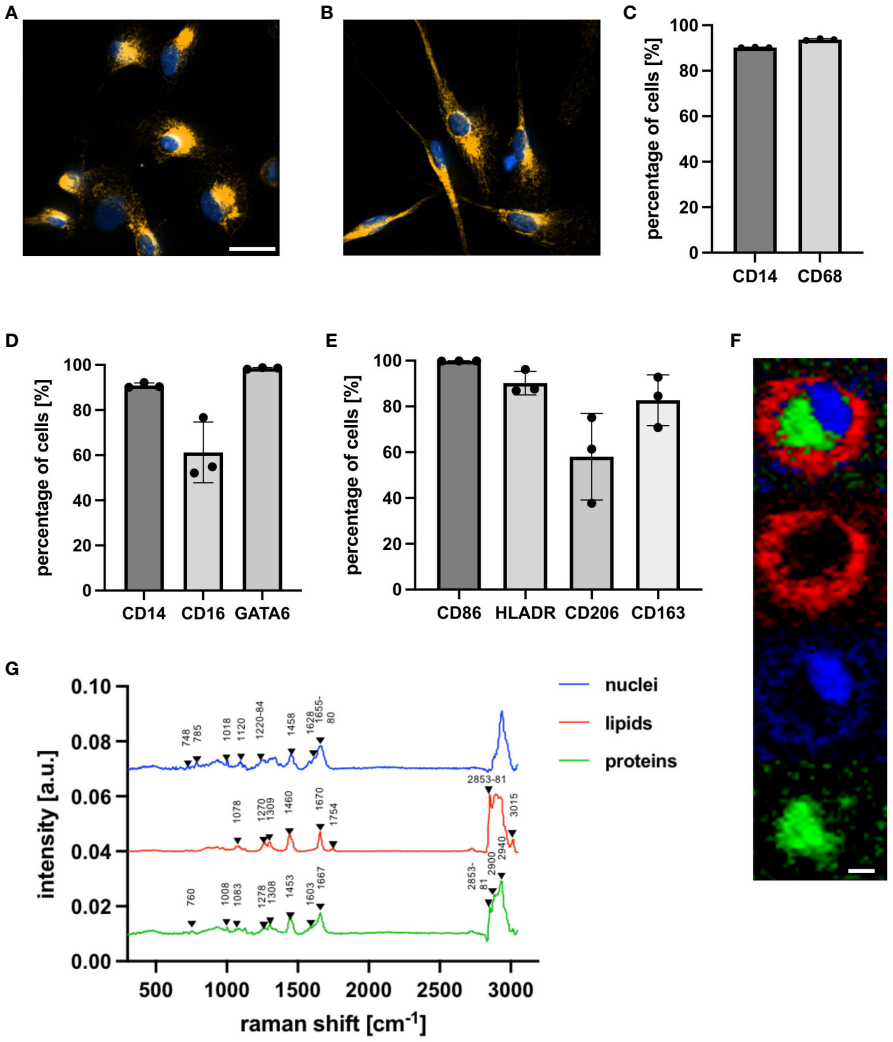

FIGURE 1
Characterization of human tissue-resident peritoneal macrophages with IF microscopy, FC staining and Raman microspectroscopy. **(A, B)** Representative IF microscopy (63 x) after staining with specific antibodies against CD68 (orange) and Hoechst, a nuclear-specific dye (blue), five days after isolation. Macrophages show round shapes and spindle-like elongation. Scale bar represents 20 µm. **(C–E)** FC analysis was used to characterize surface and intracellular markers of peritoneal macrophages. **(C, D)** shows the percentage of cells positive for the surface markers CD14 and CD16 and the intracellular pan-macrophage marker CD68 **(C)** and peritoneal macrophage-specific marker GATA-6 **(D)**. **(E)** shows the percentage of cells positive for M1 (CD86, HLADR) and M2 (CD206, CD163) surface markers. Statistical comparison was performed with paired Student's *t*-tests. Shown are the mean ± SD, n = 3. **(F, G)** Raman microspectroscopic analysis was used to characterize peritoneal macrophages at a nuclei, lipid and protein level. **(F)** True component analysis (TCA) based on specific Raman peaks facilitated identification of nuclei (blue), lipids (red) and proteins (green) by producing false color-coded intensity distribution maps. Scale bar represents 7 µm. **(G)** Average spectra of cellular structures.

related enzyme, was mildly upregulated for undiluted PAL compared to the argon-treated control (1:2-diluted: p = 0.5827, undiluted: p = 0.1008), which may explain the increased anti-oxidative potential of peritoneal macrophages.

3.3 PAL-treated peritoneal macrophages show a moderate pro-inflammatory shift by alteration of their molecular composition and cytokine release

PAL-derived RONS did appear to affect molecular composition, cytokine release and surface marker expression as shown by marker-independent Raman microspectroscopy and FC staining. Two separate PC analyses were performed for the lipidome profile of PAL-treated macrophages, as the higher wavenumber region (2700 to 3100 cm^{-1}) concealed spectral differences in the fingerprint region (600 to 1800 cm^{-1}). Score plots of the fingerprint and higher wavenumber region in Figure 4 demonstrated distinct clusters of argon-treated and undiluted PAL-treated macrophages (fingerprint: 1:2-diluted: p = 0.9965, undiluted: p <0.0001; higher wavenumber region: 1:2-diluted: p = 0.9273, undiluted PAL: p <0.0001). Raman peaks at, for example, 1270 cm^{-1} (31), 1440 cm^{-1} (32, 33), 1655 cm^{-1} (34), 2844 cm^{-1} (30) and 3010 cm^{-1} (31) in the loading plots explain spectral differences (Table 1). The aforementioned peaks can be assigned to PAL-treated macrophages, indicating the C=C double bond found in unsaturated fatty acids. Further relevant peaks are summarized in Supplementary Table S1 (35–41). Changes in fatty acid

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TABLE 1 Identified Raman peaks [cm^{-1}] and their molecular assignments.

Peaks [cm^{-1}]	Assignment	Reference
Nuclei		
785	uracil, thymine, cytosine, O-P-O backbone	(25)
1458	nucleic acid modes	(26)
1655-80	thymine, guanine, cytosine (ring breathing modes)	(25)
Proteins		
1008	phenylalanine	(27)
1308	C-N asymmetric stretching in aromatic amines	(27)
1667	protein bands	(28, 29)
Lipids		
1270	C=C groups (unsaturated fatty acids)	(31)
1440	(CH_2) (lipids), CH_2 bending (lipids)	(32, 33)
1655	C=C (lipids; not amide I)	(34)
2844	ν_s(=CH_2)	(30)
3010	unsaturated =CH stretch	(31)

composition and turnover in PAL-treated macrophages may have contributed to a moderate release of pro-inflammatory cytokines. 24 h after PAL treatment seven of the 13 analytes measured, including IL-2, IL-6, IL-8, IL-10, IL-17, IP-10 and MCP-1, were detectable in the cell culture supernatants of the PAL-treated macrophages using a bead-based immunoassay (Figures 5A–G). MFIs of the individual analytes measured were averaged (duplicates) and their respective absolute concentrations are summarized in Supplementary Table S2. Pro-inflammatory cytokines, including IL-6, IL-17 and IP-10, showed a moderate increase (undiluted PAL: IL-6: p = 0.2837; IL-17: p = 0.4288; IP-10: p = 0.1426). However, chemokine and cytokine release of PAL-treated compared to argon-treated macrophages was not significant due to a high donor-dependent variance. Further pro-inflammatory cytokines, including IL-2, IL-8 and MCP-1, showed no PAL-derived changes. The anti-inflammatory cytokine, IL-10, demonstrated a small decrease, which was higher for undiluted (p = 0.1757) compared to the 1:2-diluted PAL (p = 0.2762). IL-8, IP-10 and MCP-1 were the analytes with the highest absolute concentrations (Supplementary Table S2). FC staining of surface marker expression was also performed to analyze changes in polarization (Figure 5H). CD86 (M1) and CD206 (M2) showed no changes in MFI. However, the expression of CD163 (M2) (1:2-diluted: p = 0.9049, undiluted PAL: p = 0.1556) and HLA-DR (M1) showed a moderate, non-significant downregulation compared to the argon-treated control (1:2-diluted: p = 0.3346, undiluted PAL: p = 0.0889).

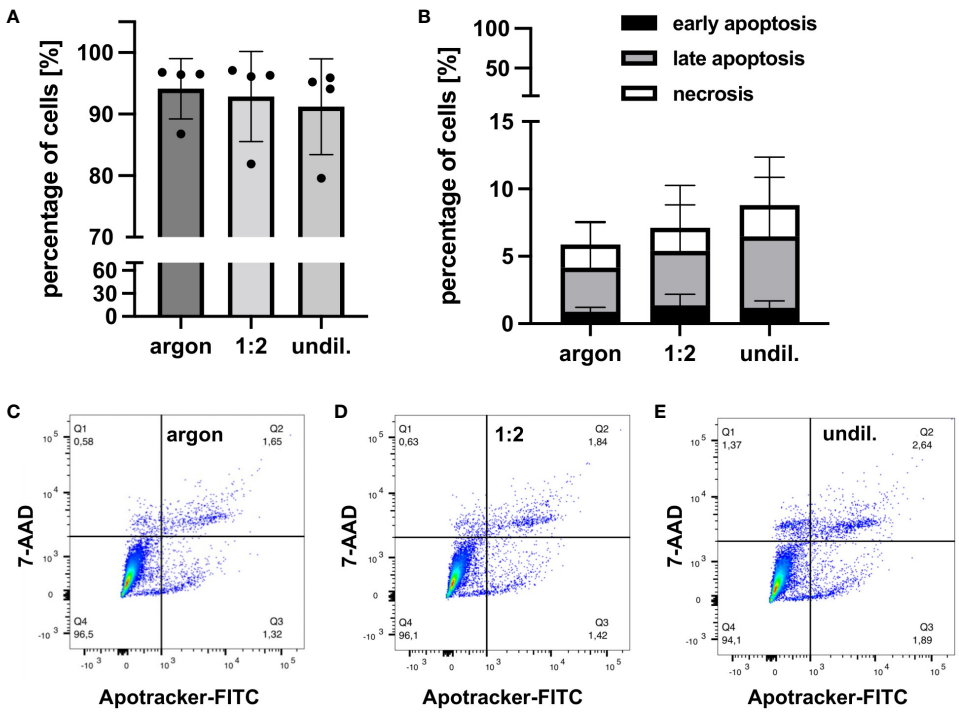

FIGURE 2
FC analysis of viability, apoptosis and necrosis of PAL-treated peritoneal macrophages. Apotracker-FITC and 7-AAD staining was performed 24 h after PAL treatment of peritoneal macrophages. **(A)** Bar graph shows high viability of the 1:2-diluted and undiluted PAL-treated macrophages. **(B)** Bar graph shows small, non-significant increase in early (black), late (light grey) apoptosis and necrosis (white) of PAL-treated macrophages. **(C–E)** Representative dot plots of one donor for the argon-treated control **(C)**, 1:2-diluted **(D)** and undiluted PAL **(E)**. Shown are mean ± SD, n = 4.

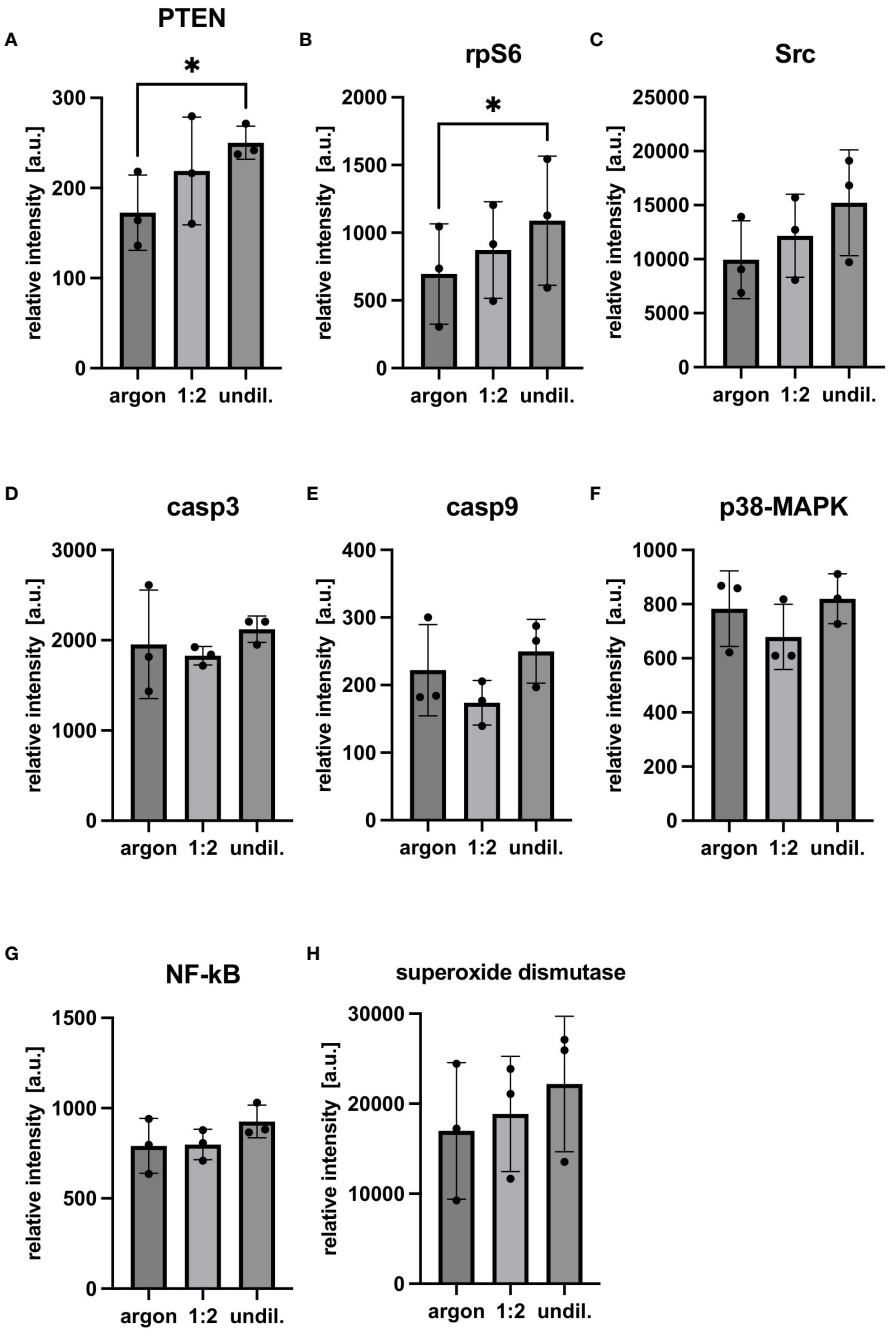

FIGURE 3
Multiplex protein profiling of PAL-treated peritoneal macrophages. Protein profiling using DigiWest technology was performed with samples frozen 24 h after PAL treatment. Antibody fluorescence intensities of the analytes were normalized to the argon-treated and their respective streptavidin loading control. **(A–C)** show cellular factors related to proliferation, immune response and survival. **(D–G)** show cellular factors and signaling pathways related to apoptosis. **(H)** shows superoxide dismutase, a redox-related enzyme. Statistical comparison was performed with paired Student's *t*-tests. Shown are mean ± SD, n = 3, * $p < 0.05$.

4 Discussion

Recent research has shown that plasma-derived oxidative stress is not only limited to selectively killing cancer cells but further modifies the tumor microenvironment, including stromal host and immune cells, and may trigger immunogenic cell death by which dying cancer cells release damage-associated molecular patterns (42–45). A NIPP-modulated immune response may thus restore immunogenicity by boosting adaptive immunity against cancer cells. Bekeschus et al., for example, revealed that NIPP treatment of CT26 colorectal cancer cells was related to a higher expression of immunogenic surface-exposed molecules (e.g., calreticulin) (46). Van Loenhout et al. further showed that as NIPP-treated pancreatic stromal host cells released less immunosuppressive signaling molecules (e.g., TGF-ß), more pro-inflammatory immune cells infiltrated the tumor microenvironment (47). These pro-

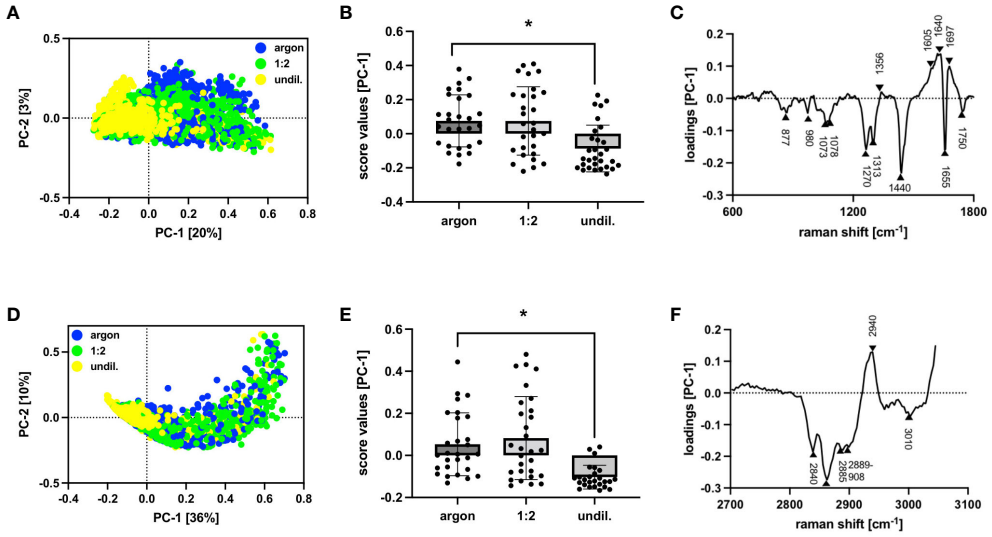

FIGURE 4
Raman and multivariate analysis of lipid composition in PAL-treated macrophages. Raman and multivariate analysis reveal spectral differences at a lipid level within the fingerprint (600 to 1800 cm^{-1}) and higher wavenumber region (2700 to 3100 cm^{-1}) in PAL-treated macrophages. **(A)** Score plot of fingerprint region demonstrated separation in PC-1 vs PC-2 of argon-treated control (blue) and undiluted PAL (yellow). **(B)** Average score values of fingerprint region show significant differences of PC-1 for argon-treated control compared to undiluted PAL-treated macrophages. **(C)** Corresponding PC-1 loading plot of fingerprint region indicates changes in lipidome profile for undiluted PAL-treated macrophages. **(D)** Score plot of higher wavenumber region demonstrated separation in PC-1 vs PC-2 of argon-treated control (blue) and undiluted PAL (yellow). **(E)** Average score values of higher wavenumber region show significant differences of PC-1 for argon-treated control compared to undiluted PAL-treated macrophages. **(F)** Corresponding PC-1 loading plot of higher wavenumber region indicates changes in lipidome profile for undiluted PAL-treated macrophages. Shown are statistical comparisons using an unpaired Student's *t*-test or Mann-Whitney *U* test of average score values ± SD for 28 single cells, n = 3, * $p < 0.05$.

inflammatory M1-like macrophages are responsible for phagocytosis of cancer cells, antigen presentation and release of cytokines (e.g., IL-6) to recruit natural killer and CD8+ T cells essential for tumor control (17). In addition, Takeda et al. showed that intraperitoneal PAL administration significantly reduced metastatic nodules within mice's peritoneal cavity without toxic effects (48). Also, ovarian cancer dissemination was suppressed *in vitro* and *in vivo* via lower MMP-9 expression, leading to better long-term survival in a mouse model (49). Compared with intraperitoneal chemotherapy (i.e., HIPEC), which may lead to severe postoperative complications (e.g., sepsis, digestive fistula and adhesive ileus) (49–51), intraperitoneal PAL administration may serve as an adjuvant treatment alternative for peritoneal metastasis with fewer adverse events and minimal cytotoxicity to healthy tissue (4, 48). This study thus aimed to identify PAL-derived molecular and immunomodulatory effects on mature human tissue-resident peritoneal macrophages. While cellular effects due to long-lived nitrates (NO_3-), nitrites (NO_2-), and hydrogen peroxide (H_2O_2) formed by plasma-liquid interactions are shown (45), other effects due to direct treatment (e.g., short-lived species, UV radiation, electromagnetic fields) could not be observed (1).

FC characterization revealed co-expression of M1 and M2 surface markers of the isolated GATA6+ macrophages. Co-stimulatory molecules, CD86 and HLADR, responsible for antigen presentation and T cell activation, are frequently identified with M1 macrophages (13, 17). Higher expression of scavenger receptors CD163 and mannose receptors CD206 indicate an M2-like phenotype (13, 16, 17). Tumor-associated macrophages strongly express CD163, and the density of these macrophages negatively influences gastric cancer growth and metastasis (52). FC characterization of the isolated macrophages showed higher expression of M1 surface markers compared to M2 surface markers. Expression of the surface markers HLADR and CD163 was moderately reduced in PAL-treated macrophages, whereas CD86 and CD206 did not differ notably from the argon-treated control. Possibly, no distinct phenotype shift was observed because of the maturity of the tissue-resident macrophages. Wang et al. demonstrated the different biological characteristics of murine macrophages derived from the peritoneal cavity, spleen and bone marrow, indicating that peritoneal macrophages with high levels of MHC II and CD86 surface marker expression were the most mature and showed lower proliferative potential (53). Alternatively, damage to the cellular membrane via PAL-derived lipid peroxidation may explain reduced surface marker expression. Superoxide radicals ($O_2^{\bullet-}$) can interfere with hydrogen peroxide (H_2O_2) and nitric oxide (NO) to trigger lipid peroxidation, leading to altered cellular membrane permeability and fluidity (54, 55).

PAL-treated macrophages further showed a high resistance towards PAL-derived oxidative stress and cellular death. Although PAL-derived RONS may alter cell membrane integrity and promote apoptosis in cancer cells (56–58), the majority of PAL-treated macrophages maintained high levels of viability and minimal, non-significant levels of apoptosis in FC Apotracker/7-AAD co-staining. Apotracker identifies externalized phosphatidylserine residues in apoptosis (59), whereas viable cells with intact cellular membranes are impermeable to 7-AAD (60). Equipped with

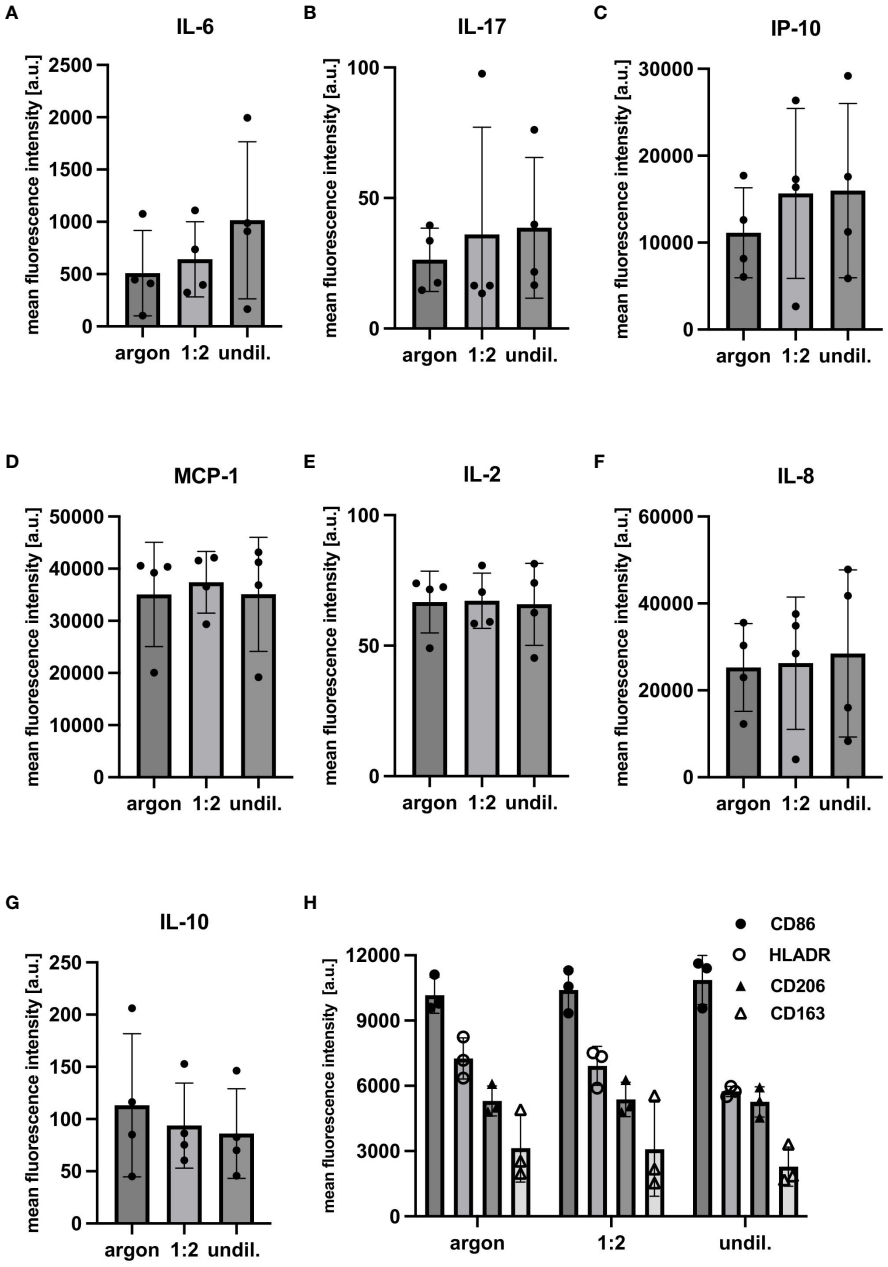

FIGURE 5
FC analysis of cytokine/chemokine release and surface marker expression of PAL-treated macrophages. FC analysis of cytokine/chemokine release and surface marker expression was performed 24 h after PAL treatment. Mean fluorescence intensities (MFIs) of cytokine/chemokine levels were measured using a bead-based immunoassay. **(A–C)** Tendential increase in pro-inflammatory cytokines and chemokines (IL-6, IL-17 and IP-10). **(D–F)** Other pro-inflammatory cytokines (MCP-1, IL-2 and IL-8) showed no PAL-derived changes. **(G)** Release of anti-inflammatory cytokine, IL-10, showed a moderate decrease. Shown are the mean ± SD, n = 4. **(H)** FC analysis of surface marker expression was performed and surface protein levels are shown as MFIs. HLADR (M1) and CD163 (M2) expression were moderately reduced for the argon-treated control compared to the undiluted PAL-treated macrophages. Shown are mean ± SD, n = 3.

increased GSH redox signaling, higher levels of DNA repair proteins and ROS reductase, macrophages have been described to be less sensitive towards higher intracellular ROS levels, which are also present in oxidative burst during phagocytosis (61, 62). As such, NIPP-treated macrophages were demonstrated to be less susceptible to oxidative stress compared to other PBMC-derived leukocyte populations (63). Protein profiling also revealed that PAL-treated macrophages mildly increased their expression of the anti-oxidant enzyme superoxide dismutase, which can catalyze the dismutation of the superoxide radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (64). Hwang et al. showed that superoxide dismutase supplementation attenuated uncontrolled inflammatory response and apoptosis via blocking of p38-MAPK/NF-κB pathways (64). Upregulation of superoxide dismutase may

also reduce apoptosis by decreasing mitochondrial release of cytochrome c (65). Further apoptosis markers and pathways (e.g., casp3, casp9 and p38-MAPK) demonstrated no significant upregulation in protein profiling of PAL-treated macrophages. Rather cell signaling and regulation pathways relevant for immune response and proliferation showed significant upregulation. PTEN, for example, promotes inflammatory responses via the release of pro-inflammatory cytokines (e.g., IL-6) (66). Src kinase, also relevant for immune response control of macrophages, is involved with their functional activation (67).

Multivariate analysis of spectral data allowed for the biomolecular characterization of cellular structures, including nuclei, proteins and lipids, of PAL-treated macrophages. The potential of Raman imaging to determine PAL-derived changes has already been analyzed in cervical tissue, peritoneal fibroblasts and mesothelial cells (6, 68). Proteins and lipids were previously identified as cellular structures most reactive to demonstrate macrophage activation in Raman imaging (69). Analysis of their lipidome profile revealed that Raman peaks at 1270 cm^{-1} (31), 1440 cm^{-1} (32, 33), 1655 cm^{-1} (34) and 3010 cm^{-1} (31) may explain the clustering behavior of the argon-treated control and PAL-treated macrophages in the score plots. These aforementioned peaks can be assigned to (undiluted) PAL-treated macrophages and describe the C=C double bond of unsaturated fatty acids, thereby indicating an altered degree of saturation in fatty acid composition. Montenegro-Burke et al. demonstrated that macrophage phenotypes have different fatty acid compositions (70). M1 macrophages are characterized by higher intensities of cholesterol esters, diacylglycerols and triglycerides, including a higher proportion of unsaturated triglycerides, especially polyunsaturated fatty acids (71). Cholesterol and triglyceride ester-containing lipid droplets are relevant for inflammatory response and may be utilized as a substrate pool for pro-inflammatory cytokines (e.g., IL-1ß, IL-6) (71, 72). Changes in lipids and their metabolites may affect macrophages' polarization and response to pathogens, phagocytosis and inflammation (73). Distinguishing in-depth between structural and molecular, as well as transient and permanent damage of cell membranes, requires further studies (i.e., mass spectrometry, electron microscopy, protein profiling) to reveal structural damage and up-/downregulation of lipid-metabolism-related factors due to PAL treatment. Nonetheless, changes in the lipidome profile of PAL-treated macrophages were consistent with observations of a tendential increase of pro-inflammatory cytokines/chemokines (IP-10, IL-6 and IL-17) and a decrease of the anti-inflammatory cytokine IL-10 in the bead-based immunoassay. However, these PAL-derived changes in the cytokine/chemokine release were not significant due to the high donor-dependent variance of primary isolated human tissue-resident peritoneal macrophages. IP-10 (CXCL10), for example, demonstrated immunomodulatory potential to recruit APCs in glioma and melanoma murine tumor models (74). However, IP-10 may also partake in tumor expansion if the receptor CXCR3 is overexpressed in cancer cells. The aforementioned PAL-derived changes in cytokine/chemokine release align with other findings (75, 76). Cheng et al., for example, showed a higher release of IL-2 and IL-6 and a lower IL-10 release in NIPP-treated peritoneal elicited murine macrophages (75).

Our findings suggest that human tissue-resident peritoneal macrophages are extremely resistant towards PAL-derived oxidative stress via upregulated pro-survival and anti-oxidative pathways. NIPP may modulate a moderate pro-inflammatory response by modifying their lipid composition and cytokine release, thereby complementing the aforementioned anti-tumoral activity of NIPP. However, the present study is limited to a 2D cell culture model. 3D cell models (e.g., organoids, spheroids, or tumor-on-a-chip) or murine models better represent the *in vivo* environment and are more predictive of PAL-derived immunomodulatory effects on solid tumors. These must validate the 2D cell culture *in vitro* findings under more *in vivo* (-like) conditions.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Institutional Ethics Committee of the Medical Faculty of the Eberhard Karls University Tübingen (protocol codes 649-2017BO2, approval: 12 January 2018 and 495/2018BO2, approval: 19 October 2018). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

LS-R: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. JM: Data curation, Formal analysis, Methodology, Writing – review & editing. DC: Data curation, Formal analysis, Methodology, Writing – review & editing. MH: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. FS-R: Methodology, Supervision, Writing – review & editing. TB: Data curation, Investigation, Methodology, Writing – review & editing. JA: Resources, Writing – review & editing. CB: Resources, Writing – review & editing. MT: Methodology, Resources, Supervision, Writing – review & editing, Conceptualization. SB: Funding acquisition, Resources, Writing – review & editing, Conceptualization, Supervision. KS-L: Funding acquisition, Resources, Writing – review & editing, Conceptualization, Supervision. MW: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing, Investigation, Project administration.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1357340/full#supplementary-material

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New insights into the role of macrophages in cancer immunotherapy

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Macrophages are the main component of the tumor microenvironment, which are differentiated from monocytes in the blood and play an important role in cancer development. Tumor-associated macrophages (TAMs) can promote tumor growth, invasion, metastasis, and resistance to anti–programmed death receptor 1 therapy by regulating programmed cell death ligand 1 expression and interacting with other immune cells in the tumor microenvironment. However, when activated properly, macrophages can also play an anti-tumor role by enhancing the phagocytosis and cytotoxicity of tumor cells. TAM is associated with poor prognosis and drug resistance in patients treated with immunotherapy, indicating that macrophages are attractive targets for combined therapy in cancer treatment. Combination of targeting TAMs and immunotherapy overcomes the drug resistance and achieved excellent results in some cancers, which may be a promising strategy for cancer treatment in the future. Herein, we review the recent findings on the role of macrophages in tumor development, metastasis, and immunotherapy. We focus mainly on macrophage-centered therapy, including strategies to deplete and reprogram TAMs, which represent the potential targets for improving tumor immunotherapy efficacy.

KEYWORDS

macrophages, tumor microenvironment, cancer, immunotherapy, PD-L1

1 Introduction

Cancer is one of the major public health issues worldwide and is the leading cause of death in many countries. According to the latest data published in 2023, approximately 1,958,310 new cancer cases were present in the United States (1). Moreover, due to the high mortality rate and low cure rate of cancer, it has brought heavy economic burden to individuals, families, and society. Therefore, the prevention and treatment of tumors were

urgent to further reduce the morbidity and mortality rates. Surgery, radiotherapy, and chemotherapy are three traditional treatment strategies for cancer, but the treatment outcome was still dismal in some patients (1, 2). In recent years, emerging treatment methods have been developed, such as Chimeric antigen receptor (CAR)-T cell therapy and immune-checkpoint inhibitors, which were considered the fourth treatment mode following traditional therapy. At present, immunotherapy has been approved for clinical use, mainly including programmed death receptor 1 (PD-1) inhibitors and CAR-T cell therapy, both of which have achieved excellent results in some advanced stage malignant tumors (3–6). However, the efficacy of PD-1 inhibitor was limited in some patients with cancer (7), and the efficacy needs to be further improved.

The tumor microenvironment was considered to be a key factor affecting tumor progression, metastasis, and treatment results (8, 9). Exploring the tumor microenvironment is the cornerstone of improving the response rate and developing new cancer immunotherapy strategies. In addition, macrophages were reported to be one of the most important immune cells in the tumor microenvironment (9). Based on the function of phagocytosis, macrophages can eliminate tumor cells at an early stage, but, under the stimulation of the stimulating factors in the tumor microenvironment, they gradually transform into tumor-related macrophages with the M2 phenotype and promote tumor growth and metastasis by inhibiting immunity, inducing angiogenesis and supporting cancer stem cells (10). To sum up, it is of great significance to explore in great depth the role of macrophages in the tumor microenvironment, and targeting macrophages may be a promising anti-tumor strategy in the future.

2 Origin, polarization, and function of macrophages

Macrophages originate from the monocytes in the circulation, and substantial heterogeneity was observed among each macrophage population (11). According to phenotype and function, macrophages can be divided into two types: classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) (12). M0 macrophages could differentiate into M1 macrophages under the stimulation of lipopolysaccharide and interferon-γ (IFN-γ), whereas they differentiate into M2 macrophages with the stimulation of interleukin (IL)-4, IL-10, and IL-13 (13). M1 macrophages could produce multiple cytotoxic substances, such as nitric oxide and reactive oxygen species, and thereby activate the function of multiple immune cells and reduce microbial activity, ultimately eliminating microbial infection (14). Meanwhile, a variety of cytokines were produced by M1 macrophages, including tumor necrosis factor–α (TNF-α), growth inhibitors, and anti-angiogenic factors, which could inhibit cancer progression (14). On the contrary, M2 macrophages often function as anti-inflammatory factors by reducing the inflammation response, promoting tissue repair and remodeling the immune system (10, 14). Tumor-associated macrophages (TAMs) were mainly thought to be M2 type in the tumor microenvironment, which could promote tumor growth, invasion, and metastasis.

3 Macrophages in the TME promote tumor progression

Macrophages are involved in different stages of tumor development. In the early stage, tumor cells release cytokines and exosomes and attract macrophages and other immune cells into the tumor stroma, where macrophages promote tumor growth, migration, and metastasis (10). As a key component of the tumor microenvironment, macrophages can produce an anti-tumor effect and cause tumor necrosis with powerful swallowing phagocytosis (15), but some studies have shown that TAM is an important driving factor of tumor progression. In the tumors formed, TAM promotes the growth and proliferation of cancer cells, angiogenesis, and lymphangiogenesis and inhibits the immune response of effector T cells (16).

TAM is considered a proinflammatory and anti-tumor phenotype (M1 type) in the early stage of lung cancer and gradually displays an anti-inflammatory and tumor-promoting phenotype in the process of cancer progression (10). TAM could promote tumor development through immune regulation and non-immune processes (17–19). For example, TAM secretes a large number of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) to promote tumor angiogenesis and metastasis (20).

In the tumor microenvironment, macrophages account for half of the total number of tumor cells and are mainly M2 phenotypes. The quantity of macrophages in the tumor microenvironment is associated with tumor micro-vessels and is negatively correlated with the survival outcome in patients with non-small cell lung cancer (NSCLC) (21, 22). In recent years, a growing body of research has revealed the TAM multifaceted regulation of the co-evolving cancer ecosystem based on next-generation technologies and single-cell sequencing technology (12, 22). Therefore, this section mainly introduces the function and mechanism of TAM in tumors.

3.1 Anti-tumor effect of M1 type TAM

Inhibition of anti-tumor immunity was reported to be the main pathogenic mechanism of TAM. TAM could downregulate the release of the immunostimulatory factor IL-12, which can trigger the tumor-killing effect of natural killer (NK) cells and cytotoxic CD4+ T cells (23). In addition, many immunosuppressive factors produced by TAM could also mediate cancer development, such as IL-10, transforming growth factor–β, and prostaglandin E2 (10, 24, 25).

TAM can also directly inhibit the function of T cells through specific enzyme activities, such as arginase 1 (ARG1), which is a hydrolase that controls the catabolism of L-arginine. ARG1 is induced by multiple signaling pathways mediated by IL-4, IL-10,

and hypoxia and affects T-cell function by limiting the activity of the semi-essential amino acid L-arginine (25). TAM can also promote T-cell apoptosis by inhibiting the expression of programmed cell death ligand 1 (PD-L1) and B7 homolog 1 on T cells (12, 25).

3.2 The function of M2 type TAM in promoting tumor development

The function of M2 macrophages in promoting tumor development depends on the proinflammatory cytokines, such as TNF-α, IL-6, and IL-11, which can activate the nuclear factor–κB (NF-κB) and signal transduction and activator of transcription 3 (STAT3) pathway in cancer cells (10, 12, 13, 18, 25). In addition, M2 TAM promoted tumor progression by promoting angiogenesis and lymphangiogenesis by increasing the expression of VEGF-A and VEGF-C (18, 20, 25).

4 Macrophages and anti–PD-1/PD-L1 immunotherapy

4.1 Effect of TAMs on PD-1/PD-L1 expression

The PD-1/PD-L1 pathway was abnormally activated in various cancers (6, 26), and anti–PD-1/PD-L1 immunotherapy has been widely used or tried in clinical trials in many solid tumors, such as lung cancer, advanced metastatic melanoma, esophagus cancer, and colorectal cancer (27, 28). However, the efficacy of PD-1 inhibitors was still dismal in some patients with high expression of PD-L1, and the concrete mechanisms remain largely unknown.

Previous studies have demonstrated that TAMs can regulate the expression of PD-1/PD-L1 through the activation of different signaling pathways (Figure 1), which, in turn, affects the efficacy of PD-1/PD-L1 inhibitors. CD163+ TAMs in the tumor microenvironment are reported to be positively correlated to PD-L1 expression in various cancers, including pancreatic cancer and liver cancer. Multiple cytokines released by TAM, including IL-6 and TNF-α, can upregulate PD-L1 expression by activating Janus kinase (JAK)/STAT3, phosphoinositide 3-kinase (PI3K)/AKT, NF-κB, or Extracellular signal-regulated kinase (ERK) 1 and 2 signaling pathways (29, 30). In addition, PD-L1 protein expression could also be upregulated by TNF-α through post-translational regulation (29).

4.2 TAMs and anti–PD-1 resistance

In addition to the PD-L1 expression on tumor cells, the tumor microenvironment was also a key factor associated with anti–PD-1 resistance. As mentioned above, cytokines released by TAMs could regulate PD-L1 protein expression, which was reported to be an important predictor for anti–PD≥1/PD≥L1 therapy. In recent years, multiple immune cells have been identified in TME, and the cancer ecosystem has evolved over time, which plays a complex role in cancer development (31, 32). The interaction between macrophages and other immune cells was explored and demonstrated to be correlated to the response to immunotherapy (31). Single-cell and spatial analysis showed that interaction between FAP^+ fibroblasts and $SPP1^+$ macrophages could promote the formation of immune-excluded desmoplasic structures and restrict T-cell which reduces the efficacy of immunotherapy (31). In triple-negative breast cancer, high levels of $CXCL13^+$ T cells are associated with the proinflammatory features of macrophages and can predict the clinical benefit of checkpoint inhibitors (32).

Exosomes are small extracellular vesicles that play a crucial role in various cell activities in cancer. Recent studies have reported that

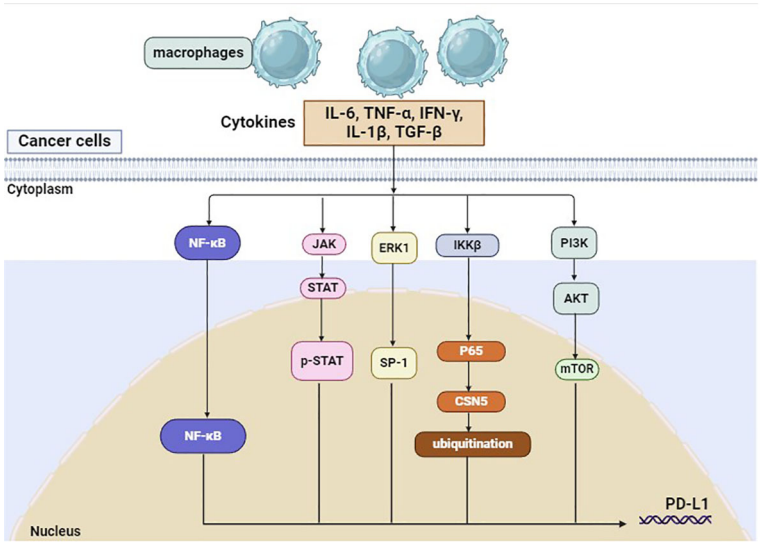

FIGURE 1
PD-L1 on tumor cells can be regulated by macrophages.

macrophage-derived exosomes may promote the formation of a pre-metastatic niche that facilitates tumor growth and metastasis. M2 macrophage–derived EVs can drive anti–PD≥1/PD≥L1 therapy resistance, promote the expression of drug-resistant genes in tumor cells, or affect the immune cell spectrum in TME (33, 34). Therefore, the interaction between TAMs and TME may contribute to anti–PD≥1 therapy resistance in cancer, providing a theoretical basis for the combination use of targeting macrophages and anti–PD≥1/PD≥L1 therapy.

4.3 Effect of anti–PD-1/PD-L1 therapy on macrophages

Previous studies have shown that PD-1 inhibitors have an impact on TME in various cancers (35). In non–small cell lung cancer, single-cell RNA sequencing demonstrated that the tumor microenvironment was remodeled after neoadjuvant PD-1 blockade combined with chemotherapy, and TAMs were transformed into a neutral type instead of an anti-tumor phenotype (36). Furthermore, anti–PD-L1 therapy can inhibit tumor growth by reducing PD-L1 expression and promoting the expression of the co-stimulatory molecules CD86 and major histocompatibility complex class II (MHC-II) (37). In addition, the phagocytic ability and immune function of macrophages were also enhanced by anti–PD-L1 therapy, which activates T cells in the TME and eradicates cancer cells (37). Therefore, anti–PD-L1 therapy may repolarize macrophages, enhance the phagocytic ability of macrophages, and ameliorate the tumor microenvironment in some patients.

5 Targeting macrophages in the tumor microenvironment

As TAM is involved in tumor immunity and tumor development, it may become a promising target in the future. Current treatment strategies targeting macrophages can be roughly divided into two categories: TAM depletion and TAM reprogramming (Supplementary Figure 1). In order to ensure treatment efficacy, targeting TAMs was frequently combined with other treatments in clinical studies, such as immunotherapy, chemotherapy, and radiotherapy (Table 1) (38–48).

5.1 Depletion of TAM

Depletion of macrophages in the tumor microenvironment may be an effective treatment strategy for cancer, either alone or in combination with chemotherapy. Inhibition of the signal transduction axis of colony-stimulating factor-1/colony-stimulating factor-1 receptor (CSF1/CSF1R), which is necessary for macrophage survival, can induce apoptosis of macrophages. On the one hand, inhibition of CSF-1R combined with radiotherapy or chemotherapy can improve T-cell responses. Blockade of CSF1R signaling can effectively deplete the immunosuppressive TAM and then stimulate the CD8+ T-cell response, resulting in prolonged survival in glioblastoma brain tumors (49). At present, CSF1R inhibitors in combination with chemotherapy are being tested in clinical trials in some cancers, such as localized prostate cancer and orthotopic glioblastoma (49, 50). In addition, blocking CSF1/CSF1R can improve the efficacy of a variety of immunotherapies, including CD-40 agonists (51) and PD-1 inhibitors (52).

As TAM was transformed from monocytes, blocking the recruitment of monocytes in the circulation to the tumor site was another method to reduce TAM in the tumor microenvironment. Recruitment of monocytes from bone marrow to the tumor site is dependent on C-C motif ligand 2 (CCL2)-CC chemokine receptor 2 (CCR2) signal transduction (53). Inhibition of CCR2 causes monocyte retention in bone marrow and leads to depletion of monocytes in the peripheral circulation, reduction of monocyte recruitment to the primary tumor sites and metastatic foci, and consequent reduction of TAM number, resulting in tumor shrinkage and survival improvement (54–56).

Other pathways involved in macrophage recruitment include CXCL12-CXCR4 and the angiopoietin 2 (ANG2)–TIE2 axis (57–59). Therefore, depletion of TEM may cause vascular destruction, neutralization of ANG2 may improve the response to vascular VEGFA blockade, and inhibition of TEM recruitment may inhibit tumor growth (60).

5.2 Reprogramming of TAM

As macrophages were the main phagocyte and antigen-presenting cell in the tumor, the immune stimulation function of macrophages was lost after the removal of TAMs. Therefore, reprogramming or repolarization of TAM to enhance its anti-tumor function and limit tumor-promoting properties is a more attractive strategy for cancer treatment. For example, in the mouse model of breast cancer, TAM represents the main source of IL-10 and inhibition of IL-10 signal transduction can significantly improve the efficacy of chemotherapy. The IL-10 secreted by TAM inhibits the IL-12 produced by APCs, thereby inhibiting the anti-tumor response of CD8+ T cells induced by paclitaxel and carboplatin (23). In addition, the repolarization of TAM makes it specifically express the proinflammatory cytokine IFN-α, which could activate NK cells and T cells in the tumor environment and significantly slow tumor growth in the mouse model (61). The epigenetic reprogramming of macrophages by inhibiting histone deacetylase (HDAC) can also trigger an immune response in T cells (62, 63). In the breast cancer model, selective class IIa HDAC inhibitor induces the anti-tumor macrophage phenotype, promotes the T-cell immune response, and increases the response to chemotherapy and immune checkpoint inhibitors (62). In addition, the activation of the PI3K signaling in macrophages can drive the immunosuppressive activity in TAM, whereas inhibition of the PI3K pathway can reprogram macrophages enhance T-cell responses (64, 65).

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TABLE 1 Selected clinical trials of agents targeting tumor-associated macrophages.

Compound	Clinical phase	Tumor type	Combination therapy	NCT identifier
Chemokine inhibitors				
Carlumab (anti-CCL2 antibodies; Centocor)	Phase II (completed)	Prostate cancer	NA	NCT00992186
BMS-813160 (CCR2/CCR5 antagonist; Bristol Myers Squibb)	Phase II (completed) [38]	Renal carcinoma	Nivolumab plus ipilimumab	NCT02996110
	Phase I/ II (completed)	Pancreatic cancer, CRC, NSCLC	Nivolumab, Nab- paclitaxel	NCT03184870
	Phase II (ongoing)	Hepatocellular carcinoma	Nivolumab	NCT04123379
PF-4136309 (CCR2 antagonist; Pfizer)	Phase II (completed) [39]	PDAC	Nab-paclitaxel, gemcitabine	NCT01413022
CCR5 antagonist (Pfizer)	Phase I (completed) [40]	CRC	Pembrolizumab	NCT03274804
	Phase I (completed)	Pancreatic cancer, CRC	Nivolumab plus ipilimumab	NCT04721301
CSF1R inhibitors				
PLX3397 (Plexxikon)	Phase I/II (ongoing)	Sarcoma, nerve- sheath tumours	Sirolimus	NCT02584647
	Phase I/ II (Terminated)	Advanced melanoma and solid tumours	Pembrolizumab	NCT02452424
	Phase I/ II (Completed)	Breast cancer	Eribulin	NCT01596751
	Phase I/II (completed) [41]	Glioblastoma	Radiotherapy, temozolomide	NCT01790503
BLZ945 (Novartis)	Phase I/ II (Terminated)	Solid tumours	PDR001 (anti- PD1)	NCT02829723
Anti-CSF1R antibodies				
LY3022855 (IMC-C S4; Eli Lilly)	Phase I/II (ongoing)	Melanoma	MEK/BRAF inhibitors	NCT03101254
Emactuzumab (RO5509554/RG7155; Roche)	Phase II (Terminated)	Gynecological neoplasms and ovarian cancer	Gynecological neoplasms and ovarian cancer	NCT02923739
	Phase I/II (ongoing)	PDAC	Nab- paclitaxel, gemcitabine	NCT03193190
	Phase I (completed) [42]	Solid tumors	Paclitaxel	NCT01494688
	Phase I (completed) [43]	Solid tumors	Atezolizumab	NCT02323191
	Phase I (completed) [44]	Solid tumors	RO7009789 (agonist anti-C D40)	NCT02760797
AMG820 (Amgen)	Phase I/II (completed) [45]	Pancreatic cancer, CRC, NSCLC	Pembrolizumab	NCT02713529
ARRAY-382 (Pfizer)	Phase I/II (completed) [46]	Solid tumors	Solid tumors	NCT02880371 (1)
Agonist anti-CD40 antibodies				
CP-870,893 (Pfizer; UPenn)	Phase I (completed)	Melanoma	NA	NCT02225002
	Phase I (completed) [47]	Solid tumors	Paclitaxel, carboplatin	NCT00607048
SEA-CD40 (Seagen)	Phase I (ongoing)	Solid and hematological tumors	Pembrolizumab, gemcitabine, Nab-paclitaxel	NCT02376699

(Continued)

TABLE 1 Continued

Compound	Clinical phase	Tumor type	Combination therapy	NCT identifier
Agonist anti-CD40 antibodies (cont.)				
APX005M (Apexigen)	Phase I (ongoing)	Melanoma, renal carcinoma	Nivolumab, ipilimumab	NCT04495257
	Phase I (ongoing)	Melanoma	Pembrolizumab	NCT02706353
	Phase II (ongoing) [48]	Oesophageal cancer	Radiation, paclitaxel, carboplatin	NCT03214250
	Phase I/II (ongoing)	Pancreatic cancer	Nab- paclitaxel, gemcitabine, nivolumab	NCT03214250
RO7009789 (Roche)	Phase I (completed)	Solid tumors	Vanucizumab (anti-A ng2– VEGF bispecific antibody)	NCT02665416
	Phase I (completed)	pancreatic cancer	Nab- paclitaxel and gemcitabine	NCT02588443
CDX-1140 (Roswell Park Cancer Institute)	Phase I (ongoing)	Breast cancer	Radiation, biological therapy, poly-I CLC	NCT04616248
NG-350A adenoviral vector (PsiOxus Therapeutics Ltd)	Phase I (ongoing)	Solid tumors	Immune-checkpoint blockade immunotherapy	NCT05165433

5.3 Macrophage cell therapy

CAR-T cells are reported to be effective in hematological malignancies, whereas the efficacy of CAR-T therapy remains dismal in solid tumors, as the entry of T cells into tumors is restrained (66, 67). However, CAR-macrophages (CAR-M) overcome this disadvantage as the macrophages in the TME could be replenished by circulating monocytes. CAR expression could enhance the antigen-dependent functions of macrophages, such as the secretion of cytokines, polarization, enhanced phagocytic ability, and anti-cancer activity (68). CAR-M cells mediate phagocytosis, exhibit M1 functions in a relatively stable way, and exert anti-tumor effects in primary and metastatic tumors (69). Currently, several clinical trials are underway or being developed to evaluate the anti-cancer efficacy of CAR-M in different tumors.

5.4 Combination of targeting macrophages and anti–PD-1 therapy in cancer

The combination of targeting macrophages and anti–PD-1 therapy in cancer has been investigated *in vitro* and *in vivo* (37, 70–72). As we have noted above, repolarization of TAM was considered a promising strategy for cancer treatment, and this approach can potentiate anti–PD-1 therapy efficacy in hepatocellular carcinoma (72). Chemotherapy and radiotherapy may reset macrophages toward an M1 phenotype and improving the efficacy of immunotherapy in cancer (71). Vinblastine can drive the polarization of TAMs to the M1 phenotype by activating NF-κB, increasing CD8+ T-cell populations, and improving the survival outcome of malignant tumor immunotherapy (71). Bi-target treatment such as PD-1–IL-2 cytokine variant (IL2v), which employs anti–PD-1 as a target moiety that is fused into an immuno-stimulatory IL2v, can improve the therapeutic efficacy by reprogramming immunosuppressive TAMs (70). In conclusion, targeting macrophages combined with anti–PD–1 therapy may be a promising strategy to overcome drug resistance in patients with cancer.

6 Conclusion

Macrophages are involved in various cell activities in cancer, and the interaction between macrophages and cancer cells or other immune cells is associated with tumor development. As an important part of the tumor microenvironment, TAMs may be a promosing target for cancer treatment. Targeting macrophages alone or combined with radiotherapy, chemotherapy, and immune-checkpoint inhibitors may produce excellent anti-tumor activity. In addition, the upstream and downstream pathways that may regulate the function of macrophages may also serve as therapeutic targets. In particular, the use of genetic engineering to reprogram macrophages to transform tumor-promoting TAM into anti-tumor macrophages is of great clinical application. Although the combination of targeting macrophages and anti–PD-1 therapy in cancer has been tried in clinical trials or preclinical experiments, this treatment approach is still in its infancy and needs further investigation. Stumbling blocks in the transformation and application of TAM-targeted therapy include the diversity and plasticity of mononuclear phagocytes in the TME (73). The dissection of the TME at the single-cell level confirmed the diversity of macrophages and their relationship with other immune cells (22, 31), which provides a rationale to selectively deplete tumor-promoting macrophages and eliminate tumors. The application of macrophage-targeted therapy in cancer is still in its infancy, and the efficacy and tolerance need to be confirmed in more experiments and clinical trials in the future.

Author contributions

LZ: Writing – original draft. TZ: Writing – original draft, Formal analysis, Data curation, Conceptualization. RZ: Resources, Project administration, Writing – original draft. CC: Methodology, Investigation, Writing – original draft. JL: Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition.

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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.2024.1381225/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Targeting macrophages in the tumor microenvironment.

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Phenotypic comparison and the potential antitumor function of immortalized bone marrow-derived macrophages (iBMDMs)

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Introduction: Macrophages are an important component of innate immunity and involved in the immune regulation of multiple diseases. The functional diversity and plasticity make macrophages to exhibit different polarization phenotypes after different stimuli. During tumor progression, the M2-like polarized tumor-associated macrophages (TAMs) promote tumor progression by assisting immune escape, facilitating tumor cell metastasis, and switching tumor angiogenesis. Our previous studies demonstrated that functional remodeling of TAMs through engineered-modifying or gene-editing provides the potential immunotherapy for tumor. However, lack of proliferation capacity and maintained immune memory of infused macrophages restricts the application of macrophage-based therapeutic strategies in the repressive tumor immune microenvironment (TIME). Although J2 retrovirus infection enabled immortalization of bone marrow-derived macrophages (iBMDMs) and facilitated the mechanisms exploration and application, little is known about the phenotypic and functional differences among multi kinds of macrophages.

Methods: HE staining was used to detect the biosafety of iBMDMs, and real-time quantitative PCR, immunofluorescence staining, and ELISA were used to detect the polarization response and expression of chemokines in iBMDMs. Flow cytometry, scratch assay, real-time quantitative PCR, and crystal violet staining were used to analyze its phagocytic function, as well as its impact on tumor cell migration, proliferation, and apoptosis. Not only that, the inhibitory effect of iBMDMs on tumor growth was detected through subcutaneous tumor loading, while the tumor tissue was paraffin sectioned and flow cytometry was used to detect its impact on the tumor microenvironment.

Results: In this study, we demonstrated iBMDMs exhibited the features of rapid proliferation and long-term survival. We also compared iBMDMs with RAW264.7 cell line and mouse primary BMDMs with *in vitro* and *in vivo* experiments, indicating that the iBMDMs could undergo the same polarization response as

normal macrophages with no obvious cellular morphology changes after polarization. What's more, iBMDMs owned stronger phagocytosis and pro-apoptosis functions on tumor cells. In addition, M1-polarized iBMDMs could maintain the anti-tumor phenotypes and domesticated the recruited macrophages of receptor mice, which further improved the TIME and repressed tumor growth.

Discussion: iBMDMs can serve as a good object for the function and mechanism study of macrophages and the optional source of macrophage immunotherapy.

KEYWORDS

macrophage, iBMDM, polarization, immunotherapy, antitumor

Introduction

Macrophages are an important component of intrinsic immunity and possess a variety of functions, including homeostasis maintenance, removal of cellular debris, elimination of pathogens and modulation of inflammatory responses (1, 2). In the tumor microenvironment, tumor-associated macrophages (TAMs) participate in immune regulation and tumor angiogenesis to affect tumor development (3–5). Due to the different stimuli in the microenvironment, TAMs present "two-sided" roles with various polarized phenotypes. Macrophages can be activated by interferon gamma (IFNγ) and Toll-like receptor (TLR) agonists to develop an inflammatory (M1-like) phenotype, thus exhibiting proinflammatory characteristics with microbial killing and tumor growth inhibition (6, 7). Conversely, in response to interleukin-4 (IL-4), IL-13 and IL-10 (M2-like activation), macrophages release anti-inflammatory factors, which promote immunosuppression, debris removal, angiogenesis, tissue remodeling and repair (7–9). Investigating the complex cellular mechanisms of macrophages in the context of disease is emerging as a fundamental step in understanding pathogenesis as well as performing macrophage immunotherapy (10).

Considering the pivotal influence of macrophage development and function on disease progression, immunotherapy based on macrophages has achieved some progress in recent years (11–15). Our previous studies have demonstrated that stimulated M1 macrophages and miR-125a-overexpressing macrophages could alleviate liver fibrosis and repress tumor growth, respectively (16). The strong plasticity and functional diversity endow macrophages with better immunotherapeutic effects and advantages. However, macrophage-based therapeutic strategies still face two limitations in terms of antitumor immunity. On the one hand, although engineered modified or gene-edited macrophages exhibit obvious antitumor potential, the repressive tumor immune microenvironment (TIME) accelerates their functional remodeling to limit immunotherapy. On the other hand, the lack of proliferation ability of infused macrophages makes gene editing and cell harvesting more difficult, which increases the treatment time and immunotherapy cost (17). How to solve the problem of microenvironment domestication and lasting proliferation ability has become the focus of macrophage immunotherapy.

Currently, the majority of macrophage sources for basic research and immunotherapy exploration consist of bone marrow-derived macrophages (BMDMs) (17–19), induced pluripotent stem cell-derived macrophages (iPS-Mφ) (20–22) and the RAW264.7 cell line (23–25). BMDMs are fully developed and function regulable and are more suitable for *in vitro* experiments and *in vivo* verification. However, the BMDMs could not achieve stable genotypic transformation due to proliferation limitations. Flexible gene editing and functional modification are advantages of iPS-Mφs in cellular immunotherapy. It is extremely costly and difficult to obtain and culture iPS-Mφs. Meanwhile, it has been reported that iPS-Mφs present an M2-like polarization phenotype, which is not appropriate for tumor immunotherapy (26). RAW264.7 is a kind of fusion-immortalized monocyte-macrophage line of BALB/c mouse origin that was established from murine tumors induced with Abelson leukemia virus by Raschke et al. in 1978 (27). The RAW264.7 cells were only used for some *in vitro* experiments of macrophage function analysis (28). Therefore, it is crucial to seek effective and safe cell sources for macrophage immunotherapy.

A growing amount of evidence highlights the intriguing possibility that macrophage immortalization may be a viable strategy for macrophage-based immunotherapy. J2 retrovirus infection-enabled immortalization has been successfully applied to fetal liver macrophages, spleen macrophages, microglia, and bone marrow-derived macrophages (BMDMs) (29–32). Immortalized macrophages express surface biomarkers of macrophages and possess typical functional characteristics. In addition, they share strong proliferation ability and long-term survival potential. Therefore, gene-edited immortalized macrophages are easy to construct, which facilitates the advancement of macrophage regulatory mechanisms. The study by Iolanda Spera et al. in 2021 detected and analyzed the functions of the immortalized BMDM

(iBMDM) cell lineage from a metabolic point of view (33). By determining intracellular and extracellular metabolites as well as the phenotypic characteristics of immortalized versus primary BMDMs, it was concluded that immortalized BMDMs exhibited similar metabolism and polarization characteristics under both classical and alternative stimulation. However, no study has systematically evaluated and compared the biosafety, immunological characteristics and antitumor functions of iBMDMs. In this study, we detected the proliferation efficiency and survival time of iBMDMs both *in vitro* and *in vivo*, indicating that iBMDMs have good biosafety and low immunogenicity. Immunology tests and coculture experiments with tumor cells were used to analyze the effect of iBMDMs on the malignant biological behaviors of tumor cells. Finally, the infusions of different macrophages into tumor-bearing mice suggested that iBMDMs present even stronger antitumor potential than primary BMDMs. Our study comprehensively explores the antitumor functions of iBMDMs *in vitro* and *in vivo* and demonstrates that iBMDMs are an optional source of macrophage immunotherapy.

Materials and methods

Animals and tumor models

Wild-type C57BL/6 mice used in this study were maintained in a specific pathogen-free facility. All the animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University to ensure the ethical and humane treatment of the animals. And all experiments used 8-week-old to 12-week-old male mice. The LLC cell line was purchased from the authenticated ATCC repository in 2014. LLC was mixed with macrophages at a ratio of 5:1 (5×10^6:1×10^6) and injected into the subcutaneous tissue of the backs of mice. The length and width of the tumor tissue were measured using a ruler and analyzed after 3 weeks of coculture. The mice were sacrificed at 2 or 3 weeks after inoculation, and tumors were digested to a single cell suspension with type V collagenase (Sigma, St. Louis, MO) and DNase I (Roche, Basel, Switzerland) for flow cytometry.

Cell culture

iBMDMs, RAW264.7 cells, and LLC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS). BMDMs were extracted from the bone marrow of C57BL/6 mice and cultured in DMEM supplemented with 10% FCS and 25 ng/mL murine macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ) for 7 days, and flow cytometry analysis was used to detect the stimulation efficiency of BMDMs. The GFP fragment was inserted into the viral vector and then infected into iBMDM to construct a stable cell line for subsequent experiments. In polarization-related experiments, macrophages were stimulated with IFN-γ (20 ng/mL, PeproTech, Rocky Hill, NJ), LPS (50 ng/mL, Sigma, St. Louis, MO) or IL4 (20 ng/mL, PeproTech, Rocky Hill, NJ) for 24 h and then used in follow-up experiments.

Immunofluorescence

The slides were placed into a 12-well plate and coated with polylysine, and the macrophages were plated on the slide to adhere to the wall, stimulated with IFN-γ, LPS or IL4 for 24 h, and stained with anti-iNOS and anti-ARG1 (CST, Danvers, MA). Mouse subcutaneous tumor tissues were removed and fixed in 4% paraformaldehyde, and then 30% sucrose solution was used to dehydrate them. The tissues were embedded and frozen for sectioning. The sections were stained with anti-F4/80 (Invitrogen, Carlsbad, CA), anti-iNOS or anti-MR and photographed with a fluorescence microscope (M5000, Thermo, Waltham, MA).

Flow cytometry

Tumor tissue was removed from the subcutaneous skin of mice, cut up, digested with 1 mg/mL collagenase V (Sigma, St. Louis, MO) and 4 mg/mL DNase I (Roche, Basel, Switzerland) at 37°C for 30 min to make a cell suspension, which was filtered through a 70-micron filter membrane and stained with flow cytometry antibody. Dead cells were removed by 7AAD. All the experimental results were analyzed by FACSCalibur and FACSCanto flow cytometry (BD Immunocytometry Systems). Data were processed by FlowJo v10 software (FlowJo, LLC, Ashland, OR).

Phagocytosis

LLC cell lines were suspended in PBS containing 0.1% serum at a concentration of 10^6 cells/ml, and the final concentration was 5 nM carboxyfluorescein succinimidyl amino ester (CFSE; MCE, NJ). The cells were stained at room temperature and shielded from light for 7 min. After staining, the same volume of serum was used to terminate the staining, and the stained LLC cells were incubated with macrophages at a ratio of 2:1 for 1 h. The phagocytosis ratio was detected by FACSCanto staining with anti-F4/80 (Invitrogen, Carlsbad, CA). The strength of phagocytosis between different cell lines was compared based on the percentage of double-positive cells in the flow-through results.

Apoptosis

Macrophages were stimulated with IFN-γ (20 ng/mL, PeproTech, Rocky Hill, NJ), LPS (50 ng/mL, Sigma, St. Louis, MO) or IL4 (20 ng/mL, PeproTech, Rocky Hill, NJ) for 24 h, the supernatant of the stimulated cells was taken. Tumor cells were seeded in 12-well plates, incubated with macrophage supernatant for 48 hours, stained with an Annexin V apoptosis detection kit (Invitrogen, Carlsbad, CA) and detected using FACS Calibur flow cytometry. Effects of macrophage supernatants with different

polarization states on early apoptosis, mid-apoptosis, and late apoptosis of tumor cells analyzed by flow data analysis.

Cell proliferation

The LLC cell line was seeded in 12-well plates, macrophage supernatants of different stimulation states were taken and incubated with tumor cells in LLC for 24 h. After fixation with 4% paraformaldehyde and staining with crystal violet (Kehao, Xi'an, China), the supernatants were washed with PBS 3 times and resuspended with acetic acid, the absorbance of the liquids was measured by an enzyme marker, and the effect of macrophage supernatants of each polarization state on the proliferation of tumor cells was compared based on the strength of the absorbance.

Cell migration

A marker was used to draw three lines on the back of the 12-well plate, and the LLC cells were inoculated into the 12-well plate. When the cells grew to 80%, the tip of the gun was used to draw a straight line perpendicular to the three lines, macrophage supernatants of different polarization states were added, photos of the scratch were taken at the intersection of the 3 straight lines according to different times, and the cell migration area was counted.

RT–PCR

Total cellular and tissue RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) reagent according to the instructions, and the concentration of the extracted RNA was determined and then transcribed into CDNA using a reverse transcription kit (Yeasen, Shanghai, China). SYBR Premix EX Taq (Yeasen, Shanghai, China) was added to the system according to its instructions, and real-time quantitative PCR was performed by QuantStudio.

Statistics

All experimental data were statistically processed by GraphPad Prism 5 software, and unpaired Student's t tests or one-way ANOVA was used for comparison. When the data results were expressed as $P<0.05$, they were considered statistically significant.

Results

iBMDMs exhibit the similar cellular characteristics of primary macrophage

CD11b and F4/80 are specific markers for macrophages (34). To identify the macrophage characteristics of iBMDMs, we performed FACS analysis by anti-F4/80 and anti-CD11b staining. As expected, iBMDMs, BMDMs and RAW264.7 cells shared similar macrophage biomarker expression patterns (Figure 1A). For further morphological comparison, the three kinds of macrophages were stimulated with different cytokines for observation by microscopy. The M1-polarized BMDMs had some appearing cogwheel, while M2-polarized BMDMs had longer pseudopodia compared with controls. The RAW264.7 cells protruded more pseudopodia after polarization stimulation. In contrast to polarized BMDMs or RAW264.7 cells, iBMDMs presented polygon or round shapes regardless of the cytokines added (Figure 1B).

Next, we verified the survival and biosafety of iBMDMs *in vivo* and *in vitro*. *In vitro*, we cultured the cells for a long time and passaged them at a ratio of 1:10 each time. According to the passage cycle, the proliferation activity of the cells was measured. The results showed that the proliferation efficiency of iBMDMs began to slow down after 3 weeks of *in vitro* culture and was almost quiescent after 6 weeks (Figure 1C). Similarly, we validated the survival cycle of iBMDMs in mice. iBMDMs survived for 3 weeks, but the number of surviving iBMDMs gradually decreased over time (Figure 1D). The results suggested that iBMDMs possessed a long-term lifespan but no immortalization capacity both *in vivo* and *in vitro*, which provided feasibility for iBMDM-based cell therapy. To ensure the biosafety of iBMDMs, we carried out HE staining by using sections of different tissues from iBMDM reinfused mice. The results showed that iBMDMs were nontoxic to mouse tissue and can be used for subsequent treatment in mice (Figure 1E). The above data suggested that iBMDMs maintained rapid proliferation and long-term lifespan, indicating potential cell sources for immunotherapy.

Normal polarization response is possible with iBMDMs

Phenotypic alterations in iBMDMs were assessed by qPCR detection of specific M1 (IL-1β and iNOS) and M2 (Arg1 and MR) polarization biomarkers. The results revealed that after LPS+INF-γ treatment, the levels of M1 genes were specifically increased in all three types of macrophages. In addition, the expression of M2 polarization markers was obviously increased in IL-4-treated macrophages. It should be noted that although the iBMDMs presented a similar polarization response to primary BMDMs or macrophage lines, the mRNA elevations of all M1-specific biomarkers (IL-1β, iNOS and TNF-α) were mild in iBMDMs compared with RAW264.7 cells or BMDMs. Compared to RAW264.7, there was no significant difference in the M2 biomarkers of iBMDM, but compared to BMDM, the increase of iBMDM was milder under IL4 stimulation (Figures 2A–C). The ELISA assay also demonstrate that iBMDM can be polarized with different stimuli, and the results indicate that M1-iBMDMs produced much higher levels of proinflammatory cytokines (TNF-α and IL-12) and lower levels of anti-inflammatory factors (IL-10 and TGF-β). Interestingly, the differences in secreted proteins between the three kinds of macrophages were not particularly significant. Under both M1- and M2-polarization conditions, iBMDMs presented the same level of inflammatory response as BMDMs (Figure 2D). M1-type macrophages have elevated aerobic glycolysis and produce inducible

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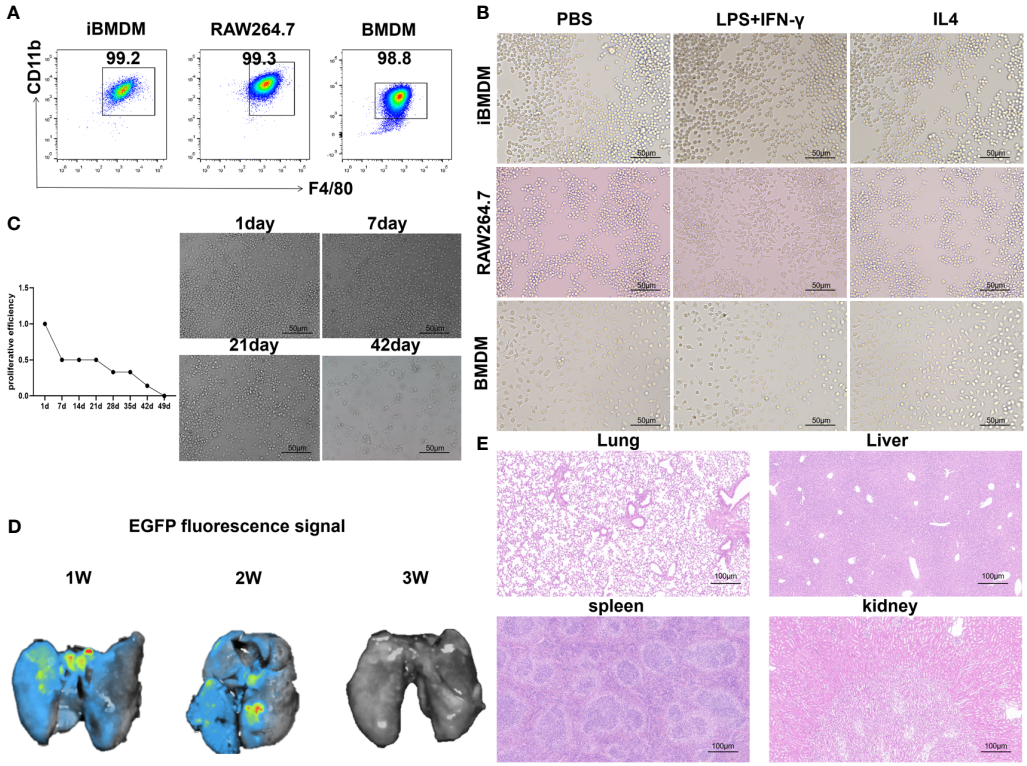

FIGURE 1
iBMDMs exhibit the similar cellular characteristics of primary macrophage. **(A)** Flow cytometry was used to detect macrophage-specific markers after staining with F4/80 and CD11b for iBMDMs, RAW264.7 cells, and BMDMs. **(B)** Using PBS, LPS+IFN-γ and IL-4 stimulated iBMDMs, RAW264.7 cells, and BMDMs, morphological changes in macrophages were observed after polarization stimulation using an inverted microscope. **(C)** Pass iBMDMs at a ratio of 1:10. An inverted microscope was used to take photos and observe morphological changes. Determine cell proliferation activity *in vitro* based on the length of its passage cycle. **(D)** iBMDMs were injected into mice, and fluorescence signal expression at different times was detected to determine the survival time of iBMDMs in mice. **(E)** Injection of ibmdm into mice and detection of the biological safety of iBMDMs in mice by HE staining of mouse tissues.

nitric oxide synthase (iNOS), which is associated with antitumor and anti-infection immunity (35). ARG1 is an enzyme involved in arginine metabolism and generation in macrophages that leads to T-cell exhaustion and functional repression (36). iNOS and ARG1 are essential markers of M1 polarization and M2 polarization, respectively. To further observe the expression of polarization markers in different cell lines, three kinds of macrophages were stimulated and stained with anti-iNOS and anti-ARG1. Meanwhile, we determined the effect of surpernatant from different macrophages on T cell activation. The results of ELISA assay suggested that M1-iBMDMs secreted higher levels of chemokines CXCL11 and CXCL12 to promote T-cell recruitment. The productions of T cells activation cytokines, including IL2 and IL15, were also increased in M1-iBMDMs (Figure 2E). The immunofluorescence results showed a similar conclusion that M1-iBMDMs expressed higher levels of iNOS, while M2-iBMDMs exhibited advantages in ARG1 expression, which was even more obvious than that of BMDMs and RAW264.7 cells (Figures 2F–H). These data suggested that iBMDMs perform a similar polarization response as other macrophage sources.

iBMDMs strongly phagocytose tumor cells

Next, we explored the phagocytosis function of macrophages, which plays a key role in tumor killing and pathogen removal (37). To examine the differences in phagocytosis among the three kinds of macrophages, iBMDMs, RAW264.7 cells and BMDMs were stimulated with polarization factors for 24 h and then cocultured with CFSE-stained LLC cells at a ratio of 1:2. Two hours later, the phagocytosis capacity was evaluated by calculating the proportion of macrophages swallowing tumor cells ($F4/80^+CFSE^+$) using flow cytometry. It should be noted that the phagocytic ability of M1-BMDMs was elevated 10-fold compared with that of quiescent BMDMs, which is equivalent to the response level of RAW264.7 cells. However, after LPS+INF-γ treatment, iBMDMs presented much stronger phagocytic enhancement. The engulfment rate of M1-loaded iBMDMs was almost 20 times that of quiescent iBMDMs (Figures 3A–D). In summary, M1-iBMDMs exhibited strong phagocytosis, which was stronger than that of BMDMs and RAW264.7 cells, and the results further demonstrated that immortalized BMDMs could interact well with tumor cells.

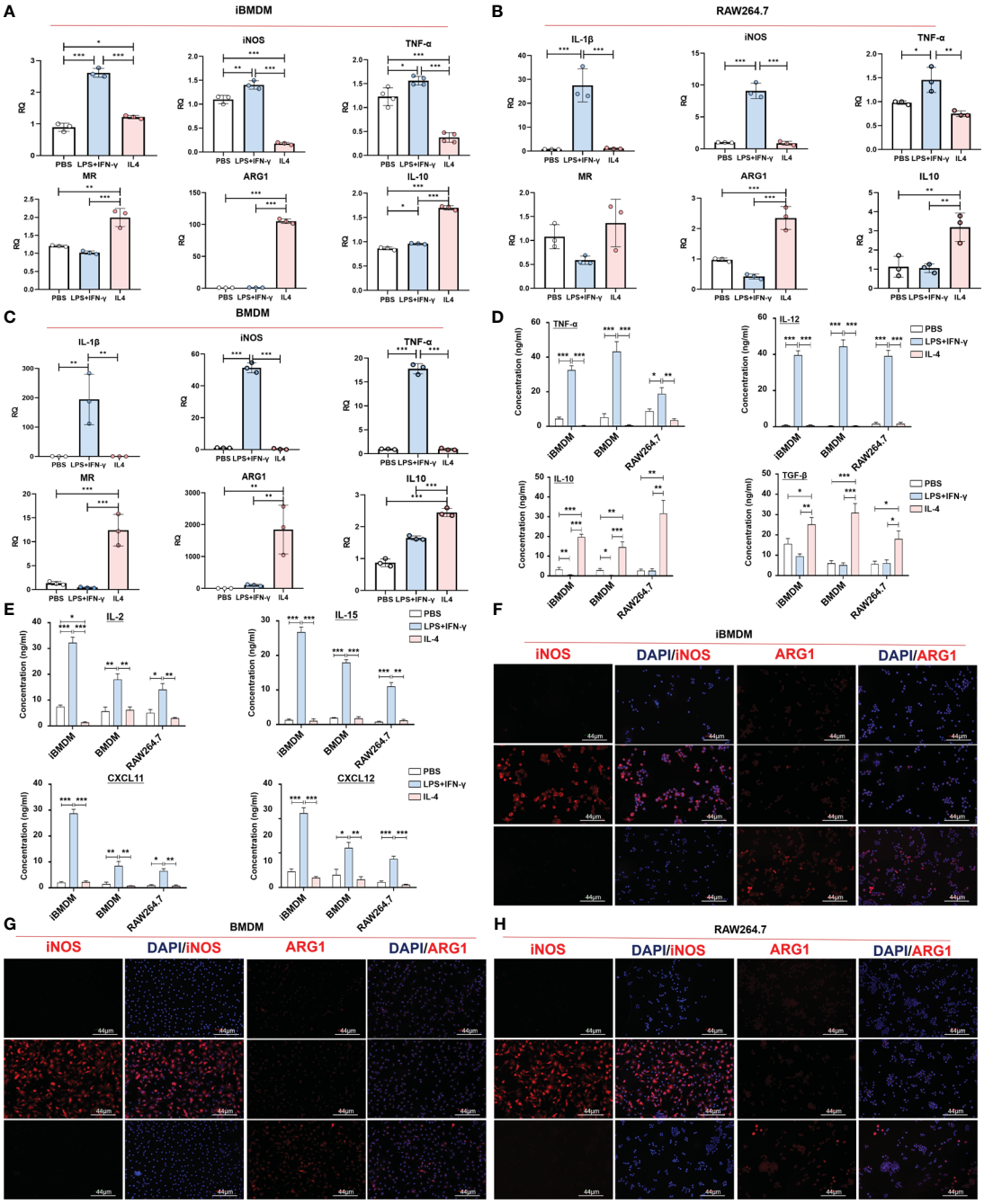

FIGURE 2
Normal polarization response is possible with iBMDM. **(A–G)** iBMDMs, RAW264.7 cells and BMDMs were treated with PBS, LPS+IFN-γ and IL4 for 24 hours. **(A–C)** QPCR was used to detect the expression of M1 polarization markers (IL-1β and iNOS) and M2 polarization markers (MR and ARG1) in iBMDMs, RAW264.7 cells, and BMDMs under different stimuli. **(D, E)** Macrophage supernatant was collected under different stimuli, and ELISA was used to detect the expression of M1 markers (TNF-α and IL-12), M2 markers (IL10 and TGF-β), T cell-associated functional factors (IL2 and IL15) and chemokines (CXCL11 and CXCL12) in the macrophage supernatant. **(F–H)** Anti-iNOS and anti-ARG1 were used as primary antibodies for immunofluorescence staining and to detect the expression of markers after macrophage polarization. Bars, mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001.

The paracrine of iBMDMs inhibits proliferation and promotes apoptosis of tumor cells

In addition to direct phagocytosis to inhibit tumor progression, macrophages can also modulate tumor cell migration, proliferation and apoptosis by secreting multiple cytokines and inflammatory mediators. To further verify the macrophage function of iBMDMs, we incubated LLC cells in the supernatant of macrophages with different treatments for 24 hours and determined cell proliferation by measuring absorbance after staining with crystal violet. As expected, the supernatant of all three groups of macrophages

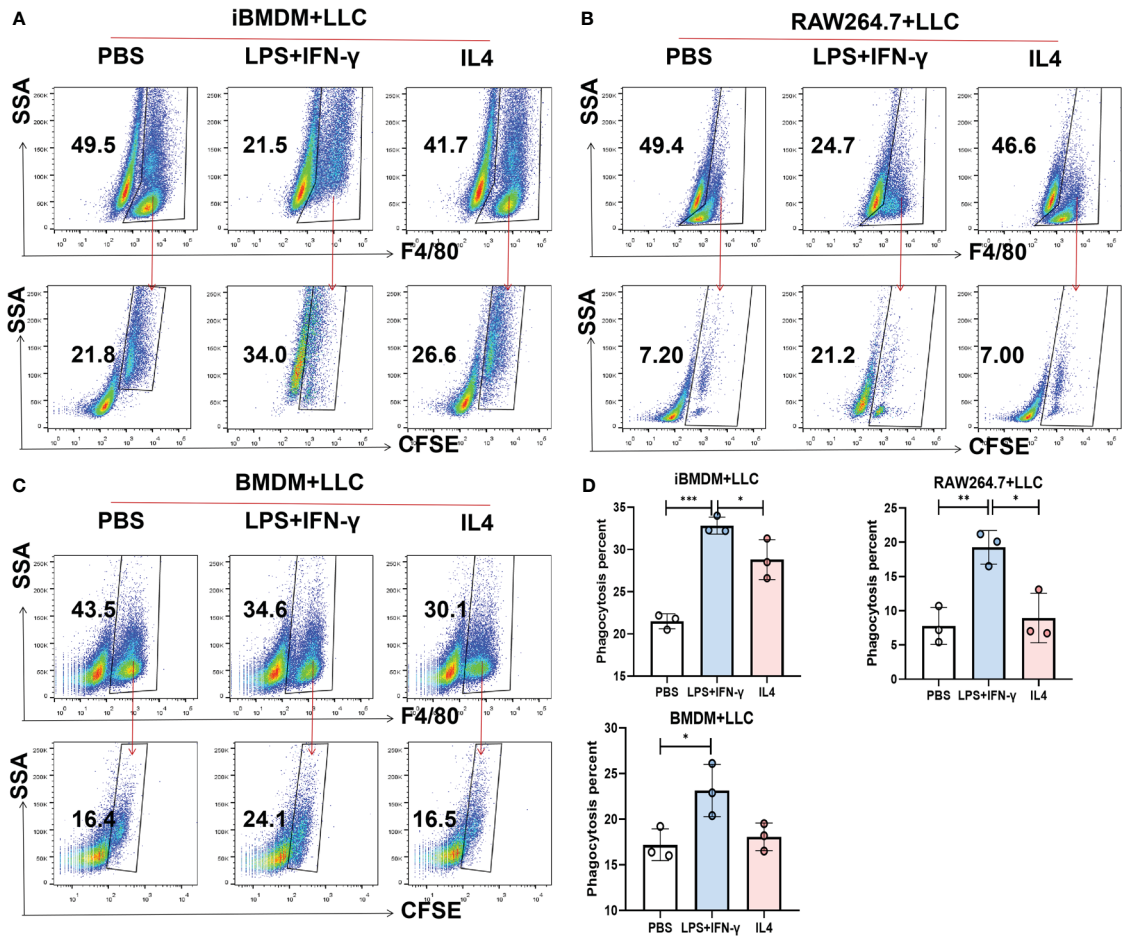

FIGURE 3
iBMDMs strongly phagocytose tumor cells. **(A-D)** iBMDMs, RAW264.7 cells and BMDMs were treated with PBS, LPS+IFN-γ and IL4 for 24 hours. Differently treated iBMDMs, RAW264.7 cells, and BMDMs were coincubated with LLCs at a ratio of 1:2 for one hour, and the phagocytosis ability of macrophages was detected by flow cytometry. Bars, mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001.

exhibited the inhibition of tumor cell proliferation, especially that of M1 macrophages. The results were consistent with previous results showing that M1-type macrophages have the ability to inhibit tumor growth. Interestingly, compared with RAW264.7 cells and BMDMs, iBMDMs showed a significant decrease in tumor cell proliferation after M1 polarization. This result proved that the iBMDMs had a stronger antitumor function (Figure 4A).

Furthermore, we cocultured LLC cells with macrophage supernatant for 48 hours and detected LLC apoptosis by using Annexin V/PI staining. We found that the control group without co-culture of macrophages showed that tumor cells almost did not undergo apoptosis in the absence of co-culture. And the supernatant of M1 macrophages promoted tumor cell apoptosis more obviously than PBS-treated macrophages. In addition, although the iBMDM supernatant displayed a certain ability to induce tumor cell apoptosis, the percentage of late apoptotic cells was much lower than that of LLC cells incubated with supernatant from BMDMs or RAW264.7 cells (Figures 4B, C). We speculate that the factors secreted by iBMDMs mainly influence the early stage of tumor cell apoptosis. In addition, we have tested the apoptosis function of tumor cells incubating with supernatant of different macrophages by qRT-PCR. The results showed that supernatant of M1-iBMDMs promoted the expression of apoptosis associated genes, including APAF1 and Caspase-9 in tumor cells, and decreased the level of protective molecule BCL2 (Figure 4D). Considered that TNF-α, which was increased in M1-iBMDM (Figure 2D), possesses the effect of promoting tumor cells apoptosis, it is reasonable that iBMDM induced apoptosis of tumor cells through cytotoxic cytokine such as TNF-α. In summary, the secreted component of iBMDMs had obvious effects on inhibiting tumor cell proliferation and promoting apoptosis, which indicated that iBMDMs could also repress tumor growth indirectly.

iBMDMs repress tumor cell migration via inhibiting EMT progress

Modulating tumor cell invasion and participating in the formation of migrated units are important functions of tumor-associated macrophages. The wound healing assay was performed by incubating tumor cells with macrophage supernatants to test the

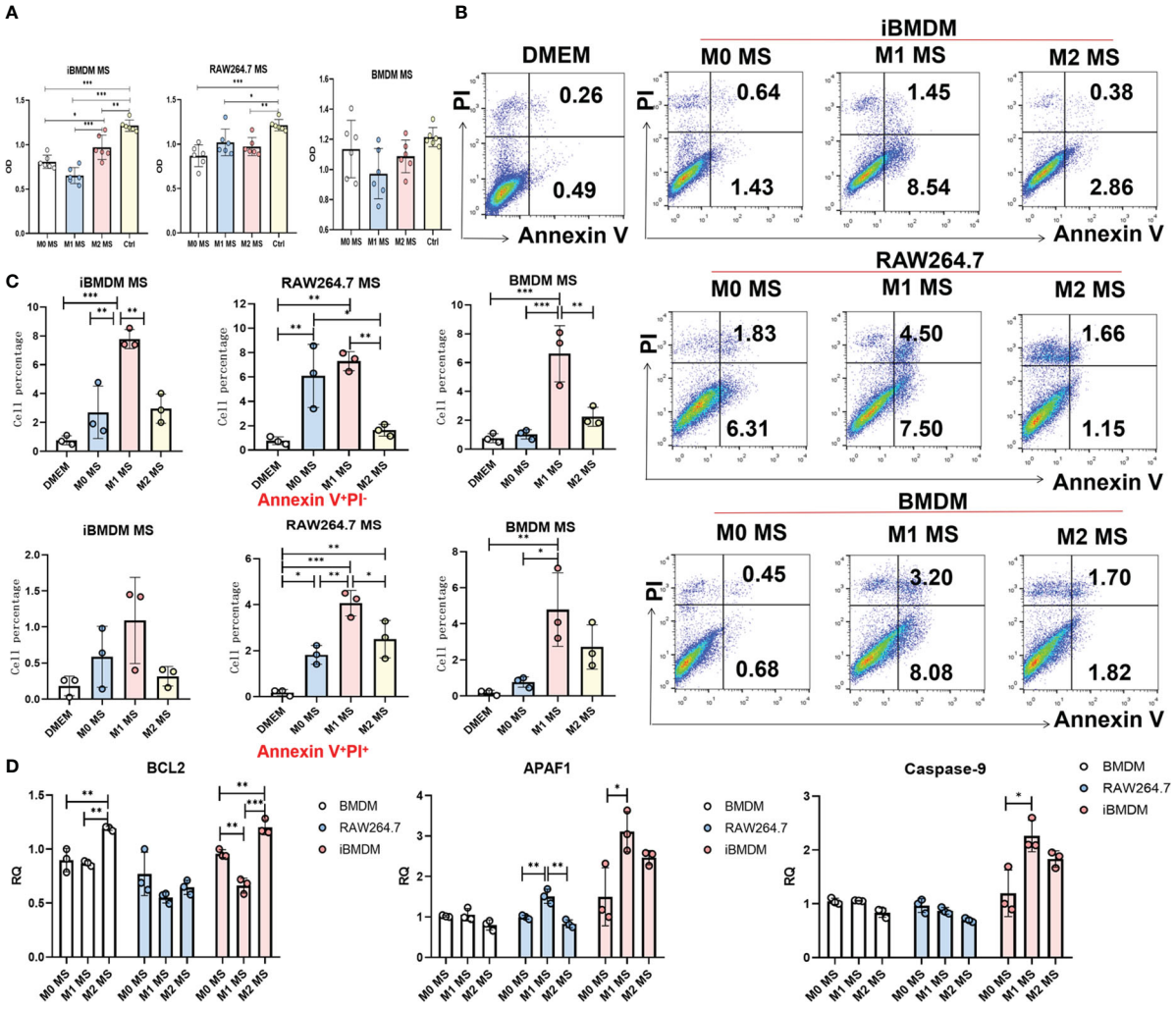

FIGURE 4
The paracrine of iBMDMs inhibits proliferation and promotes apoptosis of tumor cells. **(A–D)** iBMDMs, RAW264.7 cells and BMDMs were treated with PBS, LPS+IFN-γ and IL4 for 24 hours. **(A)** iBMDMs, RAW264.7 cells and BMDM supernatants were coincubated with LLCs in a 96-well plate for 24 h, and the absorbance was measured by an enzyme labeling instrument after crystal violet staining to detect the effect of macrophage supernatants on the proliferation of tumor cells. **(B, C)** iBMDM, RAW264.7, and BMDM supernatants were coincubated with LLCs for 48 h, and apoptosis of tumor cells was detected by flow cytometry. Bars, mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001. **(D)** Detection of apoptosis-related genes (BCL2, APAF1, Caspase-9) in LLC after macrophage supernatant treatment using QPCR.

cell motion at different time periods. The results showed that the modulation effect was not obvious after 16 h of incubation. However, tumor migration was significantly inhibited by M1 macrophage supernatant after 24 h treatment. As expected, iBMDMs, especially M1-iBMDMs, presented the most remarkable inhibitory effect (Figures 5A–E).

Multiple factors can influence tumor cell infiltration and metastasis. To investigate the mechanisms by which macrophage paracrine signaling affects LLC mobility, we cocultured macrophage supernatants with LLC cells for 24 h and then detected the expression of EMT (epithelial-mesenchymal transition) -related genes, which are responsible for tumor cell migration to some extent (38). The data suggested that iBMDMs exhibited notable repressive effects on LLC EMT. After administration of M1-iBMDM supernatant, the expression of the tight junction-related membrane protein ZO-1 and the EMT essential transcription factors Snail1 and Twist was greatly reduced, which was superior to the other two types of macrophages (Figure 5F). The above results demonstrated that the paracrine pathway of iBMDMs plays a significant role in tumor cell EMT progression and migration regulation.

M1-polarized iBMDMs rather than primary BMDMs repress tumor growth *in vivo*

Previous experiments have demonstrated the macrophage characteristics and antitumor functions of iBMDMs *in vitro*. To verify the phenotypes and effects of iBMDMs during tumor progression, we stimulated EGFP-modified BMDMs or iBMDMs into M1 polarization and mixed them with LLC at a ratio of 1:5 to inoculate them subcutaneously on the backs of mice. The tumor size and weight were monitored after 3 weeks. The tumor volume and weight in the M1-iBMDM group were smaller than those in the M0-iBMDM group, which had an inhibitory effect on tumor

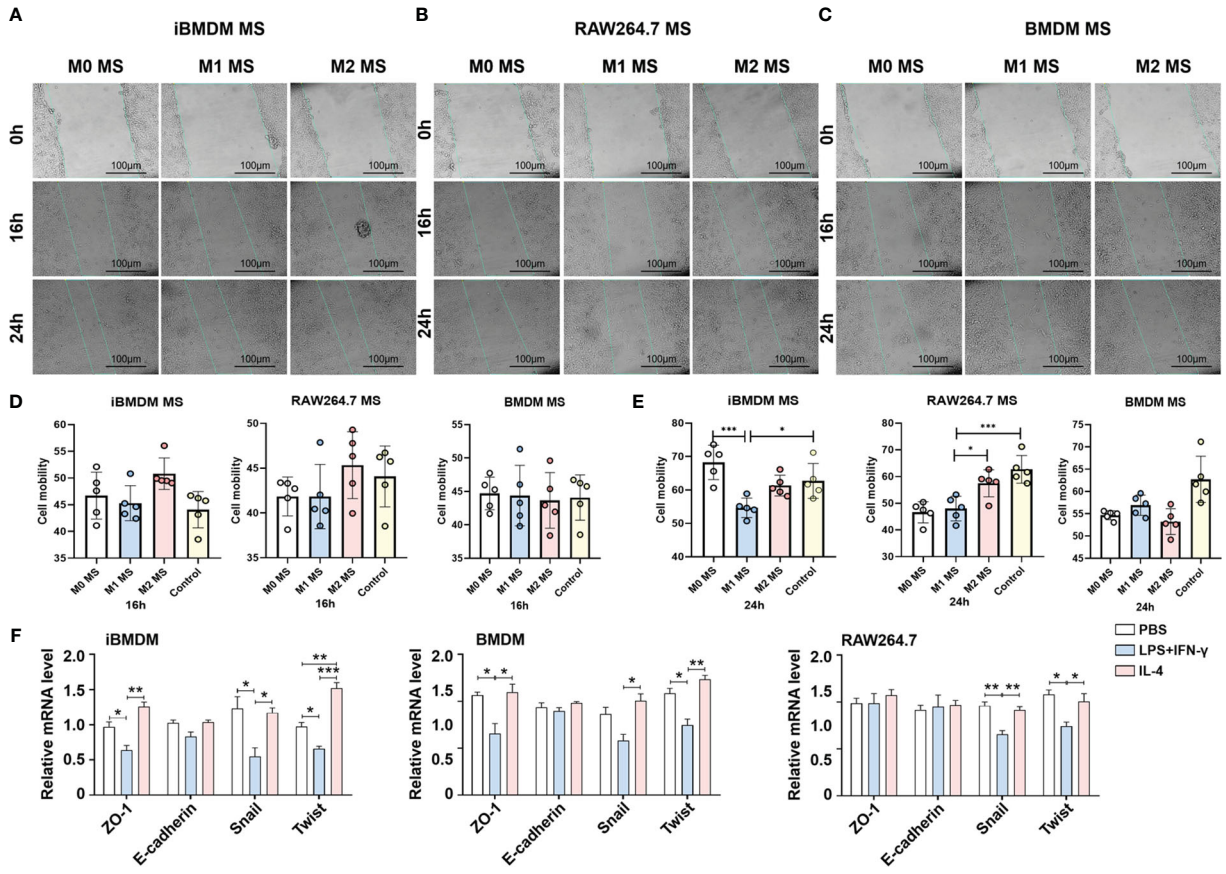

FIGURE 5
iBMDMs repress tumor cell migration via inhibiting EMT progress. **(A–C)** PBS, LPS+IFN-γ, IL4-stimulated iBMDMs, RAW264.7 cells, and BMDMs for 24 h. iBMDMs, RAW264.7 cells, and BMDM supernatants with different polarization states were coincubated with LLCs, and the effect of macrophage supernatants on tumor cell migration was detected by scratch. **(D, E)** ImageJ software was used to process the scratch results and generate statistics on the processing results. **(F)** iBMDMs, RAW264.7 cells, and BMDM supernatants were coincubated with tumor cells for 24 h, and the expression of EMT (epithelial-mesenchymal transition)-related genes was detected by QPCR. Bars, mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001.

growth. Compared with BMDMs, M1-BMDMs did not have a significant inhibitory effect and even showed an upward trend (Figures 6A, B). The results showed that the tumor size and weight of the iBMDM group treated with M1 polarization were significantly decreased compared with those of the control group (M0 group) (Figure 6A). Meanwhile, the changes in tumor size and weight in the BMDM infusion groups were not obvious and were even increased in the M1-BMDM treatment group (Figure 6B). Ki67 and TUNEL staining was also performed using tumor sections. The Ki67 staining data suggested that there was no significant change between the M1-BMDM and M0-BMDM groups. Meanwhile, M1-iBMDM treatment inhibited tumor cell proliferation and reduced tumor malignancy. (Figure 6C). TUNEL staining showed that M0-iBMDMs significantly promoted tumor cell apoptosis compared with M0-BMDMs. Interestingly, there was no significant difference between M1-iBMDMs and M1-BMDMs (Figure 6D). These results indicated that M1-polarized iBMDMs possessed significant antitumor activity *in vivo* by modulating the malignant biological behaviors of tumor cells.

Next, we examined the expression of representative molecular markers of different polarization phenotypes and macrophage function with tumor sections. The immunofluorescence results showed that there were significantly more M2-type macrophages (MR^+ $F4/80^+$) than M1-type macrophages ($iNOS^+$ $F4/80^+$) in the tumor tissues of both the iBMDM and BMDM groups. However, regardless of M0- and M1-polarized BMDM therapy, few iNOS-positive macrophages were detected after three weeks, suggesting that BMDM infusion did not domesticate the recruited macrophages. This might be because the survival time of infused BMDMs was too short to exert the immune regulatory function completely. In contrast, iBMDM infusion stimulated more M1-like TAMs to improve the immune microenvironment and repress tumor growth. Especially after the infusion of M1-type iBMDMs, the number of iNOS-positive TAMs increased significantly (Figure 6E). Most infused macrophages could not last their lifespan to 3 weeks in tumors. Even long-term iBMDMs could not be detected in the infused tissue *in vivo* (Figure 1). Obviously, the infused iBMDMs had a profound and sustained impact on the endogenous recruited

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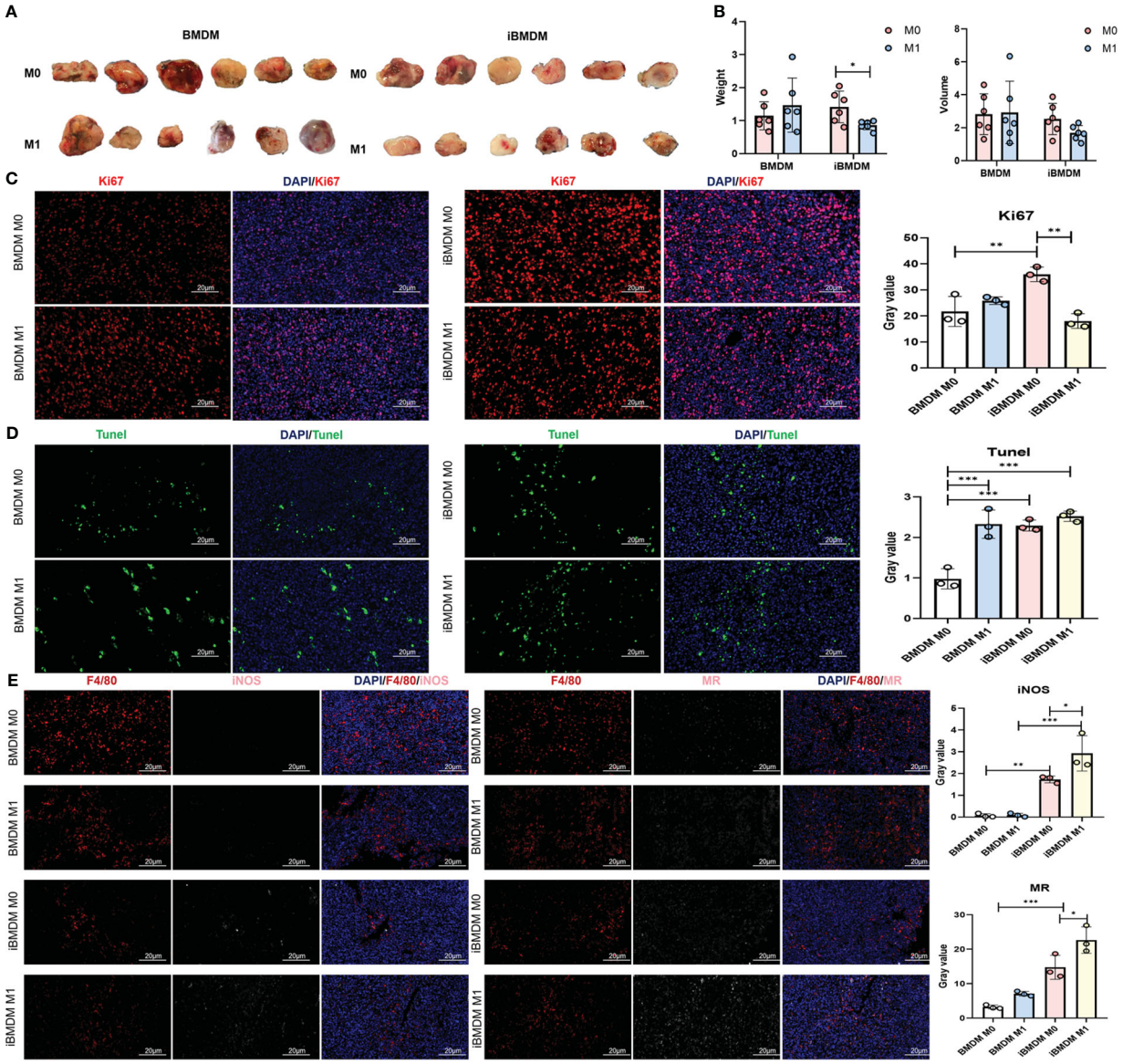

FIGURE 6
M1-polarized iBMDMs rather than primary BMDMs repress tumor growth *in vivo*. **(A, B)** iBMDMs and BMDMs were stimulated with PBS or LPS + IFN-γ for 24 h, mixed with LLC at a ratio of 1:5, and inoculated subcutaneously, and the size and weight of the tumors were measured and compared after 3 weeks. **(C, D)** Immobilization, embedding, and sectioning of subcutaneous tumor tissue were performed using Ki67 and TUNEL staining. Fluorescence microscopy was used to detect the expression of Ki67 and TUNEL in tumor tissue, and the effects of the two types of macrophages on tumor cell proliferation and apoptosis in the tumor microenvironment were compared. **(E)** Perform F4/80, iNOS, and MR staining on subcutaneous tumor sections. Fluorescence microscopy photography was used to detect the impact of the two types of macrophages on the tumor microenvironment in tumor tissue. Bars, mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001.

macrophages and tumor microenvironment. The effect of switching TAMs into the M1-like phenotype might be the reason why M1-iBMDM therapy could inhibit tumor development.

M1-polarized iBMDMs domesticate self-recruited TAMs and improve the tumor microenvironment

To further investigate the impact of iBMDMs on the tumor microenvironment, we digested the tumor tissues into single-cell suspensions for further FACS analysis. TAMs were classified into three subgroups based on Ly6C and major histocompatibility complex class II (MHCII class) expression: mature TAMs (ma-TAMs) ($Ly6C^{-}MHCII^{+}$), immature TAMs (imm-TAMs) ($Ly6C^{-}MHCII^{-}$), and TAM precursors (pre-TAMs) ($Ly6C^{+}MHCII^{-}$) (39, 40). Many studies have demonstrated that mature TAMs highly express M1 polarization-related markers and exert antitumor functions. Compared to that in the BMDM group, the proportion of mature TAMs in the iBMDM group was significantly increased, while the proportion of immature TAMs and TAM precursors was significantly decreased. Similarly, the M1 iBMDM group had a

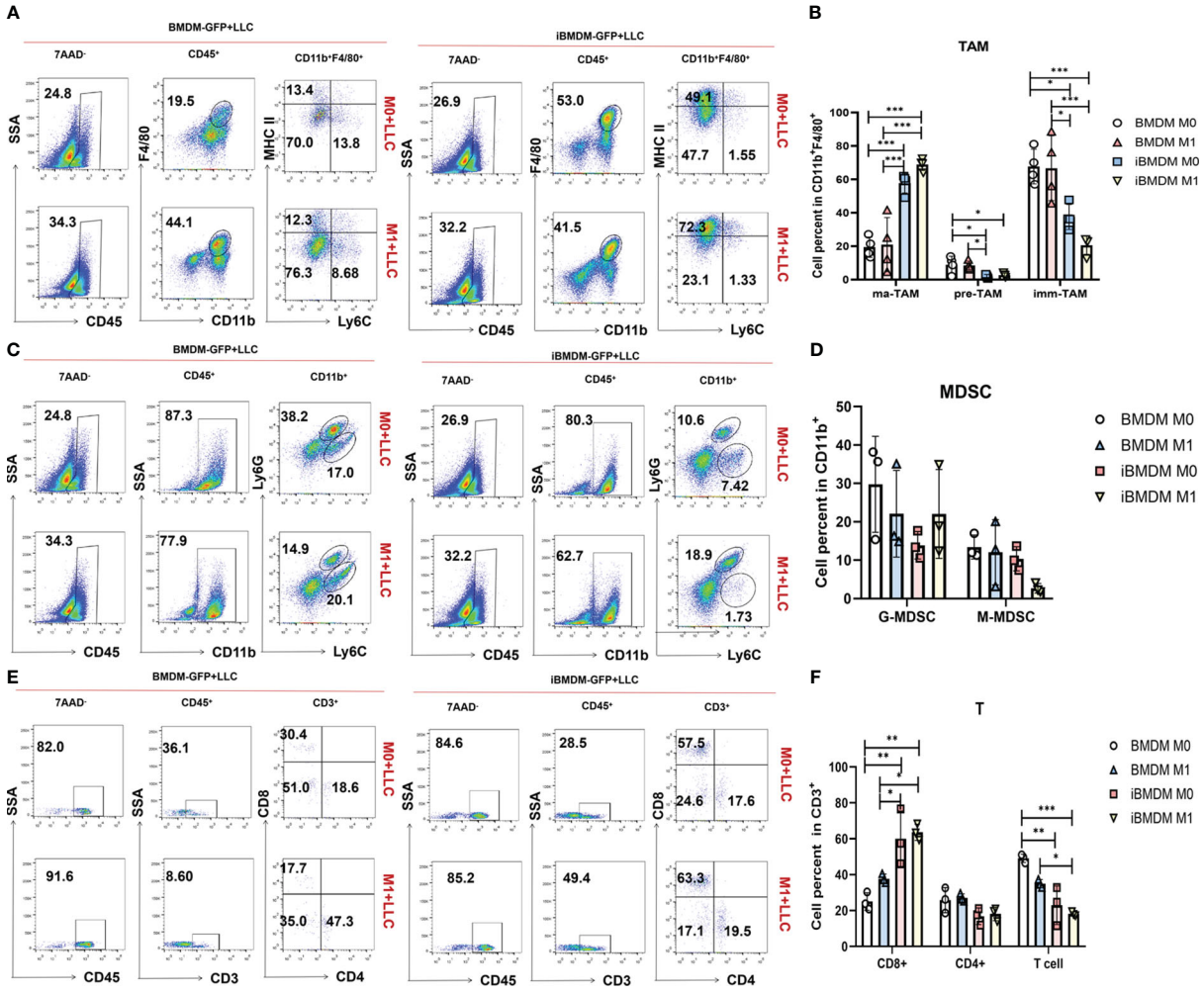

FIGURE 7
M1-polarized iBMDMs domesticate self-recruited TAMs and improve the tumor microenvironment. **(A-F)** PBS, LPS+IFN-γ-stimulated iBMDMs, and BMDMs for 24 h were mixed with LLC at a ratio of 1:5 and then inoculated subcutaneously. After 3 weeks of tumor inoculation, flow cytometry was used to evaluate the tumor immune microenvironment of mice with different treatments, and the percentages of TAMs **(A, B)**, MDSCs **(C, D)**, and T cells **(E, F)** in the tumor immune microenvironment were detected and analyzed. Bars, mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001.

significantly higher population of Ly6C$^-$MHCII$^+$ ma-TAMs than the M0-iBMDM treatment group, while BMDMs did not have this result (Figures 7A, B).

G-MDSCs and M-MDSCs play an important role in the tumor microenvironment. In tumor-related myeloid cells (CD11b$^+$), the proportions of G-MDSCs and M-MDSCs in the iBMDM group were lower than those in the BMDM group. iBMDMs can reduce G-MDSCs and M-MDSCs in the tumor microenvironment, improve the tumor microenvironment, and inhibit tumor growth. Moreover, M1 iBMDMs had a more significant inhibitory effect on M-MDSCs (Figures 7C, D). In addition, iBMDMs can promote the recruitment of CD8$^+$ T cells in tumors, increase the direct killing of tumor cells in the microenvironment, and further improve the microenvironment of tumors (Figures 7E, F). Our *in vitro* experiment results had demonstrated that the paracrine pathway of M1-iBMDM could promote the recruitment and activation of T cells (Figure 2E), which could explained the promotion of T cell population. In summary, iBMDMs not only have excellent macrophage function *in vitro* but also improve the tumor microenvironment *in vivo*, recruiting endogenous macrophages to exhibit an antitumor phenotype while reducing the proliferation of various tumor-promoting immune cells and increasing the number of antitumor CD8$^+$ T cells, thus inhibiting tumor growth. *In vivo*, the therapeutic effect of iBMDMs is superior to that of BMDMs.

Discussion

Macrophages are considered as the key immune regulator during tumor initiation and development. Macrophages not only contribute to the recruitment and activation of immune cells in the tumor microenvironment (TME) but also play an important role in tumor cell metastasis (41). Targeting tumor-associated macrophages (TAMs) is currently considered a promising strategy for combating cancer (42). How to polarize TAMs to an antitumor state without affecting macrophage activity is of great

research significance to reduce tumor growth and metastasis. The current understanding of the mechanisms involved in controlling the cancer and metastatic cascade response remains limited. Studies of mechanisms that regulate innate immune activation require *in vitro* cellular experiments or *in vivo* therapeutic validation. Currently, the most common used cell resources for macrophage research are BMDMs and RAW264.7 cells (28). BMDMs, as primary bone marrow-derived cells, need to be obtained from the bone marrow followed by stimulation and cultivation with different cytokines, which requires much more time and cost. Meanwhile, the short survival time of BMDMs is not suitable for establishing a stable transduced cell line, which limits their application for *in vivo* transfusion therapy (43). In contrast, RAW264.7 is a type of peritoneal macrophage from confluent mice that has immunogenicity and tumor-promoting properties and is not suitable for *in vivo* reperfusion therapy. Immortalized macrophages may help meet the current needs of reducing macrophage research costs and establishing stable and transmissible cell lines.

This study introduces the iBMDM cell line constructed by Elisabetta Blasi et al. in 1986 (29) and compares it with BMDM and RAW264.7 cell lines in terms of phenotype, characteristics, polarization detection, and *in vitro* and *in vivo* antitumor functions, proving the feasibility of using iBMDM cell lines as macrophages for research within a certain range. Previous studies have not systematically compared the differences in function and characteristics between immortalized macrophages and nonimmortalized cells and explored their advantages and disadvantages as macrophage therapy. This experiment validated the relative safety and effectiveness of using iBMDMs as a macrophage therapy resource. We also investigated macrophage polarization regulation and tumor microenvironment domestication reversal, especially the influence on endogenously recruited macrophages. Our data indicate that the iBMDM cell line is actually not immortalized but possesses a relatively longer survival time both *in vitro* and *in vivo*. The long-term lifespan of iBMDMs provides more possibilities for cell therapy while ensuring biological safety because they do not remain in the body after reinfusion. In addition, we also demonstrated that although iBMDMs exhibited similar characteristics as other macrophages (BMDMs and RAW264.7 cells), certain differences were found among different macrophage sources in the expression levels of polarization markers and their *in vitro* and *in vivo* antitumor functions. It should be noted that iBMDMs were demonstrated to exhibit a superior ability to improve the tumor microenvironment and repress tumor development compared to BMDMs. Our data indicated that iBMDMs facilitated T cell recruitment and activation via chemokines and cytokines secretion both *in vitro* and *in vivo* (Figures 2E, F). However, this does not fully represent the remodeling of T cell function. The role of iBMDMs on T cells and microenvironment needs to be further investigated.

In conclusion, our data indicate that these long-term iBMDMs possess macrophage characteristics and functions and are superior to other macrophages in some aspects. It can be used for *in vivo* and *in vitro* experiments on macrophages and is expected to serve as a cell resource for macrophage reinfusion therapy. Moreover, iBMDMs could be further modified with genetic editing. The edited iBMDMs presented a more stable phenotype and stronger antitumor functions. Therefore, iBMDMs have great potential for application in immune cell therapy. We also hope to further investigate the molecular mechanisms of the differences between BMDMs and iBMDMs. To advance research on macrophage therapy and its clinical application as soon as possible, more comprehensive and in-depth research needs to be implemented to identify the key molecules involved in phenotypic and functional changes. The main difficulties limiting iBMDMs-based therapy to human patients is that no human immortalized BMDMs could be used for modification or gene-editing for immune-therapies. The further investigating is required to solve how to immortalize human-derived BMDMs. Meanwhile, the quantitative systems pharmacology modeling has realized the accurate prediction of tumor therapy and the evaluation of immunotherapy effects by combining pharmacokinetics, pharmacodynamics and disease progression. The model has greatly improved the research and development efficiency of cellular immunotherapy and combination drugs (44, 45). It provides an excellent theoretical basis for the promotion of clinical immunotherapy. The use of quantitative systems pharmacology modeling could help the research focus on modified-iBMDMs and human immortalized-macrophages for immunotherapy in clinic. The revelation of the immortalization characteristics of macrophages also provides a reference for the immortalization of other immune cells (such as NK cells, dendritic cells, and T cells). and lays a foundation for further improving tumor immunotherapy.

Data availability statement

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

Ethics statement

The animal study was approved by Animal Experiment Administration Committee of the Fourth Military Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DKX: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing. JY: Formal analysis, Investigation, Methodology, Validation, Writing - original draft. PHL: Conceptualization, Formal analysis, Investigation, Validation, Writing - original draft. YWZ: Formal analysis, Investigation, Methodology, Validation, Writing - original draft. JNC: Formal analysis, Investigation, Writing - original draft.

XLC: Methodology, Funding acquisition, Writing - original draft. SLC: Investigation, Methodology, Validation, Writing - original draft. YMC: Formal analysis, Investigation, Visualization, Writing - original draft. YFH: Formal analysis, Validation, Writing - original draft. LW: Data curation, Methodology, Validation, Writing - original draft. ZHW: Investigation, Visualization, Writing - original draft. RQ: Formal analysis, Validation, Writing - original draft. JMG: Investigation, Visualization, Writing - original draft. HY: Investigation, Writing - original draft. LW: Formal analysis, Validation, Writing - original draft. ZYL: Investigation, Methodology, Writing - original draft. HH: Conceptualization, Supervision, Writing - review & editing. HYQ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing. JLZ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

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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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Macrophages: plastic participants in the diagnosis and treatment of head and neck squamous cell carcinoma

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Head and neck squamous cell carcinoma (HNSCC) rank among the most prevalent types of head and neck cancer globally. Unfortunately, a significant number of patients receive their diagnoses at advanced stages, limiting the effectiveness of available treatments. The tumor microenvironment (TME) is a pivotal player in HNSCC development, with macrophages holding a central role. Macrophages demonstrate diverse functions within the TME, both inhibiting and facilitating cancer progression. M1 macrophages are characterized by their phagocytic and immune activities, while M2 macrophages tend to promote inflammation and immunosuppression. Striking a balance between these different polarization states is essential for maintaining overall health, yet in the context of tumors, M2 macrophages typically prevail. Recent efforts have been directed at controlling the polarization states of macrophages, paving the way for novel approaches to cancer treatment. Various drugs and immunotherapies, including innovative treatments based on macrophages like engineering macrophages and CAR-M cell therapy, have been developed. This article provides an overview of the roles played by macrophages in HNSCC, explores potential therapeutic targets and strategies, and presents fresh perspectives on the future of HNSCC treatment.

KEYWORDS

head and neck squamous cell carcinoma, macrophages, tumor microenvironment, immunotherapies, engineering macrophages, CAR-M cell therapy

1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most prevalent form of head and neck cancer and ranks as the seventh most common cancer worldwide (1). Regrettably, most instances are detected at advanced stages, often involving locally advanced (LA) conditions or distant metastasis (DM). Despite the availability of various treatment options,

such as surgery, radiation therapy (RT), chemotherapy (CT), and immunotherapy (IT), a significant portion (40-60%) of LA tumors eventually experience relapse or local progression. Palliative CT for metastatic and recurrent (R/M) HNSCC tumors also presents a grim prognosis (2). The tumor microenvironment (TME) refers to the immediate surroundings of HNSCC tumors during their growth or mutation, exhibiting complexity. On one hand, alterations such as cytokine production and extracellular matrix changes occur within the TME, alongside immune surveillance which identifies and attacks tumor cells, thereby inhibiting tumor growth. On the other hand, tumor cells can interact with surrounding tissues to modify nutrient supply, generate cytokines, and suppress immune responses within the TME, thus promoting their own survival and development (3, 4).

Most patients diagnosed as HNSCC often present with locally advanced disease, requiring multimodal treatments, including immunotherapy (5, 6). Immunotherapy involves the specific recognition and targeting of cancer cells by immune cells within the TME. The TME contains various immune cells such as macrophages, effector T cells, natural killer cells, and dendritic cells (7). Among them, macrophages constitute the largest and most critical group of innate immune cells in the TME (8) (Figure 1A). Macrophages originate from bone marrow hematopoietic stem cells and embryonic yolk sac tissue (9, 10). Under the influence of different microenvironmental stimuli, macrophages exhibit heterogeneity and plasticity, allowing them to adapt their characteristics in highly specialized ways to perceive and respond to their environment. While the specific phenotypes are hard to categorize, they can be simplified into two extremes with entirely different molecular phenotypes and functional characteristics: IFN-γ/lipopolysaccharide (LPS)-induced M1 macrophages and IL-4/IL-10/IL-13-induced M2 macrophages (7).

Furthermore, tumor-associated macrophages (TAMs) refer to a type of macrophages that appear in the TME that exhibit characteristics of both M1 and M2 macrophages under different signals and stimuli (11, 12). Among these, in the TME, typical M1 activators include LPS, IL-1β, and IFN-γ, which activate macrophages to produce inflammatory factors. Conversely, M2 polarization factors such as IL-4, IL-13, IL-10, and TGF-β promote macrophage polarization towards the M2 type, exhibiting characteristics of anti-inflammatory and immune suppression (13). In HNSCC, TAMs typically display M2 macrophage features, contributing to the establishment of an immune-suppressive TME, thereby promoting tumor escape and growth (12, 14). The balance between macrophage M1 and M2 subtypes is crucial for maintaining a stable state of health in the human body. When this balance is disrupted, it can lead to disease states (15). Metabolic adaptation supports the heterogeneity of tumor-associated macrophage activities and functions, maintaining their polarization in specific environments (16, 17). In particular, in terms of energy supply, M1 TAMs primarily rely on glycolysis. The two interruptions in their TCA cycle lead to the accumulation of itaconate and succinate, resulting in the stabilization of HIF1α. This further activates the transcription of glycolytic genes, thereby maintaining the glycolytic metabolism of M1 cells (18). Conversely, M2 cells are more dependent on oxidative phosphorylation, with their TCA cycle intact and providing substrates for the electron transport chain complexes

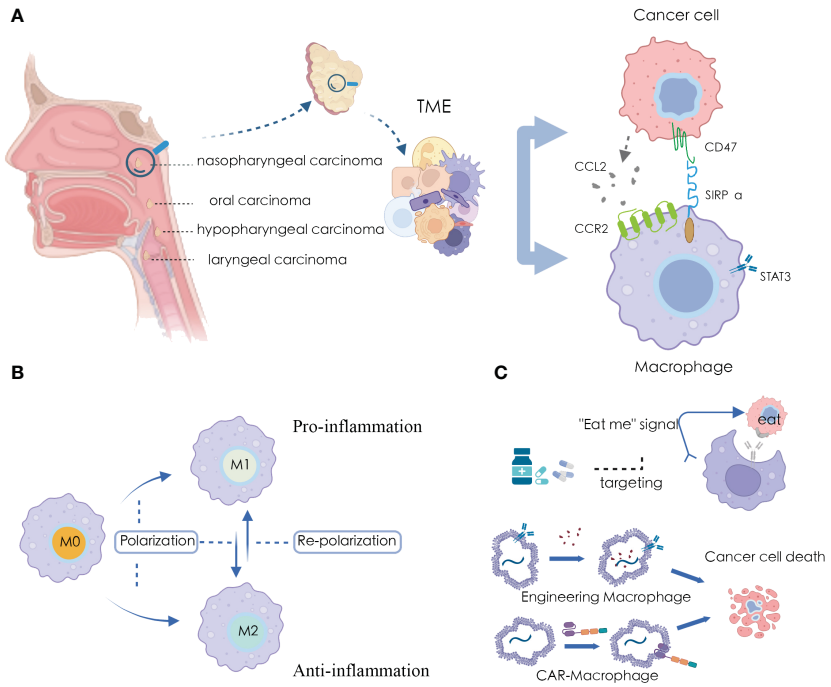

FIGURE 1
Macrophages and applications in HNSCC microenvironment **(A)** Significant crosstalk between macrophages and tumor cells in HNSCC TME; **(B)** The plasticity of macrophage polarization states; **(C)** Targeting and harnessing macrophages in disease treatment and immunotherapy.

(18). Additionally, both subtypes have specific metabolic pathways that regulate lipid and amino acid metabolism, influencing their responses (19, 20).

Due to the high plasticity of macrophages, they play a significant role in various pathological processes, further exploring macrophages is considered as an alternative approach for cancer therapy (21). Recent studies have indicated that promoting M1 macrophage polarization and inhibiting M2 macrophage polarization can exert anti-tumor effects, while promoting M2 macrophage polarization and inhibiting M1 macrophage polarization can have anti-inflammatory activity (22) (Figure 1B). Several drugs have been discovered that can modulate the polarization state of macrophages for disease treatment (23, 24), and many immunotherapeutic approaches have been developed using macrophages as carriers or tools (25, 26) (Figure 1C).

This review commences by examining the roles fulfilled by macrophages within the microenvironment of HNSCC. Additionally, explain the diverse roles and conditions they play during the development of head and neck tumors. Furthermore, we elaborate on essential therapeutic targets and the most recent related treatment methodologies, with the intent of presenting novel perspectives for forthcoming research and therapeutic strategies targeting macrophages in HNSCC.

2 Macrophage involvement in the HNSCC microenvironment

2.1 Phagocytosis and secretory regulation

In the tumor microenvironment, macrophages exhibit a dual role in HNSCC. On one hand, they can suppress cancer progression through immune actions, while on the other hand, they play a pro-cancer role by affecting several features of HNSCC, such as immune evasion, promoting invasion and metastasis, participating in angiogenesis, and influencing cancer cell proliferation (27–30).

Phagocytosis is one of the most innate capabilities of macrophages. They can actively engulf abnormal cells within the body, including cancer cells. During this process, macrophages extend pseudopodia to envelop cancer cells in vesicles formed by the cell membrane, creating a structure called a phagosome. Subsequently, this phagosome is internalized within the macrophage (31). This process helps reduce the number of tumor cells, alleviate the tumor burden, and contribute to controlling tumor growth. Currently, in cancer therapy research and development, a new generation of anti-cancer therapies has emerged based on harnessing and enhancing the phagocytic ability of macrophages, such as CAR-engineered cell therapies known as CAR-macrophages (32), which may potentially reshape the landscape of HNSCC treatment in the future.

Macrophages also regulate immune responses and tumor progression by modulating their polarization states through secretory regulation, wherein they secrete cytokines (33). For example, the secretion of certain cytokines may induce macrophages to polarize towards the M1 phenotype, exhibiting characteristics that promote immune responses and anti-tumor effects, such as TNF-α, IL-6, and IL-12 (34). Conversely, other factors may lead to macrophage polarization towards the M2 phenotype, showing features of immune suppression and promoting tumor growth, such as TGF-β and IL-10 (34). Additionally, chemokines such as CXCL9, CXCL10, and CXCL11 can attract T cells and other immune cells, thereby promoting macrophage polarization towards the M1 phenotype (35). The expression levels and activities of these factors are crucial for regulating the polarization status of macrophages, thereby influencing their functions and roles within the tumor microenvironment.

In HNSCC, TAMs typically exhibit an M2-polarized state, promoting immune evasion and growth of the tumor (12). In the early stages of tumors, TAMs release nitric oxide (NO) and reactive oxygen intermediates (ROI), causing DNA damage and genetic instability (36). Afterward, they actively participate in regulating the HNSCC microenvironment through direct secretion. These macrophages can secrete factors that enhance cell migration, including epidermal growth factor, cysteine cathepsins, and matrix metalloproteinases. Through the action of these matrix-degrading enzymes, they facilitate the movement of tumor cells (37). TAMs can also suppress T cell cytotoxicity by secreting IL-10, promote regulatory T cells, leading to immune evasion and tumor proliferation (38). Moreover, they can produce factors that promote the growth of blood vessels within the tumor, such as VEGF-A, VEGF-C, and adrenomedullin, thereby supplying oxygen for tumor development (39).

2.2 Exosomes

Extracellular vesicles, also known as exosomes, serve as vesicles originating from tumor cells, immune cells, and various other cell types. They play a role in promoting tumor proliferation, invasion, migration, modulating tumor immunology, fostering angiogenesis, and reprogramming the tumor microenvironment (40). In the progression of head and neck cancer, exosomes serve as a vital means of communication between macrophages and cancer cells. Previous studies have revealed that M1 macrophages secrete exosomes, inhibiting the proliferation, migration, and invasion abilities of head and neck cancer. These exosomes can also induce apoptosis in cancer cells, and HOXA transcripts at the distal tip (HOTTIP), as a tumorigenic specific lncRNA, is a critical molecule in these exosomes, showing the same functionality when overexpressed (41).

Additionally, in oral squamous cell carcinoma, cancer cells secrete exosomes, particularly CMTM6, which induce polarization of M2 macrophages via the ERK1/2 signaling pathway, thereby promoting malignant progression of the tumor. During this process, CMTM6 also enhances the expression of PD-L1, thereby driving tumor migration and invasion (42).

Exosomes extracted from other HNSCC cell lines, including JHU011, SNU1076, and SCC-VII, can significantly induce polarization of M2 macrophages. Exosomes carrying PD-L1 and stimulating HNSCC promote the activation of regulatory T cells (aTregs), further strengthening the positive feedback loop between aTregs and M2 macrophages, ultimately leading to immune escape in

tumors (43). The crosstalk mediated by exosomes between macrophages plays a significant role in the complex pathophysiology of head and neck cancer.

2.3 Macrophage polarization identification

Traditionally, in HNSCC surgical specimens, TAMs are typically detected using specific antibodies like CD68, CD80, and CD163. CD80+ corresponds to M1 type, while CD163+ corresponds to M2 type (44).

Recently, there has been a shift towards using the mutually exclusive gene expression of CXCL9 and SPP1, along with their ratio, as key features and standards for assessing the polarization level of macrophages within the TME. Bill et al. conducted sequencing and clinical data analysis on 52 patients with head and neck squamous cell carcinoma, revealing that the CXCL9:SPP1 expression ratio, termed CShi or CSlow, is associated with inhibition of certain pro-tumor and anti-tumor effects in head and neck tumors. For instance, CShi tumors are more prone to immune cell infiltration, promoting anti-tumor immunity (45). They propose that evaluating the CXCL9:SPP1 ratio in macrophages could serve as a comprehensive indicator for several critical aspects, such as the presence of anti-tumor immune cells in cancer, gene expression profiles of different tumor-infiltrating cell types, control or progression of communication networks influencing tumors, and the effectiveness of immunotherapy (45). This valuable insight holds profound implications for prospective studies aimed at formulating personalized treatment strategies and prognostic evaluations.

3 Diverse macrophage polarization and its implications in the progression of HNSCC

3.1 Polarization

Macrophage polarization refers to the distinct activation status of macrophages at a specific moment. This state is determined by their variable expression of surface receptors, secretion patterns, and functional roles. In cancer researches, macrophages typically exhibit an M1 pro-inflammatory profile in the early phases, but they transition to an anti-inflammatory M2 profile in later stages. Macrophage polarization is dynamic, reflecting their adaptability, and it can change in response to a variety of signals from other cells, tissues, and pathogens (46).

In the mice model of oral cancer precursor lesions exposed to nicotine smoke, the degree of M2 polarization at the disease site increased with exposure. Simultaneously, metabolic levels of compounds such as L-nicotine, D-glutamate, arachidic acid, and L-arginine also rose. Some of the mice with heightened M2 polarization even directly developed oral cancer. During this process, there was a decrease in pro-inflammatory factors (iNOS and TNF-α) that induce M1 polarization, resulting in reduced monocyte recruitment to replace them. The polarization shifted towards M2, leading to a significant increase in M2 functional factors like Arg-1 and IL-10. Further, this sustained M2 polarization is indicative of an ongoing immune response, facilitating heightened he activity of Th2 cells and instigating an immunogenic reaction (47).

In head and neck tumors, the Warburg effect manifests as excessive lactate formation, enabling cancer cells to adapt their metabolism to meet the oxygen requirements and the substantial nucleotide, amino acid, and lipid needs for cell proliferation (48). The end product, lactate, is found at higher concentrations in head and neck cancer compared to normal tissue, creating an active environment to promote cancer progression. Notably, the lactate produced by tumor cells has multifaceted effects. It can promote tumor progression by activating pro-inflammatory pathways like IL-23/IL-17 (49), while also inducing monocytes to polarize towards the M2 phenotype, thereby serving as a mediator of immunosuppression to further drive cancer progression (50, 51). Indeed, low pH can reduce the expression of iNOS, CCL2, and IL-6 in M1 macrophages, but increase the expression of M2 macrophage markers in the TME (52). Correspondingly, lactate can promote the M2-like phenotype by activating G-protein-coupled receptor 132 (GPR132) in macrophages, and genetic deletion of Gpr132 in macrophages reduces the M2-like features of tumor-associated macrophages and decreases lung metastasis in a mouse breast cancer model (53). Additionally, lactate can induce the expression of the enzyme ARG1 which indicates lactate can transform macrophages into immunosuppressive macrophages and promote M2-macrophage polarization in mice (54). The intratumoral lactate levels in human HNSCC are associated with the polarization of M2-like macrophages in the TME as well. When lactate levels in the tumor are low, more macrophages accumulate at the tumor site. Conversely, under conditions of high lactate concentration, monocyte migration is inhibited, preventing effective macrophage infiltration into lactate-rich tumors, but it promotes their polarization towards the M2 phenotype. However, unlike in mice models, both lactate and M2-polarization levels are not associated with the expression of ARG1 in human macrophages (55).

Moreover, in the interaction between cancer cells and macrophages in HNSCC, cancer cells release Apelin peptide, which promotes the polarization of M2 macrophages. Inhibiting the release of Apelin peptide by cancer cells leads to an increase in pro-inflammatory responses in co-cultured macrophages, resulting in a significant upregulation of genes like IL-1β, IL-6, and TNF-α, along with a marked reduction in anti-inflammatory cytokine levels. In the Apelin (+) group, pro-inflammatory factors are decreased, while anti-inflammatory factors are heightened (56).Tumor-derived extracellular vesicles expressing TGF-β also play a significant role in the crosstalk between tumor cells and macrophages in the HNSCC TME. These extracellular vesicles induce polarization and chemotaxis of human macrophages and also reprogram the function of primary human macrophages. This reprogramming results in increased secretion of pro-angiogenic factors, including Angiopoietin-2, MMP-9, PD-ECGF, and TIMP-1, and a shift toward a pro-angiogenic phenotype. Upon injection into mice with oral cancer induced by 4-nitroquinoline 1-oxide (4-NQO), these vesicles promote tumor angiogenesis, enhance infiltration of M2-like macrophages, and accelerate tumor progression (28).

The mechanisms through which M2-polarized cells regulate the progression of head and neck cancer are intricate. TAMs enhance the stemness of head and neck cancer cells by activating the PI3K-4EBP1 pathway. Additionally, TAMs interact with head and neck cancer cells through the CD44-VCAM-1 pathway, ultimately boosting the invasive capabilities of cancer cells (57). Furthermore, it has been demonstrated that M2 polarization can increase the expression of PD-L2 in TAMs, leading to immune evasion and tumor progression through the PD-1 signaling pathway (58).

3.2 Re-polarization

In general, M1 macrophages provide immune protection by releasing pro-inflammatory cytokines, whereas M2 macrophages exhibit anti-inflammatory properties that aid in tissue remodeling and tumor advancement (59). In cancer research, the differentiation of macrophages into M1 type from the alternative M2 type, a process known as macrophage repolarization, is a promising approach in contemporary cancer immunotherapy. Repolarizing TAMs from M2-to-M1 is considered a prospective therapeutic strategy.

To reprogram TAMs without altering the M1/M2 polarization balance within healthy organs, Xiao et al. developed a micelle nano-therapy. They released M2-targeted antagonists after exposure to the acidic tumor microenvironment, co-delivering inhibitors like STAT6 to effectively achieve M2-to-M1 repolarization, thereby inhibiting tumor growth and metastasis (60). Additionally, Wu et al. have coupled targeted drugs with tumor-specific STING agonists, finding that within the tumor microenvironment, M2 repolarizes towards M1 (61). Furthermore, statins have been found to inhibit proliferation of recurrent/metastatic HNSCC cells, enhance T cell cytotoxicity against tumor cells, and promote M2-to-M1 macrophage repolarization (62). Statins, known for their tolerability and affordability, may further enhance responses to PD-L1 checkpoint blockade and other HNSCC immunotherapies, although this potential remains to be fully explored.

Simultaneously, macrophage repolarization often broadly refers to macrophages polarizing towards different functional directions. In the previously mentioned exosomes, M1 exosomes and HOTTIP induced M1 repolarization within the tumor microenvironment, encompassing macrophage repolarization.

4 Main targets for macrophage targeting in HNSCC

4.1 STAT3

Signal transducer and activator of transcription 3 (STAT3) is frequently overactivated in various human cancers, serving as a crucial signaling node in tumor cells and the cellular components of the TME, especially in tumor-infiltrating immune cells (63). Radiation therapy, a commonly used treatment for HNSCC, aims to utilize high-energy radiation to selectively kill or control the growth of cancer cells, reducing tumor size or eliminating the tumor altogether (64). It is often employed as a treatment option for patients who are not suitable candidates for surgery.

In the circulatory system of HNSCC patients undergoing radiotherapy, there is an accumulation of therapy-resistant bone marrow cells, which affects the efficacy of radiation therapy (65). Moreira et al. found that targeting STAT3 in TAMs can enhance the therapeutic effects of radiation therapy for HNSCC. They employed the CpG-STAT3ASO strategy to target STAT3 in HNSCC-related macrophages in conjunction with TLR9 triggering. This approach can overcome radiation resistance in tumors of both HPV-positive and HPV-negative mice. The combined treatment results in reduced residual M2 macrophages in the tumor and the recruitment of activated M1 macrophages to the tumor-draining lymph nodes (TDLNs).

A single-cell transcriptomic study of oral squamous cell carcinoma has revealed an enrichment of the IL-6/JAK2/STAT3 axis in the tumor microenvironment, particularly in cell populations like macrophages, in samples induced by chemotherapy and other treatments (66). Additionally, the phosphorylation level of STAT3 can modulate the response of regulatory T cells (Tregs) to radiation therapy in head and neck cancer (67). These findings indicate that STAT3 could serve as a significant combinatorial therapeutic target to enhance the efficacy of radiotherapy and chemotherapy in head and neck cancer.

Targeting STAT3 in current research offers several advantages, including improving immune dysregulation in the tumor microenvironment, reducing endogenous proliferation of tumor cells, and enhancing the anti-tumor effects of tumor-infiltrating immune cells, among others (68, 69). As a potential target for cancer treatment, the current drug development efforts against STAT3 involve direct inhibition using peptides, small molecules, and decoy oligonucleotides (70–73), or indirect inhibition through blocking upstream signaling pathways such as the IL-6 and JAK2 pathways (74, 75).

4.2 CCL2/CCR2

CC-chemokine receptor 2 (CCR2) is primarily expressed in monocytes and macrophages and has a strong pro-inflammatory function (76). This has led to the development of CCR2 antagonists aimed at inhibiting unnecessary immune responses in inflammation and autoimmune diseases. Paradoxically, in the tumor microenvironment, CCR2-expressing monocytes and macrophages can strongly suppress immune responses (77). In recent years, researchers have explored strategies using CCR2 antagonists to selectively attract suppressive monocytes and macrophages into the tumor, with the goal of altering the tumor microenvironment and enhancing the immune system's ability to combat cancer (78).

While the mice model of HNSCC treated with radiotherapy, there showed an increase in the production of the chemotactic factor CCL2 in tumor cells, leading to the accumulation of CCR2-dependent TNF-α-producing monocytes/macrophages and CCR2+ Tregs (79). CCL2/CCR2 could potentially serve as clinical

candidates for radioimmunotherapy to counteract the radioprotective effects of macrophages and Treg cells. Currently, synthetic inhibitors of CCL2, Bindarit(Bnd) (80) and Carlumab (CNTO 888) (81), as well as CCR2 antagonists RS-50439 and MLN1202, have been developed for targeted disruption of CCL2/CCR2 signaling to intervene in the progression of various tumor types (82–85).

4.3 NRF2

NRF2, encoded by the NFE2L2 gene, plays a crucial role in maintaining cellular redox homeostasis, regulating immune responses, and detoxifying drugs (86, 87). Activation of NRF2 can lead to metabolic reprogramming, enhancing tumor proliferation, suppressing various forms of stress, and promoting immune evasion (88).

NRF2 is upregulated in HNSCC, and its expression levels are positively correlated with malignancy (89, 90). Carcinogens such as nicotine and arecoline can trigger c-myc-driven NRF2 activation in HNSCC cells, reprogramming the pentose phosphate pathway metabolism in the tumor microenvironment (90). In this metabolic pathway, glucose-6-phosphate dehydrogenase (G6PD) and transketolase (TKT) are key downstream effectors driven by NRF2, contributing to the progression of head and neck squamous cell carcinoma.

Mutations in the NRF2-encoding gene NFE2L2 can result in radiation resistance in HNSCC. Notably, in HNSCC patients undergoing radio chemotherapy, NFE2L2 mutations are significantly linked to a heightened risk of local treatment failure. In immunocompetent mice, tumors carrying NFE2L2 mutations displayed increased resistance to radiation compared to tumors with the wild-type NRF2. However, this discrepancy was less pronounced in immunodeficient mice. NFE2L2 enhances radiation resistance by diminishing the presence of M1-polarized macrophages (91).

Previously, researchers have attempted to inhibit NRF2 by studying the natural inhibitory protein Kelch-like ECH-associated protein 1 (KEAP1) that targets NRF2 (92, 93). Additionally, in order to discover new NRF2 inhibitors for targeted therapy, Singh et al. conducted a quantitative high-throughput screening in the small molecule library MLSMR and identified ML385 as a probe molecule that binds to NRF2 and inhibits its downstream target gene expression (94).

4.4 CD47

CD47 is a widely expressed cell surface protein that acts as a ligand for signal regulatory protein alpha (SIRPα) on macrophages, which, in turn, inhibits phagocytosis (95). Previous studies in various preclinical models have demonstrated that blocking the CD47-SIRPα pathway can enhance phagocytic functions, demonstrating significant anti-tumor efficacy across multiple tumor types (96, 97).

Regarding a macrophage-mediated anti-tumor immunotherapy strategy based on gene-edited nanoparticles: the first step involves blocking the CD47-SIRPα pathway, and the second step is to repolarize tumor-associated macrophages (98). Additionally, Ni and colleagues discovered in preclinical models using the IBI188 drug to block the CD47-SIRPα pathway that angiogenesis can, to some extent, limit the effectiveness of anti-CD47 antibodies against tumors. Combining anti-angiogenesis therapies with CD47 blockade can achieve higher therapeutic efficacy (99).

Recently, Lee et al. conducted macrophage phagocytosis experiments on the HN31R head and neck cancer cell line and found that the downregulation of Tristetraprolin (TTP) can induce sustained overexpression of CD47, which, in turn, inhibits the phagocytosis of head and neck cancer cells (100). Furthermore, when CD47 was expressed *in vitro* in HNSCC cell lines, both M1 and M2 macrophages exhibited a certain degree of phagocytic potential (101). However, under conditions where CD47 was inhibited, the phagocytic ability of M1 enhanced, while M2 did not (101). In summary, CD47-positive oral squamous cell carcinoma cells primarily inhibit M1 phagocytosis, leading to immune evasion.

Currently, several antibodies targeting CD47 have entered clinical trials, such as Hu5F9-G4, TTI-621, and others, for the treatment of both solid tumors and hematologic malignancies (102, 103).

4.5 TGF-β

Transforming growth factor-β (TGF-β) is a widely recognized immunosuppressive factor, playing a role in restraining excessive inflammatory responses (104–106). Additionally, TGF-β triggers macrophage M2 polarization, contributing to the alleviation of inflammation mediated by macrophages (105, 107).

PD-1 blockade therapy in the treatment of HNSCC has demonstrated a significant extension of survival in recurrent/metastatic (R/M) patients, coupled with favorable safety profiles (108, 109). However, numerous challenges persist, with a substantial portion of cancer patients exhibiting suboptimal responses to PD-1 monotherapy (110). The crucial role of TGF-β in the delicate balance between immunity and tolerance among non-responsive patients to PD-1 monotherapy has been identified (111, 112). TGF-β modulates the cancer immune cycle by altering T cell proliferation, activation, differentiation, and impeding the activity of dendritic cells and natural killer cells (113). Combining anti-TGF-β with anti-PD-1 therapy has proven effective in overcoming resistance in immune rejection models (114, 115). Subsequently, Yi et al. developed the bispecific antibody (BsAb) YM101 which targeting both TGF-β and PD-L1 (116). They observed potent anti-tumor activity of this drug in immune-inflammatory and immunosuppressive models of diverse tumors (117). Additionally, a TGF-β/PD-L1 specific antibody, the drug BiTP, has been developed and demonstrated promising anti-tumor efficacy in both *in vitro* and *in vivo* experiments (118). Simultaneously, Matos et al. have recently engineered a Polyoxazoline-Based nano-vaccine carrying a TGF-β expression regulator in combination with a PD-1 inhibitor. This combination exhibits synergistic anti-tumor effects and holds

significant potential in improving the immunotherapeutic outcomes for solid cancer patients (119).

4.6 Other targets

The regulation of macrophage proliferation, differentiation, and survival hinges on the control of CSF1R and its ligands. A multitude of preclinical investigations have underscored that the inhibition of CSF1R leads to a decreased density of TAMs, resulting in the inhibition of tumor growth and heightened sensitivity to chemotherapy (120, 121). Besides, elevated expression of CSF1R leads to increased lactate levels in HNSCC, reduces the presence of tumor-infiltrating macrophages, and promotes the induction of M2-like macrophage polarization within the tumor (55). The drugs targeting CSF1/CSF1R, such as PLX3397 and HMPL-012, have been proven to be effective in other tumors, including tenosynovial giant cell tumor and neuroendocrine tumors (122, 123).

Moreover, high expression of RACK1 in oral squamous cell carcinoma (OSCC) is associated with increased infiltration of M2 macrophages (124). OSCC cells that overexpress RACK1 promote M2-like macrophage polarization through the regulation of NF-kappa B, leading to an increase in the proportion of M2-like macrophages in xenograft mouse models (27). The corresponding targeted drug is M435-1279, a critical ubiquitin-conjugating enzyme E2T (UBE2T) inhibitor that catalyzes the proteasomal degradation of RACK1, which also has certain prospects for future applications (125). Those typical targets, pathways, and associated drugs of macrophages in the progression HNSCC are shown in Table 1.

TABLE 1 Typical targets, pathways, and associated drugs of macrophages in HNSCC progression.

Targets	Pathways	Drugs	References
STAT3	IL-6/JAK2/STAT3	LL1	(70)
		SD-36	(72)
		W1131	(73)
CCR2	CCL2/CCR2	Bnd	(80)
		CNTO 888	(81)
		RS-50439	(83)
		MLN1202	(84)
NRF2	KEAP1/NRF2	ML385	(94)
CD47	CD47/SIRPα	IBI188	(99)
		Hu5F9-G4	(102)
		TTI-621	(103)
TGF-β	TGF-β/PD-L1	YM101	(117)
		BiTP	(118)
CSF1R	CSF1/CSF1R	PLX3397	(122)
		HMPL-012	(123)
RACK1	RACK1/NF-kappa B	M435-1279	(125)

5 Applications of macrophages in the treatment of HNSCC

5.1 Conventional immunotherapy targeting

Macrophages are an important target in current checkpoint blockade immunotherapy, suppressing adaptive immune responses by expressing inhibitory counter-receptors such as PD-L1 and PD-L2. Certain chemotherapy drugs, like anthracyclines, induce the release of tumor antigens and co-stimulatory molecules, a process referred to as immunogenic cell death, engaging macrophages in a productive cancer immune cycle (126). There are also other cell-depleting therapies aimed at targeting macrophages (127). Specific strategies focused on macrophages have partly entered clinical assessment, including monocyte-derived macrophages used for cellular therapy, either through targeted recruitment and differentiation or functional reprogramming via activation or inhibition of checkpoint receptors.

In the treatment of recurrent/metastatic HNSCC patients, checkpoint inhibitors have demonstrated their effectiveness (128). However, the majority of patients do not benefit from these drugs (129). To enhance the efficacy of checkpoint inhibitors, Sato-Kaneko F et al. have established HNSCC models and employed a combination of TLR agonists and PD-1 blockade (130). They found that this approach could activate TAMs, induce tumor-specific adaptive immunity, and thus inhibit primary tumor growth and prevent metastasis. Notably, treatment with TLR7 agonists increased the M1/M2 ratio and promoted the generation of tumor-specific immune factors.

To enhance the immunotherapeutic efficacy in HNSCC, Wu et al. developed an injectable nano-composite hydrogel (131). This hydrogel is created by incorporating imiquimod-encapsulated CaCO3 nanoparticles (RC) and a cancer cell membrane (CCM)-coated mesoporous silica nanoparticle within a polymer framework (PLGA-PEG-PLGA). These components include a peptide-based protein hydrolysis targeting chimera (PROTAC) against BMI1 paclitaxel (PepM@PacC). The injectable hydrogel can selectively manipulate tumor-associated macrophages, further activating T cell immune responses.

5.2 Engineering macrophages

In response to the phagocytic and pro-inflammatory actions of M1 macrophages on tumor cells, engineered macrophages targeting cancer cells as carriers for anti-tumor therapy have been developed to modulate the tumor microenvironment (132).

For example, controlled-release biomimetic or macrophage membrane-coated nanoparticles have been developed for cancer therapy to respond to the TME (21). Rao et al. engineered cell membrane-coated magnetic nanoparticles (gCM-MNs) to enhance the affinity between the genetically overexpressed SIRPα variant on gCM shells and CD47, effectively blocking the CD47-SIRPα pathway and preserving macrophage's ability to phagocytose cancer cells (133). Meanwhile, these magnetic nanoparticles promote M2-to-M1 repolarization in the tumor microenvironment of B16F10 melanoma

mice model and the triple negative breast cancer 4T1 mice model, blocking the process of tumor cells secreting colony-stimulating factors to polarize tumor-associated macrophages into tumor-promoting M2 macrophages. This synergistically enhances macrophage phagocytosis of cancer cells and triggers anti-tumor T-cell immunity. This method effectively activates macrophages for anti-tumor immunotherapy. In addition, macrophage membrane-coated nanoparticles (cskc-PPiP/PTX@Ma) developed by Zhang et al. show enhanced therapeutic effects, homing to tumor sites and gradually controlling drug release in response to the acidic pH changes in the tumor microenvironment, releasing the hydrophobic anti-cancer drug paclitaxel to kill cancer cells. Testing the administration capability and therapeutic effects of this formulation in an orthotopic breast cancer-bearing mice model, this combination of a biomimetic cell membrane and a cascade-responsive polymeric nanoparticle yielded significant results (134).

Furthermore, Rayamajhi and colleagues developed hybrid exosomes (HE) with a size smaller than 200nm by hybridizing exosomes extracted from mouse macrophages with synthetic liposomes (135). They loaded a water-soluble doxorubicin into these HE, increasing the toxicity of drug-loaded HE to cancer cells and enabling drug release in the acidic tumor microenvironment. These macrophage-derived mimetic exosome vesicles effectively deliver bioactive molecules to recipient cells, making them suitable for drug delivery and therapy in cancer.

5.3 CAR-macrophage

Chimeric antigen receptor (CAR)-T cell therapy is an early cell-based immunotherapy designed to prevent tumor cells from evading recognition by T cell receptors. This method has been successfully used to treat hematologic malignancies, but its effectiveness in solid tumors remains limited (136). In the tumor microenvironment, macrophages, as the most abundant innate immune cells, can infiltrate solid tumor tissues and interact with almost all other cell types (137). Therefore, researchers are attempting to use CAR-modified macrophages (CAR-M) to combat solid tumors.

The first-generation CAR-M cells primarily utilize the characteristics of macrophages, focusing on their phagocytic function (138, 139). In contrast, second-generation CAR-M cells, in addition to retaining the features of the first generation, also aim to improve the presentation of tumor-associated antigens and T cell activation. In this scenario, Klichinsky et al. design murine or human macrophages through chimeric vectors and then obtain the drug after *in vitro* expansion, concentration, and purification (140). Currently, third-generation CAR-M cells are being designed by reprogramming CAR-M cells *in vivo* using non-viral vectors (141). There has been an approach to fuse nanobiotechnology with CAR-M cells, using nanocarriers to deliver the encoded CAR and interferon-gamma genes to macrophages *in vivo*, with the aim of further enhancing anti-tumor efficacy by repolarizing M2-polarized macrophages into M1 macrophages (142).

The progress of CAR-M therapy in HNSCC is currently quite limited. However, with the continuous iteration of CAR-M technology and the advancement of macrophages in head and neck squamous cell carcinoma, this field holds tremendous potential for application.

6 Discussion and prospects

Macrophages exhibit a high degree of plasticity in response to various microenvironments within normal human tissues, inflammatory stimuli, and tumor tissues. This functional diversity results in various characteristics within macrophages, making their categorization challenging. Currently, macrophages are broadly categorized into two phenotypes, M1 and M2, which are associated with pro-inflammatory and anti-inflammatory properties, respectively. Tumor-associated macrophages represent the complex interplay of various cell types within the TME and can exhibit M1 or M2 characteristics under the influence of different TME stimuli. Typically, M1-like TAMs that promote an inflammatory response against tumor cells often exhibit anti-cancer effects, while M2-like TAMs tend to support tumorigenesis (143).

Head and neck squamous cell carcinoma, being an invasive malignant tumor, is characterized by high incidence and low survival rates. Treatment options for HNSCC are limited, typically involving local surgery, radiation, and chemotherapy (144). The development of immunotherapy has harnessed the collaborative role of the TME in HNSCC progression. By understanding the processes of tumor cell evolution and immune evasion (Figure 2), immunotherapy demonstrates effective anti-cancer properties through the manipulation of self-immunogenicity or the expression of immune inhibitory mediators, ultimately enhancing the survival rates of HNSCC patients. However, it's worth noting that fewer than 20% of patients exhibit sustained responses to these treatments (145).

Within the HNSCC microenvironment, TAMs, being the most abundant group of innate immune cells, play a role in mediating immunosuppressive effects on adaptive immune cells in the TME. The polarization state of TAMs can be influenced by various signals like nicotine, Apelin peptide, and lactate. This polarization state has a strong connection with the development and immune evasion in head and neck cancer, although it doesn't necessarily impede immune responses. In the context of head and neck cancer, the M2 polarization of macrophages can impact tumor stemness, invasiveness, and the mechanisms of immune evasion. Consequently, inhibiting M2 polarization and promoting M2-to-M1 repolarization have emerged as crucial strategies that leverage the remarkable plasticity of macrophages in anti-cancer efforts. Building upon this foundational theory, more effective immunotherapeutic approaches have been further explored, including the engineering of macrophages and the utilization of CAR-M technology to eliminate HNSCC cells.

Targeting TAMs and HNSCC remains an ongoing and challenging endeavor in progress. Macrophages play a crucial dual role in different anticancer modalities, as they are actively involved not only in chemotherapy, radiation therapy, and immune

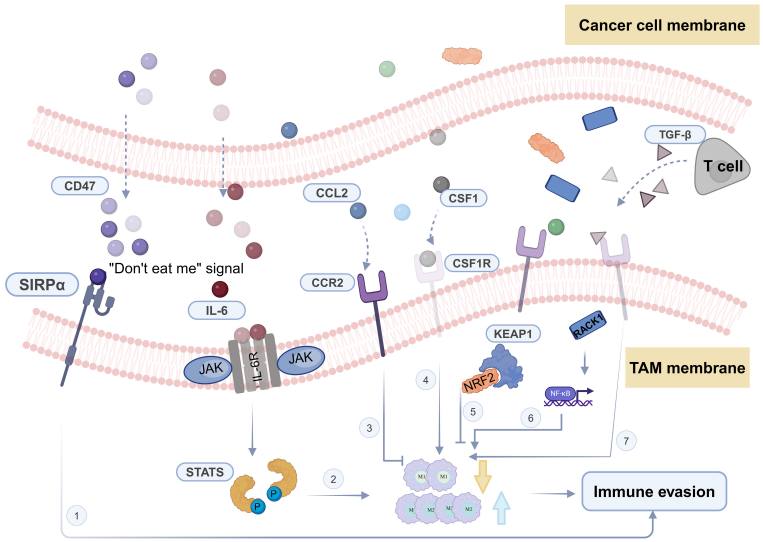

FIGURE 2
Crucial pathways and key molecules of tumor-associated macrophages in TME immune evasion.

checkpoint blockade (ICB) immunotherapy, as mentioned above, but also in anti-angiogenesis and hormone therapy. For instance, in one study, it was found that metformin reduces the accumulation of M2-TAMs in the tumor microenvironment, impeding M2-like macrophage-induced angiogenesis promotion. On the other hand, melatonin indirectly inhibits tumor angiogenesis by increasing the accumulation of M1-TAMs (146). Consequently, developing more precise targeted treatment strategies and exploring the potential of macrophage-based therapies are all research directions for further improving HNSCC survival rates and refining the approaches to HNSCC treatment in the future.

This review presents a comprehensive overview of the immune-regulatory roles played by macrophages in HNSCC. It delves into the diverse polarization states of macrophages within the tumor microenvironment and explores potential therapeutic strategies for repolarization. Recent years have witnessed significant progress in research targeting critical macrophage-related factors, along with substantial advancements and refinements in macrophage-based therapies for head and neck cancer. These developments aim to boost the efficacy of immunotherapy for HNSCC. Through this contribution, our objective is to advance macrophage-related therapeutic strategies for HNSCC, revealing more effective potential treatment methods in this evolving era.

Author contributions

CL: Writing – review & editing, Writing – original draft. YC: Writing – review & editing, Visualization, Validation, Investigation. YZ: Writing – review & editing, Visualization, Validation. SG: Writing – review & editing, Supervision, Funding acquisition. YH: Writing – review & editing, Methodology. JH: Writing – review & editing, Methodology. ZS: Writing – review & editing, Supervision, Project administration, Funding acquisition.

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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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Tumor associated macrophages in breast cancer progression: implications and clinical relevance

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Macrophages represent an immune cell population characterized by high plasticity and a range of properties and functions. Their activation status and specific phenotype are highly associated with their localization and the environmental cues they receive. The roles of macrophages in cancer development are diverse. Despite their antitumor effects at early stages of the disease, their presence in the tumor microenvironment (TME) has been linked to tumor promotion upon disease establishment. Tumor associated macrophages (TAMs) are key components of breast cancer TME and they have been associated with poor clinical outcomes. High TAM densities were found to correlate with tumor progression, increased metastatic potential and poor prognosis. Interestingly, considerably higher levels of TAMs were found in patients with triple negative breast cancer (TNBC)—the most aggressive type of breast cancer—compared to other types. The present review summarizes recent findings regarding the distinct TAM subsets in the TME and TAM involvement in breast cancer progression and metastasis. It highlights the constant interplay between TAMs and breast cancer cells and its major contribution to the progression of the disease, including such aspects as, polarization of macrophages toward a tumor promoting phenotype, induction of epithelial to mesenchymal transition (EMT) in cancer cells and enhancement of cancer stem cell properties. Further, we discuss the clinical relevance of these findings, focusing on how a better delineation of TAM involvement in breast cancer metastasis will facilitate the selection of more efficient treatment options.

KEYWORDS

breast cancer, tumor associated macrophages, surface markers, prognosis, metastasis

Abbreviations: BC, Breast Cancer; DFS, Disease Free Survival; EMT, Epithelial to Mesenchymal Transition; ECM, Extracellular Matrix; GAL8, Galectin 8; LEC, Lymphatic Endothelial Cell; HER2, Human Epidermal Growth Factor Receptor 2; LH, Lysyl Hydroxylases; LOX, Lysyl Oxidases; MDM, Myeloid Derived Monocytes; OS, Overall Survival; PoEMs, Podoplanin Expressing Macrophages; PR, Progesterone Receptor; RFS, Relapse Free Survival; TAM, Tumor Associated Macrophages; TME, Tumor Microenvironment; TNBC, Triple Negative Breast Cancer; TNM, Tumor, Node, Metastasis (T-describes the size of the tumor, N-describes whether malignant cells have spread to the lymph nodes and M-describes whether malignant cells have spread to other parts of the body, away from the primary tumor site).

1 Introduction

Breast cancer (BC) is among the most frequently diagnosed types of cancer worldwide and the second leading cause of cancer related mortality in women (1–3). It can be subdivided into different types based on the expression of estrogen (ER) and progesterone (PR) hormone receptors, HER2 expression and Ki67 (4, 5). Triple negative breast cancer (TNBC), $ER^-/PR^-/HER2^-$, accounts for 10–20% of BC and it is characterized by high aggressiveness and poor prognosis owing to the lack of targeted therapeutic strategies (6, 7). A substantial amount of evidence has supported the involvement of tumor associated macrophages (TAMs) in cancer progression and metastasis in various types of cancer, including BC (8–10). In fact, TAMs represent the dominant immune cell population of BC tumor microenvironment (TME) and they have been correlated with poor prognosis and increased metastatic potential. Distinct TAM subsets can differentially affect disease progression, and this is highly dictated by their specific phenotype, and their spatial and temporal distribution (11, 12). TAM subsets in BC TME have also been utilized as predictive tools of clinical outcomes. A thorough characterization of the TAM signature of individual BC patients could facilitate the design of personalized and more efficient treatment strategies. It could also enable a more accurate prediction of patients' response to treatment. In this review, we summarize subtypes of TAMs commonly encountered in the TME of BC, highlighting the heterogeneity and diversity of these cells. In addition, we present recent findings concerning TAM involvement in BC progression and metastasis, with particular attention to the constant crosstalk between TAMs and cancer cells and its central role in fueling and maintaining disease progression.

2 Distinct TAM subtypes in breast cancer progression and prognosis

In an oversimplified manner, macrophages in the TME were previously distinguished into the pro-inflammatory M1 type-linked to antitumor functions and the anti-inflammatory M2 type-endowed with tumor promoting capabilities (13). This M1/M2 distinction represents the two extremes, and macrophages of intermediate states are also present in the TME. More recently, the terms M1-like and M2-like macrophages were introduced to refer to anti- and pro-tumor macrophages respectively (14, 15). Still, there is a grey zone in this discrimination and macrophages of the M1-like type can occasionally exert tumor-promoting functions. A comprehensive correlation between the specific TAM phenotype and function would provide substantial information regarding the role of distinct TAM subsets in BC development/progression. Importantly, TAM spatial and temporal distribution are determining factors for their effects. TAMs located in different breast territories were reported to go through separate differentiation pathways and are characterized by distinct transcriptomic profiles (16). Additionally, TAM phenotype alters with malignancy progression, as tumor stage is one of the key determinants of spatial diversity in tumors (16). Prevalent TAM subsets in BC-based on surface marker expression-are presented next, along with their impact on disease progression and their prognostic value.

2.1 CD68 expression

Pan-macrophage marker CD68 was used in initial studies aiming to delineate TAM role in BC progression and prognosis. Increased $CD68^+$ TAM infiltration correlated with angiogenesis induction and poor clinical outcomes (17, 18). An association between high $CD68^+$ TAM infiltration in the TME and high TNM stage, increased tumor size and shorter patient survival were also reported (19). Distinct functions for TAMs located at different areas in the TME were proposed (20). High stromal $CD68^+$ TAM numbers were linked to higher tumor grade, resulting from tubular architecture modulation by TAMs, whereas high numbers of TAMs in the tumor nest were related to angiogenesis. Mahmoud et al. assessed the density and localization of $CD68^+$ macrophages in 1322 BC tissues (21). Increased total macrophage numbers were associated with high tumor grade, ER/PR negativity, HER2 positivity and basal BC, while a significant correlation between high macrophage density and reduced BC specific survival was observed. Intratumoral and stromal $CD68^+$ TAM infiltration was evaluated in hormone receptor positive and negative BC patient groups (22). High intratumoral infiltration was linked to poor disease-free survival (DFS) in both groups and was an independent DFS predictive factor in the hormone receptor positive group.

2.2 CD163/CD206/CD204 expression

CD68 pan-macrophage marker cannot distinguish between macrophages with anti-tumor effects and those with protumor functions. Additional markers were employed to better identify functionally distinct TAM subsets including CD163, CD206 and CD204-scavenger receptors. Increased $CD163^+$ macrophage infiltration in tumor stroma positively correlated with higher tumor grade, larger tumor size, Ki67 positivity and ER/PR negativity (23). In the same study, $CD68^+$ macrophages in tumor stroma positively correlated with tumor size and were an independent factor for reduced BC specific survival. High stromal $CD68^+$ and $CD163^+$ TAM infiltration was associated with BC clinicopathological features, increased tumor recurrence and reduced overall survival (OS) (24). Additionally, stromal $CD163^+$ macrophages were reported as an independent prognostic factor for relapse-free survival (RFS) and OS. Another study demonstrated that high $CD163^+$ TAM numbers were related with increased proliferation and poor differentiation of cancer cells and ER negativity (25). CD163 expression was further linked to negative prognosis and decreased recurrence-free survival. In the same study, conditioned media from the MDA-MB231 breast cancer cell line induced macrophage differentiation into $CD163^+$ TAMs *in vitro* via cancer cell secreted CSF-1. The prognostic value of $CD68^+CD163^+$ TAMs was assessed in the tumor nest and stroma of TNBC patients (26). $CD163^+$ TAMs in both locations were independent predictive factors for poor prognosis and were associated with reduced OS and RFS. In a separate study, high

$CD163^+$ TAM infiltration was correlated with increased tumor aggressiveness and reduced progression-free survival (27). The group demonstrated that $CD14^+$ blood derived monocytes were converted into $CD163^+$ TAMs, upon culture with supernatant from primary dilacerated tumors. Interestingly, distal to the tumor monocytes were refractory to M1 polarization *in vitro* and presented altered transcriptional profile, suggesting a systemic tumor effect. $CD68^+CD163^+$ TAM frequency was studied alongside the frequency of tumor infiltrating lymphocytes (TILs) in TNBC patients (28). High $CD68^+CD163^+$ TAM density, combined with reduced T and B lymphocyte presence significantly correlated with poor prognosis and reduced RFS and OS. Maisel et al., reported that $CD163^+$ TAMs in immediate proximity to cancer cells and the average number of $CD163^+$ TAMs, either adjacent to or at communicating distance with cancer cells, were independent factors of poor clinical outcomes in BC (29). A separate study in $HER2^+$ BC patients revealed a correlation of inferior clinical outcomes with high $CD163^+$ TAM density even when HER2-targeted therapy was administered (30). CD163 significance in BC prognosis was highlighted in a recent meta-analysis which used data from 32 studies and identified $CD163^+$ TAM density as superior predictor of clinical outcomes compared to $CD68^+$ TAM density (31). Strack et al., reported a relative increase to the amounts of $CD206^-$ macrophages in BC tumors compared to normal breast tissue (32). Elevated numbers of $CD206^-MHCII^{high}$ macrophages were correlating with poor prognosis, while $CD206^+$ TAMs correlated with improved survival. Similarly, Bobrie et al., reported a positive correlation between CD206 TAM positivity and improved RFS and OS (33). A higher density of $CD204^+$ TAMs compared to $CD68^+$ or $CD163^+$ TAMs was observed in patients with invasive ductal carcinoma (34). High numbers of $CD204^+$ TAMs were associated with reduced rates of RFS, distant RFS and BC specific survival. Another study reported $CD204^+$ TAM accumulation in highly aggressive breast tumors (35). $CD204^+$ TAMs were also prevalent in tumors with increased T lymphocyte infiltration and PDL1 expression and were suggested to contribute to immunotherapy resistance.

2.3 PDL1 expression

Increased numbers of $CD68^+/PDL1^+/CD163^-$ cells at intratumoral sites but not in tumor stroma were associated with favorable clinical outcomes (36). Interestingly, higher $CD68^+/PDL1^+/CD163^-$ cell density was reported in TNBC and $HER2^+$ patients compared to ER/PR^+ patients. A study in TNBC reported better prognosis in patients with high $CD68^+PDL1^+$ stromal macrophages numbers (37). Superior predictive value for $CD68^+PDL1^+$ macrophages as opposed to $PDL1^+$ macrophages was also demonstrated and was proposed as a tool to identify patients with good or poor prognosis. Similar data were obtained by Hong et al. in patients with stage I-III BC, suggesting a positive prognostic role of PDL1 expression on stromal immune cells but not on tumor cells (38). In a single-cell transcriptomic analysis, $PDL1^+$ TAMs were reported to be immunostimulatory, demonstrated a preference to localize near T cells and were associated with improved clinical outcomes (39). In another study, increased $CD163^+PDL1^+$ TAM density was associated with advanced stages of BC and metastasis, while PDL1 upregulation was proposed to occur through miRNA mediated gene regulation (40).

The abovementioned studies highlight TAM heterogeneity and underline the necessity for surface marker combinations to accurately identify functionally distinct TAM subsets. A highly specific universal TAM marker for BC prognosis is yet to be discovered. Therefore, a thorough phenotypic characterization of TAM subsets utilizing multiple surface markers remains crucial.

3 TAMs orchestrate breast cancer progression and metastasis

Epithelial to mesenchymal transition (EMT) causing loss in cell polarity and cell-cell adhesion, along with destabilization of cell junctions is a driving force of cancer cell migration and invasion. Similarly, stemness induction of cancer cells, endowing them with self-renewal capacities and multi-lineage differentiation capabilities is crucial for metastasis. Extracellular matrix (ECM) remodeling and collagen crosslinking contribute to the metastatic potential by facilitating BC cell migration. TAM involvement in all the above events has been well documented. Recent findings on TAM involvement in BC progression and metastasis are summarized below.

CXCL1, an abundant cytokine in the TME has been associated with poor BC prognosis and increased metastasis (41, 42). TAMs are the main source of CXCL1 in the TME and are involved in EMT induction and tumor cell migration. Their metastatic effect was proposed to occur through the NF-kB/SOX4 axis activation (43). SOX4 implication in EMT induction, cancer stem cell enrichment and poor prognosis in BC patients was also reported by Zhang et al. (44). Induction of CXCL1-secreting M2 TAMs through cancer cell derived visfatin (known adipokine) was reported to promote BC progression and metastasis (45). Increased tumorsphere formation and migration, along with elevated mesenchymal and stemness markers were reported after breast cancer cells were co-cultured with visfatin-treated macrophages. Breast cancer cells from the same co-cultures caused increased pulmonary metastases and high numbers of metastatic nodules in mice, while a CXCL1 blocking antibody reversed those effects. CXCL1 was reported to induce visfatin secretion by cancer cells through a positive feedback loop, thereby maintaining M2 TAM polarization (45).

CCL18 is abundantly expressed by TAMs in BC. TAMs, or myeloid derived monocytes (MDMs) activated with IL-4, promote breast cancer cell invasiveness, adherence to fibronectin and migration *in vitro*, through CCL18 secretion (46). Treatment with an anti-CCL18 antibody, or TAM/MDM transfection with CCL18-siRNAs abrogated cancer cell invasive and migratory capacities. The same group identified a membrane-associated phosphatidylinositol transfer protein 3, PITPNM3 (or Nir1) as CCL18 receptor on cancer cells. In mouse BC xenografts, intratumor rCCL18 injections enhanced vascular invasion of cancer cells and lung and liver metastasis, while breast cancer cell infection with PITPNM3-shRNA alleviated this effect. CCL18 secreting TAMs were reported to be induced by breast cancer cell derived GM-CSF, with lactate —abundant in the TME—acting as a concomitant

factor (47). GM-CSF treated TAMs induced cancer cell EMT, migration and invasiveness through NF-kB pathway activation. Importantly, the study demonstrated that both TAM secreted CCL18 and cancer cell secreted GM-CSF are required for the maintenance of cancer cell mesenchymal/metastatic phenotype and macrophage tumor-promoting polarization. Either a CCL18 neutralizing antibody, or an anti-GM-CSF antibody inhibited metastasis in a xenograft mouse model. Annexin A2 (AnxA2)-a member of the calcium dependent phospholipid binding proteins was proposed as a downstream molecule of the CCL18-PITPNM3 signaling in cancer cells (48). A recent study reported the upregulation of exosome derived miR-760 in breast cancer cells stimulated with TAM derived CCL18. This resulted in enhanced cancer cell proliferation and metastatic potential through activation of the ARF6-mediated Src/PI3K/Akt pathway, where ARF6 is a direct miR760 target (49).

IL-1β is a crucial pro-inflammatory cytokine whose aberrant levels were associated with a highly progressive and metastatic potential and poor prognosis in BC patients (50–53). Breast cancer cell lines genetically modified to overexpress IL-1β presented increased EMT and metastasis. In contrast, IL-1β signaling inhibition decreased metastases in a humanized mouse model of BC bone metastasis (51). A recent study suggested a role for IL-1β secreting TAMs in tumor progression and metastasis in TNBC (54). Based on the study, membrane derived soluble CD44 secreted by breast cancer cells triggered IL1-β expression in TAMs promoting cancer cell EMT and metastasis. In mouse models, macrophage ablation or CD44 neutralizing antibody injection, reduced IL-1β serum levels and decreased lung metastasis incidence. CD44 expression on cancer cells was shown to be up-regulated through rhIL-1β treatment, suggesting a positive feedback loop to maintain IL-1β levels. Tsai et al., described the involvement of IL-1β secreting M1 TAMs in BC cell migration and invasiveness (55). BC cell derived GLUT3 triggers lactate-mediated CXCL8 secretion by cancer cells leading to TAM M1 polarization and expression of IL-1β, TNF-α and IL-6. M1 TAMs induced EMT and BC cell migration and invasion through the produced inflammatory cytokines. A paracrine loop between cancer cells and TAMs, whereby TAM derived IL-6 activates STAT3/GLUT3 pathway in cancer cells to preserve high CXCL8 levels was suggested.

A CCL2 paracrine feedback loop between macrophages and cancer cells promotes BC growth and metastasis (56). CCL2 released by cancer cells was shown to increase macrophage migratory capacity and induce M2 polarization *in vitro*. M2 TAM derived CCL2 promoted in turn breast cancer cell stem cell properties. CCL2 expression both in cancer cells and TAMs was shown to be regulated through direct binding of β-catenin to the CCL2 gene promoter. Breast cancer cells overexpressing β-catenin demonstrated high lung metastatic potential and generated larger tumors *in vivo*. Breast cancer growth and breast cancer cell stemness were suppressed through the synergistic effect of CCR2 and β-catenin inhibition. A positive correlation was observed in the expression of β-catenin, CCL2 and CD163 in tissue microarrays from BC patients. Consistent with the above data, $CD163^+$ $CD206^+$ M2 polarized macrophages were reported to confer stem cell properties and enable EMT of TNBC cell lines through secretion of CCL2 (57). Culture of breast cancer cells in M2 TAM conditioned media enhanced their invasiveness and migratory ability, induced their mesenchymal phenotype and enriched the cancer stem cell population ($CD44^+CD24^{low/-}$ and $ALDH^+$). The study proposed a novel mechanism through which TAM secreted CCL2 activates the PI3K/Akt pathway in cancer cells upon binding to its CCR2 receptor. Subsequent elevated expression and nuclear localization of β-catenin promotes EMT and stemness. Both a β-catenin inhibitor and a CCR2 antagonist were reported to reverse these effects. Interestingly, TAM derived CCL2 has been suggested to induce invasiveness in non-neoplastic epithelial cells (58). In the study, TAM co-culture with non-neoplastic MCF10A breast epithelial cells induced EMT, invasiveness and elevated MMP9 expression in the epithelial cells, through TAM-secreted CCL2. In another study, MMP11-overexpressing TAMs promoted $HER2^+$ cell migration, induced monocyte recruitment and enhanced angiogenesis (59). These effects were mediated through CCL2 secretion by TAMs. Cancer cell migration resulted from MMP9 expression upon activation of the CCL2-CCR2/MAPK axis. Of note, MMP11 expression by TAMs can reportedly be stimulated by MMP11-overexpressing cancer cells.

Podoplanin (mucin-type sialoglycoprotein) expressing macrophages (PoEMs) were identified as a metastasis promoting TAM subset in mammary tumors (60). Podoplanin in TAMs was suggested to engage Galectin 8 (GAL8) on lymphatic endothelial cells (LEC), promoting β1-integrin activation and macrophage migration and adhesion to LECs. Upon adhesion, PoEMs were shown to induce lymphangiogenesis. They also enable transendothelial cancer cell migration and are involved in extracellular matrix remodeling through local collagen and MMP production. The same study demonstrated that either the use of anti-β1-integrin blockade or GAL8 inhibition reduced lymphatic cancer spread in mice.

Collagen crosslinking is causative of stromal stiffness and is mediated by two enzyme families, lysyl oxidases (LOX) and lysyl hydroxylases (LH or PLOD). TAM involvement in the induction of stromal stiffness and subsequent metastasis was suggested (61). TAMs in BC TME were proposed to be a source of collagen-crosslinking enzymes leading to extracellular matrix remodeling and stromal stiffness. TAM depletion before tumor invasion could reduce lung metastases in mice, while anti-CSF1 treatment (inhibiting TAM recruitment) decreased stromal LOX and PLOD secretion and reduced the collagen content and number of collagen crosslinks. Stromal PLOD2 expression correlated with poor prognosis in cancer patients.

4 Discussion

TAMs are abundant in the TME in BC and their role in promoting disease progression has been well documented. They are involved in multiple aspects of tumor progression and metastasis including cancer cell EMT, stemness induction and extracellular matrix remodeling (Figure 1). Importantly, a continuous crosstalk between cancer cells and TAMs is in place to establish and preserve TAM tumor promoting functions and perpetuate cancer cell malignant properties. Given the TAM contribution in BC progression, TAM targeting either as

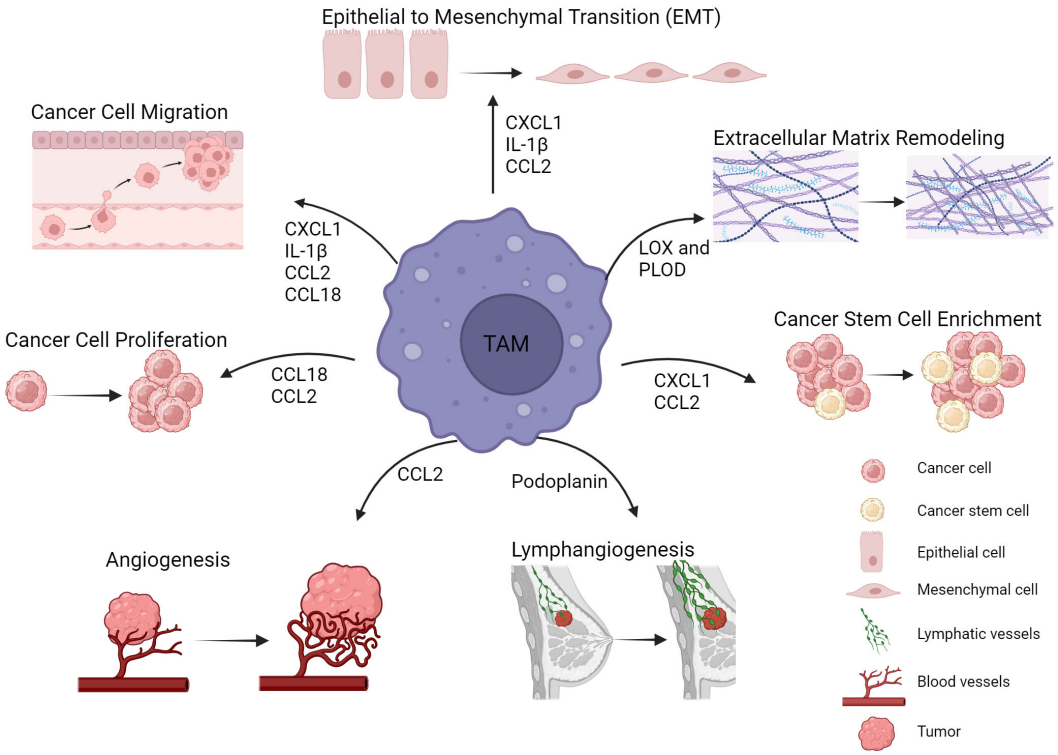

FIGURE 1
TAMs in breast cancer progression and metastasis. TAMs are involved in multiple mechanisms leading to tumor progression and metastasis. Such tumor promoting mechanisms include epithelial to mesenchymal transition, extracellular matrix remodeling, cancer stem cell enrichment and angiogenesis. Cytokines, enzymes and other factors derived from TAMs are key mediators of these processes. Figure created with BioRender.com.

monotherapy, or combined with other therapeutic modalities (chemotherapy/radiotherapy/immunotherapy) poses as a very attractive approach. TAM depletion or blockade of their recruitment to the tumor site, TAM re-education to the tumoricidal M1-like type and enhancement of TAM phagocytotic potential are among the main strategies currently explored (62, 63). Although very promising, these approaches are yet to show their high potential in the clinical setting. This could be at least in part attributed to the considerable heterogeneity of this cell population and the lack of specific and reliable markers to selectively target the desired subsets. Targeting TAMs as a general cell population would entail targeting subsets with anti-tumor effects alongside the tumor-promoting ones. More comprehensive analyses of the different TAM subsets, including analyses at the single-cell level should be considered to enable the identification of highly specific markers to discriminate between functionally different TAM subsets. Additionally, TAM spatial distribution should be accounted for when TAM targeting strategies are designed. TAM functional properties are highly influenced by their specific localization and targeting TAMs at certain sites might offer greater benefit as a treatment approach. It is also worth noting that although TAMs represent primary drivers and facilitators of metastasis, other cells in the TME including cancer associated fibroblasts are also contributing to these processes (64). Therefore, a sole focus on TAMs might not be sufficient to inhibit metastasis and disease progression since other cells could mediate a compensatory effect. Finally, better elucidation of the mechanisms used by TAMs to facilitate disease progression/metastasis along with thorough characterization of the tumor molecular landscape (i.e. expression of high levels of CD44/visfatin/miR760) could provide alternative targeted therapies for individual patients.

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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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Role of tumor-associated macrophages in hepatocellular carcinoma: impact, mechanism, and therapy

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Hepatocellular carcinoma (HCC) is a highly frequent malignancy worldwide. The occurrence and progression of HCC is a complex process closely related to the polarization of tumor-associated macrophages (TAMs) in the tumor microenvironment (TME). The polarization of TAMs is affected by a variety of signaling pathways and surrounding cells. Evidence has shown that TAMs play a crucial role in HCC, through its interaction with other immune cells in the TME. This review summarizes the origin and phenotypic polarization of TAMs, their potential impacts on HCC, and their mechanisms and potential targets for HCC immunotherapy.

KEYWORDS

hepatocellular carcinoma, tumor-associated macrophages, tumor microenvironment, treatment resistance, tumor angiogenesis, immunotherapy

1 Introduction

Primary liver cancer, including hepatocellular carcinoma (HCC) (comprising 75%–85% of cases) and intrahepatic cholangiocarcinoma (10%–15%), as well as other rare types, is one of the most frequent malignancies worldwide, with global morbidity and cancer-related mortality ranking sixth and third, respectively (1). In Asia, liver cancer is the fifth most common cancer and the second leading cause of malignant death. HCC, which is the most common histological type, accounts for the majority of incidence and mortality of liver cancer cases (2). The main risk factors for HCC are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), aflatoxin-contaminated foods, heavy alcohol intake, excess body weight, type 2 diabetes, and smoking (3). Although emerging treatments such as immunotherapies targeting the programmed death receptor 1 (PD-1) or its ligand (PD-L1) have been approved for the treatment of HCC with a major effect on patient survival (4), still there are patients who cannot benefit from them. The high

incidence and mortality of liver cancer place a heavy burden on patients, economically and mentally.

The tumor microenvironment (TME), placing great emphasis on tumorigenesis, progression, and metastasis toward HCC, also strongly contributes to the tolerogenic immune response of HCC treatment (5, 6). It comprises and can be affected by multiple components including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) (7, 8). TAMs, which are those macrophages infiltrating the TME, not only have an impact on the suppression of antitumor immune responses but also contribute to tumor immune surveillance and antitumor responses (9–12). Due to the key role that TAMs play in HCC, hepatic macrophages have long been considered as potential therapeutic targets for various HCC treatment modalities. A better understanding of the impact and mechanism of TAMs in regulating HCC tumorigenesis, progression, and metastasis is essential for the improvement of immunotherapy (13).

In this review, we summarize the origin and phenotypic polarization of TAMs, their potential impacts on HCC, and their mechanisms and potential targets for HCC immunotherapy.

2 Origin and phenotypic polarization of TAMs

According to the origin of liver macrophages, they can be classified into two types: tissue-resident macrophages, also known as Kupffer cells (KCs), and monocyte-derived macrophages (14). KCs, which are abundant in normal liver tissue, are developed from erythromyeloid progenitors (EMPs) in the yolk sac or fetal liver (15). In the progression of liver cancer, multiple protumorigenic factors would force KCs to recruit immune cells including the number of monocytes in the liver to modulate inflammation and prompt the functional differentiation of KCs since they are immunogenic in nature (16, 17). Those macrophages continue infiltrating tumors and eventually differentiate into TAMs (18).

The macrophage polarization theory indicates that TAMs undergo M1-like or M2-like activation and are divided into two types that have contrasting functions: the antitumor M1 phenotype and the protumor M2 phenotype (19, 20). M1-like macrophages are induced by interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (21). Because of their ability in antigen presentation, M1-like macrophages could promote the recruitment of type 1 helper T (Th) cells to enhance antitumor responses, kill tumor cells, and suppress tumors (19, 22, 23). M2-like macrophages are induced by transforming growth factor (TGF)-β, macrophage colony-stimulating factor (M-CSF), interleukin (IL)-10, and IL-13 (24, 25). Under the influence of those cytokines, M2-like macrophages suppress effector T-cell infiltration, activate Th2-type immune responses, and promote the progression of tumor (19, 26). It should be pointed out that the polarization of macrophages is joined in a dynamic cycle under the impact of the TME (27–29). More importantly, the M1-like/M2-like dichotomy based on *in-vitro* experiments may be defective because of the high plasticity of TAMs in the TME. Increasing evidence based on single-cell RNA sequencing (scRNA-Seq) has revealed that an M1-like/M2-like paradigm could not classify the complex phenotype of TAMs precisely, and a higher resolution than M1-like/M2-like is therefore required to categorize the molecular signatures of TAM subtypes in the TME (30, 31). Taking all these factors into consideration, TAMs, as a potential target in HCC immunotherapy, should be accorded great importance. It is essential for us to understand the role and function of TAMs in HCC and develop novel immunotherapies. Figure 1 summarizes the origin and phenotypic polarization of TAMs in HCC.

3 Impact of TAMs on HCC and their mechanism

TAMs are regulated by multiple factors in the TME of HCC. The infiltration of TAMs in HCC is related to HCC progression, therapy resistance, tumor angiogenesis, immunity, and metabolic alterations. The mechanisms of TAMs in the pathogenesis of HCC are summarized in Figure 2.

3.1 Regulation of the phenotypic polarization and infiltration of TAMs in HCC

In HCC, M1-like macrophages represent anticancer characteristics, which can suppress tumor progression through various mechanisms, while M2-like macrophages which are enriched in HCC tissue, according to The Cancer Genome Atlas Program (TCGA), are regarded as a protumoral type (19, 32). Liu N et al. identified that M2 polarization of KCs impairs hepatic enrichment of $CD8^+$ T cells, while microRNA (miR)-206 drives M1 polarization of KCs and hepatic recruitment of $CD8^+$ T cells through C-C motif chemokine ligand 2 (CCL2) production (33). The high expression of retinoic acid-inducible gene I (RIG-I) and sirtuin1 (SIRT1) in HCC regulates M1 polarization via the nuclear factor kappa-light-chain-enhancer of activated B-cell (NF-κB) pathway (34, 35). Studies conducted by Zhang Y et al. revealed that matricellular protein spondin2 (SPON2) and its integrin receptor α4β1 facilitate M1-like macrophage recruitment to the TME to prevent HCC progression (36). In addition, Wang Q et al. proved that IL-12-overexpressed monocytes could directionally differentiate into M1-like macrophages through downregulation of the signal transducer and activator of transcription (STAT) 3 and result in the inhibition of HCC growth (37).

M2-like macrophages could be divided into four subtypes based on their stimulant factors: M2a, which is induced by Th2 cytokines; M2b, which is induced by immune complexes; M2c, which is induced by anti-inflammatory cytokines or glucocorticoids; and M2d, which is induced by IL-6-like cytokines (38). Tumor acidosis could trigger regulatory

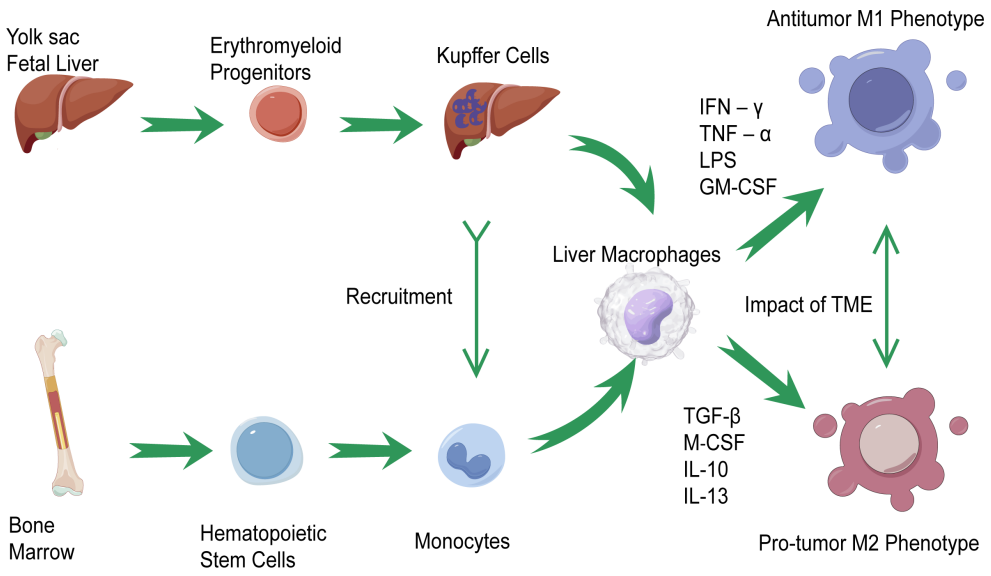

FIGURE 1
The origin and phenotypic polarization of tumor-associated macrophages (TAMs) in hepatocellular carcinoma (HCC). Kupffer cells (KCs) and monocyte-derived macrophages are the two classifications of liver macrophages. Kupffer cells, residing in tissues, are differentiated from erythromyeloid progenitors (EMPs) in yolk sac or fetal liver. Under the recruitment of KCs, monocyte-derived macrophages modulate inflammation in the progression of liver cancer and prompt the functional differentiation of KCs, which continue infiltrating tumors and differentiate into TAMs. TAMs would undergo M1-like activations through the stimulation of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), and granulocyte-macrophage colony-stimulating factor (GM-CSF). On the other hand, the M2-like activations of TAMs are induced by transforming growth factor (TGF)-β, macrophage colony-stimulating factor (M-CSF), IL-10, and IL-13. Under the impact of the tumor microenvironment (TME), the polarization of TAMs is joined in a dynamic cycle.

macrophages and enhance immune evasion (39), which eventually causes the generation of macrophages with immunosuppressive properties. Tan S et al. proved that zinc-fingers and homeoboxes 2 (Zhx2) bind to p65 protein and regulate NF-κB activation, while lactate domesticates macrophages through transcriptional regulation of Zhx2, reduces Zhx2 expression in TAMs and, in turn, attenuates the immunogenic M1-like activation of macrophage, increases the polarization of M2-like macrophages induced by IL-4, and fosters the liver tumor progression in an NF-κB–Irf1-dependent manner (40). Lu Y et al. identified that loss of xanthine oxidoreductase (XOR) increases α-ketoglutarate generation in monocyte-derived TAMs by increasing the activity of isocitrate dehydrogenase 3α (IDH3α) and drives macrophage differentiation toward the M2 phenotype (41). Wang Y et al. found that the circular RNA (circRNA) hsa_circ_0074854 is related to exosome-mediated M2-like macrophage polarization (42). Yin C et al. found that HCC cell-secreted miR-146a-5p could be delivered by exosomes into macrophages and promote macrophages toward M2-like polarization (43). Yang Y et al. demonstrated that HCC cell-derived Wnt ligands via Wnt/β-catenin signaling promote M2-like macrophage polarization (44). In macrophages, Wnt/β-catenin signaling can be activated by the long non-coding RNA (lncRNA) LINC00662 through WNT family member 3A (Wnt3a) secretion in a paracrine manner and further promoted M2-like macrophage polarization (45). Chen M et al. proved that T follicular helper (T_{FH}) cells operate via the IL-21–IFN-γ pathways to induce plasma cells and create conditions for M2b macrophage polarization, while T_{FH} cell induction is based on Toll-like receptor (TLR) 4-mediated monocyte inflammation and subsequent T-cell STAT1 and STAT3 activation (46).

3.2 TAMs affect HCC proliferation, invasion, and migration

A growing number of studies and lines of evidence have shown that TAMs are related to HCC proliferation, invasion, and migration. In the TME, TAMs and tumor cells interact through mediators such as TGF-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), M-CSF, IL-10, chemokine C-X-C motif ligand (CXCL), and extracellular vesicles (EVs) to affect tumor progression (47). For example, Gunassekaran G et al. demonstrated that IL-4R-Exo(si/mi) inhibits tumor growth by reprogramming TAMs into M1-like macrophages and increasing antitumor immunity (48). Xu M et al. found that TAMs augment the aerobic glycolysis in HCC cells and their proliferation by the extracellular exosome transmission of a myeloid-derived lncRNA, M2 macrophage polarization-associated lncRNA (lncMMPA), which could not only polarize M2 macrophage but also act as a microRNA (miRNA) sponge to interact with miR-548 and increase the mRNA level of aldehyde dehydrogenase 1 family member A3 (ALDH1A3) (49). TAMs could contribute to tumor development by inducing the expression of hepatocyte growth factor (HGF) (50). Wan S et al. and Mano Y et al. proved that TAMs could release IL-6 to enhance the expansion of human HCC stem cells, participate in tumorigenesis, and promote HCC progression via the STAT3

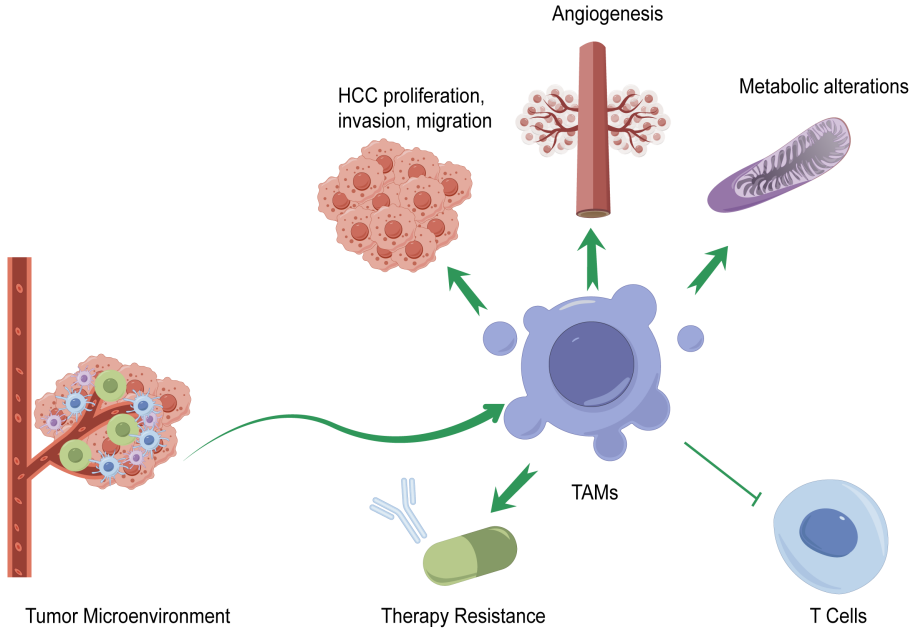

FIGURE 2
Mechanisms of TAMs in the pathogenesis of HCC. TAMs affect the progression of HCC by promoting or suppressing HCC proliferation, invasion, and migration; generating immune checkpoint blockade (ICB) therapy resistance; affecting HCC angiogenesis; regulating immune functions of various types of T cells; and altering metabolism.

signaling pathway (51, 52). M2-like macrophages could be induced by HCC-derived IL-8 and promote a pro-oncogenic inflammatory microenvironment, which would directly promote epithelial–mesenchymal transition (EMT) of HCC cells and stimulate their invasive potential (53). M2-like macrophages are also considered to promote HCC migration via the TLR4/STAT3 signaling pathway (54). Despite M1-like macrophages being thought to be tumoricidal, Zong Z et al. proved that M1-like macrophages secreted IL-1β to induce PD-L1 expression through the transcription factors interferon regulatory factor 1 (IRF1) and NF-κB in HCC cells, supporting the protumor progression role of M1 macrophages (55).

miRNAs are small non-coding molecules that can regulate gene expression at the post-transcriptional level and exhibit important regulatory roles in mediating the effects of TAMs on HCC progression. It has been proven that miR-23a-3p, highly expressed in M2 TAM-derived exosomes, enhances HCC metastasis by targeting phosphatase and tensin homolog (PTEN) and tight junction protein 1 (TJP1) (56). MiR-146a-5p, enriched in HCC exosomes, can be regulated by the transcription factor Sal-like protein-4 (SALL4) and is demonstrated to promote infiltration of M2 TAMs, which results in T-cell exhaustion and HCC progression (43). On the other hand, MiR-148b deficiency promoted HCC growth and metastasis through colony-stimulating factor 1 (CSF1)/CSF1 receptor (CSF1R)-mediated TAM infiltration (57). Ning J et al. found that the miR-17–92 cluster, originating from the extracellular EVs of M2-like macrophages, stimulated the imbalance of TGF-β1/BMP-7 pathways in HCC cells by inducing TGF-β type II receptor (TGFBR2) post-transcriptional silencing and inhibiting activin A receptor type 1 (ACVR1) post-translational ubiquitylation by targeting Smad ubiquitylation regulatory factor 1 (Smurf1), thus improving HCC cell growth and metastasis (58). Zhang J et al. demonstrated that TAM-derived prostaglandin E2 (PGE2) stimulates ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) expression by repressing miR-520d that targets the 3′-UTR of UHRF1 mRNA, while UHRF1 induces DNA hypomethylation of the CSF1 promoter and promotes CSF1 expression, thereby leading to TAM recruitment and activation which sustains PGE2 production in a self-enhancing oncogenic microenvironment to improve HCC progression (59). On the other hand, Wang L et al. proved that miR-628–5p, derived from M1-like macrophages, could inhibit the m6A modification of circFUT8, inhibiting HCC development (60).

Recently, Wu L et al. identified a 500-μm-wide zone centered around the tumor border in patients with liver cancer through nanoscale resolution-SpaTial Enhanced Resolution Omics-sequencing (Stereo-seq), referred to as “the invasive zone,” where overexpression of CXCL6 could induce activation of the JAK–STAT3 pathway, which causes SAAs’ overexpression and leads to the recruitment of macrophages and M2-like polarization, resulting in the formation of a local immunosuppressive microenvironment and the promotion of HCC invasion and migration (61).

3.3 Impact of TAMs on resistance to HCC treatment

Following the Barcelona Clinic Liver Cancer (BCLC) staging system, those with advanced-stage HCC tumors will first receive systemic therapies (62, 63). Although systemic therapies have substantially improved the reported natural history of untreated

cases at advanced-stage HCC, with median survival times of ~6 months in patients with well-preserved liver function defined as Child–Pugh A (according to the Child–Pugh score) and compensated disease (64–66), there remains a large number of HCC patients that do not respond to the treatments. Therefore, uncovering the mechanism of drug resistance and increasing the sensitivity of those drugs will be of great benefit to further improve the overall survival (OS) of patients with HCC. TAMs have been demonstrated to affect immune checkpoint blockade (ICB) therapy, especially with antibodies against the PD-1/PD-L1 signal (67). At the cellular level, an increased concentration of extracellular adenosine as well as the depletion of tryptophan and uncontrolled activation of the PI3K/AKT pathway induces an immune-tolerant TME, reducing the response to immune checkpoint inhibitors (ICIs) (68). Tan J et al. found that the number of triggering receptors expressed on myeloid cell (TREM)-2$^+$ TAMs is increased in post-transarterial chemoembolization (TACE) HCC, causing increased Gal-1 secretion to mediate the overexpression of PD-L1 in vessel endothelial cells, which turns out to compromise both the number and function of CD8$^+$ T cells and suppress the therapeutic efficacy of anti-PD-L1 blockade (69). Wei C et al. found that protein kinase C alpha (PKCα) phosphorylates zinc finger protein 64 (ZFP64) at S226 and promotes its nuclear translocation, thereby transcriptionally activating CSF1, which further induces the recruitment and M2-like polarization of macrophages, inducing immune escape and anti-PD-1 resistance in HCC (70). Lu J et al. revealed that enhanced expression of CD39 on TAMs, which is induced by HCC-secreted exosomal circTMEM181, could collaborate with CD73 to fulfill the sequential activation of the ATP–adenosine pathway, impair CD8$^+$ T-cell function, and build a PD-1 antibody-resistant tumor environment (71). M2-like macrophages are also reported to mediate sorafenib resistance in HCC by secreting HGF (72). On the other hand, TAMs are reported to cause oxaliplatin-based chemotherapy resistance by triggering autophagy and apoptosis evasion in HCC tumor cells (73). To overcome the resistance of TAMs to HCC treatment, research has been carried out to enhance the sensibility of anti-PD-1 therapy in HCC. Wang J et al. found that blockage of Calcyclin-Binding Protein (CacyBP) would inhibit the expression of C-X3-C motif chemokine ligand 1 (CX3CL1), a key chemotactic factor for the recruitment of monocyte-derived macrophages to the liver (74), and thus significantly reduce TAM infiltration and achieve synergies with anti-PD-1 treatment in HCC (75).

3.4 TAMs affect angiogenesis in HCC

Vasculature induction is regarded as one of the 14 hallmarks of tumor development (76). The hypervascular nature of most HCC tumors underlines the importance of angiogenesis in the pathobiology of HCC (77). The density of the tumor microvessel is positively correlated with macrophage counts, indicating the key role that TAMs play in HCC angiogenesis (78). Therefore, it is essential to understand the mechanism of TAMs affecting angiogenesis in HCC. MiR-223, a well-documented myeloid-enriched miRNA expressed in neutrophils, macrophages, and hepatocytes, is reported to attenuate hepatocarcinogenesis by blocking hypoxia-driven angiogenesis and immunosuppression (79). Bartneck M et al. found that C-C chemokine receptor type 2$^+$ (CCR2$^+$) TAMs are enriched in highly vascularized HCC, especially those that arise in fibrotic or cirrhotic livers, and could promote angiogenesis and tumor vascularization in those livers (80). Zang M et al. found that CD14$^+$ inflammatory macrophages in HCC tissues could alter macrophage function through persistent IL-23 generation, which are related to the higher concentrations of VEGF and the promotion of HCC development after chronic HBV infection (81). Meng Y et al. identified that the expression of C-X-C motif chemokine receptor 4 (CXCR4), a novel vascular marker for vessel sprouting in HCC tissues, can be promoted by monocytes/macrophages via the ERK pathway in hepatocellular carcinoma (82). On the other hand, combining zoledronic acid (ZA) with sorafenib could improve the antitumor efficacy by downregulating the expression of CXCR4 (83).

3.5 TAMs affect immunity in the TME of HCC

Macrophages are closely related to the immune evasion of HCC through expressing a series of immunosuppressing molecules including cytokines, chemokines, and enzymes (84). The interaction between TAMs and CD8$^+$ T cells produced an immunosuppressive microenvironment in HCC. Wu Q et al. found that hypoxia-inducible factor 1α (HIF-1α) induced increased expression of TREM-1 in TAMs, resulting in the impairment of the cytotoxic functions of CD8$^+$ T cells and the induction of CD8$^+$ T-cell apoptosis (85). On the other hand, Xiong H et al. demonstrated that increased IFN-γ signaling following anti-PD-L1 treatment can decrease Arginase-I (ARG1) expression and remodel the macrophage compartment by polarizing it toward a more proinflammatory phenotype to enhance T-cell responses (86). Liao J et al. revealed that a low dose of type I interferon could effectively reprogram human monocyte-derived macrophages to upregulate CD169 expression, and such induced CD169$^+$ macrophages exhibited significantly enhanced phagocytotic and CD8$^+$ T-cell-activating capacities (87). TAMs can also cooperate with Tregs in suppressing immunity in the TME of HCC (85). In addition, activated and exhausted mucosal-associated invariant T cells (MAITs), represented as an abundant innate-like T-cell subtype in the human liver, have been proven to be associated with disease progression and poor outcomes in HCC patients (88). Ruf B et al. demonstrated that human hepatic CD163$^+$ macrophages inhibit liver MAIT cell function through a cell-contact and PD-L1-dependent mechanism (89). Finally, Cheng K et al. proposed that since M2-like macrophages, Tregs, and MDSCs are the main components of the immunosuppressive microenvironment, eliminating TAMs may lead to the compensatory emergence of other protumorigenic immune cells (90).

3.6 Metabolic alterations of TAMs in HCC

The tumor progression of HCC is closely related to the alterations of metabolic enzymes, metabolites, and metabolic

pathways in macrophages (91, 92). TAMs actively take up and metabolize glucose to acquire immunosuppressive and protumor functions (93). Shi Q et al. revealed that the TME endowed M2-like TAMs with a high capability of glucose uptake and utilization, which enhanced the activity of the hexosamine biosynthetic pathway to enhance O-GlcNAcylation on cathepsin B (CTSB) in TAMs, leading to an elevated mature form of CTSB and its secretion in the TME, which in turn promote tumor metastasis and chemoresistance (94). On the other hand, fatty acid binding protein 5 (FABP5), a lipid-binding protein, could promote macrophage lipid accumulation and foster immune tolerance formation in HCC (95). Wu L et al. found that downregulation of receptor-interacting protein kinase 3 (RIPK3) in the TAMs of HCC facilitated fatty acid metabolism, including fatty acid oxidation (FAO), and induced M2 polarization in the TME (96). Zhang Q et al. found that FAO contributes to IL-1β secretion in M2-like macrophages, which could promote HCC cell migration (97).

4 TAMs in HCC immunotherapy

Immunotherapy is the first-line treatment for the comprehensive therapy of patients with advanced HCC in China, including atezolizumab combined with bevacizumab, sintilimab combined with a bevacizumab analog, donafenib, rovatinib, and sorafenib. Currently, the four therapeutic strategies targeting TAMs are the elimination of TAMs in tumor tissues, inhibition of TAM recruitment, promotion of TAM phagocytosis, and targeting TAM receptors (TAMRs), including Tyro3, Axl, and MerTK (98). Figure 3 summarizes the current strategies of macrophage-targeting therapies. Table 1 summarizes the preclinical studies and clinical trials that focus on macrophage-targeting therapies.

Colony-stimulating factor 1 receptor (CSF1R)-mediated signaling is crucial for the differentiation and survival of the mononuclear phagocyte system, especially macrophages (106). The intratumoral presence of $CSF1R^+$ macrophages is related to poor survival in various tumor types (107). Zhu Y et al. found that blocking CSF1/CSF1R could prevent TAM trafficking and thereby enhance the efficacy of immune checkpoint inhibitors for the treatment of HCC (99). Drugs that focus on CSF1R inhibition include RG7155 and IMC-CS4 (108, 109). On the other hand, research has found that specific targeting of $CD163^+$ TAMs, a type of M2-like macrophages, could re-educate the tumor immune microenvironment and promote both myeloid and T-cell-mediated antitumor immunity, which illustrates the importance of selective targeting of M2-like macrophages in a therapeutic context (110).

Therapeutic blocking of the CCL2/CCR2 axis inhibits the recruitment of inflammatory monocytes and the infiltration and M2 polarization of TAMs, resulting in the reversal of the immunosuppression status of the TME and activation of an antitumor $CD8^+$ T-cell response (102). However, a phase 2 study of carlumab, a human monoclonal antibody against CCL2, showed that carlumab failed to inhibit tumor growth since tumor cells compensatory increased the expression of CCL2 (111). Dual antagonists targeting both chemokine receptors simultaneously might be a strategy that could lead to a more effective TAM targeting. Chemokine receptors targeting agents need to be chosen accurately so as not to affect the recruitment of other immune cells such as natural killer (NK) cells and T cells.

CD47 has been proven to protect host cells from macrophage-mediated destruction by binding to signal regulatory protein (SIRP) 1α expressed on the surface of macrophages (112). Tang Z et al. revealed that CD47 could suppress phagocytosis not only by

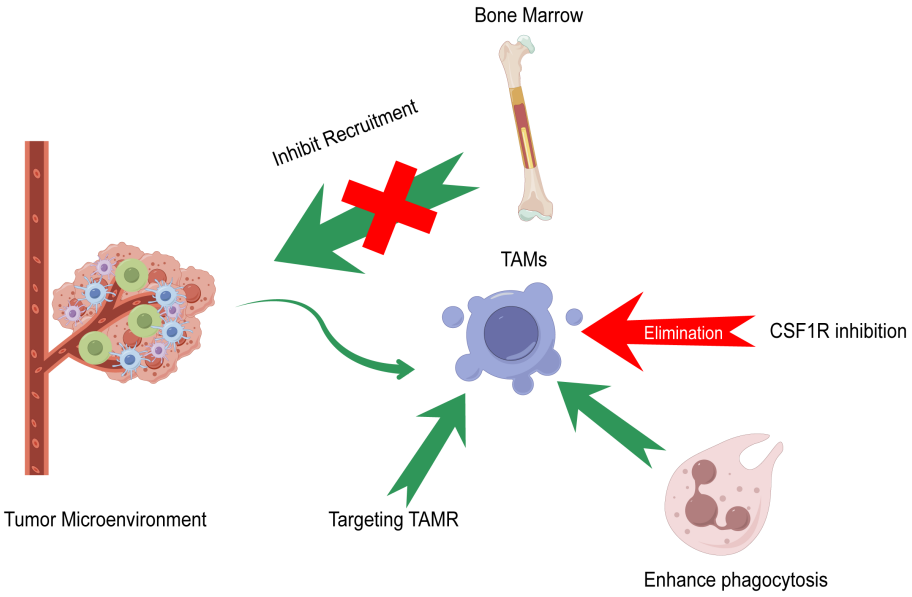

FIGURE 3
Current strategies of macrophage-targeting therapies. Elimination of TAMs in tumor tissues, inhibition of TAM recruitment, promotion of TAM phagocytosis, and targeting TAM receptors (TAMR) including Tyro3, Axl, and MerTK compose the main strategies of macrophage-targeting therapies.

TABLE 1 Studies and undergoing clinical trials of drugs targeting TAMs for HCC treatments.

Study or clinical trial number	Treatment strategy	Drug name	Results
Zhu Y et al. (99)	CSF1R inhibitor	PLX3397	Blocking CSF1/CSF1R enhances the efficacy of immune checkpoint inhibitors for the treatment of HCC.
NCT04050462	Blocking CSF1/CSF1R	Cabiralizumab	N/A
NCT03245190	Blocking CSF1/CSF1R	Chiauranib	N/A
Ambade A et al. (100)	CCL2/ CCR5 antagonist	Cenicriviroc	Ameliorates alcohol-induced steatohepatitis and liver damage
NCT04123379	CCL2/ CCR5 antagonist	BMS-813160	N/A
Yao W et al. (101)	CCR2 antagonist	747	Potentiates the therapeutic effect of sorafenib
Li X et al. (102)	CCR2 antagonist	RDC018	Blockade of CCL2/CCR2 signaling suppresses murine liver tumor growth.
Chen J et al. (103)	CD47-SIRPα blocking	Anti-CD47-Ab	Anti-CD47 antibody treatment enhances the curative effect of TACE.
Lo J et al. (104)	CD47-SIRPα blocking	Anti-CD47-Ab	Anti-CD47 antibody treatment enhances the curative effect of doxorubicin.
Xiao Z et al. (105)	CD47-SIRPα blocking	CD47mAb	CD47mAb enhances the phagocytosis ability of macrophages.

engaging SIRPα but also by masking cell-intrinsic pro-phagocytic ligands on tumor cells as well (113). Therefore, antibodies blocking CD47 and SIRPα might become an effective therapeutic strategy. The anti-CD47 monoclonal antibody B6H12 has been proven to induce macrophage-mediated phagocytosis, suppress tumor growth, and augment the efficacy of chemotherapy in HCC (104). Neutralizing antibodies against CD47 can enhance macrophage-mediated phagocytosis and activate effector T cells (114). The anti-CD40 monoclonal antibody selicrelumab is another approach to reprogram TAMs to an M1-like phenotype and enhance phagocytosis, whose main mode of action may be the induction of increased tumor-specific antigen presentation via activation of antigen-presenting cells, resulting in the production of cytotoxic T cells directed against the tumor (115–117).

TAMRs, expressed in tumors and various immune cells, exhibit diverse roles in processes such as cell fate, proliferation, migration, and regulation of tissue homeostasis and inflammation (118). Since TAMRs on macrophages have tumor-promoting roles of promoting M2-like polarization and efferocytosis, it is possible that targeting TAMRs on macrophages will be an effective therapy for treating different types of cancers (119).

5 Discussion and conclusion

As one of the most frequent malignancies worldwide, HCC is a serious threat to the lives and health of our people. The occurrence and development of HCC is a complex, multistep, and multifactor process. The polarization of TAMs, an important part and main immune cell of the TME of HCC, is affected by multiple signaling pathways and surrounding cells. TAMs participate in HCC progression by affecting HCC proliferation, invasion, and migration; mediating drug resistance; promoting angiogenesis; being involved in the formation of an immunosuppressive microenvironment; and reprogramming metabolic patterns. Owing to the crucial role that TAMs play in HCC progression, a better understanding of how TAMs regulate HCC malignancy is essential for the development of more effective TAM-targeting HCC therapies. The development and manufacture of highly selective targeting drugs will help promote the further development of antitumor immunotherapy targeting TAMs to improve clinical benefits for HCC patients.

Current investigations of TAMs in HCC remain insufficient. Although the macrophage polarization theory simplified macrophage biology by the M1-like/M2-like classification, increasing single-cell transcriptomics studies have captured a more complicated phenotype of macrophages and revealed the heterogeneous and high plasticity of TAMs at the transcriptional level. Therefore, further studies combined with genomics, proteomics, and transcriptomics analyses in both HCC *in situ* and metastasis are suggested to provide a more detailed understanding of the subtypes of macrophages in the TME of HCC and their corresponding functions in HCC.

Despite the encouraging results of clinical studies on TAMs, targeting TAMs in HCC treatment still faces some challenges. Most knowledge on how TAMs affect the TME of HCC is based on animal models. Considering the heterogeneity of tumor progression and therapy responses between animal models and humans, it is essential to explore inhibitors targeting human TAMs as well as their influence on the immunosuppressive microenvironment of HCC patients in order to enhance the applications of targeting TAM therapy strategies and improve outcomes.

In this review, we summarized the origin and phenotypic polarization of TAMs, their impact and molecular mechanism, and their potential applications in therapy strategies for HCC patients. We suggest further studies that focus on 1) identifying the diversity markers of macrophages to classify TAM subtypes, 2) revealing the heterogeneity of HCC tumors as well as the corresponding functions of TAMs in different locations such as HCC *in situ* and metastasis, and 3) enhancing the specificity of the markers for identifying TAM phenotypes. Through a better understanding of TAMs, future pharmaceuticals targeting TAMs in the specific immune environment of HCC combined with traditional immune therapy would provide a safer and more efficient treatment strategy for HCC patients to prolong survival and improve prognosis.

Author contributions

YZ: Writing – original draft. GH: Writing – original draft. JG: Writing – original draft. ZC: Resources, Writing – original draft. JW: Supervision, Writing – review & editing.

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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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Natural plant-derived polysaccharides targeting macrophage polarization: a promising strategy for cancer immunotherapy

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Tumor associated macrophages (TAMs) are the predominant innate immune cells in the tumor microenvironment (TME). Cytokines induce the differentiation of macrophages into distinct types of TAMs, primarily characterized by two phenotypes: M1-polarized and M2-polarized. Cancer growth is suppressed by M1-polarized macrophages and promoted by M2-polarized macrophages. The regulation of macrophage M1 polarization has emerged as a promising strategy for cancer immunotherapy. Polysaccharides are important bioactive substances found in numerous plants, manifesting a wide range of noteworthy biological actions, such as immunomodulation, anti-tumor effects, antioxidant capabilities, and antiviral functions. In recent years, there has been a significant increase in interest regarding the immunomodulatory and anti-tumor properties of polysaccharides derived from plants. The regulatory impact of polysaccharides on the immune system is mainly associated with the natural immune response, especially with the regulation of macrophages. This review provides a thorough analysis of the regulatory effects and mechanisms of plant polysaccharides on TAMs. Additionally, an analysis of potential opportunities for clinical translation of plant polysaccharides as immune adjuvants is presented. These insights have greatly advanced the research of plant polysaccharides for immunotherapy in tumor-related applications.

KEYWORDS

polysaccharide, tumor microenvironment, macrophage, polarization, anticancer immunotherapy

1 Introduction

Immunotherapy has emerged as a crucial adjunctive anti-tumor modality, complementing established treatments such as surgery, chemotherapy, radiotherapy, and targeted therapies. Its significance lies in the capacity to elicit sustained remission with diminished side effects (1). Immunotherapy involves the precise identification and elimination of cancer cells by immune cells within the TME, which constitutes an intricately organized ecosystem where both cellular and cell-free components possess the capability to reprogram various facets of tumor dynamics, including initiation, growth, infiltration, metastasis, and responsiveness to anticancer therapy (2). Macrophages are acknowledged as pivotal effectors of immune responses within the TME. During the development of cancer, macrophages significantly influence the inflammatory process in the TME. Given the tumor-promoting effects of TAMs, preclinical studies on strategies to counteract TAMs have made some progress. In general, these include reducing the recruitment of TAMs and "reprogramming" TAMs (3–5). Consequently, acquiring a profound comprehension of TAMs becomes imperative to enhance the efficacy of immunotherapeutic interventions.

In the innate immune system, macrophages perform a number of critical functions, such as phagocytosis removing cellular debris, controlling infections, and maintaining dynamic tissue homeostasis. Macrophages also express different functional programs in response to different signals from the microenvironment (6). This implies that macrophages have a wide range of phenotypic states and that M1 and M2 types are the extremes of macrophage functional states (6, 7). M1-like macrophages exhibiting strong cytotoxicity and antigen-raising capacity contribute to antitumor immunity. Conversely, M2-like macrophages with immunosuppressive properties promote tumor progression (8). Circulating monocytes and tissue macrophages are co-recruited into the TME and become TAMs through various soluble or mechanical factors (9–12). TAMs are also the predominant host cells in the TME. Research evidence suggests that macrophages, an important component of TME, display tumor-fighting immune responses during initiation but shift to a protumor capacity in late-stage malignancies, supporting angiogenesis and promoting tumor migration and invasion (13). Thus, TAMs can exhibit diverse responses to TME alterations. Findings demonstrate that TAMs enrichment predicts poor prognosis and drug resistance across multiple tumor types (14, 15). Therefore, targeting macrophage polarization is a promising therapeutic strategy. Acting on the TAMs in TME to change their M2 to M1 phenotype is an intriguing and promising therapeutic approach (16, 17)..

Natural products are distinguished by their abundant origins as well as innovative and diverse structures. It has been manifested that they served as a valuable resource for the discovery of anti-tumor drugs. Natural polysaccharides derived from plants, especially plant polysaccharides used in traditional Chinese medicine, have recently attracted great interest due to their broad spectrum of bioactivities, potent therapeutic potential, and low toxicity. Extensive research indicates that plant polysaccharides exhibit biological effects such as antitumor, antioxidant, immunomodulation, regulation of intestinal microbiota, and antiviral activity (18–21). More significantly, numerous studies demonstrate that plant polysaccharides exert immune-stimulating effects on macrophages, altering their polarization state for anti-tumor phenotype. For instance, *Astragalus* polysaccharides, *Panax* polysaccharides, and *Dendrobium officinale* polysaccharides have immune-stimulating or activating effects on macrophages, primarily involving cytokine secretion, production of reactive oxygen species (ROS) and nitric oxide (NO) and the regulation of numerous signaling pathways. Thus, plant polysaccharides exhibit promising potential as immune therapy modifiers for malignancy prevention and treatment.

This review discusses the classification and sources of various natural plant polysaccharides acting on macrophages and the immunomodulatory effects of plant polysaccharides targeting macrophage polarization and provides an in-depth summary of the results of clinical translational research on plant polysaccharides as potential therapeutic agents. In conclusion, we address the difficulties and constraints associated with plant polysaccharides as possible modulators and emphasize the need for further investigations.

2 Macrophage polarization and immunotherapy

Macrophages, as the principal constituents of the innate immune system and consequential contributors to the adaptive immune system, manifest noteworthy efficacy in immune responses (22). The human body harbors a considerable population of macrophages, undertaking pivotal roles encompassing phagocytosis, exogenous antigen presentation, and immunoregulation through the release of cytokines and growth factors. Importantly, macrophages demonstrate substantial adaptability, marked by functional diversity. Monocytes are no longer considered merely precursor cells to macrophages. Evidence from mice and humans that tissue macrophages originate from embryonic and adult circulating myeloid precursors (10). In many mouse tumor models, circulating monocytes are the main precursors of TAMs (13, 23). In the context of human bone marrow transplantation, lymphoma-associated macrophages were found to originate from myeloid precursors (24).

When exposed to various stimulus signals, macrophages enter a condition known as "macrophage polarization," which changes their morphology, function, and phenotype (25, 26). The classical concept divides polarized macrophages into two categories: M1 classical activated macrophages and M2 alternative activated macrophages. The two polarization states are shown in Figure 1. Depending on the type of inducer and expression marker, M2 macrophages can be categorized into a number of different subtypes, including M2a, M2b, M2c, M2d, and M2f (27). However, the expression of all subtypes *in vivo* remains unknown (28). M1 macrophages are activated by lipopolysaccharide (LPS) and cytokines (predominantly IFN-γ and IL-2) exhibiting high

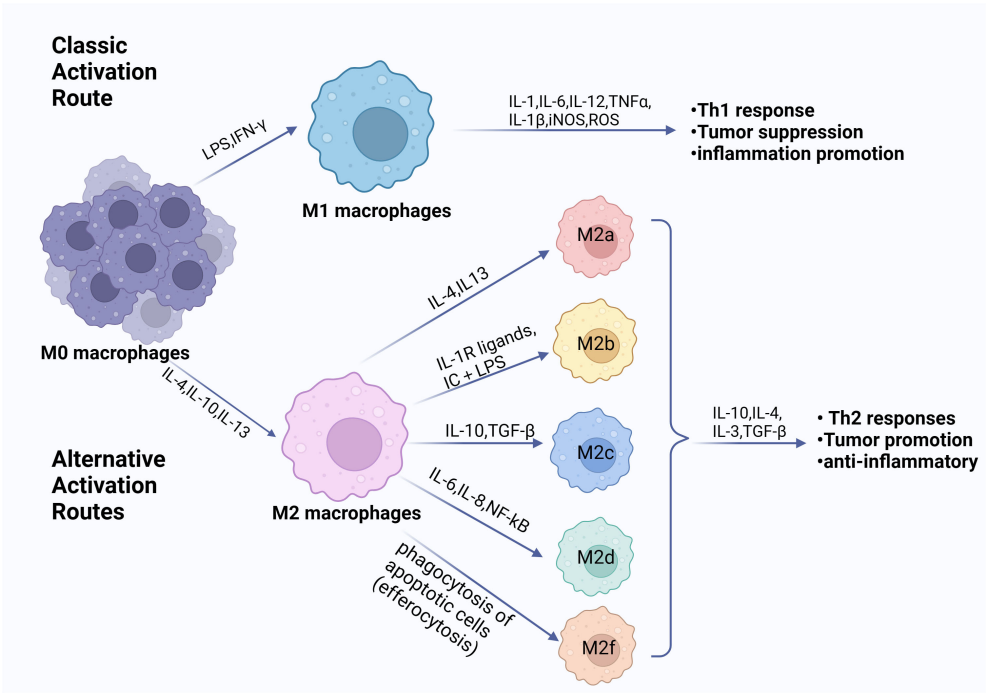

FIGURE 1
Phenotypes of macrophage polarization. Exposure to diverse cytokine environments induce monocytes' differentiation towards polarized macrophage subpopulations. When exposed to LPS, IFN-γ or other microbial products, monocytes differentiate into M1 macrophages. When exposed to IL-4, IL-10, IL-13, and immunosuppressive agents, monocytes differentiate into M2 macrophages. The M1 and M2 subpopulations are functionally and phenotypically distinct. The M1 cells exert an antitumorigenic effect. Conversely, the M2 cells contribute to a pro-tumorigenic milieu. (Created with BioRender.com).

levels of Toll-like receptors 2 and 4, CD80, CD86, and MHC class II (26). They are able to produce large amounts of inflammatory factors (IL-1β, IL-6, and TNF-α, etc.) and release NO and ROS, which play an important role in pathophysiological processes such as killing pathogens, resisting parasites and tumor cells, and pro-inflammatory responses (22, 25). M2 macrophages, induced by IL-4, IL-33, and TGF-β stimulation, usually expressing CD206 and CD163, are regulated by a variety of transcription factors and secreted cytokines in regulating tumor growth, thereby modulating inflammation, suppressing immune response, and stimulating cellular and tissue remodeling, angiogenesis and tumor progression (29, 30).

Additionally, macrophages demonstrate adaptability by modulating the TME as a tumor advances. It is noteworthy that not all TAMs manifest the M2 phenotype. Intriguingly, TAMs undergo a phenotypic transformation to M2 in hypoxic TME conditions, thereby promoting tumor progression through the secretion of immunosuppressive cytokines and consequent inhibition of immune effector cells (6, 25, 31). In addition to cytokine secretion, there are several immunosuppressive receptors on the surface of macrophages, such as sialic acid-binding immunoglobulin-type lectins (Siglecs), signal-regulating protein alpha (SIRPα), leukocyte immunoglobulin-like receptor B (LILRB), macrophage receptor with collagen structure (MARCO), and Clever-1 (32–36). Cancer cells express anti-phagocytic surface proteins CD24 and CD47 that interact with Siglec-10 and SIRPα, respectively, triggering "don't eat me" signals to evade immune surveillance and immune clearance (37, 38). Shen et al. used CD24/Siglec-10 blocking peptide (CSBP), which blocks the interaction between CD24/Siglec-10 and PD-1/PD-L1, to enhance macrophage-mediated phagocytosis of tumor cells and activate CD8 T cells (39). The molecule Clever-1 is expressed in M2-polarized macrophages. Targeting Clever-1 is anticipated to enhance existing immunotherapy approaches by enabling T-cell and macrophage-mediated anticancer immunity (36). We discuss current strategies for targeting macrophages, which include (1) altering the composition of TAM cells (2); reprogramming TAM cells to polarize M2 to M1 (3); modulation of macrophages by cytokines; and (4) functional blockade of immunosuppressive macrophages, such as Siglec-9/10, SIRPα, MARCO, LILRB2, and Clever-1. Macrophage-based immunotherapies are expected to advance immuno-oncology in the coming years.

3 Natural plant polysaccharides as modulators of macrophage polarization

Plant polysaccharides are polymers consisting of multiple monosaccharides linked by glycosidic bonds, produced by plant cell metabolism. Current research on plant polysaccharides focuses on extraction and purification, structural characterization and analysis of immunomodulatory activities (40–44). The majority of plant

polysaccharides predominantly interact with both the innate and adaptive immune systems, thereby augmenting host immunity and indirectly exerting suppressive effects on tumors (21, 45, 46). Especially, plant polysaccharides have significant effects on the regulation of immune responses by altering the activity and activities of macrophages. This, in turn, contributes to their anti-tumor and immune regulatory properties. They play a role in controlling the activity of macrophages and adjusting the levels of inflammatory cytokines, such as TNF-α and IL-1β, in order to coordinate a suitable inflammatory reaction. Moreover, these polysaccharides have the potential to improve the process of macrophage phagocytosis, therefore facilitating the elimination of pathogens or aberrant cells. Furthermore, it is believed that they regulate the polarization state of macrophages, influencing the intricate equilibrium between their M1 (pro-inflammatory) and M2 (anti-inflammatory) states. Plant polysaccharides therefore show great promise as bioactive modulators in tumor therapy and open up new options for the synthesis of novel immunomodulatory medications.

3.1 Classification, sources of natural plant polysaccharides acting on macrophages

It has been noted above that polysaccharides with the potential to modulate macrophage function have been found in a variety of plants. The fractions and biological activities of certain plant polysaccharides are listed in Table 1.

The biological activity of polysaccharides is related to their chemical composition and structure, such as molecular weight (Mw), conformation, and glycosidic bonding (89). There are large differences in the antitumor activity of polysaccharides composed of different monosaccharides. The majority of plant polysaccharides based on glucose (Glc) and rhamnose (Rha) currently exhibit strong anti-tumor action; the more Glc there is in the polysaccharide, the more anti-tumor activity there is (49–51). While some polysaccharides have only one monosaccharide component, others are made up of complicated sets of monosaccharides. In contrast to the polysaccharides isolated from *Smilax glabra Roxb*, which consisted of mannose (Man), fucose (Fuc), and Glc, all three polysaccharides derived from *Cistanche deserticola* were determined to be composed of Glc (80, 90). Furthermore, various fractions of plant polysaccharides can be isolated from a single plant, and each polysaccharide displays distinct functional effects. For example, WSRP-2a and WSRP-2b, both pectic polysaccharides, were isolated from *Rosa setate* x *Rosa rugosa* waste (47). These two fractions were mainly composed of glucuronic acid (GlcA), galacturonic acid (GalA), arabinose (Ara), galactose (Gal) and Rha, but the average molecular weights varied considerably, 56.8 and 23.9 kDa, respectively (47). WSRP-2b exhibited higher α-amylase and α-glucosidase inhibitory activities, which may be related to the higher content of glucuronides or lower relative molecular mass of WSRP-2b (91). The effect of WSRP-2a on the RAW264.7 cell proliferation and cytokine (TNF-α and IL-6) secretion with strong stimulatory effect and more immune-enhancing activity (47). The conformational relationship of pectic polysaccharides is not clear, and Wu et al. hypothesized that the different bioactivities may be due to different molecular weights (47).

Polysaccharides derived from edible or medicinal plants have several effects on macrophages, including increasing their phagocytic activity, inducing the expression of various cytokines and chemokines, upregulating ROS and NO production, and inducing either the M0 to M1 transition or the polarization of M2 to M1 states. For example, *Astragalus polysaccharide* (PG2), a principal active constituent from *Astragalus membranaceus* root, displays robust bioactivity *in vitro* and *in vivo* studies, being efficiently employed for use in the treatment of cancer and other diseases (92). Bamodu et al. demonstrated by *in vitro* and *in vivo* experiments that PG2 dose-dependently and significantly increased the polarization ratio of M1 macrophages and down-regulated IL-4- and IL-13-induced M2 polarization in non-small cell lung cancer (NSCLC) (93). RAP is a purified polysaccharide extracted from *Radix Astragali* polysaccharides containing Rha, Ara, Glc and Gal, with a backbone consisting of 1,2,4-linked Rhap, α-1,4-linked Glcp, α-1,4-linked GalAp6Me and β-1,3,6-linked GalP (94). Wei et al. demonstrated that RAP induced the expression of M1 marker genes such as iNOS, IL-6, TNF-α, and CXCL10, attenuated 4T1 cell growth, and transitioned macrophages towards an M1 phenotype or reversed M2 polarization to M1 (74).

To demonstrate the targeting of plant polysaccharides on macrophages, clodronate liposomes are a well-established method of depleting macrophages (95). Wang et al. depleted and replenished macrophages within C57BL/6 mice to further demonstrate that *Dendrobium officinale* polysaccharides can inhibit tumor growth by promoting polarization of M1 macrophages (96). In addition, studies on the mechanisms reveal that the TLRs- NF-κB pathway and the activated AMPK- PPARs pathway contribute to the anti-tumor effect of polysaccharides *in vitro* and *in vivo*. Apple polysaccharides (AP) have a relative molecular mass of 5,000-10,000 Da and their main components are GalA and Gal (76). Sun et al. found that AP not only increased macrophage M1 markers (iNOS, TNF -α, IL -23) and decreased macrophage M2 markers (TGF-β, IL -4, IL -10), but also converted M2 macrophages to M1 phenotype via TLR-4 signaling (76).

3.2 Mechanism of plant polysaccharides activating macrophages

Plant polysaccharides regulate immunity in a multifaceted modulatory manner, with a clearer mechanism observed in macrophages. Specifically, plant polysaccharides stimulate the release of cytokines such as TNF-α, IL-6, and NO, thereby promoting macrophage differentiation toward the M1 phenotype (76, 93). Simultaneously, research has elucidated the molecular mechanism of polysaccharide immunomodulation. Plant polysaccharides interact primarily with macrophage surface receptors, encompassing the mannose receptor (MR), Toll-like receptors (TLR2 and TLR4), and Dectin-1 receptor, or other derivatives (41). Macrophages are activated and stimulate signal transduction pathways leading to transcriptional activation and production of inflammatory factors.

TABLE 1 Immunomodulatory activity of natural plant polysaccharides on macrophages.

Botany	Polysaccharides	Monosaccharide composition	Models	Effects on macrophages	Ref.
Rosa setate x Rosa rugosa waste	WSRP-2a	GalA, Ara, Gal, Rha, and Man	RAW264.7	Promote proliferation, NO release, and the secretion of TNF-α and IL-6	(47)
	WSRP-2b	GalA, Ara, Gal, Rha, Man, Glc, Xyl, and GlcA			
Astragalus polysaccharide	APS	Glc, Gal, Rha, Ara, Fru, Man, and GalA	RAW264.7	Stimulate macrophages to secrete NO and TNF-α, IL-2, and IFN-γ	(48)
maca (Lepidium meyenii Walp.)	LMP-1	Glc and Ara	RAW264.7	Activate TLRs/NF-κB signaling pathway; stimulate TNF-α, IL-1b and IL-6	(40)
Asparagus officinalis L.	WASP	Rha, Ara, Gal, Glc, Xyl, and Man	RAW 264.7	Increase the release of IL-6, TNF-α, and IL-10 and improve the expression of mRNA	(49)
Hovenia dulcis peduncles	HDP3A	GalA, Gal, Rha, Ara, Xyl, Fuc, Man, and Glc	RAW 264.7	Stimulate the proliferation of RAW264.7 cells	(50)
Allium sativum L.	GPSs	Fuc, Rha, Gal, Glc, and Fru	RAW264.7	Stimulate NO	(51)
Angelica sinensis (Oliv.) Diels	APS-3a	Glc, Gal, Ara, Rha, and Man	Male BALB/c mice peritoneal macrophage		(52)
	APS-3b	Glc, Gal, Ara, Rha, and Man		Enhance the peritoneal macrophages phagocytosis; increase the release of TNF-α, NO	
	APS-3c	Glc, Gal, Ara, Rha, Man, and Xyl		Increase the release of TNF-α, NO	
Lepidium meyenii (maca)	MC-1	Ara, Man, Glc, and Gal	RAW 264.7	Enhance the pinocytic and phagocytic capacity; promote the NO, TNF-α and IL-6 secretion	(53)
	MC-2	Ara, Man, Glc, and Gal	RAW 264.7	Induce M1 polarization of original macrophages and convert M2 macrophages into M1 phenotype	(54)
Aloe vera L. var. chinensis (Haw.) Berg.	PAC	Man, Gal, Glc, and Ara	BALB/c mouse peritoneal macrophages	Stimulate TNF-α, IL-1b; stimulate peritoneal macrophage proliferation	(55)
Citrus grandis	HPP-1	Rha, Ara, Fuc, Man, and Gal	RAW264.7	Stimulate NO, TNF-α, and IL-6 secretions; activate NF-κB and MAPK signaling pathways	(56)
Nelumbo nucifera Gaertn.	LLWP-C	Rha, Ara, Gal, Glc, and GalA	RAW264.7	Stimulate NO, TNF-α, IL-1β, IL-6, and IL-12; activate MAPK and NF-κB signaling pathways	(57)
Stem lettuce	SLP	Man, Rha, GalA, Gal, and Ara	RAW264.7	Promot proliferation, phagocytosis and NO production	(58)
Rosa laevigata Michx	PPRLMF-2	Rha, Ara, Xyl, Man, Glc, Gal, and GalA	RAW264.7	Induce NO, INF-α, and IL-6; activate MAPKs and NF-κB signaling pathways	(59)
black radish (Raphanus sativus ver niger)	BRHE	Glc, Rha, Fuc, Xyl, GalA, Ara, and Gal	RAW264.7	Stimulate NO, ROS, IL-1β, IL-6, and TNF-α; stimulate iNOS and COX-2 proteins; induce TLR2/4–MAPK–NFκB–Akt–STAT3 signaling pathway; induce the promotion of macrophage phagocytosis	(60)
Gardenia jasminoides Ellis	GP2a	GalA, Ara, Gal, Glc, Rha, Man, GlcA, Xyl, and Fuc	RAW264.7	Stimulate NO, TNF-α, IFN-γ, IL-1β, IL-6, and GM-CSF	(61)
Abrus cantoniensis	ACP	Glc, Rha, Gal, GalA, GlcA, and Man	RAW264.7	Stimulate ROS, NO, iNOS, TNF-α, IL-6, and IL-1b; induce MyD88/Akt/MAPKs signaling pathway; enhance the pinocytic and phagocytic capacity	(62)
Raspberry Pulp	RPP-2a	Rha, Ara, Gal, Glc, Xyl, GalA, and GlcA	RAW264.7	Stimulate NO, TNF-α, IL-6, IL-1β, and iNOS	(63)

(Continued)

TABLE 1 Continued

Botany	Polysaccharides	Monosaccharide composition	Models	Effects on macrophages	Ref.
Lycium barbarum (L. barbarum)	LBP	Gal, Glc, Rha, Ara, Man, and Xyl	BALB/c mice peritoneal macrophages	Stimulate CD40, CD80, CD86 and MHC class II; enhance endocytosis and phagocytosis	(64)
			RAW264.7	Activate AP-1 and NF-κB; induce TNF-α, IL-1-β, and IL-12p40 mRNA expression;	
raspberry (Rubus idaeus L.)	RPP-3a	Rha, Ara, Gal, Glc, Man, and GalA	RAW264.7 murine macrophage cell	Stimulate NO, TNF-α, IL-6, iNOS, and IL-1β	(65)
Radix Aconiti Lateralis Preparata (Fuzi)	FZPS -1	D-Ara and D-Glc	RAW264.7	Promote macrophage phagocytosis; stimulate NO, IL-6, IL-1, and TNF-α	(66)
Achyranthes bidentata Blume	ABPS	Fru, Glc	J774 A.1 cell line (mouse monocyte/macrophage)	Stimulate IL-1β and TNF-α; induce TLR4/MyD88/NF-κB signaling pathway	(67)
Cyclocarya paliurus	S-CP1-8	Ara, Rha, Gal, Glc, Xyl, Man, GalA, and GlcA	RAW264.7	Stimulate NO, TNF-α, IL-1β, and IL-6	(68)
Lilium lancifolium Thunb.	LLP-1A	Man and Glc	RAW264.7	Stimulate NO, IL-6, TNF-α, and IL-1β; induce TLR4-mediated NF-κB signal pathway	(69)
Carthamus tinctorius L.	SF1, SF2	GlcA, GalA, Glc, and Ara	Female C3H/HeN (5to 6week old) mice	Stimulate IL-1, IL-6, IL-12, IFN-γ, and TLR4	(70)
Schisandra chinensis (Turcz.) Baill	SCPP11	Rha, Man, Glc, Ara, and GalA	ICR mice	Increase pinocytic activity; increase immunoglobulin levels, cytokines levels	(71)
			RAW264.7	Stimulate iNOS and TNF-α mRNA	
Glycyrrhiza uralensis fish	GP	Gal, Glc	Male BALB/c mice peritoneal macrophages	Stimulate NO, IL-6, and IL-12	(72)
Platycodon grandiflorum	PG	Fru	BDF1 mice peritoneal macrophages	Stimulate NO	(73)
Astragalus membranaceus (Fisch) Bge.; Huangqi	RAP	Rha, Ara, Glc, Gal, and GalA	RAW264.7	Stimulate NO, TNF-α, IL-6, and iNOS	(74)
Polygonatum sibiricum	PSP	Rha, Ara, Xyl, Man, Glc, and Gal	RAW264.7	Stimulate NO, IL-1β, IL-6, IL-12p70 and TNF-α; activate TLR4-MAPK/NF-κB signaling pathways	(75)
Apple	AP	Man, Rha, GalA, GalA Glc, Gal, Xyl, Ara, and Fuc	RAW264.7 murine macrophage cell	Upregulate the TLR4/NF-κB signaling pathway; switch M2 macrophages to M1 phenotype	(76)
Codonopsis pilosula endophyte	DSPS	Gal, Glc, Rha, Fuc, Ara, and Man	RAW264.7	Promote macrophage polarization toward M1 phenotype;	(77)
Ilex asprella	IAPS-2	Gal, Glc, Rha, and Ara	RAW264.7	Enhance M1 type differentiation in TAMs	(78)
			C57BL/6J mice, female	Stimulate IL-12, NO, MHC II, and INF-γ	
Cyclocarya paliurus	CPP-3	Rha, Ara, Xyl, Man, Glc, and Gal	RAW264.7	Increase the amount of NO, TNF-α, IL-1β, and PGE2 released	(79)
Smilax glabra Roxb	SGRP1	Man, Fuc, and Glc	RAW264.7	Promote the phagocytosis and increase macrophage-derived biological factors including NO, IL-6, TNF-α and IL-1β secretion	(80)
Asparagus cochinchinensis	ACMP	Man, Rha, GalA, and Xyl	RAW264.7 cells and BMDM cells	Regulate immunological function through the TLR4-MAPK-JNK/ERK/p38 signaling pathway	(81)

3.2.1 Regulation of cytokines and chemokines

Cytokines serve as crucial mediators in orchestrating the interplay between immune and non-immune cells within the TME (97). Notably, cytokines like IL-2, IL-6, TNF-α, and IFN-γ, known for their inflammatory enhancement properties, contribute to stimulating tumor cell immunity, thereby fostering anti-tumor activity (60). Conversely, cytokines such as IL-10, IL-13, and TGF-β operate by inhibiting inflammation and suppressing immune cells, consequently creating an environment conducive to tumor progression (15). Figure 2 demonstrates that natural plant polysaccharides modulate the production and secretion of cytokines involved in polarization.

Three acidic polysaccharides (APS-3a, APS-3b, and APS-3c) were extracted from *Angelica sinensis* (Oliv.) Diel by Cao et al. Among them, APS-3b and APS-3c, but not APS-3a, showed significant antitumor effects *in vivo* (52). The reason for the different anti-tumor activity functions may be related to the chemical structure (e.g., relative molecular mass, monosaccharide composition) of these acidic polysaccharides. Compared to APS-3a (5.9×105 Da), APS-3b and APS-3c had lower molecular weights (2.3×105 Da and 1.4×104 Da) (52). APS-3a and APS-3b have the same monosaccharide composition, while APS-3c contains more xylose (Xyl) (52). Each polysaccharide also contains different major monosaccharides. Glc is the primary monosaccharide of APS-3a, Ara is the main monosaccharide of APS-3b, and Man, Rha, and Glc are the major monosaccharides of APS-3c (52). In order to clarify the connection between the architectures of the three acidic polysaccharides and their functional activities, more research is required. Im et al. purified the polysaccharide SHP in *Salicornia herbacea* and found that the combination of SHP and IFN-γ synergistically inhibited the growth of mouse RAW 264.7 and stimulated the secretion of cytokines such as TNF-α and IL-1β from RAW264.7 (98). Zhang et al. identified, MC-2, a heteropolysaccharide consisting of Ara, Man, Glc and Gal extracted from Lepidium meyenii (maca) (54). MC-2 increased the concentrations of IL-6 and iNOs, whereas the levels of IL-10 and arginase-1 (Arg-1) remained unchanged, suggesting that MC-2 induces macrophage polarization toward the M1 phenotype. However, the effect of MC-2 on macrophage polarization is limited. In addition, They found that MC-2 markedly enhances IL-6 and iNOS mRNA production in IL-4-induced M2 macrophages, suggesting that MC-2 can convert M2 macrophages into M1 (54). PG2 dose-dependently enhanced M1 polarization while downregulating IL-4 or IL-13-induced M2 polarization. High M2/M1 status in TME is often associated with poor prognosis in most solid tumors (99). Consequently, PG2-induced M2 macrophage elimination offers an innovative approach to immune therapy in non-small cell lung cancer patients (93).

Chemokines regulate macrophage polarization. Studies have shown that CCL19, CCL21, CCL24, CCL25, and CXCL10 specifically induce M1 macrophage chemotaxis (100). TAMs secrete CCL3 (101), CCL5 (102), CCL15 (103), CCL18 (104), and other chemokines that can promote tumor metastasis, contribute to angiogenesis, and enhance immunosuppression and cancer cell resistance post-chemotherapy. Liu et al. concluded that macrophage-secreted CCL5 stabilizes PD-L1 *in vitro* and *in vivo*, suppressing T-cell killing of HT29 cells, and thereby promoting immune escape (105). Therefore, comprehending the function of chemokines within TME and manipulating them therapeutically offers potential strategies for cancer treatment (106).

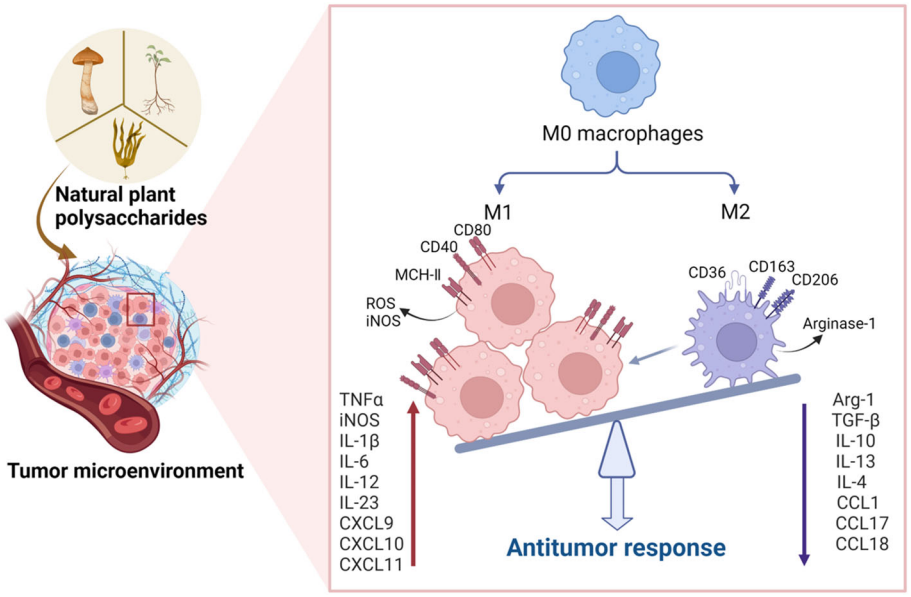

FIGURE 2
Natural plant polysaccharides act to polarize the M2 phenotype to the M1 phenotype in the TME. In addition to directly inducing apoptosis in tumor cells, polysaccharides exhibit the capacity to impede tumorigenesis and progression by influencing the TME. Specifically, these natural polysaccharides enhance the expression of M1 cytokines, including IL-6, IL-12, TNF-α, and IL-23, while concurrently inhibiting the expression of M2 cytokines such as IL-10, IL-13, TGF-β, and IL-4 within the TME. This dual action underscores the potential therapeutic efficacy of natural polysaccharides in the intricate regulation of TME, thereby presenting a promising avenue for cancer treatment strategies. (Created with BioRender.com).

3.2.2 NO and ROS generation

NO mediates cell death, eliminates infectious organisms, and functions as a signaling molecule (107). A growing number of studies reveal that iNOS mediates NO upregulation post-LPS macrophage activation, leading to mitochondrial dysfunction and tricarboxylic acid cycle disorder, resulting in macrophage transformation into M1 (108). Thus, NO has become an important marker for the transformation of M2 macrophages into M1 macrophages and enhanced tumor suppressor conditions (109). Zhou et al. reported that APS were able to directly increase NO production by macrophages *in vitro*, participate in pathogen clearance, and promote tumor cell destruction by activated macrophages (110). *Lily* polysaccharides can enhance immune function by significantly inducing NO production in macrophages in a dose-dependent manner (69). The structure of water-soluble polysaccharides extracted from *juniper cones* contains type II arabinogalactans, which were analyzed by Schepetkin et al. for their ability to induce iNOS and NO production in macrophages (111).

ROS is essential for the induction and maintenance of M1-type macrophage polarization. It has been reported that ROS promotes the expression of pro-inflammatory genes in macrophages and interferes with macrophage differentiation by stimulating the NF-κB and P38MAPK signaling pathways. BRHE, an extract isolated from black radish, was able to induce ROS production in RAW264.7 cells, and ROS are involved in immunostimulatory functions through phagocytic activation (60). The innate immune response is aided by phagocytosis, the initial reaction of an activated macrophage to invasive pathogens or microbes. Activated macrophages secrete more cytokines such as IL-6 and TNF-α, which act on pathogens and cancer cells (112). Thus, reducing the growth advantage of tumor cells is possible through balancing ROS generation and antioxidant defense (113).

3.2.3 Regulation of surface receptor expression

Plant polysaccharides primarily activate macrophages through the recognition of polysaccharide polymers by certain receptors. These receptors include TLRs, mannose receptors (MR), Dectin-1 receptors, complement receptors (CRs), scavenger receptors (SR), and others. Numerous studies have shown that TLRs play an essential role in the macrophage response to many microbial infections. Polysaccharides interacting with TLRs mainly contain glycosidic bonds of the α-(1→3), α-(1→4), β-(1→3), and β-(1→4) types (114, 115). One such receptor, TLR4, is necessary for many polysaccharide-recognition signaling events (116). In response to pathogen invasion, inflammatory cytokines such as IL-17, TNF, IFN-γ, IL-6, and IL-2 are produced when TLR4/TRAF6/NF-κB signaling is triggered (117). For example, MC-2 polysaccharides exhibit elevated glucose levels, particularly β- (1, 3)-Glc, β- (1, 4)-Glc, and α-(1→4)-Glc, which are consistently associated with TLR4 (54). In addition, TLR4 receptors-mediated signaling pathway is a common pathway for cytokine release in *Lepidium meyenii* (118), *Panax* (25), *Lycium barbarum* (119), and *Achyranthes bidentata* (67).

A crucial part of the early immune response, MR is a member of the C-type lectin receptor family and is expressed on the surface of macrophages. Due to the effect of ligands and co-receptors, MR is extensively implicated in a range of inflammatory reactions (120). The target receptor for *Aloe vera* polysaccharides may be the MR receptor of macrophages, which may bind to the MR of macrophages and lead to immune activation (55).

As pattern recognition receptors, SR work in tandem with other PRRs to identify and eradicate microorganisms in reaction to the production of cytokines. It has been shown that binding of SR and CR3 to their ligands activates phospholipase C (PLC), and the products of PLC cleavage activate protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K), leading to activation of mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), and NF-κB, which ultimately triggers gene transcription events (121). MARCO is a member of the class A scavenger receptor (SR-A) family, which is widely expressed in TAMs (35). The findings suggest that MARCO(+) TAMs is negatively associated with prognosis in some liver, lung and breast cancer cases (122–124). Eisinger et al. applied MARCO-targeting antibodies, which changed inhibitory TAM into pro-inflammatory TAMs (125). On the other hand, SR-mediated plant polysaccharides with various conformations, including α and β conformations, increase phagocytosis by macrophages and induce dendritic cell maturation. If we can find the targeting relationship between plant polysaccharides and MARCO receptors in TAMs, it provides new ideas for macrophage immunotherapy.

The primary β2 integrin that is known to aid in innate immune cells' detection of fungi is called CR3. The two ligand binding sites on CR3, the I domain and the lectin-like domain, bind to β-glucan and protein ligands, respectively (126). Most polysaccharides coupled to CR3 receptors have a β-configuration in their shape, thus stimulating polysaccharides improve phagocytosis of phagocytes, boost cytokine release, and fortify the immune system (127). Expression of CD14 in macrophages leads to pro- or anti-inflammatory responses (128). CD14 was also shown to be involved in the response to plant polysaccharides. Han et al. isolated a fructan from the radix of *Platycodon grandiflorum* and demonstrated that pretreating peritoneal macrophages with anti-CD14 or CD11b antibodies significantly reduced macrophage NO induced by tangerine polysaccharides, indicating that these surface molecules may be potential targets for polysaccharides (73). Dectin-1 is another pattern recognition receptor (PRR) that can be seen in macrophages and dendritic cells. Studies have reported that activation of Dectin-1 leads to cytokine release and ROS generation (129). In addition, Dectin-1, together with TLR2 and TLR4, can synergize to promote TNF-α production by human macrophages (130).

3.2.4 Signaling pathways

With the in-depth study of the immunomodulatory mechanisms of plant polysaccharides, attention has shifted from the extracellular to the intracellular level in the search for new targets (131). Once activated macrophage receptors can initiate a series of signaling pathways that lead to activation of transcription and production of associated cytokines that promote macrophage polarization (55, 78, 93, 132). Macrophage differentiation is

influenced by a number of variables, including some microbial products and inflammatory cytokines. Factors that stimulate M1-type macrophages include NF-κB, MAPKs, activator protein 1 (AP-1), signal transducer and activator of transcription 1 (STAT1), interferon regulatory factor (IRF) 5, and serine/threonine kinase (AKT) 2, whereas factors that stimulate M2-type macrophages include STAT6, IRF4, peroxisome proliferator activated receptor (PPAR) γ, and AKT1 (20). Figure 3 shows the action pathway of plant polysaccharides.

3.2.4.1 Toll-like receptor signaling pathway

Macrophages rely significantly on TLRs as PRRs to initiate immune responses. Notably, TLR2 and TLR4 play pivotal roles in recognizing signals associated with polysaccharides, effectively transmitting them to intracellular signaling pathways (133). Many studies have shown that plant polysaccharides can bind to TLR2 and TLR4, activate downstream signaling pathways, and exert immunomodulatory effects (69). However, TLR2 and TLR4 have different affinities for polysaccharides. Jeon et al. reported that radish polysaccharides-mediated immunomodulatory activity in RAW264.7 cells requires two major receptors, TLR2 and TLR4. The immunological response can be facilitated by both TLR4 and TLR2 signaling, which are both activated by radish polysaccharides signaling; however, the affinity of TLR4 for radish polysaccharides is much higher than that of TLR2 (60). The experiment conducted by Qu et al. demonstrated that *Abrus cantoniensis* polysaccharides (ACP) had a greater impact on TLR4 expression than TLR2, suggesting that TLR4 is the major pattern recognition receptor for ACP in macrophages (62). TLR4 expressed by macrophages is essentially involved in many natural plant polysaccharide-induced events. TLR4 signaling can be regulated through MyD88-dependent or MyD88-independent pathways (134). Myeloid differentiation factor 88 (MyD88), a key downstream signaling ligand in the TLR4 signaling pathway, drives NF-κB into the nucleus, activates related genes transcription, enhances inducible nitric oxide synthase, NO, and cytokines, and activates T cells for immune responses (135). The polysaccharide extracted from the dried rhizomes of *Atractylodes macrocephala* Koidz is a homogeneous polysaccharide composed of Glc, which is mainly connected by β-D-1→3 and β-D-1→3.6 It has a simple structure and small molecular weight. Liu et al. found that it stimulated the immune-regulatory function of the TLR4-MyD88-NF-κB signaling pathway (136). Similarly, *Achyranthes bidentata* polysaccharide, a dried root extract of *Achyranthes bidentata* Blume, as a fructan, activates TLR4 signaling through the MyD88-dependent pathway (67).

3.2.4.2 MAPK signaling pathway

The MAPK family includes three key kinases: p38, JNK, and ERK. These kinases are involved in cell proliferation, migration, invasion, and angiogenesis, and are important for cell development. Phosphorylation of particular substrates is carried out by each subclass through its own distinct activation pathway (137). The primary role of p38 is to cause cell apoptosis and initiate the synthesis of pro-inflammatory substances such as TNF-α and COX-2 (138). ERK is mainly involved in macrophage growth and differentiation (139). Multiple intracellular signaling pathways induced by plant polysaccharides ultimately converge on the MAPK pathway, which regulates macrophage NO and cytokine production

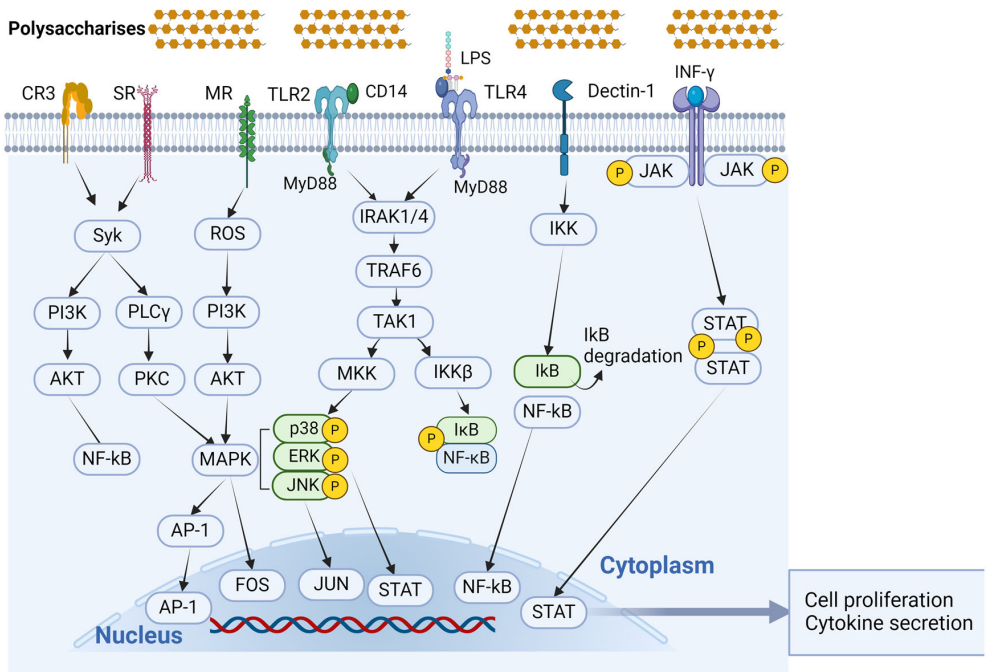

FIGURE 3
Signal transduction pathways associated with polysaccharide immunomodulation in macrophage activation. Phytopolysaccharides can activate macrophages through different receptor kinks, such as TLR4, TLR2, CR3, MR, SR, and Dectin-1. All of these receptors can function independently, and in certain cases, they may combine together to form complexes in signaling. (Created with BioRender.com).

and secretion (140). Examples include Black Radish polysaccharides (40), *Lycium barbarum* polysaccharides (119), Lotus leaves polysaccharides (57), and Aloe vera polysaccharides (141).

3.2.4.3 NF-κB signaling pathway

The NF-κB transcription factor pathway holds a pivotal role in the regulation of inflammatory diseases and immune responses (142). NF-κB is particularly instrumental in orchestrating immunological responses and governing the polarization of M1 macrophages. The target genes under the influence of NF-κB encompass IL-1, IL-2, IL-6, IL-8, IL-12, and TNF-α. It has been demonstrated that inhibiting IKKβ in tumor-associated macrophages leads to increased expression of the antitumor cytokine IL-12 and inducible nitric oxide synthase, facilitating the transition of macrophage phenotype from M2 to M1 (143). Plant extracts and isolated compounds from numerous families directly target the NF-κB signaling cascade at a molecular level. Examples of plant polysaccharides that activate the NF-κB signaling pathway and foster M1 macrophage polarization are listed below: Crocus sativus polysaccharide (144), and Pleurotus ostreatus polysaccharides (145).

3.2.4.4 JAK/STAT signaling pathways

The Janus kinase (JAK)-signal converter and activator of transcription (STAT) pathway (JAK/STAT) is activated by cytokines. Following STAT1-initiated transcription of M1 macrophage-typical genes, pro-inflammatory cytokines are released (146). The transcription factor STAT3, on the other hand, is involved in both development and tissue homeostasis. It has been found in multiple investigations that STAT3 activation can convert macrophages into M2-type (147, 148). A comprehensive analysis of the molecular mechanisms of macrophage polarization was carried out by Guo et al., who discovered that BRP regulates TAMs polarization via the STAT signaling pathway. Specifically, BRP controls M1 and M2 polarization by increasing STAT1 activation and decreasing STAT3 and STAT6 activation (149). Li et al. found that IAPS-2 polysaccharide has antitumor effects by inhibiting the phosphorylation of STAT3 in RAW 264.7 cells and S180 tumor tissues, while significantly increasing the phosphorylation of STAT1 (78).

Together, these mechanisms contribute to the regulation of macrophage polarization by natural plant-derived polysaccharides. It should be mentioned that the exact processes may differ based on the polysaccharide and the cellular environment. The signaling pathways and their molecular interactions by which natural plant-derived polysaccharides regulate macrophage polarization need to be further investigated.

4 Clinical translation and application

The development of natural products has been an important direction in antitumor drug discovery and research. This paper reviews some plant-derived crude and pure polysaccharides with clinical applications or ongoing clinical trials, aiming to provide new insights into anticancer immunotherapy. The clinical applications of four natural plant polysaccharides are summarized primarily in Table 2.

4.1 *Astragalus* polysaccharide

Preclinical studies and clinical trials have demonstrated the antitumor effects of APS (92, 150). The anti-tumor effects of APS mainly include three aspects: first, they can improve the efficacy of chemotherapeutic drugs; second, they inhibit tumor cell proliferation and promote apoptosis; and third, they play an anti-tumor role through immune mechanisms (151).

APS can induce to overcome the inhibition of cyclophosphamide, promote the proliferation of lymphocytes, increase the serum antibody gradient, and enhance the ability of vaccine antigens thus widely used in clinics (42). Kong et al. reviewed the clinical trials and laboratory studies of APS and evaluated the potential feasibility of APS for use in combination with immunotherapy in the treatment of tumors (150). They noticed that APS can regulate immune cells, such as macrophages and NK cells, through cytokines and signaling pathways. Additionally, it is involved in the immune checkpoint inhibitor signaling pathway.

TABLE 2 A review of clinical studies on plant polysaccharides.

Study model	Therapeutics	Treatment target	Mechanism	Ref.
Astragalus membranaceus	Combined with immune Checkpoint Inhibitors	NSCLC	Reduce PD-L1 expression in TME; activate and proliferate tumor-specific T cells in TME	(82)
	CCRT	HNSCC	Activate CCRT-associated AEs and deterioration in QoL	(83)
	Combined with cisplatin	nasopharyngeal carcinoma	Enhance the anti-proliferative and apoptotic effect of cisplatin by modulating expression of Bax/Bcl-2 ratio and caspases	(84)
	Combined with Apatinib	gastric cancer	Inhibit AKT signalling pathway	(85)
RG-I Pectic Polysaccharides			Enhance phagocytic activity and stimulates cytokine secretion	(86)
EPS-EPO VIIa	Combined with chemotherapy	gastric cancer	Reduce chemotherapy-induced leukopenia	(87)
Belapectin	combined with anti-PD-1 (pembrolizumab)	MM and HNSCC	Enhance anti-tumor immunity by enhancing CD8+ T-cells and repolarize M2→M1 macrophages	(88)

Immune checkpoint inhibitors (ICIs) that can activate and multiply tumor-specific T cells in TME include PD-1 and CTLA-4 inhibitors. Neutrophil-to-lymphocyte ratio (NLR) is used as a prognostic indicator in immunotherapy-treated cancer patients. Recent research indicates that patients with NSCLC who have elevated NLR are more likely to have side effects and have lower survival rates (152, 153). PG2, a polysaccharide extracted from *Astragalus membranaceus*, as a prescription drug reduces the index NLR in patients with advanced lung cancer treated with a combination of ICIs (82). This finding suggests that APS could be used in combination with immunotherapy to treat tumors (150).

Guo et al. conducted a clinical trial with 136 patients to examine the efficacy and safety of administering APS along with vinorelbine and cisplatin (VC) for advanced NSCLC. The results demonstrated that compared to patients treated with VC alone, APS combined with VC treatment led to a better quality of survival (154). In a study performed by Hsieh et al., the effect of PG2 injection on concurrent chemoradiation therapy (CCRT)-related adverse Events (AEs) and patient adherence to treatment were investigated. The results showed that PG2 has a safety profile and has the potential to ameliorate the impact of AEs in advanced head and neck squamous cell carcinoma (HNSCC) under CCRT (83). In addition to enhancing chemotherapy's effectiveness against NSCLC and HNSCC, APs have shown equal effectiveness in preclinical investigations against nasopharyngeal cancers (84), gastric (85), and ovarian malignancies respectively [132,140].

4.2 Belapectin

Proteins known as lectins bind carbohydrates and are members of the non-integrin β-galactoside-binding lectin family 6. Galactose lectin is an intracellular protein localized mainly in the cytoplasm and nucleus (155). Previous research has demonstrated that galectins have a significant role in the pathophysiology of cancer, fibrosis, and inflammation (156, 157). Galactose lectin-3 (Gal-3) is the most prominent galactose lectin secreted in disease states. Gal-3: this protein increases M2 polarization and macrophage infiltration, inhibits TCR signaling, and triggers T cell death to cause tumor-induced immunosuppression (158). Gal-3 is also upregulated by a number of cancers, and this is linked to a bad prognosis (159, 160). Several natural polysaccharides, Belapectin (GR-MD-02), Modified Citrus Pectin (MCP, PectaSol-C), and Davanat (GM-CT-01), are carbohydrate inhibitors of galactoglucan lectins (88, 161, 162). Of these, GR-MD-02 is currently being actively conducted and evaluated in various stages of clinical trials (163–165).

TCR-mediated signaling is essential for increasing effector T-cell responses to treatment with agonist anti-ox40 monoclonal antibody (aOX40) to maintain antitumor immunity (166). Sturgill et al. validated that belapectin synergizes with an agonist anti-OX40 antibody (aOX40) to promote tumor regression and improve survival by using hormonal (MCA-205 sarcoma, 4T1 breast cancer, TRAMP-C1 prostate adenocarcinoma) mice (167). Additionally, PD-1/PD-L1 involvement and overexpression of Gal-3 are key mechanisms of tumor-induced immunosuppression that contribute to immunotherapy resistance (168, 169). The researchers assessed the role of immunization in patients with metastatic melanoma (MM) and head and neck squamous cell carcinoma (HNSCC) by combining GR-MD-02) with anti-PD-1 (pembrolizumab) (88). The results of the phase I clinical trial found that the combination therapy of beraplanin + pembrolizumab was active against MM and HNSCC, and that dual blockade of PD-L1 and Gal-3 enhanced anti-tumor immunity by enhancing CD8+ T-cells, reducing MDSCs, and repolarizing M2→M1 macrophages (88).

4.3 Other polysaccharides

In a prospective study conducted by Melchart et al., EPS-EPO VIIa, a polysaccharide component isolated from *Echinacea purpurea herb* was shown to attenuate the adverse effects of chemotherapy in patients with advanced gastric cancer, but the exact mechanism remains to be investigated (87). Pectin polysaccharides rich in RG-I structure from bell peppers and carrots were proposed by Mckay et al. (86). Its ability to enhance innate immune responsiveness has been demonstrated in a series of preclinical and clinical studies to help boost immunity against infections.

In conclusion, combining chemotherapy with biological response modifiers offers a novel strategy for counteracting chemotherapy's immunosuppressive effects; however, there are still obstacles to overcome in the clinical translation of plant polysaccharides, which are naturally occurring biological response modifiers. One of the biggest problems with clinical research is the scarcity of pure chemicals and well described extracts; therefore, many more defined extracts of active compounds will be needed for future clinical trials. Second, there has to be research into both clinical and experimental settings to establish whether polysaccharides increase cancer risk. Given the toxicity of many plant derivatives, it is important to choose the safest dosage of medication and take precautions to reduce the likelihood of adverse effects.

5 Discussion

In addition to conventional approaches such as surgery, chemotherapy, targeted therapy, and radiotherapy, immunotherapy has emerged as a cornerstone in standard cancer care. Macrophages, key components of immune effector cells, exert either pro- or anti-tumor effects by modulating their polarization in response to the tumor microenvironment. This notable plasticity presents opportunities for the depletion and repolarization of TAMs. Plant-derived polysaccharide molecules, originating from sources such as plants, algae, and fungi, are identified as potent immunomodulators in this review. These compounds activate innate immune responses in macrophages, effectively suppressing malignancies. Furthermore, plant polysaccharides have demonstrated the ability to enhance radiation sensitization, augment the efficacy of vaccinations, and serve as effective adjuvants. A large number of studies have demonstrated the ability of natural plant polysaccharides in cancer prevention and treatment. However, elucidating the direct targets and

specific molecular mechanisms of natural plant polysaccharides still presents difficulties and challenges. First, the relationship between the structure and pharmacological activity of polysaccharides is unclear, and thus the study of immunomodulatory and anticancer mechanisms also poses challenges. In view of this, future research efforts may focus on identifying the optimal polysaccharide isolation technique, investigating the relationship between its chemical structure and biological activity, and exploring its role in cancer therapy. Secondly, the low bioavailability of natural polysaccharides is also a problem. Studies have shown that polysaccharides after oral administration are difficult to cross the biological barrier to act directly. Nanoparticles, characterized by favorable water solubility, stability, and biocompatibility, present a viable solution. Utilizing nanomaterials can enhance the bioavailability of polysaccharides, extending the effective duration of drugs within the body and mitigating potential side effects. In general, polysaccharides are not suitable as first-line medications in anti-cancer therapy, but only applied as adjuvant therapy. This is due to the unclear understanding of the mechanisms and targets underlying their natural pharmacological anti-tumor effects, thereby constraining their broader clinical applications.

In summary, this review provides a thorough analysis of the regulatory effects and mechanisms of plant polysaccharides on TAMs. Additionally, an analysis of potential opportunities for clinical translation of plant polysaccharides as immune adjuvants is presented. Further research on polysaccharides will lead to more efficient production and use of polysaccharide adjuvants.

Author contributions

JW: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. YD: Validation, Investigation, Writing – review & editing. NZ: Investigation, Validation, Writing – original draft. ZW: Investigation, Supervision, Validation, Writing – original draft. XT: Investigation, Validation, Writing – original draft. TY: Investigation, Validation, Writing – original draft. XJ: Formal analysis, Supervision, Writing – review & editing. SJ: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

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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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Mechanistic studies of tumor-associated macrophage immunotherapy

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Tumor-associated macrophages (TAMs) are present in the tumor microenvironment and can polarize into subtypes with different functions and characteristics in response to different stimuli, classifying them into anti-tumorigenic M1-type and pro-tumorigenic M2-type. The M1-type macrophages inhibit tumor growth through the release of pro-inflammatory cytokines, whereas the M2-type macrophages contribute to tumor progression through the promotion of tumor proliferation, angiogenesis and metastasis. Due to the duality of macrophage effects on tumors, TAMs have been a hot topic in tumor research. In this paper, the heterogeneity and plasticity of TAMs, the interactions between TAMs and other immune cells, and the effects of TAMs on tumors are reviewed, and the therapeutic strategies for TAMs are summarized and discussed. These therapeutic strategies encompass methods and approaches to inhibit the recruitment of TAMs, deplete TAMs, and modulate the polarization of TAMs. These studies help to deeply understand the mechanism of TAMs-tumor interaction and provide reference for combination therapy of tumors.

KEYWORDS

tumor-associated macrophages, TAMs, tumor microenvironment, immunotherapy, cancer

1 Introduction

The innate and adaptive immune systems in the human body are able to recognize and eliminate tumors (1, 2), but tumors may still be able to escape from the immune system and establish an immunosuppressive tumor microenvironment (TME) that is conducive to tumor progression through the modulation of immune cell function (3, 4). Macrophages are an important component of the innate immune system and are highly plastic and heterogeneous. Macrophages are polarized into classically activated M1-type and alternatively activated M2-type under different environmental conditions (5, 6). M1-type macrophages, as a potent anti-tumor immune cell, express high levels of markers (human: CD68, CD80, CD86, MHC-II, IL-1R, IL-12, TLR-2, TLR-4 and inducible nitric oxide synthase 2 (iNOS2; mice: CD68, CD80, CD86, MHC-II, IL-12, IL-23), and secrete a variety of inflammatory cytokines, such as

interleukin-1β (IL-1β), IL-6, and IL-12, to exert anti-inflammatory and tumor-suppressive effects (7). In contrast, M2-type macrophages express different markers and perform distinct roles in humans and mice. In humans, M2-type macrophages express markers such as CD86, CD163, CD206, CD200R, CD209, CD301, IL-1R, IL-10, TLR-1, TLR-8, and VEGF. In mice, they express markers like arginase-1, found in the inflammatory zone 1 (FIZZ1), and Ym1/2. M2-type macrophages are recruited by tumor cells into the TME to promote tumor progression (8). Within the TME, these macrophages are referred to as tumor-associated macrophages (TAMs), which actively produce cytokines that promote angiogenesis and support tumor cell survival and metastasis. In addition, TAMs express immunosuppressive factors, such as IL-10 and transforming growth factor-β (TGF-β), which play a crucial role in suppressing anti-tumor immune responses (9). In addition, depletion of TAMs (10) or conversion of macrophages to anti-tumor M1-type (11) significantly reduces tumor cell growth. Targeting TAMs in TME has evolved as an effective cancer immunotherapy strategy. This strategy combines traditional or emerging immunotherapies for synergistic effects and has important applications in cancer treatment.

Macrophages originate from the embryonic yolk sac, fetal liver, and bone marrow, and are categorized into two types: bone marrow-derived macrophages (BMDMs) and tissue-resident macrophages (TRMs) (12, 13). BMDMs are derived from hematopoietic stem cells in the bone marrow, while TRMs are generated from erythro-myeloid progenitors (EMPs) in the yolk sac and fetal liver (14). Macrophages from different sources within the same tissue can have distinct roles. For instance, in lung, brain, and pancreatic tumors, TAMs derived from hematopoietic stem cells are more likely to express genes associated with immunosuppression and antigen presentation, whereas embryonically-derived TAMs express genes linked to tissue remodeling and wound healing (15, 16). The heterogeneity and plasticity of TAMs, influenced by their different origins, contribute significantly to the complexity of the tumor microenvironment (TME) (Figure 1).

Activated macrophages can either kill tumor cells and induce antitumor activity or promote tumor growth and metastasis (17, 18). Further studies revealed that this duality is due to differences in macrophage stimulatory factors and secreted products resulting in both M1 and M2 phenotypes of macrophages in malignant tumors (19). Stimulated by pro-inflammatory factors such as interferon (IFN)-γ, lipopolysaccharide (LPS), and tumor necrosis factor (TNF)-α, macrophages exhibit the M1 phenotype, which is capable of generating inflammatory responses, exerting anti-tumor effects, and promoting anti-tumor immune responses through the release of IL-1β, IL-12, and reactive oxygen/nitrogen intermediates (20). In contrast, macrophages induced in TME can also exhibit M2-type characteristics. Induced by anti-inflammatory stimuli such as IL-4, IL-10, IL-13, glucocorticoids and immune complexes, macrophages secrete high levels of IL-10 and increase the expression of mannose receptors and galactose receptors (21), thus acting as an anti-inflammatory agent to promote wound healing and tissue repair, as well as to promote proliferation, metastasis, angiogenesis, and endocytosis of tumor cells (Figure 2).

A proper balance between M1 and M2-type macrophages is essential for maintaining tissue homeostasis (22). However, a large

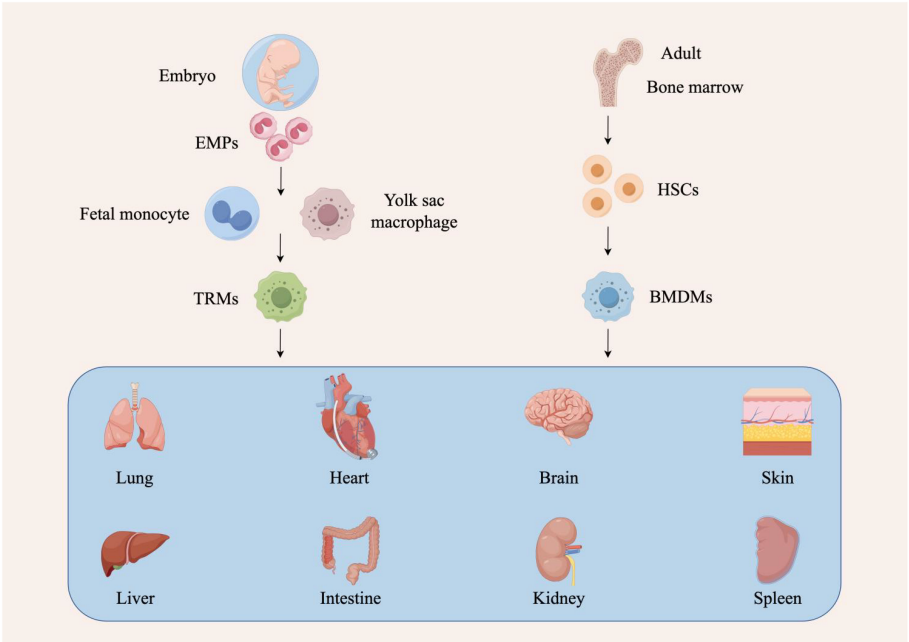

FIGURE 1
Different sources of tumor-associated macrophages. There are two sources of macrophages. The first source originates from hematopoietic stem cells (HSCs) in the bone marrow. These cells undergo developmental and differentiation steps, enter the peripheral blood as bone marrow-derived macrophages (BMDMs), and migrate to different tissues in response to stimuli. Depending on the tissue they enter, these macrophages are given different names, such as Kupffer cells in the liver, alveolar macrophages in the lungs, and microglia in the central nervous system. The second source is of embryonic origin, deriving from erythro-myeloid progenitors (EMPs) in the yolk sac and fetal liver, which develop into tissue-resident macrophages (TRMs).

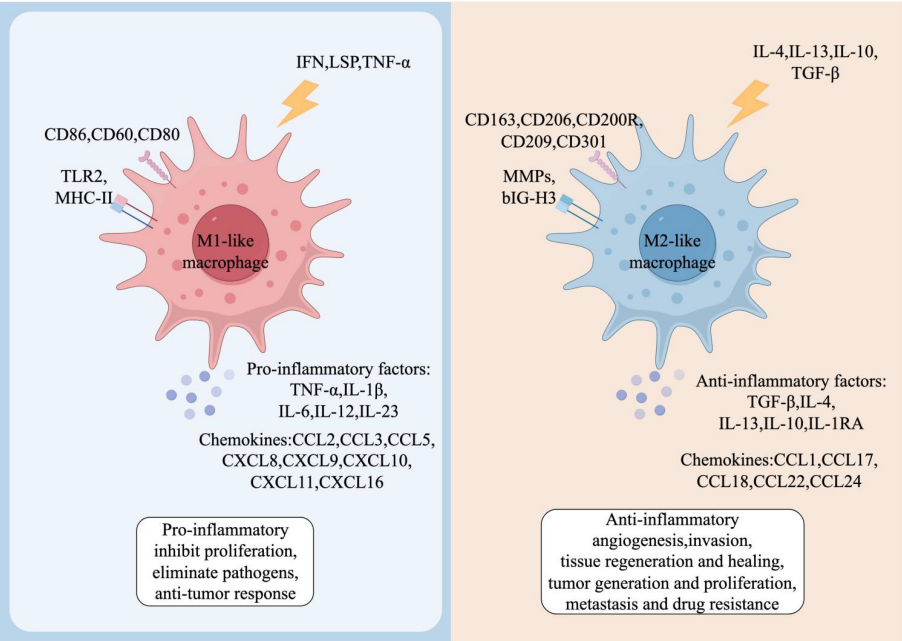

FIGURE 2
Phenotypes and functions of TAMs. Both M1-like and M2-like TAMs have distinct cell surface markers and functional factors. M1-like TAMs are induced by interferon-γ (IFN-γ), lipopolysaccharides (LPS) and tumor necrosis factor-α (TNF-α). These macrophages exhibit a pro-inflammatory phenotype and produce cytokines including TNF-α, interleukin-1β (IL-1β), and IL-6, among others. In the TME, M1-like TAMs promote inflammation, inhibit proliferation, eliminate pathogens, and contribute to anti-tumor responses. Conversely, M2-like TAMs are induced by IL-4, IL-13, IL-10 or transforming growth factor-β (TGF-β), and produce anti-inflammatory cytokines, such as IL-10 and TGF-β. M2-like TAMs in the TME are involved in anti-inflammatory activities, promoting angiogenesis, influencing tissue regeneration and healing, and fostering tumor growth, proliferation, metastasis, and drug resistance.

body of evidence suggests that the widely used ratio of M1/M2 macrophages does not accurately reflect the inflammatory state of tissues because of the stimulation of multiple pro- and anti-inflammatory factors in the tissue microenvironment. Influenced by these stimuli, macrophages do not have a defined direction of polarization when recruited to specific tissues, but rather exhibit a high degree of dynamism and heterogeneity. Thus, a synthesis of the various stimulus signals is likely to be more conducive to a comprehensive and in-depth understanding of the activated subpopulations of macrophages. Some of the more important of these signals include individual occurrence-related signals, tissue-specific signals, and other exogenous/endogenous signals (23).

2 Major molecules regulating TAM function

TAM immunoregulatory mechanisms include the colony stimulating factor 1 (CSF-1)/colony stimulating factor 1 receptor (CSF-1R) axis, IL-4/IL-13 and JAK/STAT6 transduction pathways, Toll-like receptor (TLR), and CD47-SIRPα signaling pathway (24). The CSF-1/CSF-1R axis affects tumor growth and metastasis by activating the phosphatidylinositol-3-hydroxy kinase (PI3K) signaling cascade and regulating the M1/M2 polarization of macrophages; IL-4/IL-13 and the JAK/STAT6 pathway are involved in the Th2-type immune response, inducing TAMs toward M2 phenotype and promoting abnormal tumor angiogenesis and progression; TLRs affect lung cancer metastasis and growth by recognizing pathogen-associated molecules and subsequently altering macrophage activation status; the CD47-SIRPα signaling pathway promotes tumor growth and metastasis by inhibiting macrophage-mediated phagocytosis. The study of these immunoregulatory mechanisms provides new ideas and targets for tumor therapy.

2.1 CSF-1/CSF-1R

Granulocyte-macrophage colony stimulating factor (GMCSF) regulates hematopoietic cell production and differentiation, and also plays a role in angiogenesis (25). CSF-1 binds to CSF-1R, which further promotes protein kinase B and mammalian target of rapamycin 2 (mTORC2) through activation of the PI3K signaling cascade, further promoting the activation of protein kinase B and mTORC2, thereby regulating the M1/M2 polarization axis in macrophages (26). Activation of PI3K and AKT kinases or overexpression can inhibit M1-type macrophage activation, and activation of the PI3K pathway mediates negative regulation of the nuclear factor-κB (NF-κB) signaling pathway that can promote M1 production (27). Additionally, CSF1R can be activated by binding to IL-34 (28). Therefore, when IL-34 is highly expressed with CSF-1R in tumors it marks tumor progression and lower survival. A study (29) demonstrated that high expression of IL-34 and M-CSF and their ligands was associated with lower survival in a cohort of lung

cancer patients, because lung cancers with high IL-34 and M-CSF expression were more likely to progress to advanced stages. In addition, CSF-1 can produce factors that promote tumor growth and metastasis by recruiting and reprogramming TAM (30).

2.2 IL-4/IL-13 and JAK-STAT6

IL-4 and IL-13, which are involved in Th2-type immune responses (31), are among the major stimuli that induce TAM tendency toward the M2 phenotype that promotes abnormal tumor angiogenesis and tumor progression. IL-13 and IL-4 promote the phosphorylation of JAK by binding to type I IL-4 receptor (IL-4Rα and IL-4Rγc) and type II IL-4 receptor (IL-4Rα and IL-13Rα1), which in turn phosphorylates the transcription factor STAT6. Subsequently, activated STAT6 dimerizes and translocates into the nucleus, where it binds to the corresponding site of DNA, initiating the transcription of the target gene (32, 33). STAT6 activation also promotes the expression and transcription of M2-associated specific genes, such as Arg-1, Mrc-1, and Chil3/Ym1 (34). STAT6 acts as a key factor in IL-4 and IL-13 mediated macrophage polarization towards an immunosuppressive phenotype, and is also regulated by other factors. For example, one study (35) found that TRAF3 promotes STAT6 ubiquitination and transcriptional activity as shown by ubiquitination assay and luciferase assay. Site mutation analysis revealed that ubiquitination at STAT6 K450 plays a crucial role in TRAF3-mediated STAT6 activation, which promotes increased expression of M2-associated surface markers as well as tumor progression. Bone marrow TRAF3 deficiency was found to inhibit tumor growth and lung metastasis *in vivo* using a B16 melanoma mouse model.

2.3 TLRs

The body's immune response to the environment can be divided into two types: innate immunity and adaptive immunity, and pattern recognition receptors (PRRs) are essential for the functioning of innate immunity (36, 37). In the tumor microenvironment, the interaction between pathogen-associated molecular patterns (PAMPs) and PRRs, especially TLRs, play a crucial role in tumor initiation and progression. TLRs can recognize different types of PAMPs, such as bacterial lipopolysaccharides and viral RNA. Although these PAMPs typically originate from infectious pathogens, in the tumor microenvironment, tumor cells or surrounding immune cells may also activate TLRs by releasing PAMP-like substances (38).

To date, a total of 13 TLRs have been identified in mammals, of which 11 are expressed in humans (TLR1-10). Macrophages can be reprogrammed through the activation of different TLRs, thereby altering the activation state of macrophages. For example, in a lung metastasis model, TLR4 can promote the effect of TAM on lung tumor metastasis through the NF-κB pathway. By using TLR4-deficient mice, it was found that TAM lacking TLR4 could not produce pro-inflammatory cytokines, nor angiogenic factors, and failed to activate NF-κB activity in tumors, thereby failing to inhibit (39). In addition, the Lewis lung carcinoma (LLC) cell line is a potent activator of macrophages. LLC-conditioned medium activates TLR2 and TLR6 through the extracellular matrix proteoglycan versican, leading to the production of TNF-α and IL-6 by macrophages, which strongly promotes lung cancer metastasis and growth (40). It has also been shown (41) that up to a 100-fold increase in M1-type macrophage production can be achieved by applying less toxic IFNs (including IFN-α and IFN-β) in combination with TLR agonists. This fully demonstrates their potential for anti-tumor development and suggests a new approach to TLR-related tumor immunotherapy.

2.4 CD47-SIRPα

Integrin-associated protein (IAP or CD47) is a receptor for members of the platelet-responsive protein family that regulates a range of cellular activities, including platelet activation, cell motility and adhesion, and leukocyte adhesion, migration, and phagocytosis (42). CD47 is an immunoglobulin widely distributed on the cell surface that inhibits phagocytosis of tumors by macrophages in order to promote growth and metastasis, and can be involved in the mediation of cell proliferation, migration, apoptosis and immune homeostasis (43). SIRPα, a transmembrane protein highly expressed on cell membranes, is the main ligand for the CD47 molecule (44). The NH2 terminus of its extracellular domain can bind to CD47, leading to tyrosine phosphorylation on the immunoreceptor tyrosine-based inhibitory motif (ITIM). This binding triggers the release of an inhibitory phagocytosis signal, which can inhibit macrophage-mediated phagocytosis, thereby protecting normal cells from damage caused by the immune system (45). CD47 has been shown to be highly expressed in a variety of solid tumors and correlates with a poor prognosis of tumors; therefore, inhibition of the CD47-SIRPα pathway enhances the body's adaptive immune response and increases macrophage phagocytosis.

3 Interactions between TAMs and other immune cells

Crosstalk between TAMs and other immune cells is an important aspect of TAMs affecting tumor immunity. In addition to macrophages, TMEs contain several immune cell populations such as T-cells, B-cells, natural killer (NK) cells, and neutrophils, which interact with each other through different signaling pathways (46). Macrophages and other immune cells within the TME can exhibit phenotypic plasticity in response to signals, resulting in dynamic spatiotemporal patterns that influence the immune status and tumor development of the TME. Intensive studies of the complex crosstalk between macrophages and different immune cells have led to a deepening understanding of macrophage-tumor interactions (47).

In the tumor microenvironment, type I helper T (Th1) cells, NK cells, and cytotoxic T lymphocytes (CTLs) can stimulate macrophage polarization toward the M1 type by secreting IFN-γ

(48). Polarized M1-type macrophages can release a variety of pro-inflammatory cytokines (TNF-α, IL-6, IL-12, and IL-23) and reactive oxygen/nitrogen intermediates to achieve their tumorigenic activity, and M1-type macrophages can produce chemokines (CXCL9 and CXCL10) to recruit more Th1 cells, thereby creating positive feedback and further amplifying the type I immune response (49). Therefore, M1-type macrophage-mediated immune responses can enhance the antigen-presenting ability of TAMs and effectively improve their antitumor effects.

Interactions between M2-type macrophages and other immune cells (Th2 cells, basophils, regulatory T cells) allow for an enhanced type 2 immune response and contribute to the transformation of tumor cells to malignancy (50). The latter immune cell population induces macrophage polarization towards the M2 type by producing IL-4, IL-13, or IL-10, thus recruiting more Th2 cells into the TME in response to chemokines (CCL17, CCL22, and CCL24) released from activated M2 type macrophages (51). On the other hand, Treg has been shown to promote immunosuppressive responses in macrophages by activating their programmed cell death ligand 1 (PD-L1) (52). Studies have also shown that macrophage function and diversity in TME are also influenced by tumor-infiltrating B cells. Through the production of IL-10 or immunoglobulins, B cells are able to polarize the macrophage population towards the M2 type (53). Tumor-infiltrating M2-type macrophages then inhibit the antigen-presenting ability of dendritic cells (DCs) by producing IL-10 and prevent DCs from activating CTLs, thereby causing dysfunction of DCs in the TME (54). This leads to immune escape and reduces the response of CD8+ T cells to cancer cells. Although the association between macrophages and neutrophils has rarely been reported, new evidence suggests that macrophage depletion in TME can induce the production of highly immunosuppressive neutrophils, the signaling mechanisms of which are currently unknown (55). Overall, macrophages may serve as a global target to regulate innate and adaptive immunity in the TME immune system.

4 TAMs and tumors

In early-stage tumors, M1-type macrophages play an anti-tumor immune role and inhibit tumor growth together with T cells and interferon. However, with tumor progression, macrophages gradually lose their tumor-suppressive function and exhibit M2-type tumor-promoting features (56). The role of TAMs in promoting tumor progression is multifaceted. First, TAMs are closely associated with immunosuppressive TME, which is an important cause of the poor prognosis of many human cancers (57). The main manifestation of TAMs immunosuppression is that a higher proportion of M2-type TAMs in the TME leads to increased cancer invasiveness and exacerbates the tumor by generating an immunosuppressive TME, which promotes tumor invasion, metastasis, and progression (58). Second, M2-type TAMs promote angiogenesis; M2-type TAMs are a major source of epidermal growth factor (EGF), which is a direct promoter of tumor growth. Polarized M2-type TAMs constitute a complex cell population including pro-angiogenic macrophages, immunosuppressive macrophages, perivascular macrophages, metastasis-associated macrophages, and invasive macrophages (59). Pro-angiogenic macrophages of TAMs are known to promote tumor growth through the secretion of vascular endothelial growth factor (VEGF), which is an essential component of tumor growth. VEGFA, TGF-β, and angiogenic chemokines (CXCL8 and CXCL12), which promote the activation and recruitment of endothelial cells and fibroblasts in TME. Thus, pro-angiogenic macrophages facilitate tumor angiogenesis and provide sufficient nutrients and oxygen for rapid tumor growth (60). In addition, matrix metalloproteinases (MMPs) and cathepsins produced by M2-type macrophages are able to degrade the surrounding extracellular matrix, which facilitates the migration of cancer cells from the primary tumor tissue. With the expression of angiopoietin 1 receptor, perivascular macrophages can help cancer cells to enter the blood vessels (61). Metastasis-associated macrophages (MAMs) are capable of producing VEGF receptor 1 (VEGFR1), chemokine receptors CXCR3 and CCR2, which provide protection for metastatic cancer cells from removal in the circulatory system (62). In addition to this, there is a strong crosstalk between metastatic cancer cells and MAMs in metastatic tumors. MAMs contribute to the survival of cancer cells, which in turn favors the retention of MAMs in metastatic tumors. It is these important roles of macrophages in tumorigenesis and progression that make them important targets for targeted antitumor therapy.

5 Immunotherapeutic strategies targeting TAMs

Macrophages have a dual effect on cancer cells, and their role is multifaceted, allowing for the construction of cancer therapeutic strategies targeting TAMs through multiple pathways (63). Inhibiting the recruitment of TAMs, depleting TAMs, and modulating the polarization of TAMs are all effective ways for cancer therapy (Figure 3).

5.1 Inhibition of TAMs recruitment

It has been shown that the recruitment of peripheral blood mononuclear cells into TME is achieved by a variety of chemokines and cytokines of tumor origin. These factors include CCL2, CCL3, CCL4, CCL5, and CXCL12, as well as colony-stimulating factor 1 (CSF-1) and VEGF. CCL2 is released by monocytes, tumor cells, and stromal cells in the TME, and its receptor, CCR2, plays an important role in the recruitment of bone marrow-derived monocytes into solid tumors and their development into TAMs. In breast cancer, specific monoclonal antibodies can inhibit the recruitment of TAMs by inhibiting CCL2, thereby delaying tumor progression and metastasis (64). In addition, studies on mouse ovarian cancer models have shown that the anti-tumor effects of anti-CCL2 antibody therapy can be enhanced by combining it with chemotherapy or immunotherapy (65). In conclusion, blocking the CCL2/CCR2 axis is an effective method to inhibit macrophage recruitment in animal models.

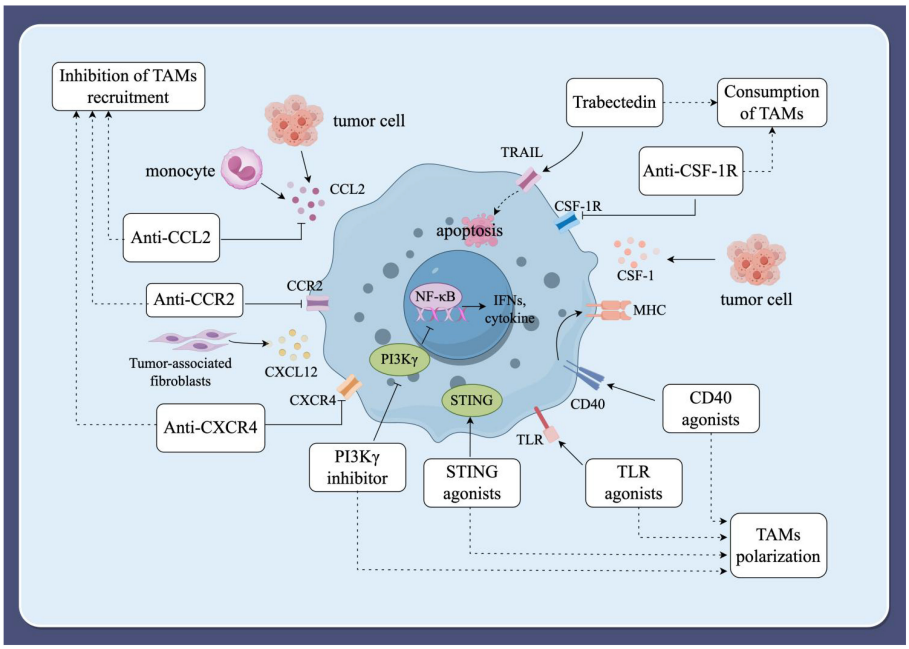

FIGURE 3
Immunotherapeutic strategies targeting TAMs. (1) Inhibition of TAMs recruitment. The recruitment of peripheral blood mononuclear cells into the TME is facilitated by various tumor-derived chemokines and cytokines, including CCL2, CCL3, CCL4, CCL5, CXCL12, colony-stimulating factor 1 (CSF-1), and VEGF. Inhibiting these factors can block the recruitment of TAMs, thereby slowing tumor progression and metastasis. (2) Consumption of TAMs. Inducing apoptosis in TAMs can also prevent tumor progression. The blockade of the CSF-1/CSF-1R signaling axis and the use of the compound trabectedin are effective strategies to deplete macrophages by inducing apoptosis. (3) Modulating the polarization of TAMs. Modulating macrophage polarization towards the M1-type is an alternative strategy for tumor immunotherapy. Current strategies under investigation include macrophage-targeting antibodies, Toll-like receptor (TLR) agonists, phosphatidylinositol 3-kinase-γ (PI3Kγ) inhibitors, specific nanoparticles, and interferon gene-stimulating factor (STING) agonists.

CXCL12, a chemokine, induces the transformation of monocytes into M2 macrophages, thereby reducing macrophage activation of T lymphocytes and enhancing macrophage migration, accumulation, and survival in tumors (66). CXCL12 from tumor-associated fibroblasts was found to be able to recruit M2-type macrophages and block CXCR4, the receptor for CXCL12, significantly reducing M2-type macrophage chemotaxis (67). Therefore, disruption of the CXCL12/CXCR4 axis may also be a strategy to inhibit recruitment of TAMs. A study showed that inhibition of the CXCL12/CXCR4 axis suppressed the accumulation of TAMs and sepsis-induced tumor progression in mice (68). However, CXCL12/CXCR4 axis inhibitors have not been reported in human cancer studies. Studies have shown that the CX3CL1/CX3CR1 axis promotes skin carcinogenesis through increased recruitment of M2-type macrophages (69). CX3CL1 is able to promote tumor growth and metastasis in TME (70). Therefore, the CX3CL1/CX3CR1 axis may be a potential target for inhibiting macrophage recruitment, which offers new possibilities for cancer immunotherapy targeting TAMs.

5.2 Consumption of TAMs

Induction of apoptosis in TAMs also prevents tumor progression (71). CSF-1, a major growth and differentiation factor released by cancer cells, interacts with its cognate receptor, CSF-1R, which is widely expressed by macrophages and monocytes (72). Blockade of the CSF-1/CSF-1R signaling axis reduces the abundance of macrophages and increases the abundance of CD8+/CD4+ T cells in the TME (73). Studies have shown that high expression of CSF-1 or CSF-1R is associated with poor prognosis in some malignant tumors, such as Hodgkin's lymphoma and hepatocellular carcinoma (74, 75). Blocking the CSF-1/CSF-1R signaling axis can convert TAMs from a tumor-promoting phenotype to a tumor-killing phenotype (76). Thus, blocking the CSF-1/CSF-1R signaling axis has emerged as a potential strategy for cancer immunotherapy. CSF-1R is a member of the tyrosine kinase receptor family that triggers its own homodimerization and activates receptor signaling upon binding to its ligands, CSF-1 or IL-34 (77). In particular, PLX3397 (pexidartinib), an orally available CSF-1R tyrosine kinase inhibitor, is the most used molecule in clinical studies (78). A study showed a significant reduction in macrophages and delayed tumor growth in mice with mammary tumors after treatment with PLX3397 (79). Tendon-synovial giant cell tumor (TGCT) has become a popular model for studying the CSF-1/CSF-1R signaling axis due to its high expression of CSF-1 and high infiltration of CSF-1R macrophages. A phase 3 trial of TGCT in 2019 demonstrated improved symptoms and prognosis in patients treated with PLX3397, the first drug to show a strong therapeutic effect in TGCT (80). PLX3397, in combination with binimetinib for advanced gastrointestinal mesenchymal stromal tumors and with paclitaxel for advanced ovarian cancer, showed

good tolerability and clinical efficacy (81, 82). It's important to note that this drug includes a boxed warning about the risk of serious and potentially fatal liver injury (83). PLX3397 has also been actively tested in other indications, including melanoma, prostate cancer, and lung cancer, among others. Unfortunately, multiple trials using PLX3397 either alone or in combination with other treatments have been terminated or withdrawn for reasons such as business decisions or insufficient clinical outcomes (NCT02452424, 01499043, 01349036, 01826448, 01090570). One previous clinical trial failed to show efficacy in glioblastoma, despite the fact that adequate drug exposure in tumors had been confirmed. A hypothesis has been proposed that the relative proportion of glioblastoma subtypes might result in treatment resistance; however, correlative studies are still needed to demonstrate this mechanism of resistance (84). This analysis suggests that targeting the CSF-1/CSF-1R signaling axis is a promising strategy for cancer treatment, and CSF-1R inhibitors have great potential to improve the prognosis of patients with advanced cancer.

In addition, some compounds such as trabectedin effectively deplete macrophages by inducing apoptosis. Trabectedin is a second-line antitumor agent that triggers apoptosis in tumor cells by binding to their DNA, resulting in cell cycle arrest and double-stranded DNA breaks (85). Germano et al. (86) found that trabectedin can induce apoptosis of TAMs via the receptor for TNF-related apoptosis-inducing ligand (TRAIL), thereby selectively depleting monocytes or macrophages in both the blood and tumors. Monocytes are highly sensitive to trabectedin-mediated apoptosis due to the low expression levels of TRAIL receptors. In preclinical models, trabectedin has been reported to inhibit the growth and invasion of cutaneous melanoma *in vitro* (87).

Although depletion of TAMs has considerable efficacy in inhibiting tumor progression, precise control of the level and duration of TAM depletion is crucial. Unselective systemic depletion of the entire macrophage population may promote tumor progression (88). Excessive macrophage depletion can disrupt immune homeostasis and increase the risk of infections and autoimmune diseases. Additionally, higher drug doses may be required for long-term TAM depletion, which can lead to adverse effects (89). Therefore, further clinical practice is needed to refine and mature this therapeutic strategy.

5.3 Modulating the polarization of TAMs

It is well established that a key feature of TAMs is their plasticity. Modulating macrophage polarization towards the M1-type is an alternative strategy for tumor immunotherapy (90). Current strategies under investigation include macrophage-targeting antibodies, Toll-like receptor (TLR) agonists, phosphatidylinositol 3-kinase-γ (PI3Kγ) inhibitors, specific nanoparticles, and interferon gene-stimulating factor (STING) agonists. Additionally, reprogramming macrophages through genetic engineering techniques, such as the CRISPR-Cas9 genome editing system, offers a promising approach to modulate macrophage polarization.

CD40 is a member of the TNF receptor superfamily expressed on the surface of macrophages. The interaction between CD40 and CD40L initiates the production of pro-inflammatory cytokines and the overexpression of MHC molecules by macrophages. As a result, the tumor-killing function of TAMs can be activated using agonistic anti-CD40 antibodies, thereby restoring their immunosurveillance against tumors (91). A recent study found that the combination of anti-CD40 antibody and anti-IL-6 antibody for glioblastoma reversed TAMs to a tumor-killing phenotype, more effectively inhibiting tumor progression (92). Macrophage receptor with collagenous structure (MARCO) is a scavenger receptor overexpressed on the surface of M2-type TAMs, making it a potential target for cancer therapy (93). Anti-MARCO antibodies can block the inhibitory Fc receptor and reprogram TAMs to the M1-type, thereby inhibiting tumor progression and metastasis.

Macrophages, a major component of the innate immune system, can be activated by pattern recognition receptors and polarized toward the M1 phenotype. Therefore, TLRs agonists can induce macrophage production in the M1 phenotype with potential antitumor effects. In a melanoma tumor model, TLR2 agonists specifically stimulate macrophage polarization toward the M1 phenotype (94). Riquimod (R848), a dual agonist of TLR7 and TLR8, is also able to induce macrophage polarization towards the M1 phenotype. Weissleder and coworkers conducted a large-scale screening assay and designed R848-conjugated cyclodextrin nanoparticles (CDNPs) (95). The R848 Due to the unique advantages of cyclodextrins, CDNPs have a high affinity for TAMs and drug binding affinity, and monotherapy with CDNPs can effectively reduce tumor size and significantly improve survival in mice by modulating the phenotype of TAMs. In 2021, Figueiredo et al. found that the use of lignin nanoparticles (LNPs) conjugated with R848 could reprogram M2 type macrophages to M1 type for enhanced chemotherapy (96). In addition, polyinosinic acid-polycytidylic acid [poly(I:C)], a TLR3 agonist, has also been widely used in cancer therapy due to its potential to activate the immune system (97). In 2020, Dacoba et al. investigated hyaluronic poly(I:C) nanocomplexes, which were shown to be effective at polarizing macrophages to the M1 type with good stability (98).

A number of metabolism-related signaling pathways are important for the altered macrophage phenotype. PI3Kγ controls the expression of arginase 1 (Arg1) and plays a central role in regulating arginine metabolism in immunosuppressed TAMs. Also, pro-inflammatory signaling pathways regulated by nuclear factor kappa-B (NF-κB) activation in macrophages are inhibited by the PI3Kγ pathway (99). Thus, during inflammation and cancer, PI3Kγ controls the switch between immune activation and immune suppression in macrophages. IPI-549 is a specific PI3Kγ inhibitor that downregulates the expression of Arg1, stimulates the activation of NF-κB, and ultimately polarizes macrophages towards the M1-type. An IPI-549 containing polymeric nanoparticles (IPI-549NP) increased the accumulation of IPI-549 at the tumor site and enhanced the anti-tumor immune response (100). In mouse models of pancreatic cancer and melanoma, IPI-549NP promotes an immunostimulatory transcriptional program that activates CD8+ T cells to exert their cytotoxic function and prevents tumor progression by prolonging host survival. In addition, checkpoint inhibitor therapy also benefited from the inhibition of PI3Kγ, as demonstrated by significant tumor regression and enhanced mouse survival in tumor-

bearing mice (101). Therefore, activation of anti-tumor immune responses by inhibiting PI3Kγ to polarize macrophages toward M1-type would be a promising therapeutic approach.

Some nanomaterials have a direct impact on immunomodulation by interacting with macrophages (102). Adriamycin, an antitumor drug composed of magnetic iron oxide nanoparticles (IONPs), reprograms TAMs to enable macrophages to exert antitumor effects, which may be useful in enhancing cancer immunotherapy mediated by macrophages (103). A study found that iron-chelated melanin-like nanoparticles could repolarize tumor-promoting M2-type macrophages to M1-type, which could be developed into specialized antigen-presenting cells (APCs) to present tumor-associated antigens induced by photothermal therapy (104). Thus, iron-chelated melanin-like nanoparticles could activate adaptive immune responses and inhibit tumor progression. In a recent study, mannose-chelated iron oxide nanoparticles (man-IONPs) were designed to reprogram TAMs into M1-type macrophages, which had a dramatic inhibitory effect on hepatocellular carcinoma progression (105). In addition, Chen et al. (106) developed an immunotherapeutic gel consisting of anti-CD47 antibody coupled with calcium carbonate nanoparticles. The nanoparticles induced the polarization of TAMs to M1-type, thereby promoting antigen presentation by macrophages to initiate T cell-mediated adaptive immune responses. At the same time, the released anti-CD47 antibody promoted phagocytosis of cancer cells by macrophages.

STING is a cytoplasmic DNA sensor present in a variety of immune cells that controls the transcription of host defense-related genes. When activated by agonists, STING stimulates signaling pathways that cause immune cells to produce a variety of pro-inflammatory cytokines and chemokines, especially type I IFNs that can promote Th1-mediated immune responses (107), and thus STING is able to polarize TAMs to M1 type. However, the route of administration of STING agonists is limited to intra-lesional injections due to their sensitivity to enzymatic degradation, which remains a barrier to successful clinical translation (108). Drug delivery systems developed from nanomaterials can overcome this obstacle. Shae et al. (109) synthesized STING-activated polymeric nanoparticles for the protection of cGAMP delivery, which could transform the tumor immune microenvironment from immunosuppressive to immunogenic and tumor-killing activity. In tumors treated with STING-activated nanoparticles, the percentage of macrophages polarized to M2 type was significantly reduced. In addition, manganese ion (Mn2+) based nano-assemblies were shown to be a STING agonist that promotes anti-tumor therapy by initiating the immune system (110). In different tumor models, significant therapeutic effects were demonstrated using very small doses of STING agonists and the population of TAMs showed an increase in the M1/M2 ratio, suggesting a conversion of TAMs to the M1 type (111).

The CRISPR-Cas9 genome editing system has great potential in cancer therapy due to its ability to precisely target key oncogenes and tumor suppressors (112). Current clinical trials using CRISPR-Cas9 for cancer therapy have focused on isolating and extracting T cells from patients, subjecting them to CRISPR-Cas9-mediated gene editing, and subsequently re-injecting them into patients. However, the safe and effective manipulation of specific genomic sequences in the tumor microenvironment remains a major challenge for the clinical application of CRISPR-Cas9 in cancer therapy. The presence of M1-type TAMs correlates with antitumor activity, whereas the presence of M2-type TAMs correlates with pro-tumor activity. Using CRISPR-Cas9, several relevant genes can be knocked out to permanently reprogram TAMs into an anti-tumor M1-like phenotype while maintaining their adaptive properties. These reprogrammed macrophages can sustain their anti-tumor effects without succumbing to the immunosuppressive tumor microenvironment, thus maximizing the efficacy of gene editing therapy. Therefore, TAMs are also important targets for enhancing the efficacy of gene editing in cancer treatment. A recent study developed an *in vivo* CRISPR-Cas9 targeting system for TAMs using bacterial protoplast-derived nanovesicles (NVs) (113). In this system, plasmid-transformed E. coli protoplasts were used as a production platform, and the vesicles were modified with pH-responsive PEG-conjugated phospholipid derivatives and galactosamine-conjugated phospholipid derivatives tailored for TAM targeting. These vesicles were loaded with DNA fragments targeting the macrophage-polarized Cas9-sgRNA ribonucleoprotein, Pik3cg, and the ligand for TLR9, CpG. The bacteriophage-derived exosomes, loaded with CRISPR-Cas9 tools, remodeled the tumor microenvironment by stabilizing the M1-like phenotype in TAMs, thereby inhibiting tumor growth in female mice. These findings pave the way for cancer immunotherapy by overcoming challenges associated with maintaining the activity, safety, and precisely targeted delivery of gene-edited cells *in vivo*.

6 Conclusion and future perspectives

In recent years, research on the diagnosis and treatment of macrophage-associated diseases, especially cancer, has made remarkable progress (114). In the tumor microenvironment, TAMs mainly exhibit M2-type tumor-promoting features, and the abundant presence of TAMs is closely related to tumor recurrence and metastasis (115). By inhibiting the recruitment of TAMs, depleting TAMs, and modulating the polarization of TAMs, targeted TAM therapy has made great progress. However, there are still many issues that need to be further studied and explored. The mechanism of macrophage differentiation and diversity in different tissues is still an important issue that remains to be resolved, and the various functional characteristics of macrophages in TME are closely related to macrophage differentiation and diversity. Currently, the assessment of heterogeneous macrophages is usually limited to the macrophage population, and elucidating macrophage heterogeneity at the single-cell level remains a great challenge. More fundamental studies of macrophage phenotype and function, and thus elucidation of the dual effects of macrophages on tumors, could inform more specific therapeutic strategies for targeting TAMs (116).

Despite the tremendous success of TAMs-targeting strategies against tumors, TAMs continue to contribute to chemoresistance in a variety of cancers due to the complexity of macrophage effects on tumors. Important mechanisms include M2 macrophage-induced epithelial mesenchymal transition, M2 macrophage production of metabolites, and M2 macrophage-induced production of anti-

apoptotic signals (117, 118). The stimulatory effects produced by M2 macrophages can severely affect the efficacy of clinical radiotherapy. Therefore, targeting TAMs as a complementary therapy, in synergy with radiotherapy, chemotherapy, or immunotherapy, may help counteract drug resistance in cancer treatment to some extent.

In conclusion, despite their negative role in tumor development, tumor-associated macrophages have great potential in tumor therapy due to their critical role as an important component of the tumor microenvironment. Targeting macrophages or integrating them with radiotherapy, chemotherapy, and immune checkpoint inhibitors has a significant impact on tumor therapy. Specifically, eliminating tumor-promoting macrophages while simultaneously administering antitumor drugs significantly improves tumor killing. Moreover, targeting pathways both upstream and downstream of macrophages offers additional therapeutic avenues to modulate macrophage function. Notably, the use of genetic engineering to reprogram macrophages to convert tumor-promoting macrophages into antitumor macrophages presents a highly promising clinical application. With a deeper understanding of tumor-associated macrophages in the future, it is anticipated that this knowledge will provide a useful reference for designing more precise treatment plans and potentially lead to new breakthroughs in the field of tumor therapy.

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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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Macrophages in tumor cell migration and metastasis

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Tumor-associated macrophages (TAMs) are a phenotypically diverse, highly plastic population of cells in the tumor microenvironment (TME) that have long been known to promote cancer progression. In this review, we summarize TAM ontogeny and polarization, and then explore how TAMs enhance tumor cell migration through the TME, thus facilitating metastasis. We also discuss how chemotherapy and host factors including diet, obesity, and race, impact TAM phenotype and cancer progression. In brief, TAMs induce epithelial-mesenchymal transition (EMT) in tumor cells, giving them a migratory phenotype. They promote extracellular matrix (ECM) remodeling, allowing tumor cells to migrate more easily. TAMs also provide chemotactic signals that promote tumor cell directional migration towards blood vessels, and then participate in the signaling cascade at the blood vessel that allows tumor cells to intravasate and disseminate throughout the body. Furthermore, while chemotherapy can repolarize TAMs to induce an anti-tumor response, these cytotoxic drugs can also lead to macrophage-mediated tumor relapse and metastasis. Patient response to chemotherapy may be dependent on patient-specific factors such as diet, obesity, and race, as these factors have been shown to alter macrophage phenotype and affect cancer-related outcomes. More research on how chemotherapy and patient-specific factors impact TAMs and cancer progression is needed to refine treatment strategies for cancer patients.

KEYWORDS

cancer, macrophages, metastasis, tumor cell migration, chemotherapy, EMT

1 Introduction

Metastasis – the systemic spread of cancer – causes the majority of cancer-related deaths (1). To metastasize, cancer cells must be able to migrate through the tumor microenvironment (TME) and intravasate. Though not all tumor cells are inherently capable of such feats, the migratory and invasive phenotypes needed to accomplish these tasks can be induced through interactions with other types of cells in the TME, including endothelial cells, immune cells, and fibroblasts. Of the cellular components within the TME, macrophages are key players in the induction of pro-metastatic phenotypes in cancer cells. In this review, we provide an introduction to macrophages and their origin, discuss macrophage polarization, and then review the latest understanding of the role of macrophages in tumor cell migration and metastasis, including the promotion of 1) epithelial-mesenchymal transition (EMT), 2) pro-tumoral extracellular matrix (ECM) remodeling, 3) tumor cell chemotaxis towards blood vessels, and 4) tumor cell intravasation. We also explore the impact of chemotherapy and host factors including diet, obesity, and race on tumor-associated macrophages (TAMs). Overall, we attempt here to summarize recent studies, discuss these new findings in the context of what is already known about the role of TAMs in tumor cell migration and metastasis, and highlight new potential avenues for refining therapeutic interventions.

1.1 Macrophage ontogeny

Macrophages have two distinct ontogenies. The first of these is monocyte-derived macrophages (MDMs) which originate from progenitors in the bone marrow and other hematopoietic niches (2), progress through several stages of differentiation, and enter the systemic circulation as monocytes. Circulating monocytes are recruited to tissues in response to locally released chemo-attractants where they differentiate into macrophages (3). Once inside the tissue, MDMs may be short- or long-lived, and their population is maintained through recruitment of new circulating monocytes as well as proliferation of pre-existing MDMs (4, 5). The second group, known as tissue-resident macrophages, arise early in embryonic development, migrating from the yolk sac or fetal liver into developing organs where they differentiate into tissue-specific macrophages, including Kupffer cells (liver), osteoclasts (bone), and microglia (brain) (5). In adults, these macrophages self-renew largely independently of the bone marrow (6, 7). Macrophages in the TME are referred to as tumor-associated macrophages (TAMs). While most TAMs are monocyte-derived, tissue-resident macrophages make up a considerable percentage of TAMs in some tumor types (8–10).

1.2 Macrophage polarization

Once inside the tumor, macrophages take on various phenotypes and functions in response to stimuli in the microenvironment. These phenotypes are referred to as "polarization states." There is a wide spectrum of macrophage polarization states ranging from pro-inflammatory (M1) to anti-inflammatory (M2).

M1 macrophages (historically called "classically activated"), are pro-inflammatory cells that participate in the host immune response against pathogens and can have anti-tumor activity. As such, environmental factors associated with infection and inflammation (including interferon (IFN)-γ, bacterial lipopolysaccharide (LPS), and granulocyte-macrophage colony stimulating factor (GM-CSF)) promote M1 polarization (11). These signals cause macrophages to express surface proteins related to antigen presentation and T cell activation (including HLA-DR, CD80, and CD86) (11–14), and secrete inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β to further enhance the immune response (11). M1 macrophages promote tumor cell killing through strong antigen presentation and effective activation of the innate and adaptive immune responses (15). Indeed, high M1 macrophage infiltration is correlated with positive outcome in cancer patients (16, 17).

M2 (or "alternatively activated") macrophages, are anti-inflammatory cells that are involved in tissue repair and immune suppression. While these cells are essential in maintaining homeostasis in healthy tissues, they can also promote tumor growth and metastasis in the TME. M2 macrophages are induced by anti-inflammatory cytokines in the microenvironment, including IL-4 and IL-10 (11). These signals cause macrophages to express surface proteins such as CD163 and CD206, which are involved in tissue "clean-up" and homeostasis, and to secrete additional anti-inflammatory factors, such as IL-10 and transforming growth factor (TGF)-β, which further suppress the immune response (11, 18, 19). M2 macrophages also express high levels of vascular endothelial growth factor (VEGF), which promotes tumor vascularization, enhancing the delivery of oxygen and nutrients to the tumor (11). M2 macrophages are poor antigen presenters and suppress both innate and adaptive anti-tumor immunity (15). Furthermore, M2 macrophages are implicated in chemoresistance and metastasis, and high M2 infiltration is associated with poor prognosis in cancer patients (20–26).

Though macrophage polarization is a spectrum with M1 and M2 on opposing ends, it is common in the literature to oversimplify this state and treat macrophage polarization as a dichotomy (M1 *or* M2). Given that many "anti-inflammatory" macrophages also participate in inflammatory signaling and vice versa, the terms "M1" and "M2" should merely give a sense of how a macrophage is predominantly functioning in a particular environment. It is also important to note that macrophage phenotype is highly plastic. Similar to other components of the innate immune system, macrophage phenotype can quickly change in response to environmental cues (27). Indeed, *in vitro* and *in vivo* studies confirm that macrophages may repolarize in response to particular stimuli (28–30), an effect that has been leveraged in several immunotherapy clinical trials (31). Promising macrophage-targeting therapies and the challenges associated with their development are reviewed elsewhere (32–37). Given their significant, plastic, and diverse roles in cancer progression, understanding the mechanisms behind macrophage-mediated

cancer progression and the effects of chemotherapy and host factors is crucial to refining cancer treatment strategies.

2 TAMs in EMT

Epithelial-mesenchymal transition (EMT) is the process by which epithelial cells lose their characteristic apical-basal polarity and tight cell-cell junctions, and gain features associated with mesenchymal cells, including the ability to migrate and invade surrounding tissue (38, 39). In healthy tissues, EMT is used in critical processes such as embryonic development and wound healing. However, cancer cells hijack this program to gain migratory and invasive phenotypes. Cells that have undergone EMT are characterized by the loss of E-cadherin and the increase in N-cadherin and vimentin. E-cadherin, often used as an epithelial cell marker, is an important cell-cell adhesion protein involved in contact-mediated inhibition of cell growth (40). During EMT, the transcription factor SNAIL directly represses E-cadherin transcription and is thus crucial in EMT regulation (41). As E-cadherin decreases, the mesenchymal cell markers N-cadherin and vimentin increase and support tumor cell survival and migration (42, 43). During this process, tumor cells pass through a series of epithelial/mesenchymal (E/M) hybrid states that reflect varying degrees of plasticity and metastatic potential (44). Tumor-associated macrophages have long been known to play a role in EMT induction (45, 46), and more recent evidence shows that TAMs also promote progression to later E/M hybrid states (44). A number of recent studies have further elucidated the mechanisms behind this relationship, pointing to feedback loops in which tumor cells undergoing EMT attract and polarize macrophages, which then secrete factors that further promote EMT in tumor cells (45–47) (Figure 1).

Macrophages can induce EMT in cancer cells by secreting various factors, including TGF-β (48), CCL2 (49), and IL-6 (50), all of which ultimately lead to SNAIL upregulation and subsequent EMT in tumor cells. For instance, IL-6 activates the JAK2/STAT3 pathway upon binding the IL-6 receptor (47, 50, 51) (Figure 1A). The JAK2/STAT3 axis is a critical signal transduction pathway that participates in many cellular functions including proliferation, differentiation, and survival, and components of this pathway are hyperactivated in many cancers (52, 53). After IL-6 receptor activation, STAT3 inhibits the transcription of tumor suppressor microRNAs including miR-34a (50, 51). MiR-34a suppresses SNAIL, and loss of miR-34a leads to SNAIL upregulation and subsequent EMT (54, 55), as well as tumor cell proliferation and migration (50, 56) (Figure 1B). Macrophage-induced mesenchymal-like tumor cells then secrete increased amounts of CCL2, which recruits macrophages (47), and IL-6, which leads to M2 polarization (51, 57) (Figure 1C), further propagating EMT in a positive feedback loop.

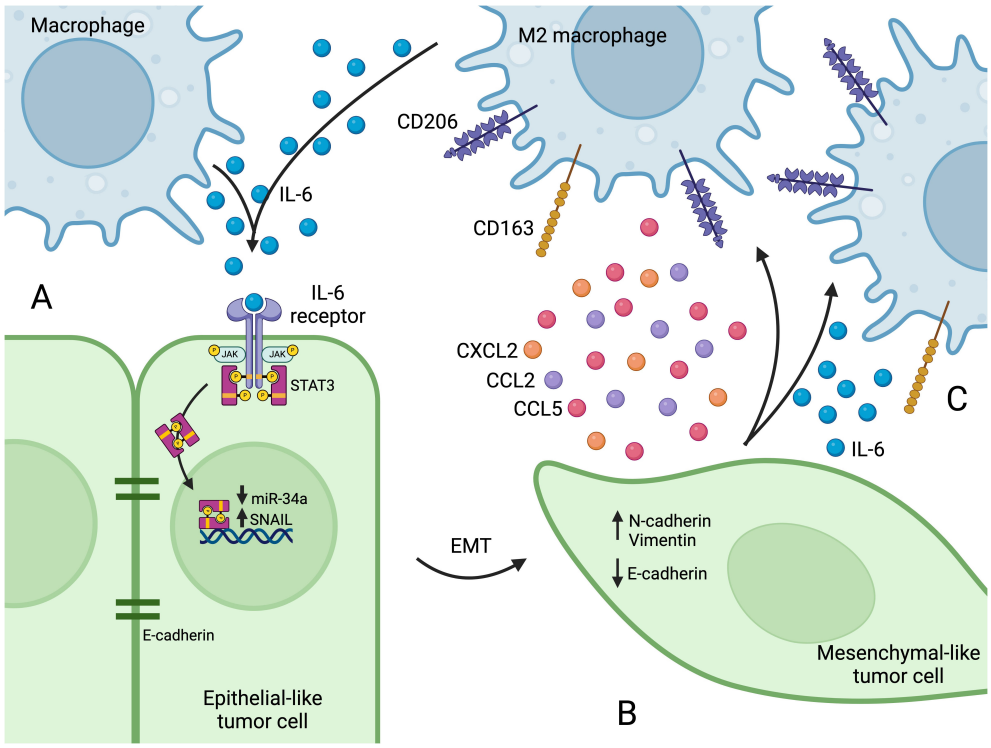

FIGURE 1
(A) Macrophages secrete IL-6, which binds to the IL-6 receptor on tumor cells, activating the JAK2/STAT3 pathway. After IL-6 receptor activation, STAT3 translocates to the nucleus and suppresses transcription of miR-34a, which leads to SNAIL upregulation. **(B)** The increase in SNAIL leads to loss of E-cadherin, and EMT programs become active and increase the expression of N-cadherin and vimentin. The tumor cell takes on a mesenchymal-like phenotype, which affords enhanced migration capacity. **(C)** Mesenchymal-like tumor cells secrete factors that recruit macrophages to the TME (e.g. CCL2, CCL5, and CXCL2) and that promote M2 polarization (e.g. IL-6). M2 polarization is characterized by the expression of surface markers such as CD163 and CD206. Figure created with BioRender.com.

These studies reveal several therapeutic targets with the potential to reduce the co-induction of EMT and M2 polarization. Inhibiting IL-6 signaling by targeting IL-6 itself, or its receptor (with anti-IL-6R monoclonal antibodies like tocilizumab), reduces EMT, decreases M2 polarization and increases M1 polarization (51). The tumor suppressor miR-34a is also a potential target. MiR-34a suppresses SNAIL and reinstates an epithelial phenotype in mesenchymal-like cancer cells (55). MiR-34a expression in tumor cells also promotes macrophage M1 polarization, demonstrating that the microRNA can favorably modify both the tumor cells and the immune microenvironment (51). Indeed, nanoparticle-delivered miR-34a has shown promise in treating several types of cancers (58, 59).In addition to promoting EMT in cancer cells, SNAIL is also involved in macrophage recruitment. SNAIL expression in tumor cells increases their secretion of CCL2, CCL5, and CXCL2, all of which attract macrophages to the TME (60–62) (Figure 1C). Indeed, SNAIL-overexpressing tumors show a significant increase in macrophage infiltration, M2 polarization, and metastasis (60, 62).

Finally, in addition to cytokines and chemokines, more recent evidence has revealed that exosomes can also mediate macrophage-tumor cell feedback loops related to EMT and M2 polarization. Tumor cells that have undergone EMT secrete exosomes containing microRNAs that are taken up by macrophages and induce M2 polarization (63). For example, it was shown that tumor cell derived exosomes contain miR-106b-5p, which upon uptake by macrophages, activates the PI3Kγ/AKT/mTOR signaling pathway to induce M2 polarization by downregulating the pathway inhibitor PDCD4 (63). Similarly, SNAIL expression directly upregulates the transcription of miR-21 in tumor cells. This microRNA is then transferred to macrophages through exosomes and also targets macrophage PDCD4, leading to M2 polarization (64).

Exosomal microRNAs can be transmitted from macrophages to tumor cells as well (65). Tumor cell uptake of M2 macrophage-derived exosomes leads to downregulation of E-cadherin and upregulation of N-cadherin and vimentin (65). Lu et al. found that these exosomes contain miR-23a-3p, which downregulates the tumor suppressor PTEN (65) – a known regulator of EMT (66). In a positive feedback loop, tumor cells treated with M2 macrophage-derived exosomes express higher levels of CCL2, leading to increased macrophage recruitment and M2 polarization (65).

In summary, tumor cell EMT and macrophage recruitment and polarization are intimately connected and co-regulated by several molecular mechanisms.

3 TAMs in extracellular matrix remodeling

3.1 TAMs in matrix stiffness

Macrophages also promote tumor cell migration and invasion through ECM remodeling. The ECM of non-cancerous soft tissue is characterized by "curly," non-dense collagen fibers that lay parallel to the epithelium (67). This soft ECM is involved in maintaining an epithelial phenotype, and matrix stiffening has been shown to play a direct role in promoting EMT (68, 69). Indeed, clinical conditions characterized by a stiff ECM – including cirrhosis of the liver (70), pulmonary fibrosis (71), and mammographically dense breast tissue (72) – are associated with a higher incidence of cancer in the respective tissues. In tumors, collagen deposition increases, and fibers become stiff, cross-linked and linearized – a process known as desmoplasia that has been associated with immune evasion and metastasis (67, 73–76). Indeed, tumors have been shown to be stiffer than healthy tissue in breast (77), pancreas (78), bladder (79), and ovarian (80) cancers. The stiffened matrix of tumors promotes malignant transformation, proliferation, and invasion of tumor cells, and acts as a "highway," guiding tumor cells towards the vasculature, where they further invade and intravasate (67, 69, 76, 77, 81–85). Recent work sheds light on the mechanistic role of TAMs in pro-tumoral matrix stiffening.

Macrophages promote matrix deposition and stiffening in both cancerous and healthy tissue (86, 87). In pancreatic cancer, macrophages foster desmoplasia indirectly by activating pancreatic stellate cells. Mechanistically, macrophages internalize and degrade surrounding collagen, which leads to an increase in inducible nitric oxide synthase (iNOS) and the production of reactive nitrogen species (RNS). RNS then activate pancreatic stellate cells leading to increased collagen deposition and desmoplasia (87).

In the desmoplastic reaction, excessive ECM deposition is followed by cross-linking, which confers increased stiffness to the TME. ECM crosslinking is mediated primarily by lysyl oxidase (LOX) and lysyl oxidase-like (LOXL) proteins, which are expressed by a variety of cells in the TME (88). In pancreatic cancer, LOXL2 expression is positively associated with tumor burden and metastasis (89). Macrophages both express their own LOXL2 and promote its expression in tumor cells (89, 90). Alonso-Nocelo et al. recently demonstrated that macrophage depletion leads to a significant decrease in LOXL2, collagen fibril orientation, and metastasis in mice, indicating that macrophages promote matrix stiffness (89). In a positive feedback loop, the stiffened matrix then promotes macrophage infiltration and M2 polarization (89). Mechanistically, macrophages increase matrix stiffness by secreting oncostatin M (OSM), which upregulates LOXL2 in tumor cells (89). In turn, the stiffened matrix activates integrin β5 in macrophages, leading to FAK/MEK/ERK activation and LOXL2 upregulation, further supporting ECM crosslinking in the TME (90). In addition to promoting tumor cell migration, stiffened matrices cause macrophages to take on a more immunosuppressive phenotype (89, 91). Indeed, macrophages cultured on stiff matrices recruit cytotoxic T cells less efficiently than those cultured on softer matrices (91).

Together, these studies indicate that macrophages support the development of a stiff ECM through direct and indirect mechanisms. In turn, the stiff ECM promotes macrophage recruitment, M2 polarization, tumor cell migration, and metastasis.

3.2 TAMs in matrix degradation

Equally as important as matrix stiffness for cancer progression is matrix degradation, which is mediated by matrix metalloproteinases (MMPs). MMPs are a group of zinc-

containing proteolytic enzymes responsible for degrading the extracellular matrix (92). MMPs are upregulated in nearly every type of cancer, and their activity has been shown to facilitate angiogenesis, tumor cell immune evasion, migration, and metastasis (92, 93). MMPs are expressed by a variety of stromal, immune, and tumor cells, and a growing body of evidence reveals the role of MMPs in the dynamic pro-metastatic interplay between macrophages and tumor cells.

MMP production can be induced through several major signal transduction pathways including STAT3, ERK, and NF-κB (94–102). Evidence shows that macrophages provide multiple ligands for these pathways that cooperate to promote MMP expression. For example, macrophages secrete AEG-1, TGF-β, and IL-6, which all increase MMP-9 expression in tumor cells by activating STAT3 (94, 95, 97). Indeed, inhibiting STAT3 in tumor cells, or its activators in macrophages, causes a significant decrease in MMP expression and migration in tumor cells (95, 97). Macrophages also secrete TNF-α and IL-1β, which activate the NF-κB pathway. Yamanaka et al. found that IL-1β activates NF-κB in gastric cancer cells, and this leads to increased MMP-9 expression and tumor cell invasion (103). Furthermore, tumor cells cultured in M2 macrophage-conditioned media express significantly increased levels of MMP-9 (98). This effect can be seen to a lesser (but still significant) extent when tumor cells are stimulated with TNF-α alone, suggesting that macrophages provide multiple ligands that stimulate MMP production (98).

MMPs expressed by macrophages also play a significant role in tumor cell invasion and metastasis. Macrophage – but not tumor cell – expression of MMP-11 is a negative prognostic marker in breast cancer (104). MMP-11-overexpressing macrophages secrete increased amounts of CCL2. CCL2 then activates MAPK signaling in tumor cells and increases tumor cell migration and MMP-9 expression (104). In Wilms' tumor and gastric cancer, MMP-9 is upregulated in M2 macrophages, and MMP-9 initiates EMT and increases tumor cell invasion (105, 106). Mechanistically, macrophage-derived MMP-9 activates the PI3K/AKT pathway in tumor cells leading to the upregulation of SNAIL and subsequent EMT (105, 106). These studies identify MMP-9 as a promising therapeutic target. Indeed, MMP-9 inhibition increased the efficacy of chemotherapy and decreased metastasis to the lungs in a mouse model of gastric cancer (106). Together, these studies identify macrophages as important regulators of tumor cell MMP production.

4 TAMs in tumor cell chemotaxis

Beyond promoting a mesenchymal phenotype in cancer cells, TAMs also supply ligands and chemotactic factors that support tumor cell migration and invasion in the tumor microenvironment (Figure 2).

In vitro and *in vivo* migration assays and intravital imaging show that tumor cells and macrophages migrate through the TME together using a CSF1/EGF paracrine loop that leads to invasion and metastasis (107–110) (Figure 2A). High levels of CSF1 (111–113) and the EGF receptor (114–117) are correlated with metastasis and poor prognosis in a number of solid tumors. CSF1 secreted by tumor cells both recruits macrophages to the tumor microenvironment and promotes macrophage expression of EGF (108). TAM-derived EGF then binds to the EGF receptor on tumor cells, leading to increased CSF1 production and activation of pathways associated with migration (107, 108, 118) (Figure 2A). Using this paracrine loop, macrophages and tumor cells migrate together along fibronectin-collagen I ECM fibers towards chemotactic gradients. Leung et al. found that in the TME, the primary chemo-attractant for the macrophage-tumor cell pair is hepatocyte growth factor (HGF), which is secreted by endothelial cells (119) (Figure 2). Within 500 μm of a blood vessel, tumor cells may perform sustained directional migration towards HGF gradients with or without macrophages. However, tumor cells at greater distances can only move towards blood vessels by co-migrating with a macrophage (119). Thus, while tumor cells may chemotax along these HGF gradients alone, co-migrating with macrophages greatly supports their ability for sustained directional migration and extends the chemoattractive influence of the blood vessels.

In addition to participating in tumor cell chemotaxis, macrophages support tumor cell migration by both promoting the formation of tumor cell invadopodia and prolonging their activity (Figure 2). Invadopodia are F-actin-rich protrusions with MMP activity used to degrade the ECM and create new physical pathways through the tumor (120, 121). TAMs promote the formation of invadopodia by secreting EGF, which activates the EGF receptor in tumor cells. EGF receptor activation initiates the assembly of invadopodial precursors through the recruitment of actin regulatory proteins such as cortactin, Arp2/3, and cofilin (118, 120, 122, 123) (Figure 2A). Phosphorylation of cortactin activates actin polymerization and leads to maturation of a precursor. The actin regulatory protein Mena (encoded by the *ENAH* gene) supports this polymerization by localizing to the barbed ends of polymerizing actin filaments and temporarily interfering with the capping proteins that block polymerization (124, 125). In non-invasive tumor cells, invadopodia can form, but do not mature, as cortactin is rapidly dephosphorylated by the tyrosine phosphatase PTP1B that is constitutively bound to Mena. This lack of maturation dramatically limits the invasive capacity of invadopodia by limiting the amount of matrix they can degrade (126).

Macrophages also play a role in promoting invadopodium maturation (thus increasing degradative activity) by stimulating the expression of a splice variant of Mena called MenaINV (Figure 2B). MenaINV prolongs the degradative activity of invadopodia by sequestering PTP1B and preventing the dephosphorylation/deactivation of cortactin and the subsequent disassembly of actin filaments (127, 128). The MenaINV-stabilized invadopodium then degrades the ECM in its path, facilitating tumor cell migration towards blood vessels (Figure 2C). Macrophages promote the alternative splicing of Mena through cooperative Notch1/NF-κB signaling (129, 130) (Figure 2B). Mechanistically, macrophages secrete TNFα, which activates the NF-κB pathway in tumor cells, leading to p65 nuclear translocation. Inside the nucleus, p65 binds to the κB binding sites on the *ENAH* promoter, initiating *ENAH* transcription. Macrophages also express the Notch1 ligand Jagged1, which

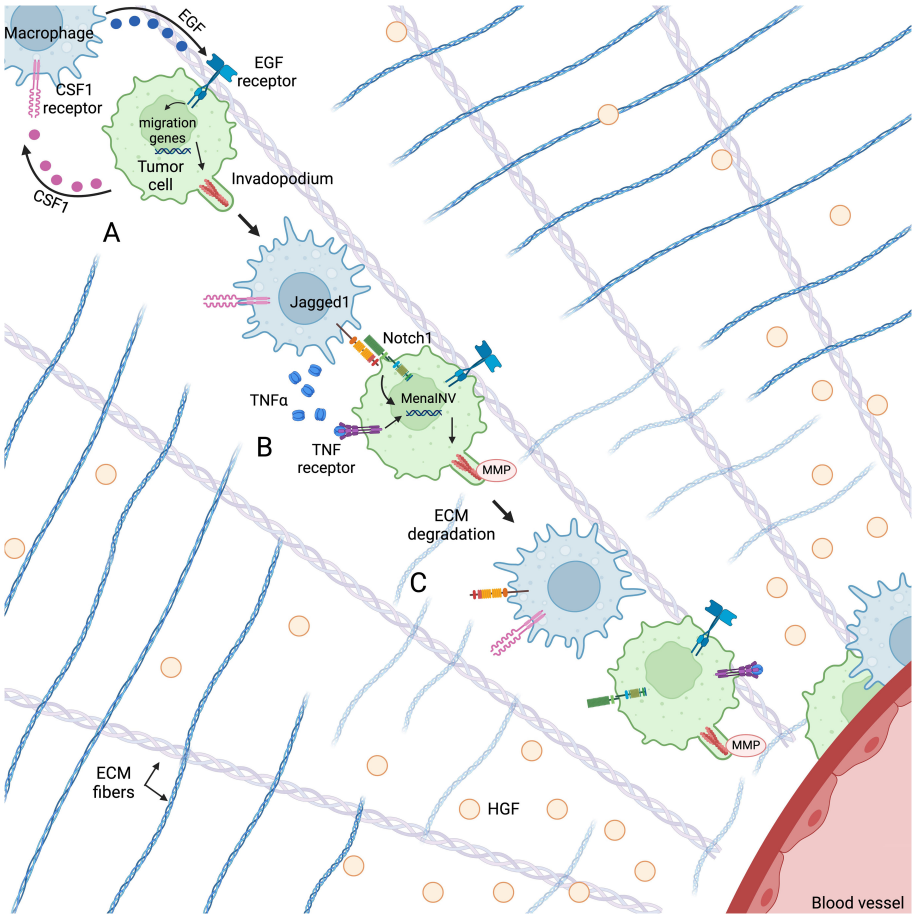

FIGURE 2
Macrophages and tumor cells co-migrate through the TME along fibronectin-collagen I ECM fibers towards HGF gradients secreted by endothelial cells using an EGF/CSF1 paracrine loop. **(A)** Macrophage-derived EGF activates the EGF receptor on the tumor cell, leading to the upregulation of genes associated with cell migration and invadopodium formation. **(B)** Cooperative Notch1/NF-κB signaling between the macrophage and tumor cell leads to an increase in MenaINV expression, which enhances invadopodium stability and degradative activity. **(C)** The invadopodium degrades ECM in its path, facilitating tumor cell migration towards the blood vessel. Figure created with BioRender.com.

engages the Notch1 receptor on tumor cells, causing nuclear translocation of the Notch1 intracellular domain (NICD). Nuclear NICD enhances the nuclear retention of p65, leading to sustained *ENAH* transcription and to alternative splicing (130). Prior work has shown that this alternative splicing is the switch that turns non-invasive tumor cells into invasive tumor cells (131).

In summary, macrophages partner with tumor cells to enhance directional migration and metastasis by guiding tumor cells towards blood vessels and promoting the assembly and invasion capacity of tumor cell invadopodia.

5 TAMs in tumor cell intravasation

Intravasation – the process by which tumor cells enter the vasculature – represents a key step in the metastatic cascade. Macrophages not only assist with tumor cell intravasation but are crucial for the process.

Breast cancer cells disseminate from the primary tumor through tumor microenvironment of metastasis (TMEM) doorways (132, 133). TMEM doorways are stable, tri-cellular structures (occurring primarily at vascular branch points) composed of a Mena-expressing tumor cell, a perivascular Tie2^{High} macrophage, and an endothelial cell in direct physical contact (132, 134–137). TMEM doorway density (hereafter referred to as TMEM doorway score) in the primary breast tumor is a clinically validated prognostic marker of distant metastasis (136, 138). Arwert et al. investigated the process of TMEM doorway assembly by systemically depleting macrophages and then tracking the fate of newly-recruited monocytes in the TME (139). They found that upon extravasation into the TME, monocytes become motile TAMs that begin to express CXCR4 and are then recruited back to the perivascular space by CXCL12-expressing perivascular fibroblasts. Once at the blood vessel, these motile TAMs become sessile, forming TMEM doorways with adjacent tumor and endothelial cells (139). Signaling between the three TMEM doorway cells results in the release of vascular endothelial growth factor-A (VEGFA) (132). The secreted VEGFA leads to the dissociation of local vascular endothelial cell-cell junctions, which causes a transient, localized vascular permeability event. Harney et al. used real-time

multiphoton intravital imaging of a murine mouse model of breast cancer to show that these transient vascular permeability events are regulated and occur concurrent with tumor cell intravasation (132). Neither transient vascular permeability nor tumor cell intravasation occurs away from TMEM doorways (132). Furthermore, TMEM doorway score increases concomitantly with circulating tumor cells, and macrophage depletion leads to a significant reduction in TMEM doorways, vascular permeability, and circulating tumor cells, highlighting the essential role of TMEM doorway-associated macrophages in tumor cell intravasation (132, 133).

In vitro and *in vivo* studies confirm that invadopodia formation is necessary for tumor cell intravasation (140–143). In addition to initiating invadopodium formation through paracrine EGF signaling, macrophages can also initiate this process through direct contact (129, 140). In TMEM doorways, contact between the TMEM doorway-associated macrophage and tumor cell induces invadopodium formation in the tumor cell (129, 140). The invadopodium then degrades the basement membrane surrounding the vascular endothelium and functionally "holds the door open" for other migratory tumor cells to enter the blood stream (129, 140). Mechanistically, macrophage-tumor cell contact activates RhoA signaling in tumor cells, which initiates invadopodium formation in the tumor cell (140, 144). Indeed, RhoA knockdown reduces tumor cell invadopodium formation, matrix degradation, and intravasation (140, 144).

Importantly, targeting TMEM doorway-associated macrophages with the Tie2 inhibitor rebastinib has shown therapeutic promise by decreasing TMEM doorway function and metastasis in preclinical studies of breast cancer and pancreatic neuroendocrine tumors (133, 145). Mice treated with rebastinib have significantly reduced TMEM doorway activity, circulating tumor cells, and metastases compared to mice treated with vehicle control (133, 145).

Together, these studies identify macrophages as key mediators of tumor cell intravasation and demonstrate that blocking crucial macrophage signaling pathways may be a strategy to block tumor cell dissemination in patients.

6 TAMs in response to chemotherapy

Cytotoxic chemotherapies are characterized by their ability to directly prevent proliferation and promote apoptosis of dividing cells. Chemotherapeutic agents, including anthracyclines, platinum-based drugs, and other alkylating agents induce apoptosis by damaging DNA and preventing DNA replication and repair (146). Taxanes and vinca alkaloids prevent cell division by interfering with the mitotic spindle, and antimetabolites – structural analogs of nitrogenous bases – prevent DNA synthesis by getting fraudulently inserted into replicating DNA, as well as by preventing the synthesis of proper bases (146). Increasing evidence suggests that many of these drugs also exert indirect effects by modulating the immune microenvironment. While many of these indirect effects support tumor cell killing, some promote drug resistance and metastasis. In this section, we review the current understanding of how common chemotherapies affect macrophages in anti- and pro-tumoral ways.

6.1 Anti-tumor TAM response to chemotherapy

Paclitaxel – a microtubule stabilizing agent in the taxane group – has been shown to increase the immune response and tumoricidal activity of murine macrophages. In mice, paclitaxel treatment leads to a robust increase in macrophage expression of TNFα and IL-1β, pro-inflammatory cytokines associated with the M1 phenotype (147–149). Paclitaxel also increases macrophage expression of IL-12, a Th1-type cytokine involved in activating the innate and adaptive immune response (148, 150). As a LPS mimetic, paclitaxel activates toll-like receptor (TLR) 4 on murine macrophages leading to NF-κB activation and increased production of pro-inflammatory signals (148). Though some studies show that paclitaxel also activates TLR4 in human cells (151–153), others show that species-specific differences in the TLR4 accessory protein, myeloid differentiation factor 2 (MD-2), do not allow this activation (154–159). Interestingly, some studies indicate that docetaxel – another taxane – has more potent effects on human macrophages than paclitaxel. Millrud et al. found that docetaxel, but not paclitaxel, promoted an M1 phenotype in human monocytes (160). Furthermore, a clinical study assessing immune responses to taxanes in breast cancer patients showed that, while both docetaxel and paclitaxel lead to an increase in serum M1-associated markers (including IL-6, GM-CSF, and IFN-γ), the effects were significantly more pronounced in patients who received docetaxel (161). The effects of taxanes on macrophages are also highly context dependent. For instance, IFN-γ has been shown to "prime" macrophages for tumoricidal activity, and paclitaxel affords macrophages increased cytotoxicity after macrophage exposure to IFN-γ (162). While the exact mechanisms have yet to be elucidated, these studies indicate that taxanes can promote anti-tumor M1 polarization in a context-dependent manner.

In addition to taxanes, platinum-based drugs, antimetabolites, and alkylating agents have also been shown to promote M1 macrophage polarization. The combination of platinum-based agents with antimetabolites is a common first-line treatment for gastric cancer (163). In studies analyzing the TME of matched pre- and post-treatment biopsies from gastric cancer patients, post-treatment samples harbored significantly more M1-polarized macrophages, and this increase was associated with a favorable response to treatment (164, 165). Furthermore, when given at high doses, the alkylating agent cyclophosphamide is highly immunosuppressive. However, lower doses of the drug strikingly improve anti-tumor immunity (166). This has led to the development and use of metronomic schedules of administration, in which low doses of the drug are administered more frequently (166). In line with observations that low-dose cyclophosphamide improves anti-tumor immunity, several studies show that low-dose, metronomic cyclophosphamide increases macrophage M1 polarization and decreases tumor burden (167–169).

Leukemia and lymphoma are often treated with monoclonal antibodies. While these treatments are largely effective at targeting cancer cells in many niches, cancer cells often become resistant to such antibodies in the bone marrow (170–172). Several studies found that combining antibody therapy with cyclophosphamide prevented antibody therapy resistance in the bone marrow in part by promoting macrophage phagocytosis of antibody-targeted cancer cells (170–172).

In summary, these studies show that in some circumstances, chemotherapy reprograms macrophages to increase anti-tumor activity.

6.2 Pro-tumor TAM response to chemotherapy

The macrophage response to chemotherapy is a double-edged sword. While some studies show that chemotherapy promotes the anti-tumor activity of macrophages, others show that chemotherapy causes a macrophage-mediated pro-tumoral response.

Chemotherapy causes tumor cell death and tissue damage followed by a cytokine storm that promotes the release of endothelial and immune progenitor cells from the bone marrow (173–175). In response to this tissue damage, cells in the TME initiate a wound healing response by increasing their secretion of CSF1, CXCL12, and other chemokines that recruit these circulating progenitor cells to the tumor (176, 177). One result of this response is that perivascular TAMs increase following chemotherapy (133, 177). These newly recruited perivascular TAMs express high levels of VEGFA and the angiopoietin receptor Tie2, which have been shown to promote relapse and metastasis following chemotherapy (132, 133, 177–180). Several studies show how chemotherapy induces a macrophage-mediated pro-tumoral effect that can be abrogated by targeting macrophages.

Hughes et al. used mouse models of breast cancer to show that treatment with cyclophosphamide causes an increase in CXCR4-expressing perivascular macrophages, which promote tumor revascularization and regrowth via VEGFA signaling (177). Blocking CXCR4 signaling prevents the accumulation of perivascular macrophages and delays tumor regrowth (177).

In neuroblastoma, chemotherapy leads to the selective expansion of CCL2-expressing mesenchymal-like tumor cells and macrophage infiltration in patients, which promotes relapse and chemo-resistance (181). In mouse models, combining chemotherapy with CSF1R inhibition prevents macrophage infiltration and tumor regrowth (181).

Furthermore, while chemotherapy increases the infiltration of Tie2+ macrophages, Tie2 inhibitors have been shown to work synergistically with chemotherapy to delay tumor growth (145) and relapse (182).

In addition to promoting tumor relapse, macrophages can also increase tumor cell dissemination following chemotherapy. We have previously shown that treatment with paclitaxel causes a robust, macrophage-dependent increase in MenaINV expression, which promotes tumor cell migration, intravasation, and metastasis (130, 133). This indicates that paclitaxel causes a macrophage-mediated increase in metastasis-competent tumor cells, though the exact mechanism behind this effect remains unknown.

Furthermore, chemotherapy significantly increases the assembly and function of TMEM doorways, which are portals for tumor cell intravasation. Indeed, circulating tumor cells and lung metastases are more prevalent in mice treated with paclitaxel compared to vehicle control (133). Concerningly, TMEM doorway assembly is also increased in patients with ER+/HER2- breast cancer following neoadjuvant chemotherapy (133), thus increasing their risk of distant metastasis (136). As mentioned in Section 5, targeting TMEM doorway-associated macrophages with the Tie2 inhibitor rebastinib dramatically decreases the pro-metastatic effects of chemotherapy in pre-clinical studies, indicating that Tie2 inhibition in combination with a cytotoxic agent may improve patient outcomes (133, 145).

Another mechanism by which TAMs promote metastasis in response to chemotherapy is by upregulating the enzyme heparanase. Heparanase cleaves heparan sulfate, which is an important structural component of the ECM (183). Similarly to MMPs, this matrix-degrading enzyme is upregulated in many cancers and correlates with increased metastasis and poor prognosis (184). Unfortunately, heparanase has been shown to increase in some patients following chemotherapy (185). Mechanistically, treatment with chemotherapy leads to an increase in VEGFR3-expressing TAMs which secrete cathepsins that activate heparanase and promote ECM remodeling, lymphangiogenesis, and metastasis (186). Notably, blocking VEGFC/VEGFR3 signaling inhibits chemotherapy-induced lymphangiogenesis and metastasis (186).

In summary, chemotherapy can act on macrophages to promote relapse and metastasis in a variety of ways. Recent pre-clinical studies show that targeting macrophage recruitment or function is a promising approach to optimize cancer treatment. Indeed, there has been a 3-fold increase in clinical trials on macrophage-targeted therapies in the past 10 years (31). However, due to the diversity of patients, chemotherapies, and macrophage phenotypes, more research is needed to clarify the exact mechanisms of chemotherapy-induced cancer progression to refine treatment strategies and determine biomarkers that can identify good – and bad – candidates for different treatments.

7 Host factors governing TAMs

7.1 Diet and natural compounds

Evidence supporting the role of a healthy diet in cancer prevention and treatment is ever-increasing (187, 188). While natural compounds are known to have a profound role in regulating EMT in cancer cells (189, 190), numerous recent studies have also shed light on how dietary agents and natural compounds target tumor-associated macrophages.

7.1.1 Antioxidants

Perhaps the most well-known dietary anti-cancer agents are antioxidants – compounds that neutralize free radicals that would

otherwise damage DNA and other cellular structures and lead to carcinogenesis. Foods high in antioxidants include berries, fruits, vegetables, walnuts, and pecans (191). Recently, Latronico et al. demonstrated that dietary antioxidants act on macrophages and inhibit the expression and activity of macrophage-derived MMP-2 and MMP-9, which have pro-tumor ECM remodeling activity (see Section 3.2) (192). Macrophages mediate the production of reactive oxygen species (ROS) in the TME (193), and there is an established relationship between ROS and MMP production (194, 195). The authors posit that dietary antioxidants prevent MMP production by removing ROS in the microenvironment (192).

Propolis, a natural resin produced by honeybees, also has antioxidative properties (196–198). It is widely used as a natural additive in both ingestible (i.e. capsules, throat lozenges, food) and topical (i.e. lotions, cosmetics) products. Propolis induces the depolarization and repolarization of M2 macrophages to M0- and M1-like states, respectively. M2-polarized macrophages treated with propolis also express decreased levels of IL-8, IL-10, CCL2, VEGF, and MMP-9 (199). Consistent with this shift of M2 to M1 macrophages, propolis significantly decreases EMT and tumor cell migration and invasion (199).

For centuries, cloves have been used not only as a spice, but also as an herbal remedy due to their antimicrobial and antioxidative properties (200). Kumatakenin, a flavonoid isolated from cloves, has recently shown significant anti-cancer effects by acting on both tumor cells and tumor-associated macrophages (201). In addition to inducing apoptosis in human ovarian cancer cells, kumatakenin reduces tumor cell expression of CCL2 and CCL5 – both implicated in macrophage recruitment, cancer progression and metastasis (201–203). Kumatakenin also prevents M2 polarization and macrophage expression of IL-10, VEGF, MMP-2, and MMP-9 (201).

Together, these studies implicate antioxidants in reducing macrophage-mediated pro-tumoral effects including immunosuppression, angiogenesis, and pro-tumoral ECM remodeling.

7.1.2 Vitamin D and omega-3 poly-unsaturated fatty acids

Vitamin D is a steroid hormone precursor that can come from the diet or be endogenously synthesized in the skin upon exposure to UV radiation (204). Vitamin D exerts its effects by binding to the vitamin D receptor and is primarily responsible for regulating calcium and phosphate levels in the body (205, 206). Though there is no clear consensus on the impact of vitamin D on cancer, a recent study showed that vitamin D may exert anti-cancer effects through macrophages. *In vitro* studies showed that vitamin D reverses M2 polarization, decreases macrophage secretion of TGF-β1 and MMP-9, and reduces the macrophage-induced proliferation and migration of ovarian cancer cells (207).

Omega-3 poly-unsaturated fatty acids (ω-3 PUFAs) – long lauded for their anti-inflammatory properties – also exhibit anti-cancer effects on tumor-associated macrophages (208). Studies on mouse models of castrate resistant prostate cancer show that a diet rich in ω-3 (vs. ω-6) PUFAs significantly delays tumor progression, decreases M2 polarization, and increases M1 polarization and infiltration of CD4+ T cells. M2 macrophages from tumors in mice fed a high ω-3 diet also show a significant decrease in MMP-9 and VEGF expression (208).

Together, these studies have begun to reveal the mechanisms by which a healthy diet can induce anti-cancer changes in macrophages and the TME more broadly.

7.2 Obesity

Obesity is a fast-growing global health crisis that is associated with an increased risk of cancer, as well as general morbidity and mortality (209, 210). In fact, women with obesity who are diagnosed with breast cancer have an increased risk of distant metastasis and are less likely to respond to some cancer treatments (211–214). Unsurprisingly, adipose tissue macrophages are thought to play a key role in creating a pro-tumorigenic microenvironment (86). CCL2 expression is significantly increased in the adipose tissue of obese compared to lean mice (215) which leads to the accumulation of macrophages. Indeed, it is estimated that macrophages make up <10% of cells in the adipose tissue of lean individuals and nearly 40% of cells in the adipose tissue of obese individuals (216). These recruited macrophages surround dead and dying adipocytes, forming crown-like structures (CLS) that are characteristic of adipose tissue inflammation (217). Overweight and obese patients (BMI ≥ 25 kg/m^2) with breast cancer are more likely to have CLS compared to patients at a healthy weight (BMI < 25 kg/m^2), and BMI ≥ 25 kg/m^2 is associated with a shift in CLS macrophage phenotype that may be indicative of metabolic dysfunction and poor treatment outcomes under some conditions (218). These macrophages also interact with pre-adipocytes and prevent their differentiation, instead causing them to take on a fibroblastic phenotype and enhance the synthesis and deposition of ECM components (219, 220). This indicates that macrophages cause increased ECM density in obese adipose tissue, including in the breast where ECM density is a significant risk factor for cancer (72, 221). Further implicating adipose tissue macrophages in breast tumor development, a study on transgenic mice that overexpress CCL2 in the mammary epithelium showed that CCL2 overexpression causes increased macrophage density, stromal density, and ECM crosslinking enzyme LOX compared to non-transgenic controls (222). CCL2-overexpressing mice also had an increased susceptibility to DMBA-induced mammary tumors, demonstrating a relationship between macrophages, ECM density, and cancer risk (222).

Though much is still unknown about how obesity shapes cancer development, these studies suggest that obesity promotes macrophage-mediated ECM stiffening that is known to support tumorigenesis (see section 3.1).

7.3 Race

There are widespread racial disparities in the diagnosis, treatment, and outcome of cancer patients (223–226). While some of these disparities are due to systemic racism in the medical field, some studies identify biological differences that could be contributing to this effect (227). Indeed, Black men and

women with prostate and breast cancer, respectively, have significantly worse outcomes than their white counterparts even after socioeconomic and other mediating factors are accounted for (228, 229). We previously evaluated distant recurrence-free survival (DRFS) in breast cancer patients following neoadjuvant (NAC) versus adjuvant (AC) chemotherapy and found that treatment type had no impact on DRFS for white women (230). However, Black women had significantly worse DRFS when treated with NAC (230). Differences in TAMs may contribute to this. Black breast cancer patients have significantly increased macrophage infiltration and M2 polarization compared to white patients, and this is prognostic of progression-free survival (231, 232). Black, compared to white women treated with neoadjuvant chemotherapy for ER+/HER2- breast cancer also have a higher TMEM doorway score and macrophage density in the residual tumor tissue, which may contribute to poorer outcomes (223). In summary, racial disparities in cancer development and outcome may be mediated in part by macrophage infiltration and activity.

8 Conclusion

The diversity and complexity of tumor-associated macrophages leaves their functions highly context-dependent and variable. Despite this complexity, a consensus is emerging in the literature that tumor-associated macrophages support tumor cell migration and metastasis in many ways (Figure 3). TAMs confer migratory abilities in tumor cells by activating EMT. They remodel the ECM to facilitate tumor cell migration and provide ligands to promote invadopodium formation and chemotaxis. Finally, TAMs participate in the signaling cascade that opens TMEM doorways and allows tumor cells to intravasate and disseminate.

The plastic nature of TAMs means that their phenotypes and functions can dramatically change in response to environmental factors, including controllable factors such as chemotherapy, diet, and obesity and immutable factors such as race. Future research elucidating just how these factors play a role in macrophage function and cancer progression are crucial for refining treatment strategies.

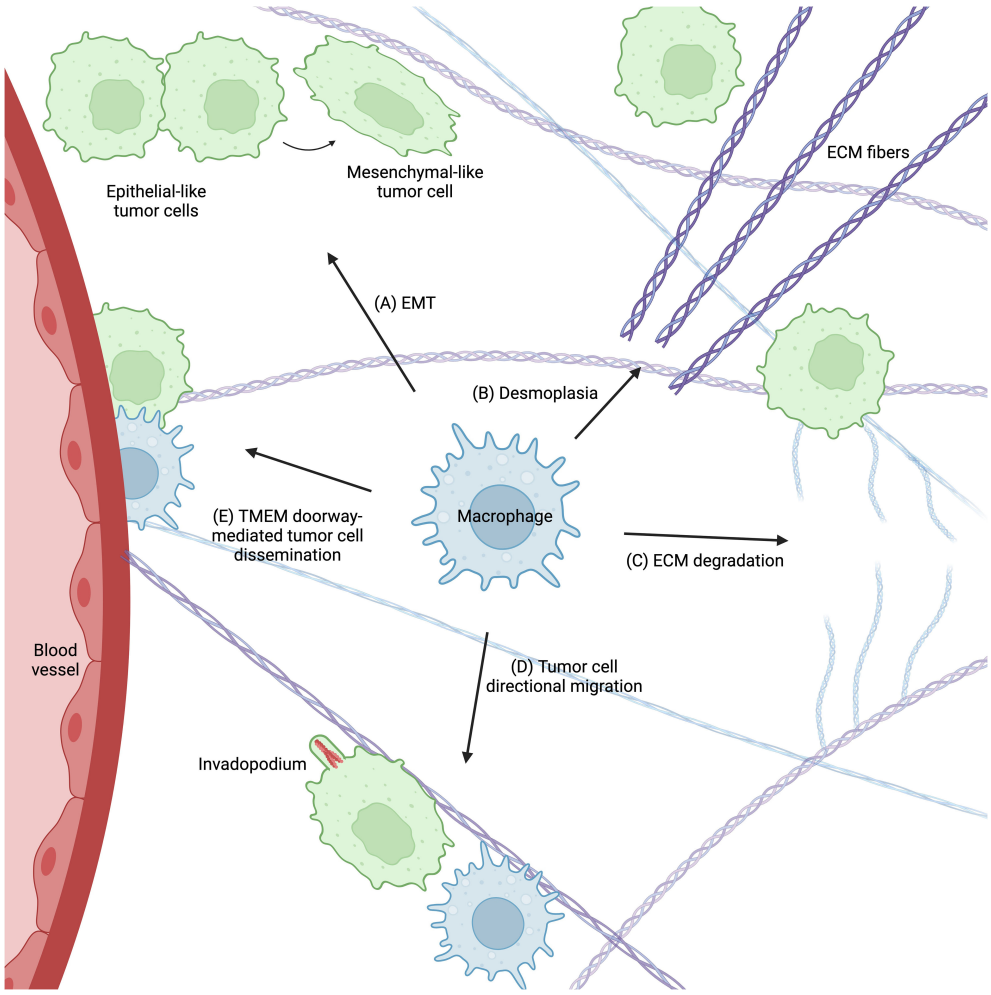

FIGURE 3
Macrophages support tumor cell migration and metastasis in many ways. **(A)** Macrophages promote EMT in tumor cells, which confers a migratory phenotype. They regulate ECM remodeling by enhancing both ECM stiffness (desmoplasia) **(B)** and degradation **(C)**, which supports tumor cell migration and metastasis. Macrophages also provide signals that promote tumor cell invadopodium formation and directional migration towards blood vessels **(D)**. Finally, macrophages participate in TMEM doorway-mediated tumor cell intravasation **(E)**, which allows tumor cells to disseminate throughout the body. Figure created with BioRender.com.

Author contributions

MF: Writing – original draft, Writing – review & editing. GK: Writing – review & editing. JC: Writing – review & editing. MO: Writing – review & editing. DE: Writing – review & editing.

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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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GPER1 signaling restricts macrophage proliferation and accumulation in human hepatocellular carcinoma

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Background: Sex hormones and their related receptors have been reported to impact the development and progression of tumors. However, their influence on the composition and function of the tumor microenvironment is not well understood. We aimed to investigate the influence of sex disparities on the proliferation and accumulation of macrophages, one of the major components of the tumor microenvironment, in hepatocellular carcinoma (HCC).

Methods: Immunohistochemistry was applied to assess the density of immune cells in HCC tissues. The role of sex hormone related signaling in macrophage proliferation was determined by immunofluorescence and flow cytometry. The underlying regulatory mechanisms were examined with both *in vitro* experiments and murine HCC models.

Results: We found higher levels of macrophage proliferation and density in tumor tissues from male patients compared to females. The expression of G protein–coupled estrogen receptor 1 (GPER1), a non-classical estrogen receptor, was significantly decreased in proliferating macrophages, and was inversely correlated with macrophage proliferation in HCC tumors. Activation of GPER1 signaling with a selective agonist G-1 suppressed macrophage proliferation by downregulating the MEK/ERK pathway. Additionally, G-1 treatment reduced PD-L1 expression on macrophages and delayed tumor growth in mice. Moreover, patients with a higher percentage of $GPER1^{+}$ macrophages exhibited longer overall survival and recurrence-free survival compared to those with a lower level.

Conclusions: These findings reveal a novel role of GPER1 signaling in regulating macrophage proliferation and function in HCC tumors and may offer a potential strategy for designing therapies based on understanding sex-related disparities of patients.

KEYWORDS

macrophages, GPER1, proliferation, hepatocellular carcinoma, tumor microenvironment

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent tumors and is associated with increasing mortality worldwide, posing a significant threat to human health (1). Epidemiological observations have reported sex disparities in the incidence and progression of HCC (1–3). Generally, males have a higher risk of HCC and a worse prognosis than females. However, the mechanisms underlying these differences are not fully understood.

Evidence has suggested that distinct immune responses in males and females contribute to variations in the incidence and efficacy of treatment for infections, autoimmune diseases and cancers (4). For instance, females generally display a stronger response to pathogens (5), have greater vaccine efficacy (6) and are more susceptible to autoimmune diseases compared to males (7, 8). In the context of tumors, there have been reports of sex disparities in the functions of tumor-infiltrating immune cells mediated by sex hormone signaling in melanoma and colorectal cancer (9–11). Macrophages constitute a major component of the leukocyte infiltrate in HCC. Educated by signals in the tumor microenvironment, macrophages undergo polarization and acquire a phenotype that promotes tumor growth (12, 13). Our previous study has demonstrated that self-replication serves as an important mechanism for macrophage accumulation, and that proliferating macrophages exhibit an immunosuppressive phenotype in HCC tumor tissues (14). However, the influence of sex disparities on the proliferation and accumulation of macrophages in HCC remains unclear.

Sex hormones and their receptors play a crucial role in mediating the differences between males and females, both in normal physiological conditions and in pathological situations. G protein–coupled estrogen receptor 1 (GPER1), a non-classical estrogen receptor, is found in various cell types and is involved in regulating a wide range of physiological and pathological responses (15–17). For instance, GPER1 is essential in protecting fetal health from maternal inflammation caused by pathogen infections through suppressing IFN signaling in fetal tissues (16). Additionally, the activation of GPER1 signaling has been shown to regulate the proliferation of tumor cells in various types of cancer (18, 19). However, the role of GPER1 in regulating macrophage proliferation remains unclear.

In this study, we discovered that the accumulation and proliferation level of macrophages in tumor tissues of HCC were significantly higher in male patients compared to females. Our mechanical study demonstrated that the activation of GPER1 signaling restrained macrophage proliferation and accumulation. It also led to a decrease in PD-L1 expression on macrophages and a delay in tumor growth in mice. Moreover, patients with a higher percentage of GPER1$^+$ macrophages exhibited longer overall survival and recurrence-free survival compared to those with a lower level. These findings reveal a novel role of GPER1 signaling in the tumor microenvironment that regulates macrophage proliferation and function in HCC.

Abbreviations: HCC, hepatocellular carcinoma; GPER1, G protein–coupled estrogen receptor 1; ERα, estrogen receptor α; ERβ, estrogen receptor β; AR, androgen receptor; E2, 17β-estradiol; OS, overall survival; RFS, recurrence-free survival; ATCC, American Type Culture Collection; PCR, polymerase chain reaction; QPCR, Quantitative real-time PCR; IHC, Immunohistochemistry; ELISA, Enzyme-Linked Immunosorbent Assay; EdU, 5-ethynyl-2'-deoxyuridine; CCK-8, Cell Counting Kit-8; TSN, tumor culture supernatants; PBMC, peripheral blood mononuclear cell; EPIC, Estimating the Proportion of Immune and Cancer cells; ICI, immune checkpoint inhibitor.

2 Materials and methods

2.1 Patients and specimens

Liver tissue samples were obtained from patients with pathologically confirmed HCC who had not received any anticancer therapy prior to sampling. Individuals with a concurrent autoimmune disease, HIV, or syphilis were excluded. Samples from 48 HCC patients who had complete follow-up data were included to assess overall survival (OS) and recurrence-free survival (RFS) using immunofluorescence staining of GPER1 and CD68. OS was defined as the time between surgery and either death or the last observation for patients who survived. RFS was defined as the time between surgery and either the first recurrence or death, or the last observation for patients without recurrence. The clinical characteristics of these patients are summarized in Supplementary Table S1. In addition, fresh biopsy specimens from 7 HCC patients were used to isolate tumor-infiltrating leukocytes for flow cytometry analysis. All samples from HCC patients were coded anonymously in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki). Written informed consent was obtained from all participants prior to study onset. The use of human subjects for this study was approved by the Institutional Review Board of Sun Yat-sen University Cancer Center.

2.2 Culture of HCC cell lines and preparation of tumor culture supernatants

The hepatoma cell lines (HepG2, Huh7, SK-Hep-1and Hepa1-6) were obtained from the American Type Culture Collection (ATCC). All cells were regularly tested for mycoplasma contamination using the single-step polymerase chain reaction (PCR) method. The cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 U/mL penicillin (Sigma-Aldrich Corp), and 100 μg/mL streptomycin (Sigma-Aldrich Corp) in a humidified 5% CO2 incubator at 37°C. To prepare tumor culture supernatants (TSN), 5×10^6 tumor cells were plated in 10 ml complete medium in 10-cm dishes for 24 hours. Then, the medium was changed to phenol red and serum-free DMEM. After 48 hours, the supernatant was collected, centrifuged, and stored in aliquots at -80°C (14).

2.3 Monocyte purification from human peripheral blood and macrophage generation

Human monocytes were isolated as previously described (20, 21). Briefly, peripheral blood mononuclear cells (PBMCs) were

isolated from the buffy coats derived from healthy donors' blood by Ficoll density gradient centrifugation. $CD14^+$ monocytes were then purified from PBMCs using magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. The purified monocytes were cultured in DMEM supplemented with 10% human AB serum for 7 days to generate macrophages. Afterward, the culture medium was replaced with phenol red-free DMEM (Procell) containing 2% AB serum and 20% TSN to mimic a relatively nutrient-deficient tumor microenvironment. Meanwhile, in certain experiments as indicated, macrophages were treated with biochemical reagents, including G-1 (10008933, Cayman), G-15 (14673, Cayman) or 17β-estradiol (E8875, Sigma-Aldrich).

2.4 Mouse tumor models and treatments

All animal experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (Guangzhou, China). Male C57BL/6J mice were purchased from the Guangdong Medical Laboratory Animal Center and maintained under specific pathogen-free conditions. The mice used in the experiments were between 6 and 8 weeks old. For the subcutaneous tumor model, a total of 1×10^6 Hepa1-6 cells were subcutaneously transplanted into the flanks of mice. For the orthotopic tumor model, a total of 1×10^6 Hepa1-6 cells were suspended in 25 μl of 50% basement membrane extract (354234, corning) and injected into the left lobe of the liver of anesthetized mice. The mice began receiving daily subcutaneous injections of G1 (4mg/kg) dissolved in a solvent containing 10% DMSO (MP), 30% PEG300 (TargetMol), 5% Tween 80 and 55% ddH2O on day 4 (orthotopic tumor model) or day 5 (subcutaneous tumor model). The control mice received a 200 μl dose of the solvent.

2.5 Immunohistochemistry

Formalin-fixed and paraffin-embedded HCC samples were cut into 4-μm sections and processed for IHC as previously described (14). Following incubation with anti-human CD3 (MA514524, Thermo Fisher Scientific), anti-human CD20 (ab78237, Abcam), anti-human CD15 (ZM-0037, ZS), anti-human CD68 (ZM-0060, ZS), anti-human CD163 (ab182422, Abcam), anti-human CD204 (KAL-KT022) or anti-mouse F4/80 (70076S, CST) antibodies (Abs), the sections were then stained with the corresponding secondary Abs and visualized using 3,3'-diaminobenzidine (Nichirei). An automatic slide scanner (KF-PRO-020) was used to scan the sections, and then positive cells were quantified by HALO image analysis software (Indica Labs).

2.6 Immunofluorescence staining

Formalin-fixed and paraffin-embedded HCC sections were processed as described previously (14). For human tumor sections, anti-human CD68, anti-human Ki67 (ZM-0167, ZS) and anti-human GPER1 (PA5-109319, Thermo Fisher Scientific) Abs were used. For mouse tumor sections, anti-mouse F4/80 (700767, CST) and anti-mouse Ki67 (ab15580, Abcam) Abs were used. Immunofluorescence signals were amplified by a tyramide signal amplification kit (PANOVUE) as instructed by the manufacturer for visualization. Sections were scanned using the Polaris Fully Automatic Digital Slide Scanner (Akoya Biosciences), and then positive cells were quantified using HALO image analysis software (Indica Labs).

For immunofluorescence staining of cultured cells, the cells growing on coverslips were fixed with tissue/cell fixation buffer for 15 minutes at room temperature. Afterward, they were rinsed with PBS and permeabilized and blocked with PBS containing 5% BSA and 0.3% Triton X-100 for 1 hour at room temperature. The cells were then incubated overnight at 4°C with the primary antibody against human Ki67. This was followed by exposure to Alexa Flour 488-conjugated anti-mouse IgG. For double staining of Ki67 and GPER1 or ERα, the cells were simultaneously incubated with primary Abs against human Ki67 and GPER1 (PA5-109319, Thermo Fisher Scientific), or ERα (ab16660, Abcam). They were then exposed to Alexa Flour 488-conjugated anti-rabbit IgG and Alexa Flour 555-conjugated anti-mouse IgG. Nuclei were counterstained with DAPI. The immunofluorescence staining images were visualized using a high-resolution confocal laser microscope (LSM880 with fast airyscan, Zeiss).

2.7 EdU incorporation assay

Human monocyte-derived macrophages were either left untreated or treated with 20% Huh7-TSN for 48 hours, in the presence or absence of G-1 (1 μM), and then the cells were cultured with EdU (5-ethynyl-2'-deoxyuridine) at a final concentration of 1 μM for 5 hours. Afterward, the cells were fixed, permeabilized, and dyed following the manufacturer's instructions (C0071S, Beyotime). Images were then visualized using an inverted fluorescence microscope (Nikon ECLIPS Ti2).

2.8 Isolation of leukocytes from tissues

Tumor-infiltrating leukocytes were obtained from fresh tissue samples as described previously (20). Briefly, fresh biopsy specimens from HCC patients were cut into small pieces and digested in RPMI 1640 medium supplemented with 0.002% DNase I (Roche), 0.05% collagenase IV (Sigma-Aldrich) and 10% FBS for 45 minutes at 37°C. The dissociated cells were passed through a 70-μm cell strainer and then erythrocytes were lysed and removed. The remaining cells were thoroughly rinsed and resuspended in PBS supplemented with 1% FBS (Gibco) for flow cytometry analysis.

2.9 Flow cytometry

Flow cytometry was performed as previously described (20). Before antibody staining, the cells were incubated with the Zombie Fixable Viability reagent for 15 minutes at room temperature. For surface

staining, the cells were stained with fluorochrome-conjugated Abs for 30 minutes. For intracellular staining, the cells were fixed using the Fix/Perm solution (eBioscience), washed with the Perm/Wash buffer (eBioscience), and then incubated with fluorochrome-conjugated Abs for 30 minutes. For GPER1 staining, cells were incubated with GPER1 antibody before being stained with Alexa Flour 488-conjugated anti-rabbit IgG. Data was acquired using Cytoflex flow cytometer (Beckman Coulter) and analyzed using CytExpert software. Representative plots were created using Flowjo software 10 (Tree Star). The reagents and Abs used for flow cytometry are listed as follows: Zombie NIR™ (423105, Biolegend), Zombie Violet™ (423113, Biolegend); anti-human Abs including CD45-PE (304008, Biolegend), CD14-AF700 (557923, Biolegend), Ki67-PE (556027, Biolegend), Ki67-APC (350514, Biolegend), PD-L1-PC7 (558017, Biolegend) and GPER1 (PA5-109319, Thermo Fisher Scientific); anti-mouse Abs including CD45-BV605 (103140, Biolegend), CD11b-FITC (101206, Biolegend), Ly-6G-ECD (562700, BD Biosciences), F4/80-PE (123110, Biolegend), PD-L1-PC7 (124314, Biolegend) and Ki67-APC (652405, Biolegend).

2.10 Cell Counting Kit-8 assay

Human monocytes were seeded in 96-well plates with a density of 12,500 cells per well, and cultured in DMEM supplemented with 10% human AB serum for 7 days. Then the monocyte-derived macrophages were either left untreated or treated with 20% Huh7-TSN for 48 hours, in the presence or absence of G-1 (1 μM). Afterward, the cells were incubated with the CCK-8 (CK-04, KYD bio) solution for 2 hours, and absorbance was measured at 450 nm.

2.11 Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent, and then 1μg RNA was used to synthesize cDNA with Color Reverse Transcription Kit (A0010CGQ, EZBioscience). Sequences of the primers used are listed as follows: *ESR1* (ERα), Forward: 5′-GCTTACTGACCAACCTGGCAGA -3′, Reverse: 5′- GGATCTC TAGCCAGGCACATTC -3′; *ESR2* (ERβ), Forward: 5′- AGAGT CCCTGGTGTGAAGCAAG, Reverse: 5′- GACAGCGCAGA AGTGAGCATC-3′; *GPER1*, Forward: 5′- TCTAAACTGCGG TCAGATGTGGC-3′, Reverse: 5′- TGTGAGGAGTGCAA GGTGACCAG-3′; *AR*, Forward: 5′- GACGACCAGATGGCTGT CATT-3′, Reverse: 5′- GGGCGAAGTAGAGCATCCT-3′. QPCR was performed in triplicate according to a standard protocol using Color SYBR Green qPCR Master Mix (A0012-R2, EZBioscience) with the LightCycler 480 System (Roche). To determine the levels of the analyzed RNA, their expression was normalized relative to human *GAPDH*.

2.12 Immunoblotting

human monocyte-derived macrophages were either left untreated or treated with 20% Huh7-TSN for 48 hours, in the presence or absence of G-1(1 μM). Then the protein was extracted for immunoblotting analysis. Immunoblotting was performed as described previously (14). Primary Abs used are listed as follows: anti-human Cyclin E1 (4129T, CST), Cyclin D1 (2978T, CST), CDK2 (2546T, CST), CDK4 (12790T, CST), p-AKT (13038S, CST), AKT (4685S, CST), p-Erk1/2 (4370T, CST), Erk1/2 (4695T, CST), β-actin (4970S, CST). HRP-linked anti-rabbit/mouse IgG Abs were purchased from CST.

2.13 Enzyme-Linked Immunosorbent Assay

Fresh biopsy specimens from HCC patients were weighed and fully ground to generate tissue homogenate. The cells were then lysed completely using an ultrasonic crusher, followed by centrifugation to obtain clarified supernatant. Methanol was added to the supernatant, and the mixture was incubated at room temperature for 10 minutes. After centrifugation, the supernatant was transferred to a clean tube and evaporated to dryness using centrifugal concentration drying system (Eppendorf). Assay buffer was added to reconstitute the precipitate, and then the estradiol content was immediately measured using an ELISA kit (501890, Cayman), following the manufacturer's instructions.

2.14 Estimating immune cell scores of HCC tumor samples

The gene transcription expression data of HCC was obtained from The Cancer Genome Atlas (TCGA) database (22). Then the data was used to estimate the accumulation levels of various immune cells in 371 HCC tumor samples. This was achieved using the "IOBR" R package, employing the "Estimating the Proportion of Immune and Cancer cells" ("EPIC") and the "xCell" methods (23).

2.15 Statistics

The statistical tests used are indicated in the figure legends. Two-tailed Student's t-test or one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of two or multiple groups, respectively. Survival curves were calculated using the Kaplan-Meier method and analyzed with the log-rank test. The statistical analyses mentioned above were performed using GraphPad Prism 6. Univariate and multivariate analyses were performed using the Cox proportional hazards model (SPSS Statistics 21, IBM). $P < 0.05$ was considered statistically significant for all tests.

3 Results

3.1 Sex disparities in the accumulation and proliferation of macrophages in HCC tumors

To investigate the sex-related differences in the immune microenvironment of HCC tissues, we compared the density of

various immune cells, determined by IHC, in tumor tissues derived from male and female patients in our previous cohort (24). While there were no significant differences in the density of $CD3^+$ T cells, $CD20^+$ B cells or $CD15^+$ neutrophils between male and female patients, we observed a higher accumulation of $CD68^+$ macrophages in the tumor tissues of male patients compared to female patients (Figures 1A–C; $n_{male} = 381$; $n_{female} = 52$). We also found higher levels of the markers CD204 and CD163, which are associated with a pro-tumor phenotype of macrophages in HCC (25, 26), in the tumor tissues of male patients compared to female patients (Figures 1D, E). These differences were not observed in the non-tumor regions. Additionally, we examined the immune cell infiltrations in HCC tumor samples from TCGA dataset using the "xCell" and the "EPIC" methods, and found that the level of macrophages, particularly M2-like macrophages, also showed the same sex discrepancy (Supplementary Figures S1A, B; $n_{male} = 250$; $n_{female} = 121$).

Self-replicating macrophages are enriched in HCC tumors and serve as an important mechanism for macrophage accumulation (14). Therefore, we set out to investigate whether sex difference was involved in the variation in macrophage self-replication. We conducted double immunofluorescence staining of Ki67 and CD68 in HCC tumor tissues from our previous cohort (14), and compared the level of $Ki67^+CD68^+$ cells between male and female patients (Figure 1F). The results revealed that both the percentage and the number of proliferating macrophages in tumor tissues were significantly higher in male patients compared to female patients (Figure 1G).

These results collectively reveal that there are sex disparities in macrophage proliferation and accumulation in HCC tumor tissues.

3.2 GPER1 expression negatively correlates with macrophage proliferation in HCC tumors

To investigate the mechanism behind this phenomenon, we established an *in vitro* model by incubating human monocyte-derived macrophages with tumor culture supernatants (TSN) to

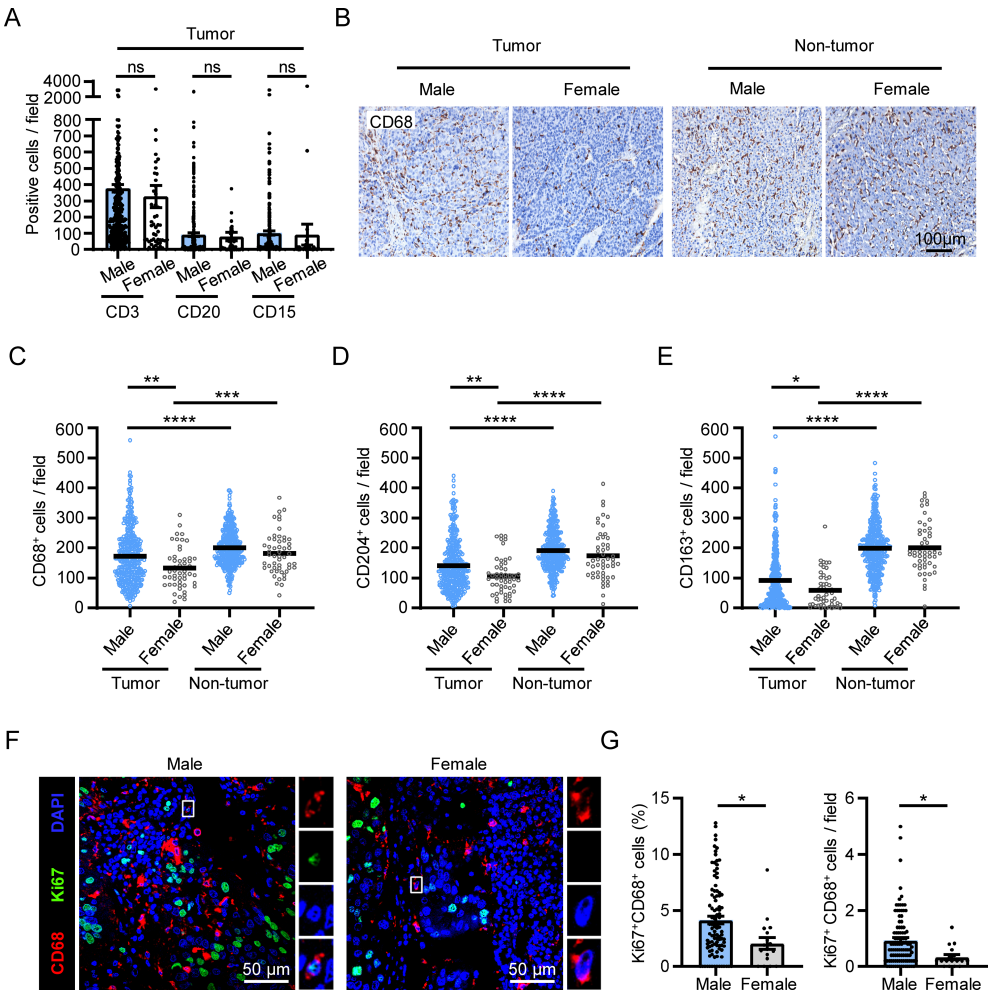

FIGURE 1
Sex disparities in the accumulation and proliferation of macrophages in HCC tumors. **(A–E)** IHC staining was performed on paraffin-embedded tissues from HCC patients (male, n = 381; female, n = 52). Statistical analysis of CD3, CD20, CD15 staining in the tumor tissue of HCC patients was shown in **(A)**. A representative CD68 staining is shown in **(B)**. The scale bar is 100 μm. **(F, G)** Fluorescence visualization and quantification of proliferating macrophages ($Ki67^+CD68^+$) among the total $CD68^+$ macrophages in the tumor tissues of HCC patients (male, n = 100; female, n = 17). The scale bar is 50 μm. The results shown are represented as mean ± standard error of the mean (SEM), *p* values were obtained using nonpaired two-tailed Student's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant.

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induce macrophage proliferation, as previously described (14) (Figure 2A). Then, qPCR was utilized to examine the expression of sex hormone receptors, specifically estrogen receptor α (ERα), estrogen receptor β (ERβ), GPER1 and androgen receptor (AR), which are known to play roles in sex-related differences in physiological and pathological conditions. The results showed that both GPER1 and ERα were significantly downregulated by TSN, whereas ERβ and AR were not affected (Figure 2B). Next, we compared the protein-level expression of GPER1 or ERα between proliferating and non-proliferating macrophages. Flow cytometry and immunofluorescence staining revealed that proliferating macrophages exhibited significantly lower expression of GPER1 when compared to non-proliferating cells in the presence of TSN (Figures 2C–E). However, no significant difference was observed in the expression of ERα between proliferating and non-proliferating macrophages (Figure 2F and Supplementary Figure S2).

The association between GPER1 expression and macrophage proliferation was examined in human HCC tumor tissues. Flow cytometry analysis of fresh tumoral leukocytes isolated from HCC patients showed that the proliferation level was significantly lower in $CD14^+GPER1^+$ cells compared to $CD14^+GPER1^-$ cells (Figure 2G). GPER1 expression in macrophages was also detected *in situ* using

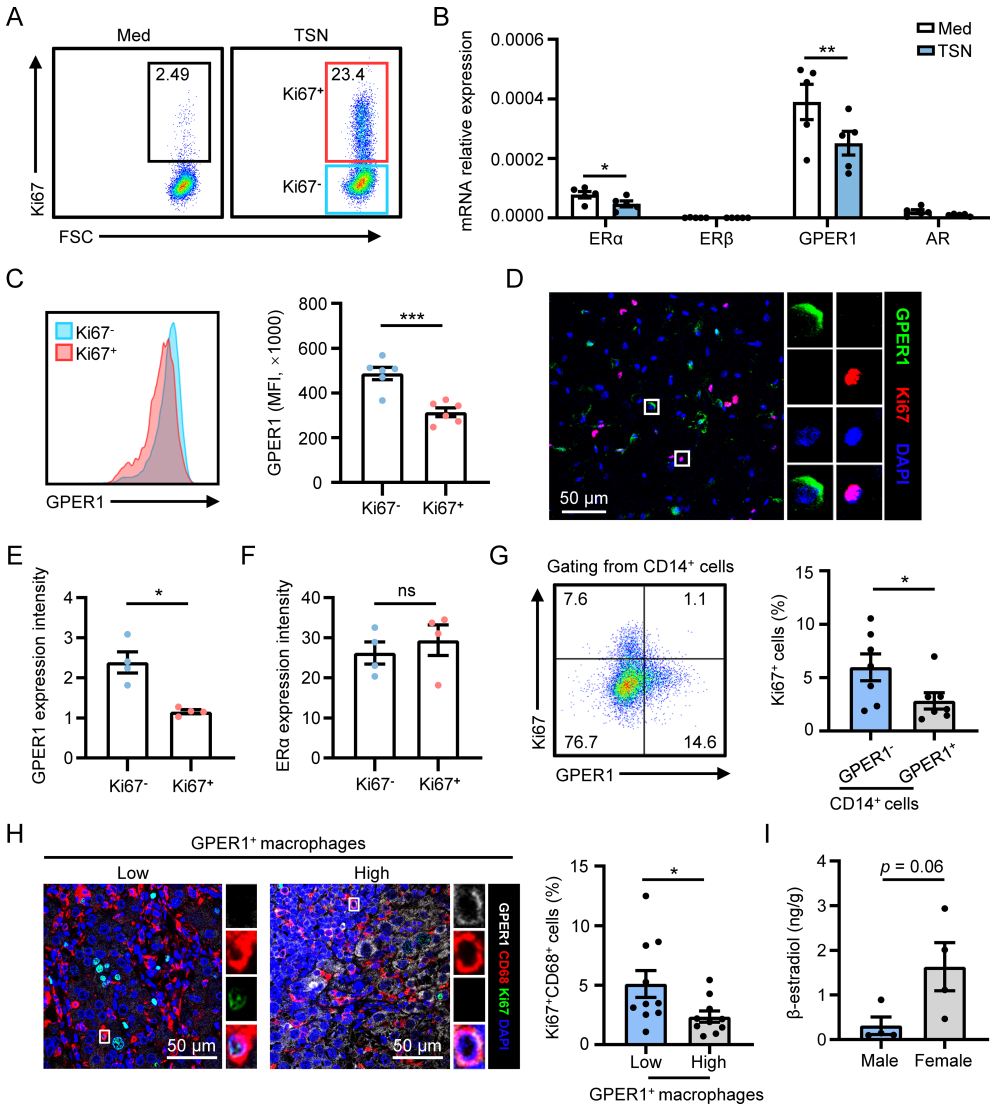

FIGURE 2
GPER1 expression negatively correlates with macrophage proliferation in HCC tumors. **(A–F)** Human monocyte-derived macrophages were treated with 20% culture supernatant from SK-Hep-1 cells (TSN) or control media (Med) for 48 hours. The proliferation level of macrophages was detected using flow cytometry analysis, and $Ki67^-$ and $Ki67^+$ macrophages were gated **(A)**. The expression levels of sex hormone receptors were examined using qPCR **(B**, n = 5). The expression levels of GPER1 in $Ki67^-$ and $Ki67^+$ macrophages gated in **(A)** were determined using flow cytometry **(C**, n = 6). The expression of Ki67, GPER1 and ERα in TSN-treated macrophages was visualized using confocal microscopy. Then the fluorescence intensity of GPER1 or ERα staining was compared between $Ki67^-$ and $Ki67^+$ macrophages **(D-F**, n = 4). The scale bar is 50 μm. **(G)** Representative dot plot and statistical analysis of the proliferation level of $GPER1^-$ or $GPER1^+$ macrophages isolated from fresh tumor tissues of HCC patients (n = 7). **(H)** HCC tumor samples were stained with anti-human CD68, GPER1 and Ki67 antibodies, and were then analyzed using confocal microscopy. The scale bar is 50 μm. Patients were divided into two groups according to the median frequency of $GPER1^+$ macrophages among the total $CD68^+$ macrophages, and the percentage of $Ki67^+$ macrophages among the total $CD68^+$ macrophages was compared between the two groups (n = 20). **(I)** 17β-estradiol concentrations (ng/g tissue) in HCC tumor tissues were examined using Enzyme-Linked Immunosorbent Assay (ELISA) (male, n = 4; female, n = 4). The results shown are represented as mean ± SEM. *P* values were obtained using paired or nonpaired two-tailed Student's t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; ns, not significant.

confocal microscopy. As shown in Figure 2H, the GPER1 positive signal was less prominent in Ki67$^+$ macrophages compared to Ki67$^-$ macrophages. Statistical analysis revealed that patients with a higher frequency of GPER1$^+$ macrophages exhibited a significantly lower level of macrophage proliferation in tumor tissues. Additionally, the levels of 17β-estradiol (E2), the primary natural ligand for GPER1 (27, 28), were measured in HCC tumor tissues, and the result indicated that tumor tissues from female patients tended to exhibit a higher level of E2 compared to those from male patients (Figure 2I). Therefore, there is a negative correlation between the expression of GPER1 in macrophages and their proliferation, both *in vitro* and in HCC tumor tissues.

3.3 GPER1 activation restricts macrophage proliferation

Next, we set out to investigate the impact of GPER1 signaling on macrophage proliferation using G-1, a GPER1 specific agonist (29). The results showed that G-1 significantly suppressed the proliferation of macrophages induced by TSN from various hepatoma cell lines (Figures 3A–D). Moreover, the inhibitory effect of G-1 on TSN-induced macrophage proliferation was observed to be dose-dependent (Figures 3C, D). Immunofluorescence staining confirmed that G-1 treatment resulted in a notable decrease in the percentage of Ki67$^+$ macrophages (Figures 3E, G). To determine

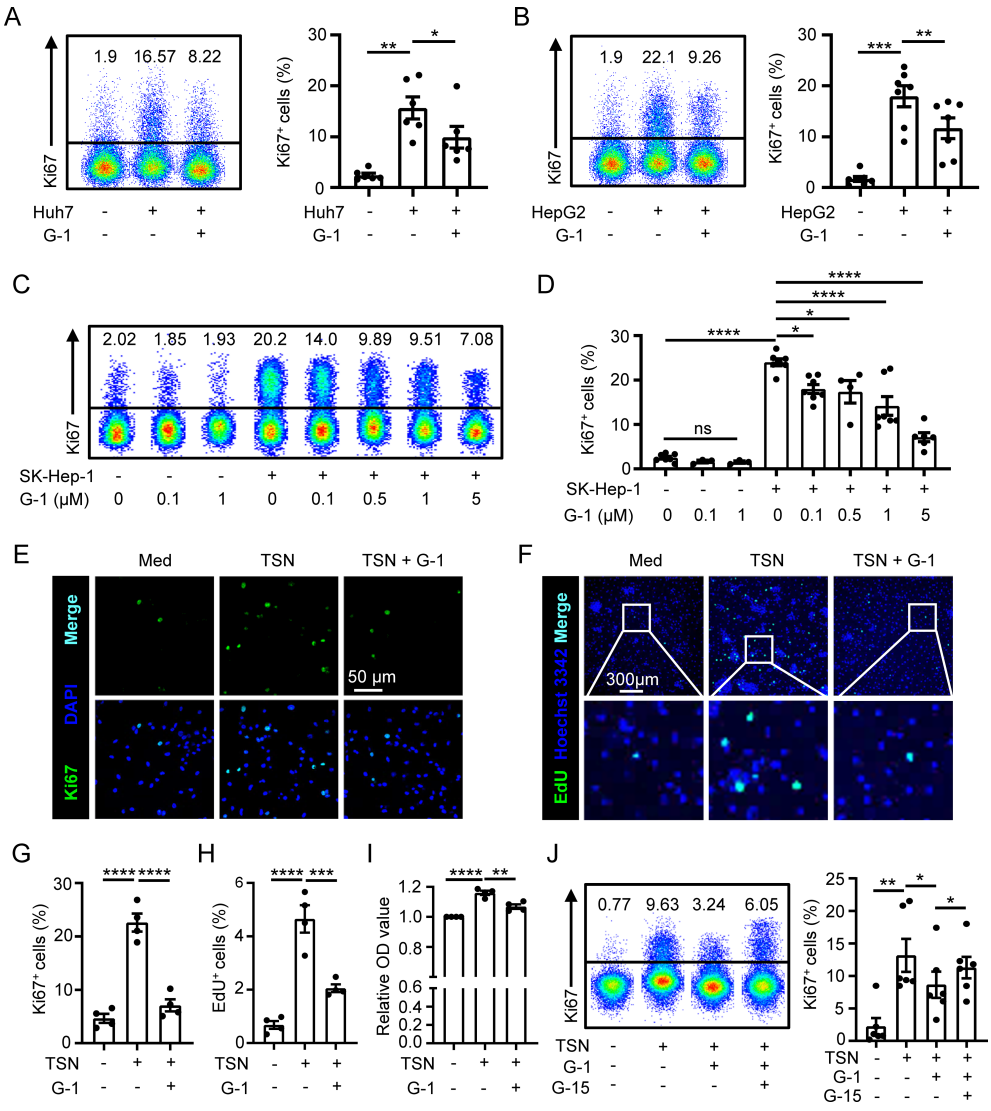

FIGURE 3
GPER1 activation restricts macrophage proliferation. **(A–D)** Human monocyte-derived macrophages were either untreated or treated with 20% TSN from Huh7, HepG2 or SK-Hep-1 cells for 48 hours, in the presence or absence of indicated concentrations of G-1 (1 μM for **A** and **B**). The percentages of Ki67$^+$ macrophages were assessed using flow cytometry (n = 7). **(E–I)** Human monocyte-derived macrophages were either untreated (Med) or treated with 20% Huh7-TSN for 48 hours, in the presence or absence of G-1 (1 μM). Ki67$^+$ **(E)** and EdU$^+$ **(F)** macrophages were visualized using confocal microscopy. The scale bar is 50 μm in **(E)** and 300 μm in **(F)**. The statistical analysis of the percentages of Ki67$^+$ (**G**, n = 4) and EdU$^+$ (**H**, n = 4) macrophages is shown. Densities of macrophages were determined using a CCK-8 assay, and the optical density (OD) values of each donor were normalized relative to the corresponding value of the group without TSN treatment and then compared among different groups (**I**, n = 4). **(J)** Human monocyte-derived macrophages were either untreated or treated with Huh7-TSN for 48 hours, in the presence or absence of G-1 (1 μM) or G15 (0.1 μM). Ki67$^+$ macrophages were assessed using flow cytometry (n = 6). The results shown are represented as mean ± SEM. *P* values were obtained using one-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant.

DNA synthesis in macrophages, we conducted an EdU incorporation assay, which revealed an elevated frequency of EdU^+ cells in macrophages exposed to TSN. However, this frequency was markedly reduced by the treatment of G-1 (Figures 3F, H). Additionally, the impact of GPER1 signaling on macrophage accumulation was examined using a CCK-8 assay. The results indicated that TSN increased the number of macrophages, while G-1 significantly decreased it (Figure 3I).

We further used a GPER1 specific antagonist G-15 to inhibit GPER1 signaling (30) that was activated by G-1. The results displayed that the suppression of macrophage proliferation mediated by G-1 was attenuated by G-15 treatment (Figure 3J and Supplementary Figure S3). In addition, we also examined the effect of E2 on macrophage proliferation, and the results showed that E2 inhibited TSN-induced macrophage proliferation, which was rescued by G-15 (Supplementary Figure S4).

Taken together, these data suggest that activation of GPER1 signaling restricts macrophage proliferation.

3.4 GPER1 signaling restrains macrophage proliferation by inhibiting the MEK/ERK/cyclin pathway

The PI3K/AKT and MEK/ERK pathways play crucial roles in regulating macrophage proliferation (14). Therefore, we investigated whether these signaling pathways are involved in the GPER1-mediated downregulation of macrophage proliferation. As expected, the levels of p-ERK and p-AKT significantly increased in macrophages exposed to TSN. G-1 treatment reversed the level of p-ERK, while the level of p-AKT was not significantly affected (Figures 4A, B). This indicates that GPER1 activation restricts macrophage proliferation by inhibiting the MEK/ERK pathway.

The ERK-mediated cyclin-dependent pathway is involved in cell-cycle progression and proliferation (31). Considering the reduced activity of DNA synthesis in TSN-treated macrophages after GPER1 activation (Figures 3F, H), we examined the expression levels of cyclins and cyclin-dependent kinases that regulate the entry of the DNA synthesis phase. Immunoblotting results showed that G-1 treatment significantly decreased the TSN-induced upregulation of cyclin D1, cyclin E1 and CDK4 (Figures 4C, D). These data suggest that GPER1 activation may restrict macrophage proliferation by downregulating the MEK/ERK/cyclin signaling pathway.

3.5 G-1 treatment inhibits macrophage proliferation and accumulation in HCC mouse models

Next, we investigated whether the activation of GPER1 signaling with G-1 affects macrophage proliferation and accumulation in mouse models of HCC. We found that G-1 treatment significantly suppressed tumor growth in a subcutaneous Hepa1-6 tumor model (Figures 5A–C). Flow cytometry analysis displayed a significant reduction in macrophage proliferation in G-1-treated tumors (Figure 5D). Immunofluorescence staining of tumor tissues showed that G-1 treatment significantly decreased both the proliferation and the density of macrophages compared to the control group

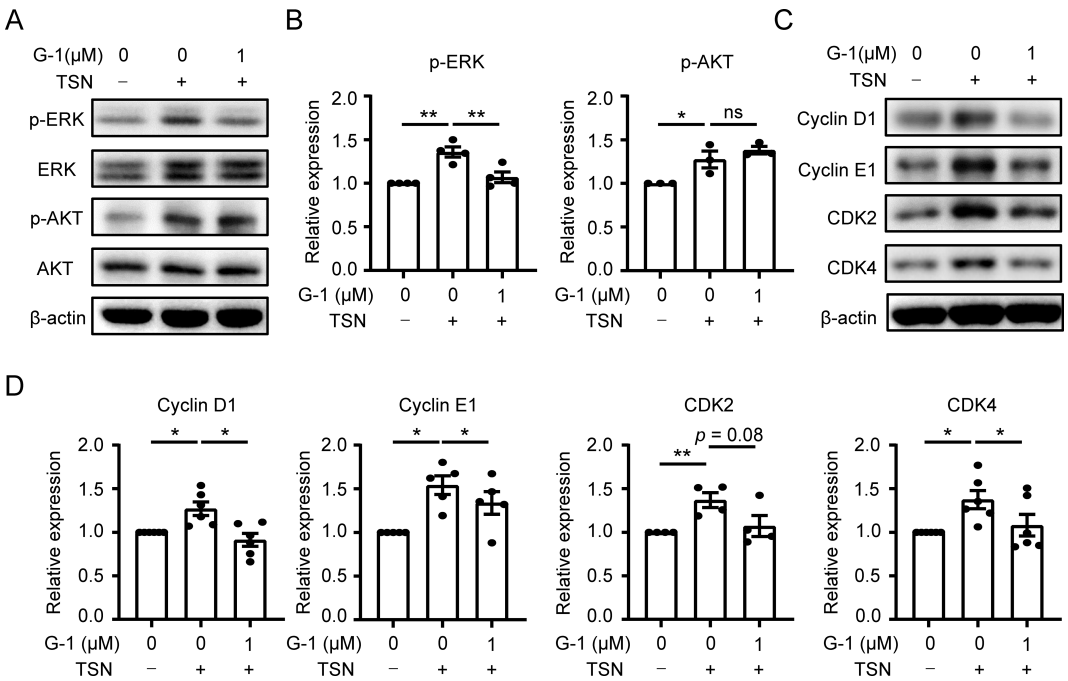

FIGURE 4
GPER1 signaling restrains macrophage proliferation by inhibiting the MEK/ERK/cyclin pathway. **(A–D)** Human monocyte-derived macrophages were either untreated or treated with 20% Huh7-TSN for 48 hours, in the presence or absence of G-1(1 μM). The levels of p-ERK, ERK, p-Akt, Akt, cyclin D1, cyclin E1, CDK2 and CDK4 were determined using immunoblotting **(A, C)**. Quantitative analysis of protein expression levels, normalized to β-actin, was performed and plotted (**B, D**, n = 4 or 6). The results shown in **(B, D)** are represented as mean ± SEM. *P* values were obtained using one-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$; ns, not significant.

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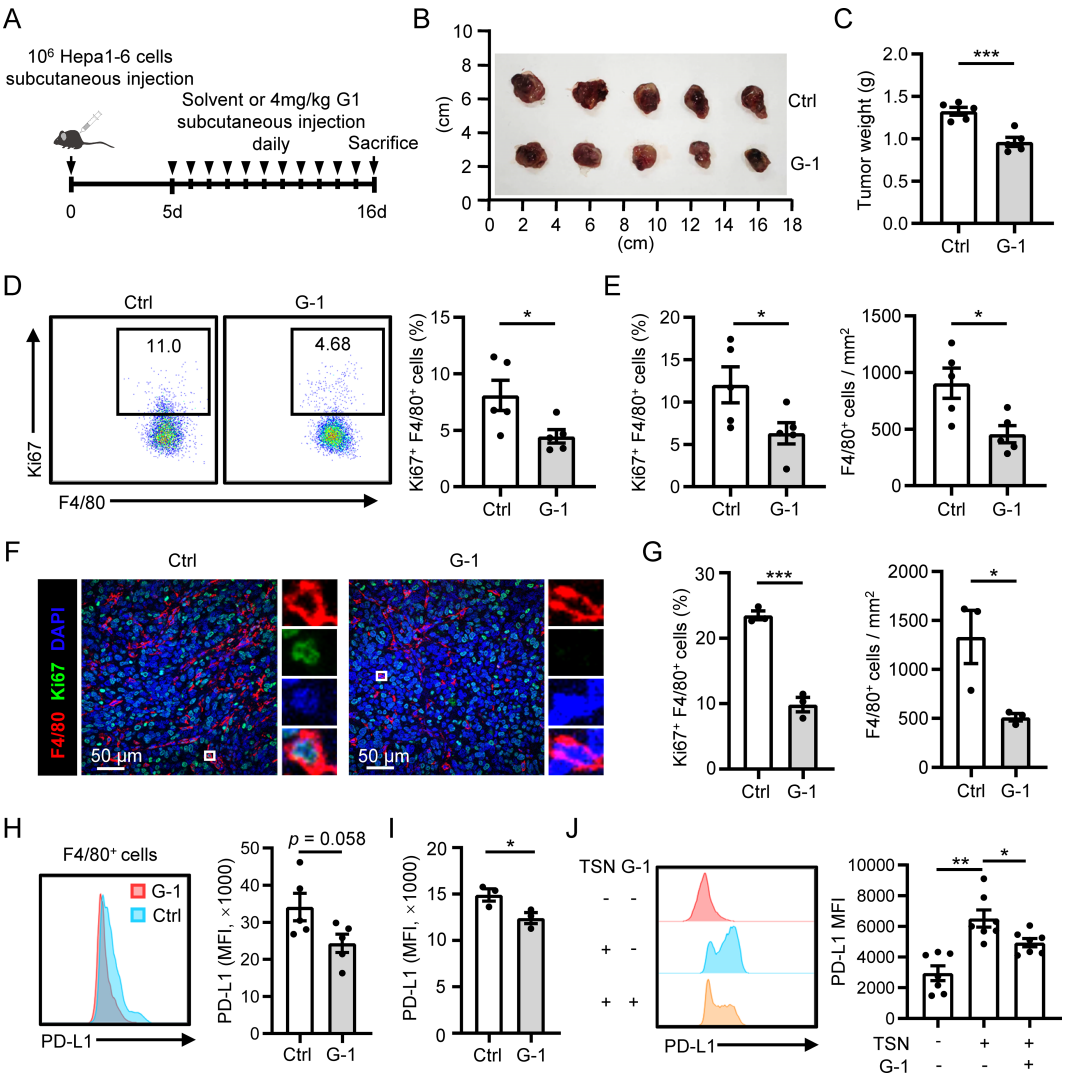

FIGURE 5
G-1 treatment inhibits macrophage proliferation and accumulation in HCC mouse models. **(A–F)** The subcutaneous tumor model was established (n = 5 for each group). Tumors were excised at the indicated time point, and their weights were analyzed **(B, C)**. Proliferation levels of F4/80$^+$ macrophages in the tumor tissues were examined using flow cytometry **(D)**. **(E, F)** Immunofluorescence staining of F4/80 and Ki67 was performed on tumor sections. The proliferation level or density of F4/80$^+$ macrophages was compared between the control and G-1 treated group. The scale bar is 50 μm. **(G)** The orthotopic tumor model was established (n = 3 for each group). Proliferation levels of F4/80$^+$ macrophages in the tumor tissues were examined using flow cytometry (left). Densities of F4/80$^+$ macrophages in the tumor tissues were analyzed using IHC (right). **(H, I)** PD-L1 expression on F4/80$^+$ macrophages in tumor tissues were examined using flow cytometry and compared between the control and G-1 treated group in the subcutaneous tumor model **(H)** and orthotopic tumor model **(I)**, respectively. **(J)** Human monocytes were left untreated or treated with TSN in the presence or absence of G-1 for 7 days, and the expression of PD-L1 was examined using flow cytometry (n = 7). The results shown are represented as mean ± SEM. For **(C–I)**, *p* values are obtained by nonpaired two-tailed Student's t test. One-way ANOVA with Tukey's multiple comparisons test was used for **(J)**.

(Figures 5E, F). This inhibition of macrophage proliferation and accumulation by G-1 was also confirmed in an orthotopic Hepa1-6 tumor model (Figure 5G). Furthermore, we found that the macrophages in G-1-treated tumors tended to exhibit a lower level of PD-L1 in both the subcutaneous (Figure 5H) and the orthotopic HCC model (Figure 5I). The decrease in PD-L1 expression was also observed on human macrophages cultured *in vitro* when treated with G-1 (Figure 5J). Collectively, these results suggest that activating GPER1 signaling by G-1 restricts the proliferation and accumulation of macrophages in HCC mouse models.

3.6 GPER1 expression positively correlates with the survival of HCC patients

To investigate the clinical significance of GPER1$^+$ macrophages, percentages of GPER1$^+$CD68$^+$ cells in HCC tumor tissues were determined using immunofluorescence staining and confocal microscopy analysis. The patients were divided into two groups based on the median percentage of GPER1$^+$CD68$^+$ cells. As shown in Figures 6A, B, patients with a higher percentage of GPER1$^+$ macrophages exhibited significantly longer OS and RFS compared

to those with a lower level. Multivariate analysis indicated that the percentage of GPER1$^+$ macrophages was an independent prognostic factor for both OS and RFS (Figures 6C, D, Supplementary Table S2). There was no obvious association between GPER1$^+$ macrophages and the clinical characteristics of the patients (Supplementary Table S3).

These findings suggest that the presence of GPER1$^+$ macrophages in HCC tumors predicts a better prognosis for HCC patients.

4 Discussion

The present study demonstrated the sex disparities in macrophage accumulation and proliferation in HCC tumor tissues. We found that GPER1, a 7-transmembrane G protein-coupled estrogen receptor, exhibited lower expression in self-replicating macrophages. When GPER1 signaling was activated through G-1 treatment, macrophage proliferation and accumulation were significantly suppressed in murine HCC tumors. Furthermore, patients with a higher percentage of GPER1$^+$ macrophages exhibited longer OS and RFS compared to those with a lower level. These findings highlight the significant role of intrinsic sex hormone receptor signaling within macrophages in regulating macrophage proliferation and accumulation in HCC.

Sex-related differences in the tumor immune microenvironment have been observed in various cancers and been considered as potential explanations for the disparities in cancer incidence, prognosis, and response to treatments between sexes (4). For instance, the androgens/AR signaling has been found to suppress T cell immunity against cancer in males, resulting in faster tumor progression compared to females in colorectal cancer and melanoma (10, 11). On the other hand, the estrogens/ERα signaling has been shown to skew the polarization of macrophages towards an immune-suppressive state, leading to sex-specific differences in response to immune checkpoint inhibitors (ICIs) in melanoma (9). However, the sex-related mechanisms in determining the composition and function of the tumor microenvironment of HCC remain elusive. Macrophages, which are a major immune component in tumor tissues, have been widely reported to promote the progression of various types of cancer, including HCC (32–34). In this study, we demonstrated a higher accumulation of macrophages in tumors from male patients in two independent HCC cohorts. We observed that the accumulation of macrophages in tumors could be reduced by selectively targeting the GPER1 signaling with G-1 in mouse models of HCC, suggesting a potential strategy to modulate the macrophage pool by targeting estrogen receptor signaling.

Multiple environmental cues and intrinsic pathways influence the accumulation of macrophages in tumor tissues (35). We and other groups have demonstrated that macrophage proliferation within tumor tissues is a significant characteristic of malignancy (14, 36, 37) and an independent prognostic factor for poor survival of HCC patients (14). Therefore, our current study focuses on the proliferation of tumor-associated macrophages. We found that macrophages from male HCC patients displayed a higher level of proliferation compared to those from females. It should be noted that chemotaxis is also a key factor in the accumulation of monocytes/macrophages in tissues. The regulation of chemokines has been discussed in several studies, such as the CCL2/CCR2 axis, which promotes the trafficking of monocytes/macrophages into

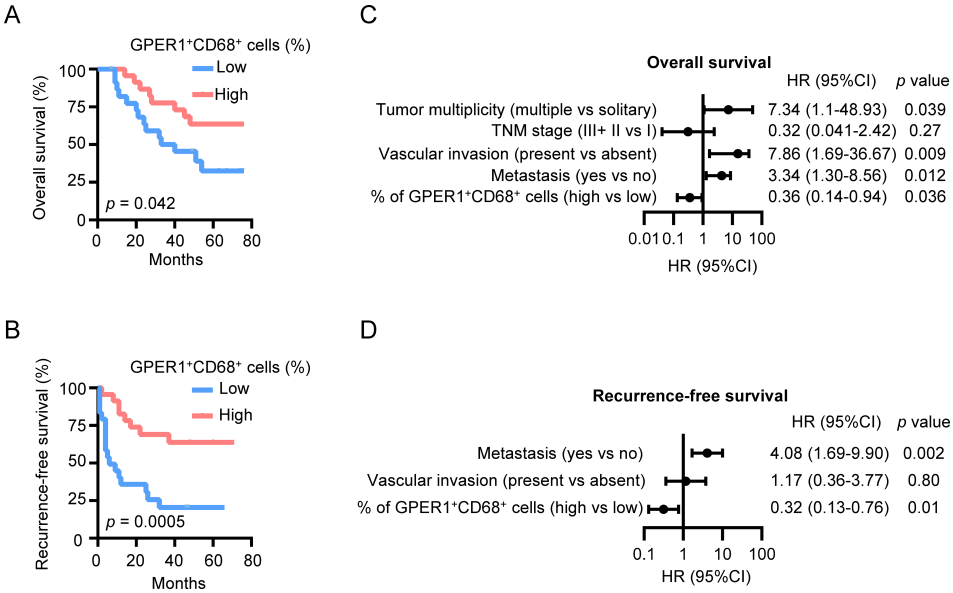

FIGURE 6
GPER1 expression positively correlates with the survival of HCC patients. **(A, B)** HCC patients were divided into two groups according to the median percentage of GPER1$^+$ macrophages among the total macrophages in the tumor tissues. The cumulative overall survival **(A)** and recurrence-free survival **(B)** of patients were analyzed using the Kaplan-Meier method and compared by the log-rank test (n = 48). **(C, D)** Forest plot illustrating the associations between overall survival **(C)** or recurrence-free survival **(D)** and the clinical characteristics of HCC patients. A multivariate cox proportional hazards regression model was applied, incorporating variables that exhibited a significant univariate association with the outcome ($p < 0.05$). HR, hazard ratio; CI, confidence interval. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

tumor tissues (38). Further exploration is warranted to determine whether sex disparities also contribute to these factors that influence the accumulation of macrophages in HCC tissues.

GPER1, a non-classical estrogen receptor, differs in structure, intracellular localization and functions from classical nuclear estrogen receptors (ERα, ERβ) (28). The estrogens/GPER1 signaling plays important roles in various physiological contexts and has been found to mediate sex differences in the incidence and severity of cardiovascular and autoimmune diseases (39–41). While the regulatory role of GPER1 in tumor cell proliferation has been explored in various cancers, including HCC (18, 19, 42), there has been less study on its effects on immune cell proliferation. In this study, we analyzed GPER1 expression in primary human macrophages *in vitro* and in HCC tumor tissues, and found lower levels of GPER1 in proliferating macrophages. Functional experiments demonstrated that macrophage proliferation was hindered by GPER1 specific agonist G-1. Moreover, we measured the levels of E2, the primary physiological ligand for GPER1, in the tumor tissues of HCC patients, and the results showed that E2 levels tend to be higher in female patients compared to males. Although the potential effects of G-1 treatment on tumor cells require further exploration, these findings may partially explain the aforementioned sex differences in macrophage proliferation and accumulation in HCC tumors, and highlight a novel role of GPER1 signaling in the regulation of the HCC microenvironment.

Several studies have shown that GPER1 interacts with other sex hormone receptors to regulate cell proliferation and function (43, 44). For instance, AR suppresses GPER1 signaling to promote cell proliferation in triple-negative breast cancer (43). Additionally, the balance between GPER1 and ERα plays a role in regulating vascular remodeling (44). We have observed that macrophages treated with TSN exhibited reduced expression of ERα which is another receptor for E2; however, no significant difference in ERα expression was found between proliferating and non-proliferating macrophages. It remains unclear whether this estrogen receptor can act antagonistically or synergistically with GPER1 to regulate macrophage function and phenotype.

In addition, we observed a decrease in PD-L1 expression on macrophages in the HCC mouse models treated with G-1. We also confirmed this effect in cultured human macrophages, suggesting that G-1 can modulate immune function. It has been reported that G-1 inhibits PD-L1 expression on the tumor cells of pancreatic ductal adenocarcinoma and melanoma, which enhances the efficacy of PD-1 targeted immunotherapy (45, 46). Clinical trials have recently been conducted on a GPER1 agonist called LNS8801, specifically for its use in combination therapies with ICIs in cancer treatment. These trials have demonstrated that LNS8801 exhibits a favorable safety profile when used alone or in combination with pembrolizumab (47, 48). Therefore, gaining a deep understanding of the influence of estrogen receptor signaling in modulating the tumor microenvironment may help in designing novel therapeutic strategies and selecting patients who may benefit from them.

In conclusion, our study provides insights into the role of intrinsic GPER1 signaling within macrophages in regulating their accumulation and function in the tumor microenvironment of HCC, and underscores the potential to develop tailored therapies for HCC treatment by considering the sex-related disparities among patients.

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 studies involving humans were approved by the Institutional Review Board of Sun Yat-sen University Cancer Center. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Institutional Animal Care and Use Committee of Sun Yat-sen University (Guangzhou, China). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YY: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Formal analysis, Project administration. YCW: Investigation, Methodology, Writing – review & editing. HZ: Investigation, Writing – review & editing, Project administration. ZL: Investigation, Writing – review & editing. WC: Writing – review & editing, Investigation. ZH: Writing – review & editing, Investigation. YLW: Writing – review & editing, Investigation. XY: Writing – review & editing, Resources. JX: Writing – review & editing, Funding acquisition, Conceptualization, Visualization. LZ: Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1481972/full#supplementary-material

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The role of macrophage migratory behavior in development, homeostasis and tumor invasion

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Tumor-associated macrophages (TAMs) recapitulate the developmental and homeostatic behaviors of tissue resident macrophages (TRMs) to promote tumor growth, invasion and metastasis. TRMs arise in the embryo and colonize developing tissues, initially to guide tissue morphogenesis and then to form complex networks in adult tissues to constantly search for threats to homeostasis. The macrophage growth factor, colony-stimulating factor-1 (CSF-1), which is essential for TRM survival and differentiation, is also responsible for the development of the unique motility machinery of mature macrophages that underpins their ramified morphologies, migratory capacity and ability to degrade matrix. Two CSF-1-activated kinases, hematopoietic cell kinase and the p110δ catalytic isoform of phosphatidylinositol 3-kinase, regulate this machinery and selective inhibitors of these proteins completely block macrophage invasion. Considering tumors co-opt the invasive capacity of TAMs to promote their own invasion, these proteins are attractive targets for drug development to inhibit tumor progression to invasion and metastasis.

KEYWORDS

tumor-associated macrophages, motility, invasion, HCK, PI3K p110δ, breast cancer, melanoma

1 Introduction

Tumor-associated macrophages (TAMs) are now widely understood to play a range of mostly deleterious roles in cancer progression. In doing so, TAMs recapitulate behaviors of normal macrophages during embryogenesis, homeostasis and repair. Macrophage behaviors such as growth factor secretion, immune regulation, extracellular matrix (ECM) remodeling and guidance of other cells in developing and healing tissues are subverted by cancers to encourage their growth and dissemination (1, 2).

While macrophages have long been known for their phagocytic and host defense capacities, more recently we have come to understand that they have many non-immune roles, some common to all macrophages and some highly specific to their tissue of residence (3–5). These

tissue resident macrophages (TRMs) integrate tightly into all tissues with their heterogeneous phenotypes reflecting different environments and demands. TRM specification begins very early in embryogenesis soon after macrophage precursors (pMacs) migrate into developing organs and differentiate in response to local cues (6). Differentiated TRMs also migrate within tissues during development to guide formation of structures such as the mammary gland ductal network (7). In adult organisms, TRMs patrol their local territory to maintain tissue homeostasis and initiate wound repair by either migrating through or extending long dendrites or shorter finger-like pseudopodia into tissue structures (8, 9). Many of the same mechanisms regulating interstitial migration also control dynamic cell projections in macrophages, which express a unique set of motility molecules for this purpose (9, 10). In invasive cancer, the migratory activity of TAMs is hijacked by tumor cells with TAMs guiding tumor cells out of the tumor and into surrounding tissue, thereby recapitulating embryonic TRM behavior (11, 12).

Many excellent reviews have recently been published on the general biology of TAMs in cancer, including a masterful historical overview of TAMs and their many roles in cancer promotion (2). Rather than undertaking a comprehensive overview of how macrophage behaviors are subverted in cancer development and progression, this review examines the role of macrophage motility in normal development and homeostasis and, with a particular focus on the mammary gland, how cancers co-opt this core function to enable local tumor invasion, which leads to distant metastasis.

2 Macrophage biology

The mechanisms by which TRMs contribute to tissue development and homeostasis indicate how TAMs contribute to tumor invasion and metastasis. The need for macrophages in normal development was first revealed by the discovery of multiple congenital abnormalities in organisms lacking expression of either the primary macrophage growth factor, colony-stimulating factor-1 (CSF-1), or its receptor (CSF-1R) (13–16). Macrophages were subsequently shown to colonize embryonic tissues very early to help shape organogenesis and then help maintain tissue homeostasis and restore it after various disturbances (3, 5, 17). In response to local cues, newly arrived TRMs functionally integrate with parenchymal cells to carry out both common core housekeeping functions and highly tissue-specific functions. TRMs make up 8-18% of tissue mass in adult tissues (18). After tissue-specific adaptations, TRMs can undergo additional phenotypic changes in response to perturbations such as injury, infection and disease. In other words, macrophages are chameleon-like in their ability to respond to both short and long term cues in their host tissue. With this finely tuned responsiveness to the local environment, it is not surprising that macrophages are co-opted in a number of ways by disease processes, including cancer.

2.1 Macrophage ontogeny

Before fate mapping approaches revealed that macrophages arise from several distinct hematopoietic origins in the embryo, all macrophages were thought to be derived from pluripotent hematopoietic stem cells (HSCs) that differentiated into progenitor cells of the mononuclear phagocytic lineage under the influence of a cocktail of hematopoietic factors, CSF-1 being the most important (19, 20). According to this model, highly proliferative progenitor cells in the bone marrow differentiate into circulating monocytes that enter tissues where they differentiate into macrophages. Once *in situ*, macrophages were considered incapable of further proliferation and were replenished by incoming monocytes (19). However, over the last 15 years, fate mapping studies have revolutionized our understanding of macrophage biology, using lineage markers of macrophages or their progenitors to demonstrate that embryonic TRMs arise from non-monocytic yolk sac macrophage progenitors and fetal liver-derived monocytes (2, 6, 21–23). These embryonic TRMs are long-lived and proliferate locally to maintain numbers (22, 24). Indeed, some TRMs, notably microglia and Langerhans cells, rely entirely on life-long self-renewal although they can be replaced by monocytes if profoundly depleted (23, 25, 26). Distinct TRM populations in the same organ can demonstrate different replacement kinetics with embryonically-derived Kupffer cells replaced by self-renewal in the healthy liver while liver capsular macrophages are monocyte-derived (5, 17, 27). In contrast, macrophages in the gut and dermis, which undergo rapid turnover, rely on circulating monocytes to maintain their numbers (28, 29). In a fitting twist that exemplifies the developmental role of macrophages, embryonic macrophages shape the architecture of the hematopoietic niche for HSCs in the fetal liver and the adult bone marrow such that their depletion leads to premature differentiation of HSCs (30).

Unlike most organs, development of the mammary gland largely occurs postnatally (31). A study that used CD11b as a marker of mammary gland macrophages revealed persistence of fetal macrophages in the stroma of the postpubertal mammary gland (32). However, a CD11b-/Cd11c+ ductal macrophage population was recently identified lying between the luminal and basal ductal epithelial cells in mouse mammary ducts (33, 34). The same intraductal population of TRMs is seen between the epithelial layers in human mammary ducts and their branched morphology is very different to that of the large, circular macrophages seen within the duct lumen (Figures 1B, D). Compared to stromal macrophages, ductal macrophages form a small proportion of TRMs in the virgin mouse mammary gland but expand 40-fold during pregnancy, through both local proliferation of embryonic macrophages and recruitment of bone marrow-derived monocytes, before decreasing to baseline numbers in involution (33). Lineage tracing was used to show that initially both stromal and ductal TRMs are embryonically derived with stromal macrophages slowly replaced over time while embryonic ductal macrophages are largely replaced by monocyte-derived macrophages during puberty after which they self-renew (33, 34). In general, however, circulating monocytes do not act as a supply reservoir for most TRM populations in steady-state. Rather they are recruited in large numbers to sites of inflammation, infection or injury then typically disappear unless inflammation persists (5, 27). As 'wounds that never heal', tumors attract and retain monocyte-derived TAMs, often in huge numbers if the tumor

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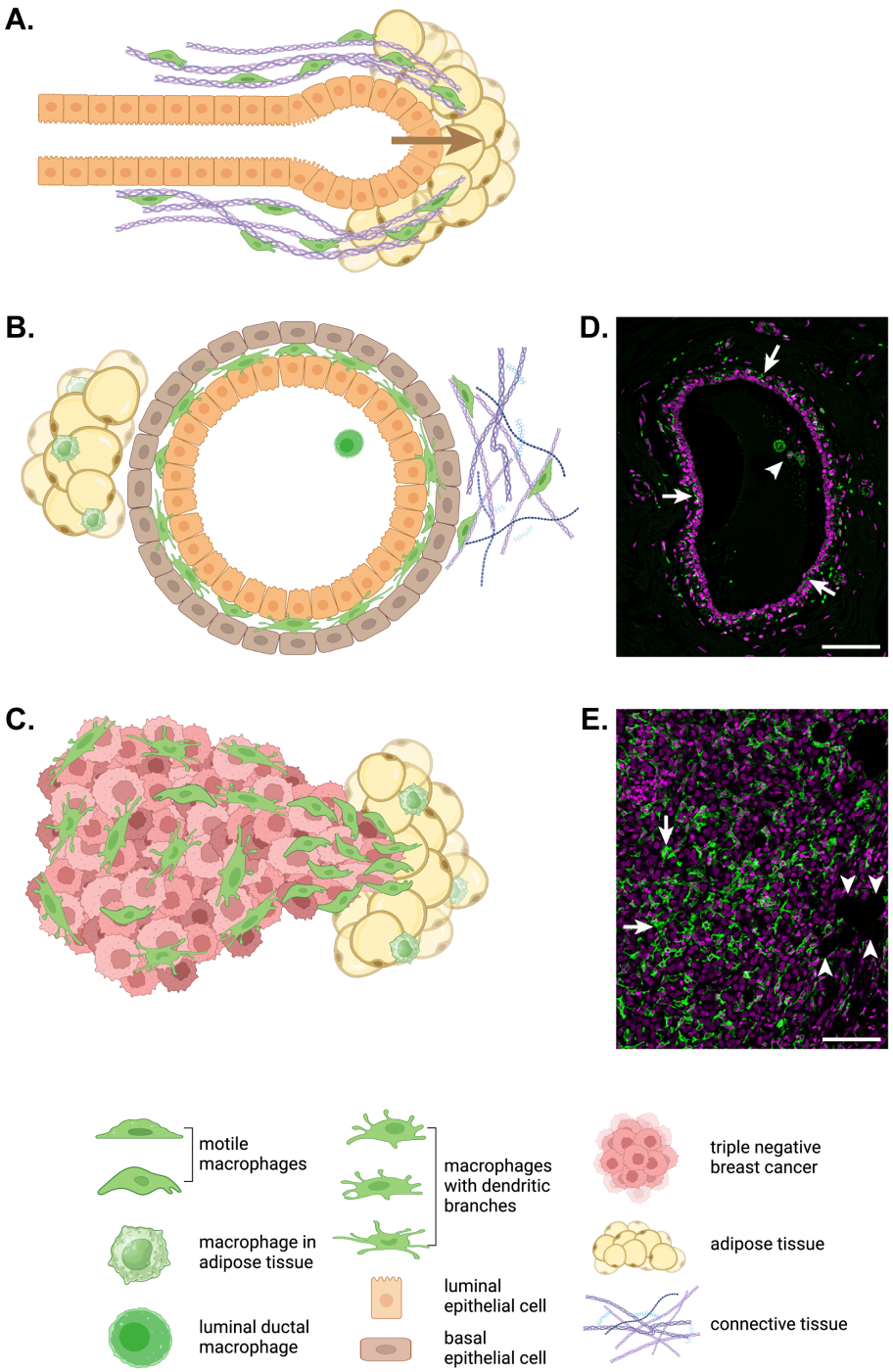

FIGURE 1
Macrophage motility in development, homeostasis and breast cancer. **(A)** In the developing mammary gland, terminal end bud outgrowth and elongation into the mammary fat pad is guided by macrophages (green) that move along and remodel collagen fibrils surrounding the developing duct. **(B)** In the adult mammary gland, elongated and branched macrophages (green) are found intercalated between the luminal and basal epithelial layers of mammary ducts embedded in a mix of adipose and connective tissue with their own tissue resident macrophage populations. While ductal macrophages do not migrate through tissue, their dendritic branches routinely patrol the ductal epithelium (33). **(C)** In invasive triple negative breast cancer, macrophages (green) accumulate in large numbers particularly at the invasive front. For the immunofluorescent immunohistochemistry images of a normal human mammary duct **(D)** and human triple negative breast cancer **(E)**, ionized calcium binding adaptor molecule (IBA)1+ macrophages are shown in green and nuclei are magenta. Arrows indicate ductal macrophages and the arrowhead points to a group of luminal macrophages in **(D)**. In **(E)**, arrows indicate TAMs and arrowheads point to adipocytes at the invasive front. Scale bars represent 100μm. The schematic diagrams in this figure were created in BioRender.com.

cells secrete CSF-1 and other macrophage or monocyte chemokines such as CCL2 (35, 36).

2.2 TRM plasticity: specification, activation, morphology and function

While lineage tracing and single cell technologies have confirmed the extraordinary heterogeneity of TRMs, striking morphological differences between tissue-specific macrophages such as Kupffer cells, microglia and alveolar macrophages had long been recognized (37). Morphologically disparate TRMs also exist within organs, for example ramified microglia, spindle-shaped meningeal macrophages and stellate choroid plexus macrophages in the brain, reflecting the niche-specific demands placed on TRMs (38, 39). As noted earlier, embryonic tissues are colonized by pMacs, which express a set of core macrophage genes under the influence of CSF-1 and the macrophage lineage-determining factor PU.1 (5, 6). As pMacs migrate into tissues, they rapidly differentiate into tissue-specific TRMs in response to local cues, the process driven by upregulated expression of tissue-specific TRM lineage determining factors (6, 40–42). Hence, Kupffer cells upregulate ID3, microglia SALL1 and alveolar macrophages PPARγ with a host of other lineage determining factors driving specialization in other TRM populations (5, 6, 39, 43).

After tissue specific adaptation, macrophages can undergo additional phenotypic changes in response to exposure to external cues such as cytokines, microbial products and other modulators (44). For some time, these changes were believed to occur in a binary fashion with a 'classical' or M1 phenotype developing in response to interferon (IFN)γ or toll-like receptor (TLR) ligands and an 'alternatively activated' or M2 phenotype arising after exposure to interleukin (IL)-4, which was thought to reflect the transition from inflammation to repair (45). However, this is now known to be a very simplistic representation of the full range of macrophage activation states in response to a panoply of modulators. Transcriptional analyses of human macrophages activated *in vitro* by a diverse range of stimuli or in different murine TRMs *in vivo* indicate that many distinct gene expression changes occur between each macrophage population (40, 46). Indeed, the full spectrum of macrophage activation states is very complex and appears to be of limited use in the context of human health and disease (1, 2). Yet the oversimplified classification of TAM activation phenotypes into M1-like or anti-tumoral and M2-like or pro-tumoral unfortunately lingers despite strong evidence of TAM phenotypic diversity in a range of different cancers such as breast cancer and glioblastoma (47–49).

Morphological changes are central to TRM differentiation and specialization. Microglia form a highly ramified, regularly spaced network in the brain (37, 38). Several other TRM populations create similarly complex tissue surveillance networks such as epidermal Langerhans cells whose dendrites and migration towards lymph nodes resulted in their misidentification as dendritic cells for decades (50). Early immunohistochemical studies also demonstrated highly dendritic morphologies in bone marrow stromal macrophages (37). Similar if less complex membrane extensions are seen in other TRMs such as Kupffer cells, which use finger-like extensions to sample liver sinusoidal fluid, and lymph node subcapsular macrophages, which extend fingers upwards into the subcapsular space to capture antigens as well as long branches downwards into underlying follicles to interact with B cells (51, 52). Even yolk sac pMacs have a stellate morphology (18). While it is difficult to observe dynamic behavior of TRM dendritic networks deep in tissues, high resolution live imaging in the mammary gland has shown that mammary ductal macrophages move their dendrites constantly to survey the entire ductal epithelium within a two hour cycle (33). Similarly ramified ductal macrophages can be seen lying between the luminal and basal epithelial layers of the collecting ducts and in lobules in human breast tissue. Dynamic TRM responses to injury have also been captured by intravital imaging in the peritoneum (53).

However specialized, TRMs still carry out core macrophage functions such as phagocytosis and immune surveillance (3). Migration is also an essential core function, which is used by pMacs to colonize embryonic tissues and by differentiated TRMs to guide tissue morphogenesis (9). Although mature TRMs, considered by some to be sessile, may no longer move through tissues routinely, dynamic dendrite movement in interstitial or sinusoidal spaces is unceasing and, when tissue injury occurs, TRMs can extend pseudopods to cloak microlesions and limit inflammation or move into larger wounds to orchestrate repair (33, 53, 54). Thus, TRMs retain the capacity for interstitial migration, which can be coopted to facilitate tumor invasion.

The molecular mechanisms that underpin formation of protrusive membrane structures in macrophages such as the leading edge of a migrating cell, a phagocytic cup or a probing dendrite are similar and involve actin polymerization and coordinated formation of specialized adhesions to enable rapid responses (9, 55). TRMs selectively express a complex array of adhesion and actin cytoskeletal remodeling proteins to enable them to extend, maintain and restructure these processes (9). Moreover, TRMs are embedded in ECM and express a huge number of matrix metalloproteinases (MMPs) and cathepsins to enable protease-dependent mesenchymal migration (10, 56). CSF-1R signaling is essential for the expression of this unique set of motility and matrix degrading proteins as evidenced by the myriad changes in expression of genes regulating adhesion, actin cytoskeletal remodeling and matrix degradation seen with CSF-1-induced differentiation of non-adherent progenitor cells into mature, adherent macrophages (9, 10). The dependence on CSF-1R signaling for macrophage motility is underscored in zebrafish with an inactivating *Csf1r* mutation. Yolk sac-derived macrophage progenitors in the mutant zebrafish are unable to migrate into the cephalic mesenchyme to become microglia (15). Hence, to acquire full tissue-specific functionality, TRMs require CSF-1R signaling as well as niche-specific signals.

2.3 CSF-1R signaling to macrophage morphology and motility

CSF-1R signaling is essential for TRM survival and self-renewal as well as differentiation, morphology and function, as demonstrated by the almost total depletion of most TRM populations following administration of a CSF-1R blocking antibody (20, 57). CSF-1 was originally considered the only CSF-1R ligand but the more severe developmental abnormalities of the CSF-1R-deficient mouse led to the discovery of an additional ligand, IL-34 (16, 58). However, CSF-1 drives the expansion of the majority of TRMs required for normal development and homeostasis, microglia and Langerhans cells excepted (14, 59). The striking effects of CSF-1 on macrophage morphology are easily observed in bone marrow-derived macrophages *in vitro*. CSF-1 triggers actin polymerization and adhesion formation to cause ruffling and spreading within a minute followed by further spreading, polarization and finally migration over the ensuing 10 minutes (60–62). The CSF-1R is a class III receptor tyrosine kinase that autophosphorylates multiple tyrosine residues to create binding sites for docking and activation of downstream signaling proteins (20, 63). A macrophage cell line system with individual CSF-1R tyrosine mutants was used to identify two autophosphorylated CSF-1R tyrosine residues, Y721 and Y974, primarily responsible for triggering signals to macrophage motility (62, 64, 65).

Loss of signaling from Y721 in the CSF-1R greatly reduces CSF-1-induced actin polymerization and adhesion formation, resulting in a striking reduction in macrophage motility (62). CSF-1R pY721 binds and activates the class IA phosphatidylinositol 3-kinase (PI3K) to produce a rapid pulse of PI 3,4,5-trisphosphate (PIP3) at the leading edge membrane (62, 66). PIP3 then triggers membrane translocation of pleckstrin homology domain-containing molecules such as AKT/PKB to activate growth, survival and proliferation as well as migration signals (67, 68). The three catalytic isoforms of PI3K, ubiquitous p110α and p110β and hematopoietically restricted p110δ, all of which are expressed by macrophages, have non-redundant biological roles and isoform selective inhibitors indicate that only PI3K p110δ activates CSF-1-induced macrophage motility and matrix degradation signals (68–70). Reflecting the importance of macrophage motility on tumor invasion, PI3K p110δ inhibition also completely blocks co-migration and invasion of co-cultured macrophages and tumor cells in an *in vitro* invasion model (36). Although the precise motility pathways downstream of PI3K p110δ have not been fully elucidated, AKT, Rho family GTPases Rho, Rac and Cdc42, along with Src family kinases (SFKs) regulate actin cytoskeletal remodeling and phosphorylation of adhesion proteins such as paxillin and leupaxin (9, 10). However, because PI3K-activating motility signaling involves bifurcating pathways, direct inhibition of PI3K p110δ is likely to be a more successful strategy to target macrophage motility.

Adhesion and motility in macrophages are also regulated by SFKs (65, 71). Macrophages express no less than five SFKs, each of which has overlapping and unique functions (65). Expression of HCK and LYN increases as macrophages differentiate from non-adherent precursors under the influence of CSF-1 while SRC and FGR decrease (10). CSF-1R pY974-based signaling regulates at least some of these changes as FGR expression is dramatically increased in CSF-1R Y974F mutant macrophages, which spread and move slowly in response to CSF-1 (65). Of the SFKs expressed in macrophages, only HCK and LYN associate with the CSF-1R, HCK in a CSF-1 dependent manner, suggesting it transduces signaling from the activated CSF-1R (65). Confirmation that HCK is the primary SFK transducing the CSF-1R motility signal in macrophages was provided by the observation that macrophages expressing constitutively active HCK move faster, digest matrix more efficiently and encourage greater tumor cell invasion *in vitro* than control macrophages while a HCK selective inhibitor, RK20449, blocks motility, degradation and invasion of both control and constitutively active HCK macrophages (72). Constitutive activation of HCK also drives increased invasion *in vivo* in a gastric tumor model (72). Thus, HCK is an attractive target for macrophage motility inhibition as a therapeutic strategy.

2.4 CSF-1R signaling and macrophage motility in the mammary gland

The importance of CSF-1R signaling and macrophage motility is evident in the developing mammary gland. During puberty, mammary epithelial structures called ductal terminal end buds grow into the mammary fat pad then elongate and branch to fill the fat pad with a complex ductal tree (Figure 1A) (31). CSF-1-dependent TRMs are recruited in large numbers to the neck of terminal end buds to help guide ductal morphogenesis as ductal length and branching are reduced in CSF-1-deficient female mice while transgenic over-expression of CSF-1 produces increased branching (31, 73, 74). Intravital imaging has shown that mammary gland macrophages associate with collagen fibers found alongside growing terminal end buds and that these macrophages migrate along the fibers and fuse shorter fibers to promote their elongation, thereby shaping ductal outgrowth into the mammary fat pad (Figure 1A) (7). Mammary macrophages also shape lobular morphogenesis during the estrous cycle and pregnancy and phagocytose apoptotic epithelial cells during involution (75). There are large increases in ductal TRM numbers during puberty and pregnancy to facilitate these processes (32, 33). Importantly, ductal and not stromal macrophages are thought to be co-opted by tumor cells to become TAMs in breast cancer (33, 34).

3 TAMs and tumor progression

Tumors are aberrant organs with their own integrated populations of resident macrophages known as TAMs. Indeed, most solid tumors contain large numbers of TAMs with a high correlation between TAM density and poor outcome in many types of cancers in humans, including breast cancer (76–79). Single cell transcriptomic studies have confirmed both the abundance and heterogeneity of TAMs within and between tumor types in a range

of human cancers, including breast cancer (43, 79–82). Consistent with these observations, TAM density is striking highly in triple negative breast cancer, which has the lowest survival of breast cancer subtypes (Figures 1C, E) (82, 83). Furthermore, macrophage heterogeneity is increased in tumors compared to nearby normal tissue (81).

It is now well understood that TAMs co-evolve with cancers and contribute to their development and progression in several ways, including support of tumor growth through production of growth factors, promotion of angiogenesis through secretion of pro-angiogenic factors and immunosuppressive effects on the adaptive immune system (2, 49, 84–87). However, perhaps the most lethal contribution TAMs make to tumor progression is their promotion of tumor invasion and metastasis (2, 88–90). Consistent with this notion, TAMs have been shown to accumulate at the invasive front of breast cancers (Figures 1C, E) (91).

3.1 CSF-1R signaling in TAMs and tumor invasion

The importance of CSF-1R signaling in cancer progression was originally hinted at by the association of high circulating levels of CSF-1 with poor outcomes in breast, ovarian and endometrial cancers and further supported by the co-localization of CSF-1 expressing carcinoma cells with CSF-1R+ TAMs in invasive breast cancer (91–94). Confirmation that CSF-1-dependent macrophages promote tumor progression, particularly to invasion and metastasis, was provided by an experimental model in which the CSF-1-deficient osteopetrotic mouse was crossed with the polyoma middle T (PyMT) mouse, an autochthonous model of breast cancer to produce CSF-1-deficient PyMT mice. Multifocal mammary tumors arise and progress steadily to pulmonary metastasis in the female mice and, while initiation and early progression of mammary tumors are unchanged, late stage progression is slowed and pulmonary metastasis is all but halted in the absence of CSF-1-dependent TAMs (88, 95). Inhibition of pulmonary metastasis is due in part to the failure of tumor cells to disrupt the basement membrane unless macrophages are present (88, 89). This is because TAMs set up a paracrine chemokine interaction with tumor cells to activate tumor invasion via a mechanism of relay chemotaxis (89). Tumor cells secrete CSF-1 and TAMs secrete epidermal growth factor (EGF) to enable both cell types to co-migrate along collagen fibers in an alternating fashion (11, 89). Notably, either CSF-1R or EGFR inhibition signaling completely stop invasion of both TAMs and tumor cells (89, 96).

In vitro live imaging of co-cultured mammary tumor organoids and bone marrow-derived macrophages enables a closer examination of relay chemotaxis and reveals that tumor cell invasion from the organoids only occurs after motile macrophages that had previously exited the organoid make contact with the tumor cells to activate their motility then lead them into the surrounding matrix (36). Flow cytometric analysis of the invasive cells revealed a 3:1 ratio of tumor cells to macrophages (36). In this co-invasion assay, selective inhibition of either HCK or PI3K p110δ are equally as effective as CSF-1R inhibition in shutting down macrophage-led tumor cell invasion while macrophages expressing constitutively active HCK promote increase tumor cell invasion (36, 72). Consistent with a requirement for matrix degrading activity in invasive macrophages that lead tumor cells out of tumors, upregulation of cathepsin protease activity increases the invasion-promoting activity of TAMs (97). Underlining the central role of motility in this interaction between macrophages and tumor cells, gene expression studies of co-migrating tumor cells and TAMs show upregulation of motility genes in the tumor cells with upregulation of trophic genes in the already motile macrophages (98, 99). Thus, motile TAMs appear to be an essential component of tumor cell invasion in at least some invasive tumors such as breast cancer. In a form of what has been labeled 'oncofetal reprogramming', interstitial migratory TAMs recapitulate the motile and matrix remodeling behavior of embryonic TRMs and activate tumor cell motility to lead them through the basement membrane and into nearby tissue (100). This leads to the notion that not only does TAM-dependent invasive activity lead to metastatic spread due to incidental breaching of blood or lymphatic vessels but, by releasing physical constraints on primary tumor growth, invasive TAMs contribute to primary tumor growth, i.e. invasive growth.

3.2 TAM ontogeny and heterogeneity

Until the identification of self-renewing embryonic TRMs, all TAMs were thought to be derived from circulating monocytes. It is important to note, however, that maintenance of TRM populations through self-renewal relies on steady state conditions as perturbations such as extensive tissue injury or experimental macrophage depletion can lead to replacement of TRMs by circulating monocytes that differentiate into TRMs, albeit with distinct phenotypes (2, 6, 23, 101). As flagged earlier, monocytes are also recruited in large numbers to sites of inflammation and, since chronic inflammation is a consistent feature of cancer, it is not surprising that monocyte-derived TAMs are found in large numbers in many tumors (5, 35). However, as TRMs are proliferative, TAMs can also arise from local TRM populations. Consistent with this possibility, parabiotic studies in a mouse model of pancreatic ductal carcinoma showed that embryonic TRM-derived TAMs appear to predominate and they drive the strong fibrotic response so typical of these tumors (102). In contrast, TAMs in spontaneous PyMT mammary tumors are predominantly monocytic in origin (103). Other tumor types display a mix of monocyte-derived and TRM-derived TAMs, for example early non-small cell lung cancers contain mostly TRM-derived TAMs that are gradually replaced by monocyte-derived TAMs as the tumor progresses, and brain cancer (86, 104). Thus, it would appear that monocytes and TRMs account for distinct proportions of TAMs in different mouse models of cancer and these proportions can change over time as the cancers progress (101, 105).

TAM ontogeny in human tumors is more difficult to tease apart for obvious reasons. Nevertheless, a meta-analysis of single cell transcriptomic data from lung, colon and liver cancers and nearby

normal tissues demonstrated an increase of monocyte-derived macrophages compared to normal tissue, although TRMs contributed to TAM numbers in liver cancer (43). Consistent with the largely postnatal development of the mammary gland, lineage tracing and other approaches used to map the ontogeny of TAMs in experimental models indicate that TAMs are largely monocyte-derived in breast cancer. In the PyMT mammary tumor model, TAMs are phenotypically distinct from TRMs and are recruited from the bone marrow through tumor cell secretion of CSF-1 and the monocyte chemokine CCL2 (36, 101, 103). Continuous seeding of monocyte-derived TAMs was also demonstrated in three additional models of breast cancer (47). Whether monocyte-derived TAMs also predominate in human breast cancer is currently not clear, due in part to TAM heterogeneity. Nevertheless, it is likely that the majority of TAMs in human breast cancer are monocyte-derived while TRMs contribute to one or more TAM subtypes (106). Metastasis-associated macrophages are functionally distinct from primary tumor TAMs and are also predominantly monocyte-derived and recruited by tumor cell-secreted CCL2 in the PyMT and other models of breast cancer that give rise to pulmonary metastases (2, 107). However, as both monocyte-derived TAMs and TRM-derived TAMs have the capacity to proliferate, it is likely that both populations contribute to the abundant TAMs that accumulate in human tumors and their metastases, with the balance of each contribution differing between tumor types (2).

Just as there are diverse TRMs within individual organs, human tumors contain heterogeneous TAMs. A study comparing breast and endometrial cancer revealed that TAMs within tumors are more diverse than and distinct from TRMs in neighboring normal tissue and also that they differ between tumor types (79). Considerable inter-patient variation in TAM abundance and phenotypes is also seen in individual cases of human breast cancer (81). Similarly, distinct transcriptomic profiles of diverse TAM subtypes can be seen across many different human cancer types (80, 87). Spatial transcriptomics has added yet more complexity to the classification of TAMs and this has been further complicated by evidence that progressive differentiation of TAMs can occur within tumors. For example, invasive TAMs that co-migrate hand in hand with tumor cells out of tumors transition into sessile perivascular TAMs in the vicinity of blood vessels in PyMT breast cancers (108). Similarly, in an orthotopic PyMT model of breast cancer, the adipose tissue-rich environment of the mammary gland induces a lipid-associated phenotype in all TAM clusters whereas this phenotype only occurs in specific subtypes in other cancers (Murrey at al., under review) (82, 109, 110). Thus, the influence of the tumor environment and nearby tissues on TAM phenotypes is critical. Moreover, while transcriptomic analysis might classify particular TAM subtypes as pro-angiogenic or immunosuppressive, other subtypes also express angiogenic and immune suppressing genes.

Despite this heterogeneity, consistent subtypes are found across different tumor types such as angiogenic TAMs that accumulate in hypoxic, necrotic regions and immunosuppressive TAMs that inhibit cytotoxic T cells and NK cells and recruit immunosuppressive regulatory T cells (2, 47, 111). Because of this, two groups recently attempted to develop a consensus nomenclature for TAM molecular subtypes using single cell and spatial transcriptomic data extracted from several pan- cancer data sets. Six subtypes plus proliferating TAMs were identified with broad agreement across four subtypes - interferon-primed/interferon-mediated regulatory TAMs, immune regulatory TAMs, inflammatory TAMs and proangiogenic TAMs – with disagreement on whether lipid-associated TAMs constitute a specific subtype, which may reflect the adiposity of the tumor environment (109, 110).

A couple of additional points regarding single cell studies of TAM heterogeneity deserve consideration. Firstly and relevant to interpretations of mouse models of cancer, TAM heterogeneity is significantly greater in spontaneously arising PyMT breast cancers, which develop within ductal tissue, than in orthotopic PyMT tumors, which develop as a ball of tumor cells outside the normal ductal architecture (34). This is an important consideration as the former reflects the natural history of human breast cancer and because ductal macrophages are believed to be the TRM population that contribute to breast cancer development (33, 34). Secondly, concomitant expression of M1 and M2 markers in various TAM subtypes across several studies indicates that the concept of anti-tumoral M1-like and pro-tumoral M2 TAMs-like is well and truly outdated (80, 81).

3.3 Therapeutic targeting of TAMs

The broad range and prolonged effects of the tumor promoting activities of TAMs have made them obvious therapeutic targets. In addition, TAMs interfere with patient responses to chemotherapy and radiotherapy as cytotoxic therapies result in increased macrophage infiltration or altered TAM behavior (112–115). One particular mechanism of therapeutic interference was revealed when paclitaxel treatment of PyMT mice was shown to increase tumor cell secretion of CSF-1 and IL-34 and addition of a CSF-1R inhibitor to the treatment regime reduced tumor growth and rates of pulmonary metastasis (116). TAMs can also interfere with treatment response by directly secreting growth and angiogenic factors to maintain tumor cell survival and, through their promotion of immune evasion, they are potent inhibitors of responses to immunotherapies (117, 118).

Because macrophages depend on CSF-1 for survival, TAM drug development was initially focused on depleting them through inhibition of the CSF-1R, either by small molecule inhibitors or antibodies targeting the CSF-1/CSF-1R axis. CSF-1R inhibition reduces tumor growth in several mouse models of cancer, including PyMT-driven mammary cancer, cervical cancer, glioblastoma and melanoma (119–121). Surprisingly, CSF-1R inhibition in the glioblastoma model does not reduce TAM numbers but alters their phenotype from pro-tumoral to anti-tumoral in response to tumor secretion of granulocyte macrophage (GM)-CSF (120). However, while a CSF-1R blocking antibody is clinically useful in tenosynovial giant cell tumors driven by constitutive synovial CSF-1 production, clinical trials of CSF-1R inhibitors as single agents have proven disappointing (118, 122). Moreover, long term administration of these agents also depletes

TRMs with adverse consequences and there is evidence that TAMs can also promote cytotoxic T cells responses such that wholesale TAM ablation can reduce anti-tumoral immunity (118, 123).

Hence, rather than eliminating TAMs altogether, attention turned towards blocking monocyte recruitment to tumors or reprogramming TAM behavior within tumors (111, 118). Monocyte recruitment can be inhibited by targeting either CCL2 or its receptor, CCR2. However, a CCL2 neutralizing antibody did not show any clinical benefit in a trial of advanced solid cancers and more recent CCL2 inhibitor trials have also been disappointing, perhaps because monocytes continue to be recruited by alternative chemokines (118, 124). Concerningly, interruption of CCL2 inhibition in several metastatic mouse models of breast cancer models appears to accelerate bone marrow monocyte release and increase the rates of metastasis and death, suggesting that caution should be exercised with CCL2/CCR2 inhibitor development (125).

Thus, a number of TAM drug development programs have been directed towards targeting specific pro-tumoral behaviors of TAMs. For example, tumor cells express a 'don't eat me signal' CD47, which interacts with TAM-expressed signal regulatory protein (SIRP)α to stop tumor cells from being phagocytosed (126). Antibodies targeting CD47 enhance tumor cell phagocytosis to reduce tumor growth in xenograft models and a number of clinical trials of anti-CD47 antibodies and small molecule inhibitors have produced good results in lymphomas (127). However, their therapeutic use has been limited by adverse hematological effects (127). Although a range of other TAM-reprogramming therapeutics are currently under development, they are not reviewed here (118, 128). Considering the lethal contribution of TAMs to tumor invasion and metastasis, it is worth examining whether TAM migration could be targeted to inhibit tumor invasion. As outlined earlier, TAMs depend on the CSF-1/CSF-1R axis not only to stimulate their migration via PI3K p110δ/AKT and HCK signaling but also to acquire the molecular machinery supporting macrophage interstitial migration. There are several clues that macrophage motility plays an important role in tumor growth and invasion and that targeting macrophage motility might be a useful therapeutic approach. Firstly, selective inhibition of either PI3K p110δ or HCK with idelalisib or RK20449 respectively shut down both motility and matrix degradation in macrophages *in vitro* (65, 70, 72). These findings can be extended in a complex *in vitro* co-invasion assay using mammary tumor spheroids pre-infiltrated with macrophages, where tumor cell invasion is completely blocked by inhibition of macrophage motility signaling (36, 72). Finally, both RK20449 or acalisib, another PI3K p110δ inhibitor, produce striking reductions in orthotopic PyMT mammary tumor growth *in vivo* (Murrey et al., manuscript submitted) (129). Inhibition of macrophage motility is, therefore, an alternative and potentially powerful approach to therapeutically target the invasion and metastasis-promoting behavior of TAMs.

4 Concluding comments

We now understand that macrophages have critical immune and non-immune functions in the body, beginning in embryogenesis and lasting throughout life. Embryonic macrophages infiltrate into every tissue and organ system where they rapidly differentiate into highly specific TRMs that contribute to normal development and then actively monitor the local environment for signs of perturbation of homeostasis. In order to undertake regular and comprehensive tissue surveillance as well as activate repair mechanisms, TRMs either move or extend dynamic dendritic branches to explore their regions of responsibility. CSF-1 is the most important cytokine regulating TRM survival, differentiation and migration. Tumors, which are aberrant organs, secrete CSF-1 to recruit monocyte-derived macrophages as well as subvert the normal housekeeping activities of TRMs to promote tumor invasion and metastasis, angiogenesis, immunosuppression and resistance to cytotoxic therapies. Therefore, it is not surprising that TAMs form a compelling target for drug development in the treatment of cancer, especially in combination with other therapies. While blockade of the CSF-1R itself has not proven helpful in the clinic except for tenosynovial giant cell tumors, the many deleterious behaviors of TAMs can be specifically targeted, including the particularly dangerous effect they have on tumor invasion and metastasis.

Author contributions

MM: Data curation, Writing – review & editing. IN: Writing – review & editing. FP: Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

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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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