CELLULAR DYNAMICS WITHIN THE TUMOR MICROENVIRONMENT

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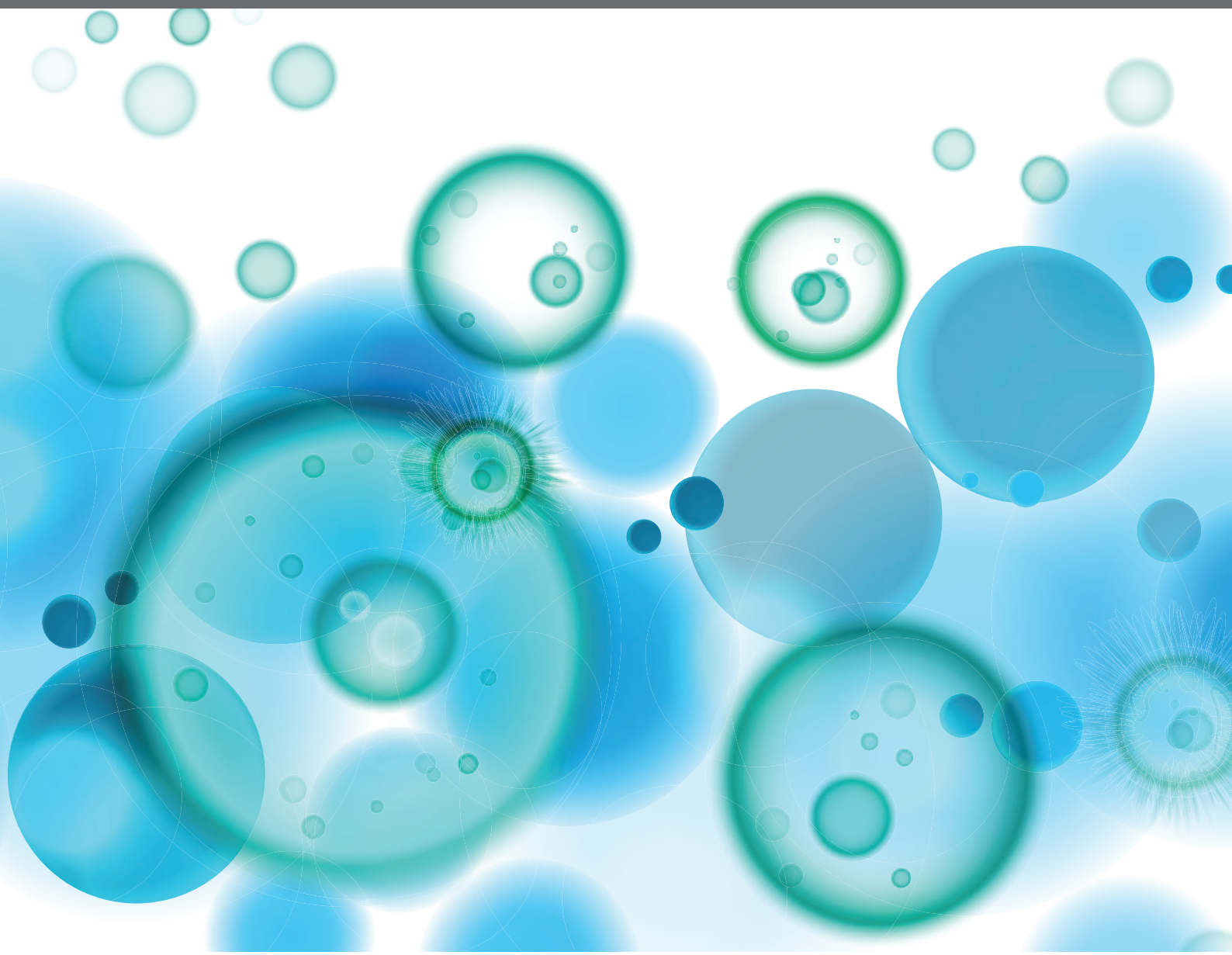

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CELLULAR DYNAMICS WITHIN THE TUMOR MICROENVIRONMENT

Topic Editors:
Daniela F. Quail, McGill University, Canada
Leila Akkari, The Netherlands Cancer Institute (NKI), Netherlands

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REVIEW
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Immune Tumor Microenvironment in Breast Cancer and the Participation of Estrogen and Its Receptors in Cancer Physiopathology

*Mariana Segovia-Mendoza and Jorge Morales-Montor**

Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico

Breast cancer is characterized by cellular and molecular heterogeneity. Several molecular events are involved in controlling malignant cell processes. In this sense, there is an overriding importance to study the multiple cell alterations within this pathology. That the immune response can vary depending on sex is a widely identified fact. Steroid hormones and their receptors may regulate different functions and the responses of several subpopulations of the immune system. Few reports are focused on the function of estrogen receptors (ERs) on immune cells and their roles in different breast cancer subtypes. Thus, the aim of this review is to investigate the immune infiltrating tumor microenvironment and the prognosis conferred by it in different breast cancer subtypes, to discuss the current knowledge and to point out the roles of estrogen and its receptors on the infiltrating immune cells, as well as to identify how different immune subsets are modulated after anti-hormonal treatments in breast cancer patients.

Keywords: immune infiltration, breast cancer, estrogen receptor, estrogen receptor inhibitors, tumor microenvironment

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****Correspondence:***
Jorge Morales-Montor
jmontor66@biomedicas.unam.mx

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INTRODUCTION

Breast Cancer and the Microenvironment of Infiltrated Immune Cells

Breast cancer is the most frequently diagnosed malignancy in women worldwide, and it represents the second most common cause of cancer deaths (1). Epidemiological studies have indicated that steroid sexual hormones play important roles in the initiation and progression of breast cancer. Other risk factors are also associated with this disease such as diet, ethnic differences, age, early menarche, not bearing children, having a first pregnancy at over 30 years of age, obesity, genetic mutations, exposure to oral contraceptives, consumption of alcohol or cigarettes, and environmental contaminants, among others. It is estimated that more than 1,000,000 women are diagnosed with breast cancer every year, and more than 410,000 will die from the disease (2, 3). The above indicates that breast cancer represents an important worldwide health problem.

On the other hand, breast cancer is a heterogeneous disease, which is traditionally classified into three phenotypes: luminal [estrogen receptor (ER) positive], human epidermal growth factor receptor type 2 (HER2)-positive, and triple negative (ER-negative/HER2-negative) (4). Moreover, breast cancer is characterized by a highly inflammatory microenvironment, which is supported by the infiltrating immune cells, cytokines, and growth factors (5, 6). In addition, immune infiltration

of breast tumors has been shown to be related to clinical outcome through the modulation of treatment response. Breast tumors with immune infiltration are associated with different patterns based on ER presence; however, a common negative immune feature is that regulatory T cells (T regs) are associated with poor prognosis in both ER-positive and ER-negative breast tumors, conferring an immunosuppresive environment (7, 8). Such a feature is a characteristic that highlights the importance of the immune tumor microenvironment in breast cancer.

With respect to other infiltrating immune cells in breast cancer phenotypes, a strong proportion of natural killer cells (NK) and neutrophils have been found in ER-positive breast tumors, while cytotoxic T cells ($TCD8^+$) as well as naïve and memory T cells ($TCD4^+$) are found in smaller proportions. Interestingly, eosinophils and monocytes are associated with a good response after chemotherapy, and B lymphocytes are also associated with good prognosis in this phenotype. Recently, activated mast cells have additionally been correlated with good prognosis (9). However, the presence of this population is still controversial (10). Moreover, in this phenotype, tumor-associated macrophages (TAMs) 1 and 2 and T reg lymphocytes displayed poor prognosis due to their inflammatory, immunosuppressive, and pro-tumorigenic roles (11–14). In ER-negative breast tumors, the major component of immune infiltration cells are T regs, TAM2, and activated mast cells, which are also associated with negative prognosis. In contrast, $TCD4^+$, $TCD8^+$, B lymphocytes, and dendritic cells (DCs) are related to better prognosis, but they are found in lower numbers and can be associated with a favorable response to neoadjuvant chemotherapy (7, 14–21). With respect to the HER2-positive breast cancer type, there are not many reports about the infiltrating immune mass. However, it is mainly represented by DCs, mast cells, γδ T lymphocytes, T regs and neutrophils—interestingly, all of them confer poor prognosis, disease relapse, and metastasis in this phenotype (see **Figure 1**) (14, 22, 23).

This intra-tumoral immune pattern establishes a complex relationship between the heterogeneity of immune infiltrating cells, the tumor phenotype, and the treatment response in breast cancer.

ESTROGEN SIGNALING AND ESTROGEN EFFECTS IN BREAST CANCER CELLS

Estradiol (17β-estra-1,3,5 (10)-triene-3,17-diol) E2 is a steroid hormone produced by theca and a granulosa cell in the ovaries. E2 regulates several physiological and pathological processes, including cancer. Classical or genomic E2 signaling is mainly mediated by two isotypes of the receptor: ERα and ERβ, both of which are nuclear transcription factors that bind to their specific ligand or several estrogens in general; and, subsequently, they form homo- or heterodimers that bind to estrogen response elements (EREs) contained in the promoter region of specific genes in order to activate or suppress their expression. These actions are mediated by the recruitment of distinct co-activators or co-repressors or through the interaction with other transcription factors (**Figure 2**) (24). E2 actions are also mediated by other non-classical pathways, known as ligand-independent ERα signaling, by a membrane-anchored receptor called G protein-coupled estrogen receptor 1 (GPER1), in which target gene transcription occurs through second messengers and several transcription factors. Thus, GPER1 mediates the increase of different second messengers such as cyclic adenosine monophosphate (cAMP) and diacilglycerol (DAG) levels, mobilization of intracellular calcium (Ca^{2+}), and the activation of extracellular signal-regulated kinase (ERK)1/2 and the phosphoinositide 3-kinase (PI3K/AKT) pathways by the trans-activation of the different growth factor receptors (GFRs). Moreover, activation of GPER1 can induce the release of several growth factor ligands such as heregulin, which results in a direct activation of GFRs, depicted in **Figure 2** (25–28). It is important to mention that different antagonists or ER inhibitors, such as ICI182,780 and tamoxifen, can mimic the effects of estradiol and induce GPER1 activation.

In breast cancer, E2 can act in different ways. For instance, in immortal cell lines of breast cancer, E2 via ERα signaling is seen to stimulate proliferation, while ERβ activation inhibited cell proliferation and promoted apoptosis (29, 30). Interestingly, estrogen can also undergo several metabolic processes, and its metabolites exert genotoxic effects that contribute to the development of breast cancer through adduct DNA formation (31–33). Many reports on the effects of E2 in breast cancer cells have reported the transcriptional modulation of different genes that are affected; among which are proliferation regulators, growth factors, cell cycle, and apoptotic modulators (29, 34, 35).

Importantly, both classic and membrane ERs have been implicated in several effects of immunity and autoimmunity (36, 37). It is known that the immune system shows remarkable sex-differential responses; thus, this fact potentially suggests that sex hormones such as estrogens address these events. Following this, many reports mention that women respond more aggressively to self-antigens, being more susceptible to autoimmune diseases through of the activation of ER signaling (38). In general, ERs participate in many immune system functions—ERα has been

Abbreviations: BPA, bisphenol A; cAMP, cyclic adenosine monophosphate; CAT, catalase; CG, cathepsin G; Da, daltons; DAG, diacilglycerol; DCs, dendritic cells; DNA, deoxyribonucleic acid; E2, estradiol; ERK, extracellular signal-regulated kinase; ER, estrogen receptor; EREs, estrogen response elements; G-CSF, granulocyte colony-stimulating factor; GFRs, growth factor receptors GPER1; GM-CSF, granulocyte macrophage colony stimulating factor; GPER-1, G protein-coupled estrogen receptor 1; GSH-Px, glutathione peroxidase; GSTP, glutathione S-transferase P; HER2, epidermal growth factor receptor type II; IFN-γ, interferon-gamma; Ig, immunoglobulin; IL, interleukin; iNOS, inducible nitric oxide synthase; IRF4, interferon regulatory transcription factor 4; LPS, lipopolysaccharide; MCs, mast cells; MCP-1, monocyte chemo-attractant protein 1; MMPs, metalloproteinases; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NF-κB, nuclear factor-B; NK, natural killer cells; NO, nitric oxide; PAMPs, pathogen-associated molecule patterns; PI-9, proteinase inhibitor 9; PI3K/AKT, phosphoinositide 3-kinase; PR3, proteinase 3; PTGS2, prostaglandin-endoperoxide synthase; ROS, reactive oxygen species; SERM, selective estrogen receptor modulator; SOCS3, suppressor of cytokine signaling 3; SOD, superoxide dismutase; TAMs, tumor-associated macrophages; TET1, ten-eleven-translocation 5-methylcytosine dioxygenase; TCD4, helper T cells; TCD8, cytotoxic T cells; TGF-β, tumor growth factor beta; TLRs, Toll-like receptors; TNFα, tumor necrosis factor alpha; T regs, regulatory T cells; VEGF, vascular endothelial growth factor.

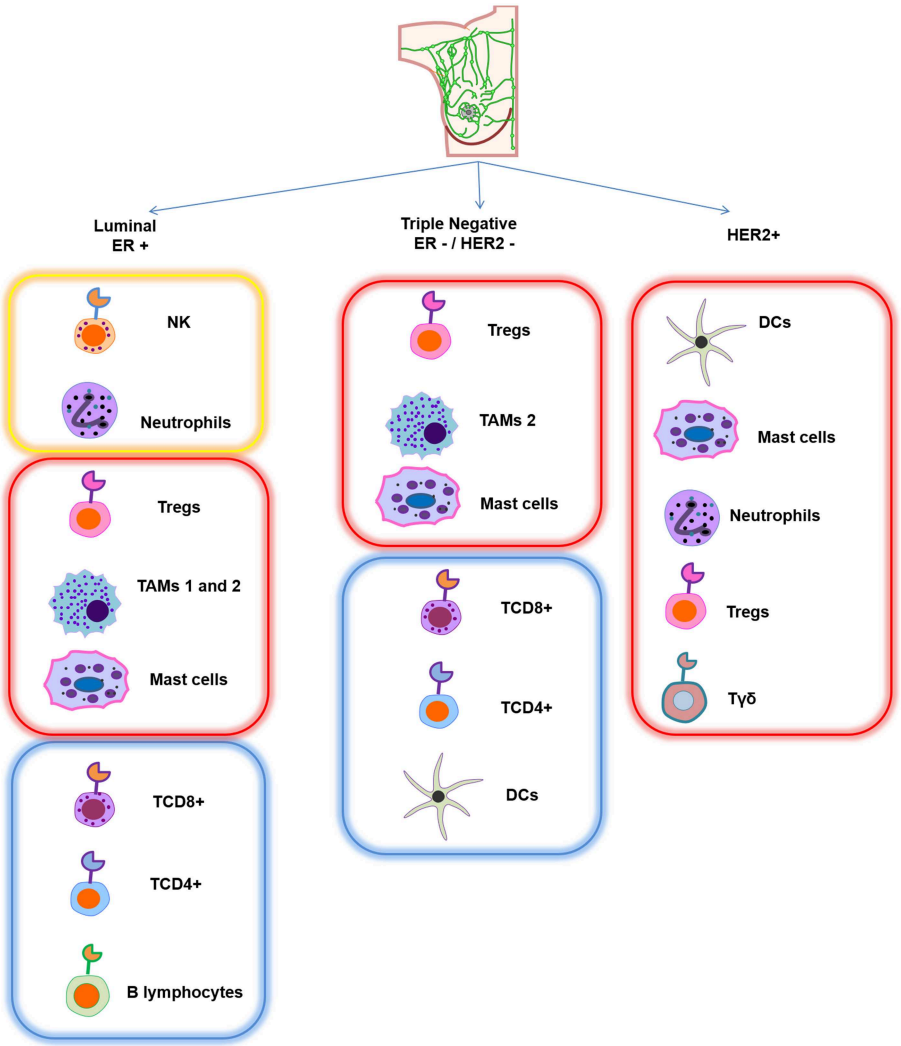

FIGURE 1 | Schematic representation of the main infiltrating immune cell pattern in different breast cancer subtypes. Each subtype has a different composition of immune cells. Yellow frame represents strong presence of specific immune cells that confer good prognosis, red frame indicates that this infiltrating signature is associated with poor prognosis, and blue frame corresponds to a lower proportion of immune cells, which is also associated with good prognosis.

related to spleen and thymus function while ERβ is important for bone marrow functions (24). Both types of ERs are expressed on innate and adaptive immune system cells, indicating an important role for this hormone and its receptor signaling regarding correct immune performance (39).

We describe below the modulation of the most common tumor-infiltrating immune cells by estradiol action upon binding to its receptors in these immune cells of the tumor microenvironment.

ESTROGEN EFFECTS ON IMMUNE SYSTEM CELLS

ER in Dendritic Cells (DCs)

DCs are involved in several processes such as immune tolerance, autoimmunity, stimulation, and differentiation of naïve T cells. They are considered as potent antigen presenting cells (APCs) and are mainly activated by stress or damage signs from pathogens that are recognized mainly by Toll-like receptors (TLRs). Following their stimulation via TLRs, DCs secrete pro-inflammatory cytokines to stimulate T lymphocytes and initiate innate immune response. In this sense, ER participates in the favoring of DC function. These cells contain the presence of ERs; when its ligand binds to ERs in these cells it can trigger migration and activation processes. In addition, in mouse *in vitro* models of DCs, estrogen can induce differentiation, survival, and increase the expression of co-stimulatory molecules (39). It has been reported that pre-treatment of E2 in co-cultures of mature DCs with T cells resulted in the stimulation of T cell proliferation (40). Besides, E2 up-regulates the expression and secretion of different pro-inflammatory cytokines and chemokines such as tumor necrosis factor alpha (TNFα), interleukin (IL)-6, CXCL-8 (IL-8), and monocyte chemo-attractant protein 1 (MCP-1) (40). This concept can be directly related to the improvement

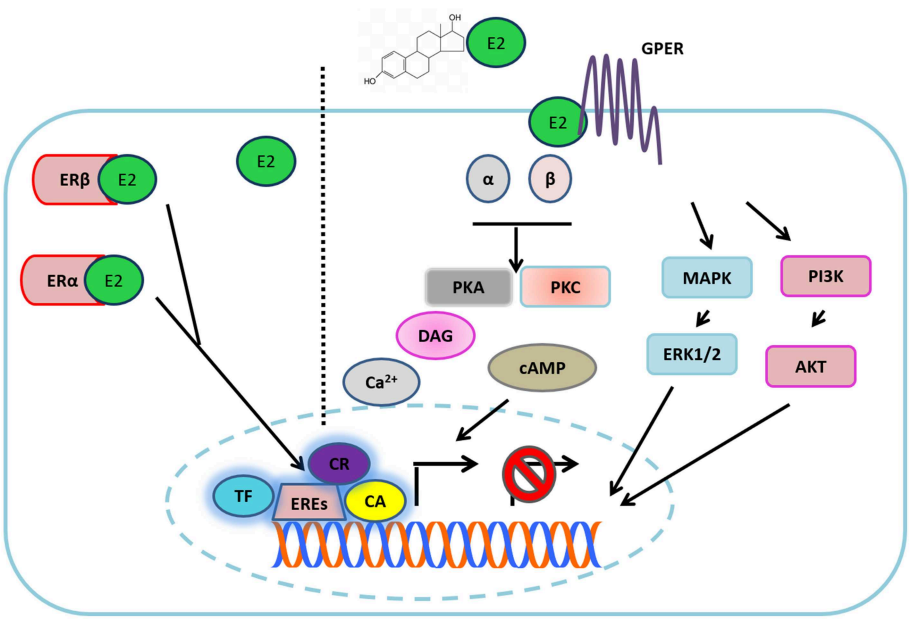

FIGURE 2 | Estradiol signaling. Estradiol (E2) can bind to its different receptors to activate the genomic pathway or the non-genomic pathways. In the first one, E2 binding to ERα and ERβ, each complex is directed to the nucleus where it joins with EREs in the DNA, recruiting different transcription factors (TF), co-activators (CA), or co-repressors (CR) in order to activate or suppress the transcription of target genes. In the non-genomic pathway, E2 binds to GPR30, triggering the activation of G proteins. The above turns out in the increase of different second messengers (cAMP, Ca^{2+}, DAG). Additionally, E2 can activate different growth factor receptor (GFR) activity through the non-genomic pathway, which results in the activation of different downstream signaling pathways (MAPK and PI3K) and in the release of different ligands of GFRs.

of DCs' capability to mediate the presentation of self and foreign antigens, and, potentially because of this, the immune system response against tumors is better in early stages of the disease. Nevertheless, the presentation process is disrupted by E2, since after hormone exposure, production of INF-γ and IL-2 is decremented in mature DCs (41). This suggests that the effects of E2 in DCs depend on their maturation stage. Thus, it would be interesting to determine the degree and phenotype of DC maturation in tumors. In addition, differentiation of functional DCs from bone marrow can also be modulated by this hormone since it favors their migration to lymph nodes, an effect that was reverted with the use of specific ERα antagonist (ICI 182,780) (42–44). Supporting this notion, E2 induces myeloid DC differentiation through the activation of two inflammatory-related proteins, the interferon regulatory transcription factor 4 (IRF4) and the participation of granulocyte macrophage colony stimulating factor (GM-CSF). Interestingly, it was reported that the exacerbated activation of these two factors by E2 at some point can lead to a tolerogenic phenotype for DCs (45). The association of ERα with other proteins such as thiolase and glutathione S-transferase P (GSTP) is also linked with DC differentiation. In addition to this, metabolic function, several growth factors, and accessory proteins in bone marrow derived from mice DCs are also affected. On the contrary, the absence of GSTP enhanced DCs' metabolism, their proliferative and differentiation rates, and their effector functions (46). It is important to note that not only does E2 have effects in DCs, an estradiol metabolite, estriol also generated tolerogenic DCs in an *in vivo* model that protects against autoimmunity (47). The above highlights the need to monitor the effects of ER inhibitors on different immune cell functions, favoring not only the inhibition of cancer cells but also the migration of the immune cells to lymph organs or avoiding their anergic phenotype.

ER in Macrophages (Mø)

Macrophages are a fundamental part of the innate defense mechanisms against foreign pathogens, and they can promote specific immunity by inducing T cell recruitment and activation. Their role is essential for triggering adaptive immune response. Macrophages collaborate with T and B cells based on the release of cytokines, chemokines, and reactive radicals, among other proteins. Despite this fact, their presence within the tumor microenvironment has been associated with enhanced tumor progression and promotion of cancer cell growth, angiogenesis, and immunosuppression (11, 48).

Several articles have reported the presence of ER in monocytes and macrophage precursor cells (49, 50), that the expression of this hormone receptor varies between stages of differentiation, and that monocyte expresses ERβ while macrophages express ERα (51). Recently, however, both receptors have been found in macrophages (52). E2 treatment has been shown to modulate different macrophage actions and their metabolism; for example,

it is well-known that production of nitric oxide (NO) into the macrophages allows them to exert antimicrobial and antitumor actions (53). Related to this concept, hormone treatment stimulated NO release in human peripheral monocytes and in a murine macrophage cell line via GPER activation coupled with intracellular calcium influx (54, 55). In line with this, stimulation with LPS in isolated peritoneal macrophages from young female rats resulted in elevated NO release; this effect was not observed in macrophages derived from the middle-aged animals, where circulating E2 levels were diminished (56). Moreover, macrophages produce and use arachidonic acid and its different metabolites for the recognition of pathogens and to enhance or suppress inflammatory response (57). E2 has been shown to modulate the lipid metabolism of macrophages since it elicits an increase of arachidonic acid release and prostaglandin E2 production (a derivative of arachidonic acid) in human monocytic cell lines (58). In addition, the phagocytic activity of macrophages is performed in part by reactive oxygen species (ROS)—which cause DNA or cell membrane damage—and the interplay between intracellular ROS and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) is important in the macrophage phagocytic function, activation, differentiation, and recruitment process (59). In this context, it has been reported that E2 administration in rats modulated CAT activity in *ex vivo* macrophages (60). Part of the bacterial killing mechanism of macrophages induced by LPS is the activation of metalloproteinases (MMPs); gene expression of MMP-9 especially was dramatically reduced after E2 treatment in rat cell lines of macrophagic origin, and this effect was blocked with ICI 182,720 treatment (61). This hormone also modulates macrophage survival, and this effect was reported in an *in vitro* culture of human macrophages where E2 treatment induced the anti-apoptotic protein Bcl-2, action mediated by the modulation of the intracellular Ca^{+2} concentration, the activation of protein kinase C, and ERK phosphorylation (62, 63). Furthermore, macrophages can recognize distinct pathogen-associated molecule patterns (PAMPs) which contributes to activating several signaling cascades and diverse cytokines and chemokines (64). E2 via ERα reduced gene and protein expression of the pro-inflammatory IL-8 in monocytes previously challenged by LPS (65). The modulation of this chemokine impacts not only the macrophage's function but also the neutrophil's recruitment to inflammation sites, mediating pathogen clearance (66). E2 can also modulate other functional macrophage cytokines; its treatment decreased IL-6, TNF-α, and IL-1β expression in whole blood cultures derived from healthy postmenopausal women, in bone marrow cell cultures, and in *ex vivo* rat macrophages (56, 67–70). The modulation of these cytokines was confirmed to be an E2-dependent effect, according to the opposite event found in these cells when they were treated with ICI 182,780 (69). A similar result from E2 treatment related to the decreased expression of the TNF-α gene was reported in an ER-positive murine monocytic cell line through of the down-regulation of Jun NH (2)-terminal kinase activity, with a consequent decrease of AP-1 transcription factor, affecting TNF-α transcription (71). In addition, E2 modulates the macrophage's activation (72), which is mainly classified into two categories: classical activation (macrophages kill microbes and act as anti-tumor effector cells), which is promoted by IFN-γ, TNF-α, and TH2-related cytokines or alternative activation (macrophages lay down extracellular matrix components to promote wound healing, angiogenesis, and sustain tumor progression). This type of macrophage activation is promoted by TH1 cytokines, being an IL-4/IL-13-dependent mechanism (73). The effect of this hormone in macrophage activation was clearly observed in a murine wound healing model in ovariectomized mice. In this sense, macrophages coming from ovariectomized animals show preferentially a classical activation. In addition, the gene expression of two alternative activation macrophages markers (Fizz1 and Ym1) was reduced, and the ovariectomized mice also presented a reduction in both macrophage numbers in the wound area and the inflammatory environment through the reduction of monocyte-associated TNF-α secretion as compared with the intact group. In contrast, E2 supplementation in ovariectomized mice restored the expression of both markers, leading to alternative macrophage activation, wound repair, remodeling, and angiogenesis (72). Furthermore, the alternative macrophage activation promoted by E2 has been documented in other assays. With respect to this notion, the gene expression of arginase 1, another established alternative activation macrophage marker, was up-regulated with ERα agonist treatment in an *in vitro* culture of bone marrow-derived macrophages (74). This work also evaluated the role of E2 in wildtype or in mice with ERα and inflammatory gene deletion (LysM-ERα) subjected to incisional wounds with a subsequent exogenous E2 replacement. Of note, in the absence of the hormone, healing was delayed (74) as has been previously reported in an ovariectomized wildtype mouse model (72). However, the hormone treatment revealed increased recovery in healing response, whereas in ERα knockout mice it resulted in a marked healing delay. The above highlights the role of estradiol-ERα action in the induction of alternative macrophage activation (74). Additionally, the role of E2 in favoring alternative macrophage activation was corroborated in an *in vitro* and *ex vivo* study on human blood-derived macrophages. In fact, classical lipopolysaccharide (LPS)/IFN-γ stimulus on un-polarized macrophages induced the down-regulation of two markers of alternative activation (CD163 and CD206); these effects were avoided through treatment via the modulation of NFκB transcription factor (75). Interestingly, much evidence supports the notion that macrophages, especially alternatively activated macrophages, shape immune tumor infiltration and have influence in high vascular grade associated with metastasis (76–79). In this sense, breast cancer phenotype can also regulate the type of infiltrating macrophage phenotype (80). Current evidence suggests that this population of macrophages regulates at the same time ERα expression in an epigenetic manner through the modulation of a DNA hydroxymethylation marker, ten-eleven-translocation 5-methylcytosine dioxygenase (TET1). The above was demonstrated in co-cultures of endometrial cancer cells with alternatively activated macrophages, with the results showing that alternatively activated macrophages enhanced both E2-driven endometrial cancer cell proliferation and up-regulation in

ERα expression, a mechanism dependent on IL-17A expression (81). The above highlights the importance of the interplay among sex steroids, the immune system, and tumor progression.

ER in Mast Cells

Mast cells (MCs) are tissue-resident immune cells that form part of the innate immune system. They are commonly associated with allergic reactions and parasitic infections. These cells are characterized by the presence of granules loaded with different inflammatory mediators that they release depending on the time and the type of stimulus (82). Additionally, secretion of serine proteases such as tryptase or chymase define what phenotype of the mast cell will be activated, which means that mucosal mast cells produce tryptase and the connective tissue mast cells secrete tryptase, chymase, and carboxypeptidases (83). These enzymes—in conjunction with the release of IL-8, tumor growth factor beta (TGF-β), and TNF-α–have been associated with angiogenesis trough vascular endothelial growth factor (VEGF) and MMP modulation in different breast cancer phenotypes (9, 84). Mast cells can be activated by the direct recognition of pathogen-associated molecular patterns (PAMPs) or by immunoglobulins and immunoglobulin E receptor (Fcϵ RI) interaction; both cases result in the release of different molecules from their granules, recruiting different immune cells.

On the other hand, several studies have reported the presence of ERα but not ERβ in mast cells; however, it was recently described that these cells have the presence of both nuclear receptors (85–88). In this sense, treatment of E2 or an endocrine disrupting compound such as bisphenol A has been demonstrated to induce the release of histamine (an important biomolecule involved in allergic reactions) from rat mast cells in a concentration-dependent manner (89). Of note, the histamine release is also important in breast cancer promotion since this protein or its receptors (H3R and H4R) have been associated with the induction of breast cancer cell proliferation and migration. Importantly, these molecules have been identified to a greater extent in breast tumor samples as compared with non-tumor samples (90). The above suggests that the inhibition of this molecule could result in an interesting target in this disease. E2 has an important role in inducing the release of asthma mediators such as leukotriene and β-hexosaminidase in a rat mast cell line. The release of β-hexosaminidase has also been described in both the human mast cell line and in a primary culture (non-transformed) of mast cells. This action was blocked with the addition of tamoxifen or ICI 182,780, demonstrating that ERα is responsible for these actions (89, 91, 92). In relation to breast cancer progression, tryptase release from mast cells has been closely associated with an increased number of carcinoma-associated fibroblasts in breast tumor samples, favoring the tissue remodeling and angiogenesis (93). Related to this, E2 up-regulates tryptase secretion in the human mast cell line HMC-1 (88), assuming that it induces the degranulation of these cells. In addition, E2 in an *ex vivo* model induces the expression of two chemokine receptors (CCR4 and CCR5), which are implicated in the migration of periphery mast cells to the uterus (88, 94). The above highlights the effects of E2 in mast cell function with the purpose of favoring breast cancer progression. On the other hand, there are few reports with respect to E2 function by the non-genomic pathway in mast cells. In this regard, it has been shown that estradiol induces the release of intracellular calcium, which is important for degranulation and leukotriene synthesis in mast cells (95). Recently, the role of mast cells in breast cancer has been largely studied (10); however, many of their functions and components in their granules in relation with breast cancer progression are still little addressed, and this makes them an important population for study in the cancer microenvironment.

ER in Neutrophils

Neutrophils, which are other fundamental pathogen-fighting immune cells, constitute the first line of host defense. They can be recruited to infection sites and eliminate microbes by classical phagocytosis or degranulation, and they also produce ROS, release antimicrobial peptides, or expel their nuclear content in order to form neutrophil extracellular traps (NETs) (96, 97). Neutrophils collaborate with other immune cells such as macrophages or DCs and secrete many chemokines and cytokines that regulate the immune response (98). It has been described that neutrophils as well as other immune cells present both nuclear receptors (99). In this regard, E2 through ERα binding has been shown to regulate neutrophil survival, function, and number. E2 exposure delayed apoptosis in human neutrophils, and this effect was correlated with a significant decrease in active caspase 3 protein expression and was reverted by ICI 182,780 treatment (100). This represents a possible explanation of sexual dimorphism, being that neutrophil number differs between men and women (101). One effect of E2 on the function of neutrophils is that it enhances NO production and nitric oxide synthase, demonstrated previously in human neutrophils (102, 103). Additionally, neutrophils secrete several serine proteases (NSPs), including neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G (CG), which are essential for the elimination of infectious agents and the modulation of inflammation (104). Neutrophils derived from splenocytes of mice administered with E2 showed incrementation of NE, PR3, and CG in gene and protein expression as compared with placebo-treated mice. Moreover, E2 administration in these mice increased the number of neutrophils in different lymphoid tissues (splenocytes, peripheral blood, and bone marrow) and the gene and protein expression of myeloperosidase, a major component of neutrophil granules (105). E2 via ERα modulated inflammation, and the actions mentioned above were associated with an autoimmune disease as an increase in neutrophil number and NSPs were found in mice with lupus (105). Moreover, G1-GPER1 activation also participates in neutrophil polarization (analogous concept of macrophage activation) (106), promoting the gene expression of the pro-inflammatory phenotype (N2) and its lifespan, actions mediated by the activation of the cAMP/PKA/CREB, MAPK, and p38 signaling pathways (107). This work also shows that IL-1β, IL-8, the prostaglandin-endoperoxide synthase (PTGS2), the suppressor of cytokine signaling 3 (SOCS3), and granulocyte colony-stimulating factor (G-CSF) gene expression, were enhanced after stimulation of G1-GPER1 in a dose-dependent manner. Additionally, the release of IL-8 was significantly increased as compared with non-treated

human neutrophils and with neutrophils stimulated with LPS. Furthermore, this hormone–receptor interaction up-regulated the surface expression of two markers of neutrophil activation (CD11b and CD62L) (107), supporting the notion that G1-GPER1 interaction is responsible for IL-8 neutrophil release. Other work proved that 17β-estradiol-ERα did not induce the release of this chemokine; in fact, the estradiol treatment had the opposite effect in the release of this chemokine in human neutrophils pre-stimulated with LPS (108). In addition, this classical activation may participate in the attenuation of neutrophil activation. E2 reduced the shedding of a surface adhesion neutrophil molecule (CD62L or selectin) (108), which is normally implicated in diapedesis at sites of tissue injury and inflammation (109). Also, E2 treatment blocked the neutrophil chemotaxis promoted by IL-8, and the generation of superoxide anion by neutrophils was diminished with this hormone treatment (108, 110, 111), affecting their host defense function (112).

It is well-known that a certain type of breast cancer is dependent on E2 action; coupled with this notion, this hormone can promote inflammation through the induction of neutrophil infiltration and the expression of pro-tumoral cytokines/chemokines and tissue-remodeling enzymes in mammary neutrophils (113). In a mammary involution mice model, E2 administration induced mammary neutrophil infiltration and neutrophil pro-tumoral activity signature, as at least 10 inflammatory genes were up-regulated in mammary resident cells; interestingly, neutrophil depletion reversed the expression pattern of these inflammatory genes. Moreover, in this mammary involution mice model, the mice were administrated with E2 and injected with a triple negative breast cell line (4T1). Again, the hormone treatment induced mammary neutrophil infiltration—however, neutrophil depletion with a specific antibody resulted in the marked abolition of estrogen-induced mammary tumor growth (113). The mammary neutrophil recruitment induced by this hormone was observed in other *in vitro* and *in vivo* breast cancer research, in which it promoted N2-neutrophil polarization, correlated with the overexpression of integrin LFA-1 and TGF-β, intra- and extravasation and trans-endothelial breast cancer cell migration, and with major breast tumor growth; this last effect was reversed by ICI 182,780 treatment. In fact, E2 treatment transformed a non-metastatic breast cancer cell line into one that was metastatic-associated in the presence of neutrophils (114). The previous observations provide the presence of mammary neutrophils and its activity—which are importantly regulated by E2—with a significance regarding cancer progression.

ER in NK Cells

NK cells are central components of the innate immunity and they participate in preventing and controlling infections, tumor growth, and metastasis (115). Usually, in tumors there is a downregulation of self-ligands and expression of stress-induced ligands which can be recognized by NK cells (116). Their activation also leads to secretion of stimulatory cytokines and chemokines such as IFN-γ, TNF-α, GM-CSF, MIP1-α, and RANTES, which participate in the stimulation of the adaptive immune system. Moreover, their biological importance lies in their ability to exert a cellular cytolytic effect through the liberation of granzymes and perforin (117).

Since the 1990s, it has been known that E2 causes a reduction in NK cell cytotoxic activity in mice models in a dose-dependent manner (118, 119). This data was confirmed when the hormone was administered in postmenopausal and premenopausal women, resulting in a reduction in NK cell activity (120). In fact, the use of oral contraceptives, which bind to sex steroid receptors, has been associated with changes in NK cytotoxic activity and with an increase in infections (121). Interestingly, the suppressive effect of E2 on the NK cells was attributed to the enhancement of metastasis in a fibrosarcoma and melanoma cell model, where immunosuppressed mice treated with this hormone also exhibited deficient NK cell activity and increased susceptibility to develop metastasis of allogeneic tumor cells (122). Additionally, synthetic non-steroidal estrogens such as diethylstilbestrol showed the same effects regarding inhibitory NK cell activity and the mice's susceptibility to generating tumors derived from this NK cellular inhibition. Of note, NK inhibitory activity was dramatically affected with only neonatal administration of diethylstilbestrol into the mice (123). On the other hand, it has been described that E2 can induce or suppress NK cell activity in mice, with the actions being dependent on time. At short time intervals it acts in a stimulating way, and at long time intervals it suppresses NK cell activity (124). Estrogen can also inhibit NK cell-mediated apoptosis due to the fact that this hormone induced a granzyme inhibitor, named proteinase inhibitor 9 (PI-9) (125). Today, there are few reports that evaluate the effects of E2 in NK cells. However, it is known that the reduction of their activity is related to the promotion of tumor growth (126); therefore, NK cells might be considered as a target for immune therapies in order to avoid the estrogen-mediated increase in breast tumor incidence.

ER in B Cells

B lymphocytes are part of the adaptive immune system that is specialized in antibody production, which is part of humoral immunity (127). It has been described that B lymphocytes have the expression of both nuclear ERs in all B cell subsets (39, 128). In this sense, E2 has stimulatory effects on B-differentiated lymphocytes derived from human PBMCs. It increased immunoglobulin (Ig)G and IgM production in a dose-dependent manner, and this effect was enhanced by the addition of IL-10, an anti-inflammatory cytokine, to B cells previously treated with E2 (129), and the above becomes relevant in an autoimmune context. The stimulatory effect of E2 on antibody titers has been observed since the 1980s in *in vitro* studies and in the serum of rats administered with this hormone, where an increase in IgM antibodies was reported (130, 131). Of note, it has been reported that IgMs have a direct cytotoxic effect on transformed cells through the activation of the complement pathway (132, 133). This is relevant since the increases on IgMs levels due to E2 exposure are important for breast cancer suppression. Besides, they also might serve as diagnostic indicators of the phenotype or stage of this pathology due to the fact that they are well-correlated with the clinical score and

disease spread of breast cancer patients (134); however, more studies are necessary to confirm this fact. Added to that, E2 through the ERα pathway also impacts the activation and survival of B cells through the modulation of several genes. These effects were observed in splenic B cells derived from ovariectomized mice (or not) administered with it. Interestingly, these results were reverted in mice treated with ICI 182,780 (128). Regarding the effects of GPR30 on B lymphocytes, some reports have mentioned that different chemokines can activate it, triggering different roles of B subsets such as migration, chemotaxis, proliferation, and apoptosis, among others. In fact, this receptor has been correlated with different B cell malignancies such as leukemia and lymphomas (135, 136). Nevertheless, more information or mechanisms of action related to this topic would be interesting in relation to the pathogenesis of breast cancer.

ER in TCD4$^+$ and TCD8$^+$ Cells

Lymphocytes have important roles in immune protection; traditionally, these cells are divided into two subtypes, TCD4$^+$ and TCD8$^+$. The first subtype can help B cells to produce antibodies, in order to induce immune response through activation of macrophages and recruitment of different immune cells to specific sites with inflammation. The second type is important for defense against cellular pathogens, among other functions. These immune populations can contribute to attenuate inflammation, production of antibodies, and protection of pathogens (137). Based on the different cytokine secretion profiles, TCD4$^+$ is divided into different subsets—for instance, T helper (Th)1 and Th2. Th1 is characterized by secretion of INF-γ, IL-2, IL-12, and TNFα, which are cytokines that stimulate macrophages' functions and cellular response; meanwhile, Th2 cells secrete IL-4, IL-5, IL-6, and IL-10, which are important cytokines for B cell antibody production and humoral response.

It has been described that E2 affects the size, maturation, and development of T cells, a process known as thymic atrophy (37, 138), and this effect is mainly caused by ERα signaling (139). Moreover, it can also influence the expression of the phenotype of CD4$^+$/CD8$^+$ T cells and their subsets' functions (140), and it also contributes to the development of other T cell subtypes from the lymph nodes, such as Th17 cells (141). Interestingly, the proliferation and generation of active T cells are governed by different metabolic glycolytic demands (142). In this sense, the orphan nuclear hormone receptor, estrogen-related receptor-α (ERRα), is a key regulator that supports T cell functions, since the inhibition of ERRα decreased several glycolytic genes implicated in inflammatory cytokine production and T cell proliferation in an *in vitro* and in an experimental autoimmune encephalomyelitis mouse model, and a similar effect was found in ERRα-deficient T cells (143). Several studies have demonstrated that E2 modulates IFNγ-secretion of Th1 cells in both human and mice cells, which is potentially mediated by direct interaction of ER with its EREs in the promoter region of the IFNγ gene (38, 144, 145). This cytokine has a pivotal role against intracellular infections as well as autoimmune and inflammatory disorders. Furthermore, E2 inhibits the production of Th1 pro-inflammatory cytokines such as IL-2, IL-12, IFN-γ, and TNFα (146). In accordance with this notion, the decline of ovarian function related to menopausal state in women and reduction in the production of this hormone have been associated with an increment in pro-inflammatory cytokine production (147). In line with that, Th1-related cytokine levels such as IL-2 and IFN-γ were augmented in postmenopausal women, and hormone replacement therapy in this population caused a significant decrease of these cytokines (148). On the other hand, the effects of E2 in Th2 cells are related to the increment of anti-inflammatory cytokines such as IL-10, IL-4, and TGF-β (146, 149). In addition, IL-4 incrementation has been correlated with the increase of an essential Th2 transcription factor (GATA-3) (150). Interestingly, E2 administration in a mammary involution mice model diminished CD4$^+$ and CD8$^+$ T cells in mammary tissue, highlighting the effects of this hormone on the function of these immune cells' type (113).

ER in Regulatory T Cells (Tregs)

Tregs are involved in self-tolerance, suppression of immune cell functions, down-regulation of self-reactive lymphocyte action, and prevention of transplant rejection through activation of a lineage-specific transcription factor that governs Treg development, differentiation, maintenance, and function—forkhead/winged helix transcription factor (FoxP3) (151). The T regs' immunosuppressive T cell inflammatory activity includes IL-10 secretion and the induction of programmed cell death 1 receptor (PD-1) (137, 152). In breast cancer, these cells are associated with a high rate of relapse and with favoring the tumor microenvironment (7, 16).

E2 *in vitro* and *in vivo* mouse models have been shown to induce the gene expression of FoxP3 and IL-10. These effects were reversed with the treatment ICI 182,780 (153, 154). It also modulates the Tregs' inhibitory capacity, since estradiol treatment increased intracellular PD-1 levels in Tregs coming from splenocytes of wildtype mice, whereas an opposite effect was seen in ER knockout mice (155). E2 treatment has also been shown to promote the proliferation and the number of human Tregs. In addition, it favors the change of CD4$^+$, CD25$^-$ cells to a CD4$^+$, CD25$^+$ Treg phenotype (156). Interestingly, a recent work demonstrated that infiltrating Tregs derived from human cervical cancer contain elevated levels of estrogen. Additionally, E2 through ERα signaling binds in the EREs of the Tregs' FoxP3 promoter. In this way, a loop is formed and leads to the activation of FoxP3 activity (157). As in other works, ICI 182,780 treatment reverted effects of E2 in Tregs and resulted in the ablation of FoxP3 protein expression and a decrease in TGF-β secretion (157). Another study supports the notion that in addition to ERα signaling, GPER with the estrogenic small molecule (G-1) is critical for the expansion of Tregs and the induction of the Foxp3 protein in *ex vivo* cultures of purified TCD4$^+$ mouse cells. In addition, G-1-GPER activation was able to maintain the Tregs' phenotype and to induce the expression of two proteins implicated in the control of immune homeostasis, PD-1, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) in the presence of Th17 cytokine inflammatory polarization conditions (158). It has been described that Tregs secrete immunomodulatory cytokines such as TGF-β and IL-10 (159).

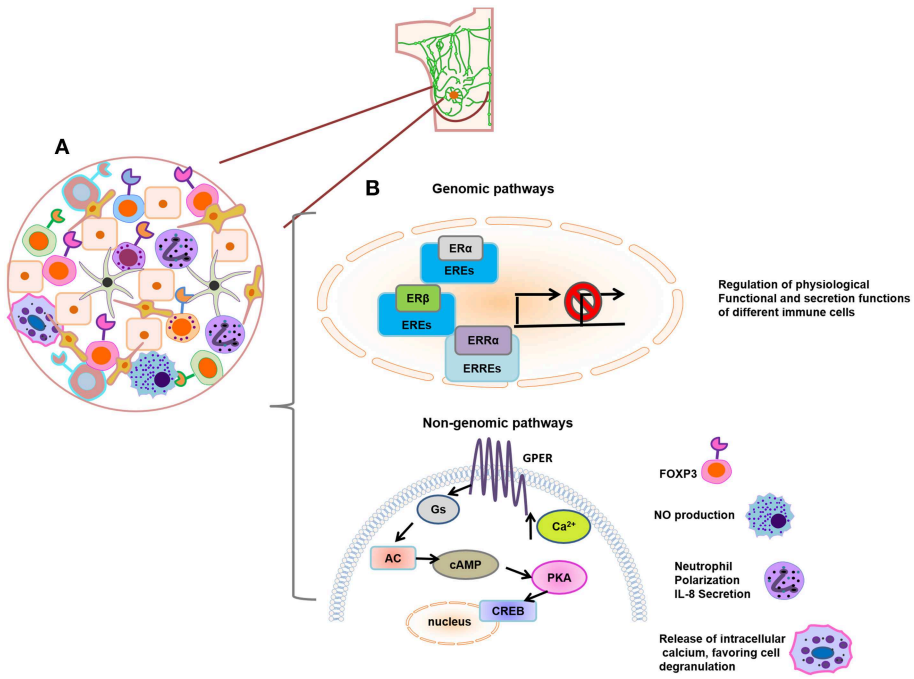

FIGURE 3 | (A) Schematic representation of immune infiltrating tumor cells. **(B)** Genomic and non-genomic estrogen pathways on immune cells. Estrogen regulates the physiological, functional, and secretion actions of different immune cells; these effects are mainly studied by the activation of genomic pathways such as ERα, ERβ, or ERRα. In addition, little effects of Tregs (increase of Foxp3 expression), macrophages (NO production), neutrophils (neutrophil polarization and IL-8 secretion), and mast cells (mobilization of intracellular calcium, favoring cell degranulation) have been described by the action of non-genomic pathway.

This cytokine secretion pattern was favored with E2 treatment in Treg cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy women (160). The previous data highlight the fundamental role of the estradiol-ERα / G1-GPER pathway in Treg physiology.

The estrogen pathways on immune cells studied in the basal condition described above are illustrated in **Figure 3**, which highlights that few reports have evaluated the effects of Tregs, macrophages, neutrophils, and mast cells mediated by non-genomic pathways. It also aims to represent how immune infiltration is found in breast cancer. In addition, **Table 1** summarizes the effects of estrogen on the immune cells that we described in the previous section.

REGULATION OF IMMUNE CELL FUNCTIONS BY ER INHIBITORS TREATMENT IN BREAST CANCER PATIENTS

It is widely known and accepted that the use of inhibitors of ER in the treatment of patients with estrogen-positive breast cancer has offered high survival rates (161). However, their use in other breast cancer phenotypes and their effects on the immune system cells in clinical stages have not been addressed.

In the previous section, we described *in vitro* and *in vivo* data that clearly show how the tumor infiltrating immune cells could play an important role in the development, progression, and response of breast cancer through ER signaling activation. However, they also encourage focusing on the modulation of their antitumor functions with ER inhibitors. In this regard, few studies have reported the effect of ER inhibitors on immune infiltrating breast tumor cell functions in clinical phases. As we described previously, Tregs have been found to be up-modulated in breast tumors, and a high number of these cells were present in high-grade ER-negative breast cancer patients. Also, they were associated with ER-positive breast tumors identified with high-risk patients (7, 162). It is known that Tregs give valuable information about breast cancer prognosis and progression, since a high number of Tregs can identify patients at risk of relapse after 5 years. Nevertheless, there was no relationship between the number of Tregs and the type of therapy that patients received (7). Interestingly, in 2009, Generali et al. reported that the number of Tregs was significantly decreased in patients who received an aromatase inhibitor treatment alone (letrozole) and in combination with an antineoplastic agent (letrozole + cyclophosphamide) (163). Another *in vivo* model reported that ICI 182,780 could reverse the estradiol actions for inducing Treg phenotype (154). These facts possibly indicate that E2 inhibition is an important antitumor strategy for manipulating the tumor microenvironment through inhibiting the function and number of Tregs; additionally, letrozole might also be useful in combination treatments in patients with ER-negative tumors regardless of ER expression in the tumor cells. Returning to the fact that this hormone can inhibit NK activity, an interesting work reported that post-menopausal

TABLE 1 | Estradiol effects of different immune cells.

Type of immune cells	Modulation	Reference
DCs	Increase expression of co-stimulatory molecules such as INF-γ Stimulation of T-cell proliferation and differentiation Induction of pro-inflammatory cytokines and chemokines; TNFα, IL-2, IL-6, IL-10, IL-8, MCP-1 DCs migratory response to lymph nodes after LPS stimulation Induction of DC differentiation via GM-CSF and the IRF4 Generation of tolerogenic DCs affecting their cell antigen presenting function	(39–47)
Macrophages	Stimulate NO release Modulate the lipid metabolism of macrophages through the release of arachidonic acid and prostaglandin E2 production Modulate catalase CAT activity Reduce MMP-9 expression Increase macrophage survival through Bcl-2 activation Reduce IL-8 expression Decrease IL-6, TNF-α, IL-1β expression Reduce TNF-α gene expression Induce alternative macrophage activation through the modulation of activity and expression of several markers such as Fizz1, Ym1 and arginase 1, CD163 and CD206	(49–81)
Mast cells	Induction of histamine, leukotriene, β-hexosaminidase and tryptase release Induction of chemokine receptors (CCR4 and CCR5) Release of intracellular calcium favoring degranulation	(10, 85–94)
Neutrophils	Enhance NO production and the neuronal nitric oxide synthase Promote neutrophil pro-inflammatory phenotype through GPER- cAMP/PKA/CREB, MAPK activation Increase IL-1β, IL-8, PTGS2, SOCS3, and G-CSF gene expression Increase IL-8 release via G1/GPER Up-regulation of two markers of neutrophil activation (CD11b and CD62L) Reduce IL-8 neutrophil release and CD62L expression via ERα Reduce neutrophil chemotaxis and superoxide anion production Increase the number of neutrophils in different lymphoid tissues and the NSPs including NE, PR3, and CG Increase MPO expression	(100–114)
NK cells	Reduction of NK cells' cytotoxic activity over long period of exposure Enhancement of tumor susceptibility and metastasis Stimulation of NK cell activity in short period of exposure Induction of PI-9	(118–126)
B lymphocytes	Enhancement of IgG and IgM production Increase survival, proliferation, migration, and chemotaxis	(39, 128–136)
$TCD4^+$ and $TCD8^+$Th1Th2	Promotion of $CD4^+$ /$CD8^+$ T phenotype expression Induction of glycolytic genes implicated in the inflammatory cytokine production and T cell proliferation via ERRα Inhibition of pro-inflammatory cytokines IL-2, IL-12, IFN-γ, and TNF-α Negative regulation of IFNγ promoter Increment of IL-10, IL-4, and TGF-β Induction of Th2 transcription factor GATA-3	(37, 38, 113)
Tregs	Induction of FoxP3 and IL-10 gene expression Maintenance of Tregs phenotype Activation of FoxP3 activity via estradiol-ERα-EREs Induction of FoxP3, PD-1, and CTLA-4 protein expression via GPER Increase of immuno-modulatory cytokines such as TGF-β and IL-10	(153–160)

stage I breast cancer patients who received tamoxifen for 1 month showed a statistically significant increase in NK activity; however, NK activity could not be related to ER expression in breast tumors due to the limited number of patients included (164). This fact correlates with mice models and estrogen actions in NK cell activation (124). It is also important to mention that some studies have reported a low proportion of NK cells in late stages of breast tumors (165); therefore, the work of Berry et al. suggests that in the early stages of breast cancer, patients treated with tamoxifen could benefit from the activation of NK cells instead of using this drug in the late stages, concluding that these cells could be considered as therapeutic targets.

With respect to E2 modulation on the TAMs' function, there are not any reports that have evaluated its inhibition effect in clinical trials. We described before that E2 promotes alternative macrophage activation (72, 74). Interestingly, Hollmén et al. found that ER-positive and ER-negative tumors induced different macrophage phenotypes with different biological functions, morphology, and cytokine and chemokine secretion. In fact, alternatively activated macrophages present in triple negative breast cancer have a down-regulation in citrulline metabolism

(80). From this concept, it would be interesting to study the effects of this hormone on citrulline metabolism, since it is known that nitric oxide synthase (iNOS) expression is enhanced by E2 action (166) and, simultaneously, this enzyme is associated with citrulline and arginine metabolism, determining the macrophages' activation phenotype in breast cancer (167). The number of neutrophils *in situ* in breast tumors is positively correlated with poor prognosis (102), so the modulation of their number could be interesting for breast cancer patients. In 2017, Dai et al. clearly demonstrated that estradiol treatment increased the number of neutrophils in the spleens of mice (105). An increased neutrophil number was also found in the complete blood of prostate cancer patients treated with estramustine (168), an antineoplastic agent with ER affinity (169). However, at present there are not any reports on neutrophil modulation in breast cancer tumors by ER inhibitors. On the other hand, it is known that in neutrophils, NETs formation is relevant for pathogen death, and a selective estrogen receptor modulator (SERM), raloxifene, inhibited NETs formation of human neutrophils, interfering with bacteria clearance after the treatment of the NET inducer phorbol 12-myristate 13-acetate (PMA) (170). This was opposite to the effect that was found with tamoxifen treatment (171). With respect to other immune populations, there are not any reports regarding their function modulation by ER inhibitors in breast cancer patients. In addition to the data described above, our workgroup reported that endocrine-disrupting compounds such as bisphenol A (BPA) have a significant effect on the modulation of ERα expression in T lymphocytes, macrophages, and NK cells of breast cancer tumors as well as in tumor growth. Impressively, a single administration of BPA in neonatal mice resulted in important changes in the presence of Tregs infiltrated into breast tumors in the adult stage (172). These facts provide new approaches to studying the effect of various compounds with estrogenic activity on the modulation of immune cells as well as in the selective inhibition of ER.

On the other hand, although different immunohistochemical studies as well as DNA sequencing data have given promising landscapes of infiltrating immune cells in this neoplasm for its therapy (13, 21), and despite the extraordinary efforts to reach a consensus on the study of the invasive population in breast cancer in daily histopathological practice (173, 174), different techniques such as flow cytometry must be applied in the clinic in order to guarantee precise studies. This is because it has been described that, according to the tumor area, the presence of infiltrating lymphocytes can vary (175). The above would allow offering personalized, predictive, and effective combined breast cancer treatments.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The main aim of this paper is to stand out the components of immune cells within the tumor microenvironment in different phenotypes of breast cancer, and the participation of E2 and its receptors in their function. As described above, E2 modifies the functions of different immune populations. Although the effects of this hormone were described in a particular way in each immune lineage, it is known that all of them are interconnected by cytokines, maintaining a dynamic interaction in the tumor microenvironment. Several reports have mentioned that immune infiltrating cells play a positive role in avoiding the progression of breast cancer and have a significant clinical impact on the response to treatment in a manner independent of the cancer phenotype (176, 177). However, little is known about their percentage and their grade of activation or anergy in different advanced clinic stages of this pathology, which might be modified due to the intratumoral E2 concentration. Based on the role that E2 and its signaling have in different populations of the immune system, we consider it important to evaluate or measure the intratumoral levels of this hormone and/or different compounds such as endocrine disruptors mainly in the advanced stages of this disease, which could be associated with their pro-anergic state. It has been documented that the concentrations of E2 as well as the enzyme that produces it (aromatase) are elevated inside the tumor (178, 179), affecting not only epithelial cell growth but also the immune cell effects. Taking into consideration the previous fact, we also regard the use of intratumoral therapy using ER inhibitors in the different types of breast cancer as an integral adjuvant approach for heightening both other therapies and immune response. The previous concept has taken on importance in cancer therapy; indeed, new studies on this topic are being done with different treatment schemes (180). Finally, the immune cells' function and their cytokines are key factors whose modulation should be studied, and they should also be considered as predictive markers and important therapy targets in different subtypes of breast cancer.

AUTHOR CONTRIBUTIONS

MS-M was in charge of all compilation of information, drafting of the manuscript, and participated in its conception. JM-M participated in the critical revision of the content of the manuscript and made a substantive intellectual contribution to drafting it. All authors read and approved the final manuscript.

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Erratum: Immune Tumor Microenvironment in Breast Cancer and the Participation of Estrogen and Its Receptors in Cancer Physiopathology

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***Correspondence:**
Frontiers Production Office
production.office@frontiersin.org

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SUPPLEMENTARY MATERIAL

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The Tumor Immune Contexture of Prostate Cancer

Natasha Vitkin[1], *Sarah Nersesian*[1,2], *David Robert Siemens*[1,3] *and Madhuri Koti*[1,2,3,4*]

[1] Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada, [2] Cancer Biology and Genetics, Queen's Cancer Research Institute, Kingston, ON, Canada, [3] Department of Urology, Queen's University, Kingston, ON, Canada, [4] Department of Obstetrics and Gynecology, Queen's University, Kingston, ON, Canada

One in seven men in North America is expected to be diagnosed with prostate cancer (PCa) during their lifetime (1, 2). While a wide range of treatment options including surgery, radiation, androgen deprivation and chemotherapy have been in practice for the last few decades, there are limited treatment options for metastatic and treatment resistant disease. Immunotherapy targeting T-cell associated immune checkpoints such as CTLA-4, PD-L1, and PD-1 have not yet proven to be efficacious in PCa. Tumor mutational burden, mutations in DNA damage repair genes, immune cell composition and density in combination with their spatial organization, and expression of immune checkpoint proteins are some of the factors influencing the success of immune checkpoint inhibitor therapies. The paucity of these features in PCa potentially makes them unresponsive to contemporary immune checkpoint inhibition. In this review, we highlight the hallmark events in the PCa tumor immune microenvironment and provide insights into the current state of knowledge in this field with a focus on the role of tumor cell intrinsic events that potentially regulate immune related events and determine therapeutic outcomes. We surmise that the cumulative impact of factors such as the pre-treatment immune status, PTEN expression, DNA damage repair gene mutations, and the effects of conventionally used treatments on the anti-tumor immune response should be considered in immunotherapy trial design in PCa.

Keywords: prostate cancer, tumor immune microenvironment (TIME), immunotherapy, immune checkpoint, DNA damage response, PTEN, hormone therapy

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Edited by:
Leila Akkari,
The Netherlands Cancer Institute (NKI), Netherlands

Reviewed by:
Ekaterina Jordanova,
Center for Gynaecologic Oncology Amsterdam, Netherlands
Zong Sheng Guo,
University of Pittsburgh, United States

***Correspondence:**
Madhuri Koti
kotim@queensu.ca

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INTRODUCTION

Prostate cancer (PCa) is the second most commonly diagnosed malignancy in men; each year, ~220,000 men in the United States are diagnosed with PCa (3). Newly diagnosed PCa is assessed using a combination of typical cancer staging (TNM), histological characteristics of a prostate biopsy, as well as prostate specific antigen (PSA) levels (4). In men diagnosed with lower risk, localized cancer, treatment options include active surveillance, radical prostatectomy (RP) or radiation therapy (RT) (4). Those with higher risk but still potentially curable disease will often require multiple interventions including RP +/- RT, as well as androgen deprivation therapy (ADT) as an adjuvant (4). However, these treatments are not curative for all patients, and biochemical recurrence occurs in approximately 25% of patients (5). Following recurrence, or for those presenting with metastatic disease, ADT is the current standard of care to remove circulating androgens that drive PCa growth and survival (6). Despite an initial clinical response, the majority of patients fail ADT and develop castration-resistant PCa (CRPC), a state of disease

progression which occurs despite surgical or medical castration (7). Short term responses to systemic chemotherapy or other androgen receptor targeted therapies may occur, however, CRPC is ultimately lethal and results in the death of ∼29,000 American men each year (3, 7). The high morbidity of this disease urgently necessitates the development of novel treatment strategies.

One such promising approach under investigation for PCa therapies is immunotherapeutic treatments that harness and exploit the body's intrinsic anti-tumor immune response. The recent success of immune checkpoint inhibitors (ICIs) in cancers such as melanoma and bladder cancer, has led to renewed interest in the tumor immune contexture to identify prognostic and predictive biomarkers as well as to direct novel immunotherapy combinations and sequencing toward precision cancer therapies (8). Several investigations on spatial and molecular profiling of tumors have attempted to define a pan-cancer immune landscape ranging from broad classifications as immunologically cold or hot (9), to six molecular subtypes; wound healing, interferon (IFN)-γ dominant, inflammatory, lymphocyte depleted, immunologically quiet, and transforming growth factor (TGF)-β dominant (10). Such comprehensive classification of the tumor immune microenvironment (TIME) in prostate cancer (PCa) is currently unavailable. Attributed to the disease complexity and significant heterogeneity, a deeper view of the PCa TIME is currently lacking and is needed to inform the design of immunomodulatory treatments and drug sequencing. In-depth knowledge regarding the cumulative effects of oncogenic drivers in distinct TIME states is critical to guide selection of therapies exploiting the anti-tumor immune responses. In this review, we focus on the immune features associated with localized and metastatic PCa to allow a knowledge-driven approach for future immunotherapy-based treatments.

The PCa Tumor Immune Microenvironment (TIME)

Immune responses, involving both secreted and cellular factors in the TIME, can drastically impact the balance between tumor progression, tumor clearance, and treatment response. Specifically, the variability in response has shifted the focus of rational design of ICIs to incorporating the features of the TIME such as infiltration and localization of tumor infiltrating lymphocytes (TILs) and presence of immunosuppressive cell populations (11). Interestingly, among the genitourinary cancers, PCa exhibits a unique TIME profile with distinct features of these populations (12).

The presence of cytotoxic and helper T lymphocytes within tumor margins has been associated with favorable prognoses and clinical implications across a multitude of cancer types (13). Identifying the critical function of TILs in cancer progression led to the establishment of the "immunoscore" as a standardized metric to assess the tumor immune contexture based on the density and location of CD3+ and CD8+ T cells (13). Given that the compartmentalization of TILs within the tumor is a critical feature associated with response and outcomes, only TILs within the tumor center and invasive margins are considered in the immunoscore (14). Using this classification in combination with tumor inflammation signature, solid tumors can be broadly classified into T cell inflamed/ "hot," and non-T cell inflamed/ "cold" tumors (15). ICI trial outcomes in some solid cancers such as melanoma urothelial and lung cancer, show that favorable responses are observed in hot tumors, which have a pre-existing higher density of TILs and expression of an IFN-associated gene signature (8, 16). Patients with an inflamed TIME also exhibit better responses to traditional therapies such as radiation and chemotherapy. Both treatment strategies are known to stimulate immunogenic cell death and consequently enhance the efficacy of immune checkpoint inhibitor therapy (14, 17).

In many solid tumors, high CD8+ TIL infiltration, especially their activated state, correlates with better prognosis due to their cytotoxic functions (18, 19). However, the prognostic relevance of CD8+ TILs is unclear in PCa, with some studies demonstrating that a high tumor TIL infiltrate is detrimental to patient survival. Indeed, one study reported that a higher density of stromal CD8+ TILs associates with poor prognosis in radical prostatectomy specimens and demonstrated a significant correlation between immunosuppressive CD73 expression and CD8+ TIL density (20). Another report showed that infiltration by CD8+ TILs within the invasive margins and stromal compartment of tumors associates with poor clinical outcomes and a shorter time until BCR in PCa patients (21). Another study evaluated tumor infiltrating CD8+ TILs and programmed death-ligand 1 (PD-L1) immune checkpoint expression in 51 node-positive PCa samples and reported that both CD8+ TIL density and PD-L1 expression were independent predictors of clinical progression (22). Most recently, an analysis of gene expression profiles of 1,567 prostatectomy specimens showed that high tumor TIL infiltrates were associated with worse distant metastasis-free survival (23). These findings may be due to improper TIL functionality; previous studies suggest that CD8+ TILs in the PCa TIME may be dysfunctional or suppressed, contributing to impaired cytotoxic responses despite tumor antigen stimulation (21, 24). It is currently unknown whether PCa-infiltrating TILs are in a state of anergy, exhaustion, or senescence; all of these are characterized by low or negligent levels of effector function (25). Further research is needed to characterize the functional status of TIL infiltrates in PCa to definitively assess the impact of their localization on prognosis.

The immune response is a balance between immunostimulatory and immunosuppressive factors; accordingly, functional TIL activity in PCa could be impaired by the magnitude of impact of secreted and cellular immunosuppressive factors. When looking at other T-cell populations in PCa, studies have noted high proportions of both CD4+ and CD8+ forkhead box P3 (Foxp3+) regulatory T cells (Tregs), within the tumor margin and epithelial compartment in PCa (26, 27). Another report examining changes in TIL infiltrates in PCa biopsies at diagnosis and subsequent relapse showed that increased infiltrates of Foxp3+ TILs were significantly associated with worse progression-free survival and overall survival (28). Preliminary data suggests that the presence of other receptors such as CCR4 on Tregs may impact PCa patient survival, although further research is required to support this

claim (29). Previous reports in gastric cancer show the positive association of CD8+Foxp3+ T cells with favorable prognosis which is in contrast to findings in PCa (30). In addition to the presence of immunosuppressive lymphocytes, multiple reports have demonstrated that high tumor-associated macrophage (TAM) infiltration in the PCa TIME is pro-tumorigenic (31), however, most do not differentiate between the M1 (tumor suppressive) and M2 (tumor promoting) phenotypes of TAMs. Notably, co-culturing of naïve monocytes with PCa cells resulted in decreased expression of co-stimulatory molecules and reduced endocytic ability compared to monocytes stimulated with M-CSF (31). Furthermore, these macrophages secreted high levels of M2-associated immunosuppressive cytokines and chemokines, with TGF-β2 being the most highly expressed (31). Given the established role of TGF-β in immune exclusion, this may be one of many factors contributing to poor TIL infiltration in PCa (32). In addition to providing insights into the association between M2 macrophages and poor prognosis in PCa, the immunosuppressive role of TGF-β is critical in the context of current ICI, where targeting TGF-β prior to ICI treatment has been suggested as an approach to improve response (32).

Factors Affecting the PCa TIME

The factors underlying evolution of an immunologically cold PCa TIME may be attributed to hormonal influence, genetic alterations, selective pressures of treatment. Further, immune exclusion and/or evasion mechanisms as a result of malignant progression could also lead to a cold TIME state (33). Several tumor intrinsic factors contribute to the evolution of a unique pre-treatment TIME in PCa, in addition to host physiological factors such as age and hormones. Low tumor-associated antigen expression, DDR defects, decreased MHC Class I expression, loss of PTEN protein, and dysfunctional IFN1 signaling are some of the mechanisms thought to be important in determining the features of the PCa TIME (**Figure 1**).

Tumor Mutational Burden

A feature of PCa important to the immune landscape is its relatively low somatic mutation burden and consequently diminished neoantigen expression compared to many other cancers (34). Overall rates of mutation in PCa cells are low; one study revealed a mean mutation frequency of 0.9 per megabase, about 10 times lower than that of melanoma (35). A lack of tumor neoepitopes is associated with reduced immune cell attraction to the tumor site, with fewer tumor-specific epitope-MHC interactions, resulting in reduced antigen presenting cells (APCs) cross-priming to TILs. The lack of these key interactions underlies the evolution of a non-inflamed TIME. In this scenario, transformed cells could evade immune cell-mediated elimination and proliferate freely (36). Consequently, treatment with immunotherapies would be ineffective as a pre-existing active immune contexture would be lacking. Indeed, ICI therapies such as those targeting the PD-L1/PD-1 immune checkpoint axis have the largest clinical impact in cancers with the highest numbers of somatic mutations such as melanoma and non-small cell lung cancer (37).

DDR Gene Defects

DDR is an important cellular pathway initiated to drive timely and accurate repair of genetic material damaged by mutagens such as ionizing radiation. Lack of cellular DDR mechanisms can lead to the accumulation of genetic aberrations, resulting in tumor evolution and progression (38). While fostering genetic instability, these alterations are also thought to skew the TIME toward an inflamed state, partly by increasing interactions of tumor-specific antigens with infiltrating immune cells or through altering cellular IFN pathways (39). The field of DDR in PCa is relatively understudied because of its low prevalence in this cancer. However, recent next generation sequencing based profiling efforts from The Cancer Genomic Atlas Network highlight these defects in both primary and advanced PCa (40). This study, conducted on primary PCa and localized disease, showed the presence of mutations in the DDR genes *BRCA2, BRCA1, CDK12, ATM, FANCD2, RAD51C* in 19% of cases (40). Similarly, an enrichment in DDR gene mutations in the metastatic scenario was reported in 23% of cases (41). Analyses based on 150 primary and mCRPC cases showed an enrichment in aberrations in *TP53* (53%), *RB1* (21%), the *PTEN-PI3K* pathway (49%), and *AR* (63%) in mCRPC compared to localized disease (41). The presence of many molecular subtypes with different mutations in DDR pathways and driver mutations makes generalizing the TIME status in patients challenging (**Figure 2**).

In line with these observations, a recent trial reported that PCa patients with DDR deficiencies (*BRCA1*, *BRCA2*, and *ATM)* had significantly better responses to Olaparib with corresponding increases in overall survival and progression free survival (42). No differences in these metrics were reported between patients with germline mutations compared to somatic aberrations, suggesting that by the time CRPC occurs, the impacts of germline and somatic DDR defects are functionally equivalent. In localized PCa, percentage of men with germline DDR defect was lower (4.6%), and odds ratios also support a higher proportion of DDR defects in men with mCRPC compared to localized PCa (43). These results are especially promising for patients who have failed multiple treatments, as they implicate late stage PCa patients with DDR deficiency as better responders to therapy. In a study of over 600 mCRPC cases, 11.8% had a germline mutation in a prominent DDR gene, compared to only 4.6% in localized PCa patients (43). Furthermore, the presence of germline mutations in *BRCA2*, *ATM* and *CHEK2* were associated with histologically advanced disease (43). The challenges of mapping the primary and metastatic sites make it difficult to assign a clear trajectory of these events as secondary to treatment pressures vs. progression of an inherently aggressive cancer.

It has been established that DNA damage induces AR activity, which feeds back to activate gene expression program promoting DNA repair; both *in vitro* and *in vivo*, activating AR signaling can promote resistance to DNA-damaging agents (44). Synergistic effects of second-generation ADT and radiotherapy to decrease PCa cell survival has been shown to be mediated partly by PARP1 (45). Since recurrent PCa is treated with ADT, sensitizing tumors to radiotherapy is common, however, it may also contribute to

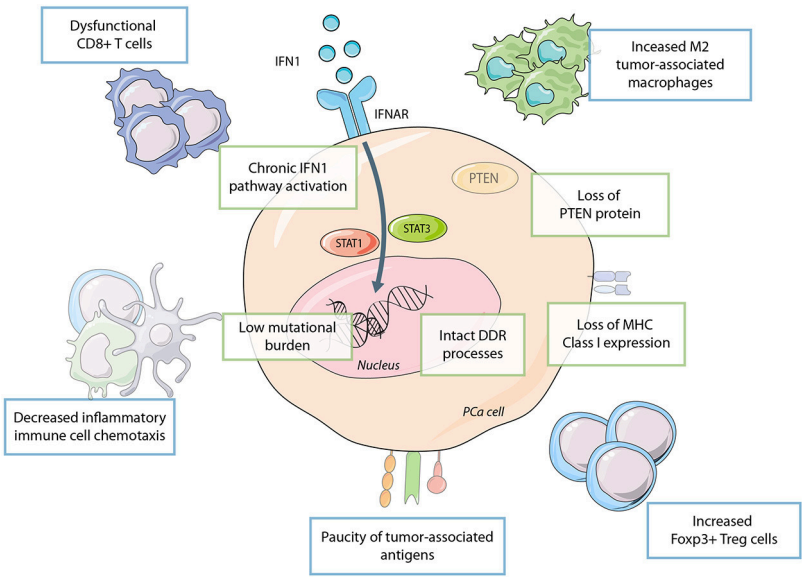

FIGURE 1 | Schematic illustrating the various factors impacting the PCa TIME and their propose on the TIME. Numerous cancer cell-intrinsic factors drive the evolution of a the heterogenous pre-treatment TIME. In PCa, loss of PTEN could lead to a dysfunctional IFN1 signaling. Other features include low tumor-associated antigen expression, decreased MHC Class I expression, which could potentially contribute to immune escape. These, in combination with the pre-existing immune contexture led by other factors, are thought to be critical in determining the balance between activated and suppressive states of TILs including classical or alternatively activated tumor-associated macrophages.

clonal evolution and newer mutations. Regardless, as seen in other cancers (32, 46), DDR defects are indeed beneficial from an immune perspective and could potentially form the basis for immune sensitization of PCa to ICIs.

Loss of MHC / HLA Expression

MHC Class I proteins are normally expressed on nucleated cells and present cytosolic peptides to T lymphocytes, triggering an immunostimulatory signal cascade resulting in T cell proliferation and target cell lysis (47). Accordingly, loss of MHC Class I expression is a common immune evasion mechanism employed by a variety of cancer types (47). Defective MHC Class I may result from aberrations in multiple pathways including HLA synthesis and transport, antigen processing, or loss of critical accessory proteins (47). Preliminary evidence also suggests that epigenetic silencing of MHC Class I genes is important in PCa (48). This loss of MHC Class I expression has been documented in both metastatic PCa cell lines and clinical specimens (49, 50). Different signaling pathways including the IFN axis can also impact MHC Class I expression; in a syngeneic mouse model of PCa, treatment with IFN-γ led to increased survival and heightened expression of proteins important in MHC Class I production such as TAP1 (51). Cell line experiments have also demonstrated that radiation increases MHC Class I expression and leads to unique MHC Class I binding antigenic peptides (52). Increased MHC Class I expression in tumors is predicted to facilitate the activation and expansion of CD8+ TILs within the invasive margins of the tumor, eliciting a more robust immune response. However, in the context of an immunosuppressive TIME lacking a dense TIL infiltrate, heightened expression of MHC Class I proteins in isolation is unlikely to shift the TIME toward an immunoactive state, especially in cases with concurrent immunosuppressive features.

PTEN Loss

A well-characterized molecular aberration in PCa is the loss of the tumor suppressor protein PTEN. PTEN is generally known as a lipid and protein phosphatase encoded by the *PTEN* gene which antagonizes the pro-growth PI3K signaling pathway and is deleted in up to 30% and mutated in 2-5% of primary PCa cases (53). Emerging literature suggests that the immune regulatory functions of PTEN are mediated through modulating the activation of cellular IFN1 pathways (54). In other cancers such as melanoma, patients with PTEN loss exhibited significantly poorer responses to PD-1 ICI and had lower TIL infiltration compared to patients with >10% of tumor cells positive for PTEN staining (55). Furthermore, the therapeutic activity of tumor-specific TILs from adoptive T cell therapy was significantly reduced in mice with *PTEN*-silenced melanoma cells compared to those with an intact *PTEN* gene, indicating that PTEN can confer sensitivity to T-cell-based immunotherapy (55). Other alterations may also cooperate with PTEN loss to drive distinct tumor immunological phenotypes. Using *in vivo* models, a recent study demonstrated the qualitative and quantitative impact of *Pten* loss in the TIME. Specifically, myeloid-derived suppressor cell (MDSC) infiltrates in $Pten^{-/-}$; $Zbtb7a^{-/-}$ prostate tumors exhibited a distinct phenotype affecting NF-κB signaling whereas MDSCs within $Pten^{-/-}$; $Tp53^{-/-}$ tumors were associated with Treg

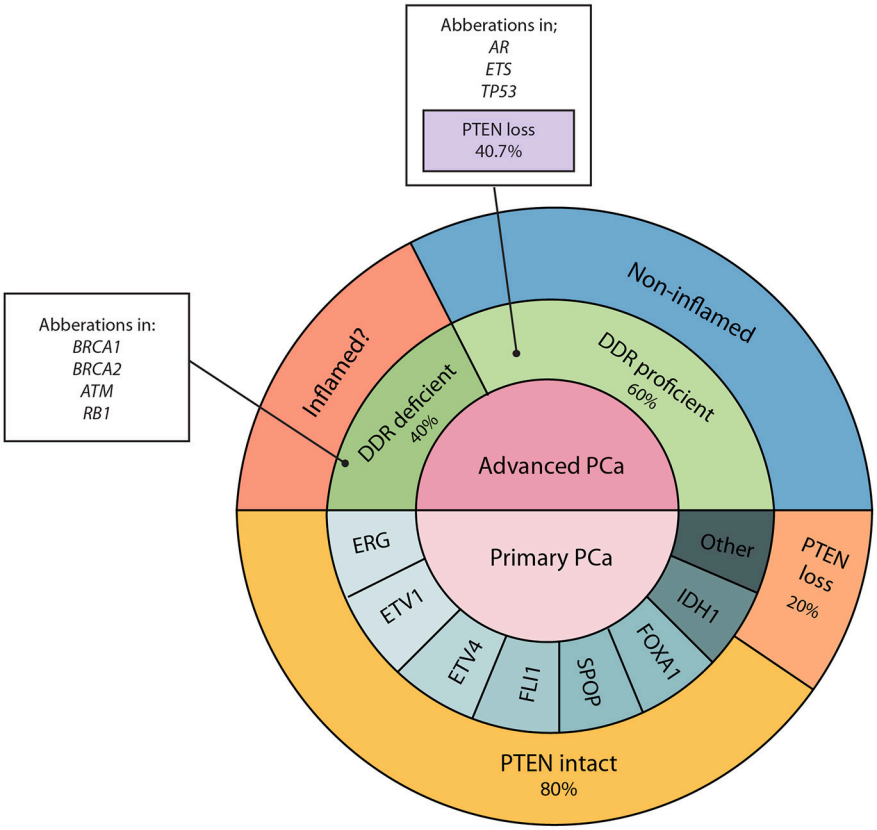

FIGURE 2 | Genetic aberrations associated with primary and advanced PCa. PTEN loss is associated with 20% primary and 40.7% of advanced PCa. Increased proportions of mutations in DNA damage repair genes, *BRCA1, BRCA2, ATM,* and *RB1*, have mostly been reported in advanced PCa. While DDR deficient tumors may exhibit increased numbers of oncogenic mutations, we speculate that this may result in a more immunogenic phenotype and give rise to an inflamed TIME as observed in some other solid tumors.

immunosuppression (56). These findings implicate PTEN as a tumor suppressor which, in addition to regulating the PI3K-Akt-mTOR signaling network, can govern the tumor immune milieu and response to immunotherapy, however, these findings must be validated in PCa. These data provide compelling evidence for an undefined mechanistic role of PTEN in altering the immune contexture of the PCa TIME. Recent studies conducted in phosphatase inactive PTEN cells have highlighted its phosphatase independent tumor suppressive functions, specifically in DNA repair and apoptosis (57, 58). An area relatively understudied in PCa, however, is the specific effect of altered levels of nuclear, cytoplasmic and secreted PTEN proteins in mediating an aggressive disease and immunosuppressed TIME state. Given that all three isoforms of PTEN exert different regulatory functions (59), in processes that alter cancer progression and immune response in the TIME, future investigations should incorporate these in scenarios where PTEN deficiency is not attributed to loss of 10q region harboring the *PTEN* gene. A precise definition of these genotype and associated immunophenotype relationships will allow the development of alternate targeted therapies and improved patient stratification.

IFN1 Signaling

Few studies have characterized the functional status of immune cell populations in the PCa TIME, but preclinical and clinical data supports that IFN1 signaling in the TIME exerts protective anti-tumor effects in PTEN-deficient tumors. IFN1 is an important group of immunostimulatory cytokines released in response to direct binding of IFN1 to its extracellular receptor, or from cellular detection of invading pathogens by innate pattern recognition receptors (60). It is established that IFN1 is crucial to mounting an efficient anti-tumor immune response, which is accomplished by a variety of mechanisms such as cytokine and chemokine production, increasing the expression of immune costimulatory molecules, activating adaptive immune cells, and facilitating CTL killing (61). The activation of transcription factors STAT1 and STAT3 drive canonical IFN1 signaling by mediating the transcription of over 2000 interferon-stimulated genes, which serve a diverse array of functions involved in stimulating and regulating the innate and adaptive immune responses (62). Combined prostate-specific *STAT3* and *PTEN* deficient mice exhibited accelerated cancer progression and metastasis compared to *PTEN*-deficient mice; these animals had tumors up to six times larger than $PTEN^{-/-}$

mice (63). The authors show that these effects are mediated through the ARF-MDM2-p53 axis, and suggest that PTEN-deficient tumors cannot effectively activate this axis, resulting in tumor metastasis (63). However, conflicting evidence has demonstrated that STAT3 inhibition results in decreased PCa cell growth and tumor metastasis, both *in vitro* and animal models of PCa (64, 65). Chronic IFN1 signaling has been associated with immunosuppression and therapy resistance; both unphosphorylated STAT1 and STAT3 (U-STAT1/3) can serve as active transcription factors and mediate the expression of specific subsets of ISGs (66, 67). The subset of ISGs activated by U-STAT1 after prolonged IFN1 exposure render cancer cells insensitive to radiation and chemotherapy (68) Multiple studies have demonstrated that in addition to contributing to therapeutic resistance, these genes also promote cancer growth and metastasis (69). The ability of IFN1s to modulate the expression of distinct sets of ISGs through differences in signal duration and STAT activation provides a mechanism to account for the opposing roles of IFN1 in immune stimulation and regulation. Furthermore, it is likely that cellular and environmental cues such as PTEN loss, DDR defects, TIL infiltration and activity, and the presence of immunosuppressive factors reflect these divergent findings.

Impact of Therapy on the PCa TIME

Androgens and their receptors play a critical role in both progression and treatment of PCa. Antagonists of androgen receptor (AR) such as bicalutamide and enzalutamide are therefore widely used as part of ADT therapy in PCa (70). As the immune response is a dynamic process affected by environmental factors, PCa treatments can also affect the tumor immune contexture. Complex mechanisms of androgen blockade mediated effects on the PCa TIME, ranging from thymic enlargement, increased lymphocyte migration, to GABA-A receptor mediated off-target effects leading to impaired T cell priming have been reported (71). Due to the dependency of PCa cells on androgen signaling, ADT treatment results in cancer cell apoptosis, failing to release immunostimulatory signals (72). In a syngeneic murine model, increased CD3+ T cell infiltration in tumors post orchiectomy (surgical castration) with corresponding tumor regression was observed, albeit eventual relapse (71). This response was associated with a thymic T cell wave, which is typically short-lived, and may be accompanied by increases in regulatory immune cell populations (73). Suppression of both cell mediated and humoral immune responses by AR antagonists (medical castration) has been reported in syngeneic murine models of PCa (74, 75). A key finding is the contrasting impact of medical vs. surgical castration on T cell priming, which is a critical factor in anti-tumor immune response. While treatment with gonadotrophin-releasing hormone analogs has similar effects as orchiectomy, opposite effects were observed using AR antagonists. Clearly, more longitudinal studies in patients are warranted to define these precise correlations for effective sequencing of AR antagonists and immune based therapies. Similarly, given their predictive importance (76), and expression of PD-L1, defining the TAM phenotypes that associated with pre- and post ADT treated tumors will be crucial for determining the proper sequencing of ICI treatment. Another important question that remains unanswered pertains to how these changes correlate with the pre-treatment TIME states, specifically with regard to stromal and epithelial localization of cytotoxic TILs.

Treatment-induced ICD leads to the release of cancer cell antigens to which the immune system can respond (77). This mediates the influx and activation of dendritic cells (DCs) and TILs, which can facilitate a more robust anti-tumor immune response. Notably, the presence of an active immune contexture predicts a favorable response to chemotherapy, implicating that cells of the TIME are critical for an individual's response to treatment (72, 78). Docetaxel, an effective systemic chemotherapy used for men with metastatic CRPC, does not initiate classic ICD although studies suggest that it can augment TIL-mediated tumor killing and decrease MDSC populations (79, 80). In a Phase II clinical trial, metastatic CRPC patients receiving a PSA vaccine and subsequent docetaxel had a median progression-free survival of 6.1 months while patients taking docetaxel alone survived 3.7 months (81). These results suggest that while not directly inducing ICD, docetaxel treatment for CRPC patients may potentiate the immune response and mediate an inflamed TIME.

Radiation therapy is another therapeutic modality, utilized for both curative and palliative indications, that also has been demonstrated to have immunomodulatory properties. Radiotherapy has been shown to increase the number and diversity of tumor-specific surface peptides and expression of MHC Class I molecules in a dose-dependent manner, which increased the efficacy of TIL-mediated cancer cell killing (52). Immuno-potentiation may also be attributed to the release of immunostimulatory cytokines and danger-associated molecular patterns (DAMPs) due to radiation exposure (73). The abscopal effects of radiation on distant metastases in PCa have also been documented; metastatic patients who received first-line radiotherapy had significantly higher overall survival compared to patients who did not receive this treatment in one retrospective study (82). It could be hypothesized that these outcomes could be secondary to radiotherapy-instigated immune activation, which would mediate a systemic anti-tumor immune response targeting distant metastases as well as the primary tumor.

Another relatively understudied area in PCa is the difference in TIME profiles in primary tumors compared to metastatic disease. A recent landmark study comparing 150 matched primary and metastatic CRPC reported novel clinically actionable aberrations, including higher frequencies of aberrations in DDR genes such as *BRCA1, BRCA2*, and *ATM* (41). Given the availability of tumor molecular profiles from immunologically distinct sites of metastasis in studies such as this, a comprehensive characterization of the spatial and molecular immune profiles of metastatic lesions could provide an improved understanding of immune evasion mechanisms in PCa.

Current State of Immunotherapy in PCa

Two vaccine-based immunotherapy approaches have shown moderate success in PCa treatment. Sipuleucel-T is a

personalized treatment constituting the *ex vivo* expansion and activation of patient-derived peripheral blood mononuclear cells (PBMCs) with a recombinant prostate-specific fusion protein (83). The registration trial involved CRPC patients and those receiving this treatment had in a median survival was 4.1 months longer than placebo-treated patients (83). Other additional immunotherapeutic approaches, including several vaccine trials including GVAX, and PROSTVAC, however did not demonstrate a survival benefit compared to placebo in phase 3 trials despite encouraging early results (84–86).

ICI treatment in PCa has to date demonstrated less than exciting results; a Phase III trial testing CTLA-4 blockade (Ipilimumab) did not observe any differences in overall survival compared to placebo in CRPC patients (87). Ipilimumab, analyzed in two Phase III studies, did not show any survival benefit in this tumor. The KEYNOTE-199 study analyzed the role of pembrolizumab for post-docetaxel mCRPC patients and concluded that pembrolizumab had antitumor activity and acceptable safety in these patients (88). Its activity was observed both in PD-L1 positive and PD-L1 negative cohorts, however, the response rate was low, with a complete and partial response of <5% (88). To date, immune checkpoint inhibitors have yet to be FDA-approved for the management of metastatic PCa (86).

These and other data suggest that ICI alone may not be enough to facilitate a robust anti-tumor immune response in PCa patients, rather, activating tumor-specific TILs may provide more benefit. Future clinical trials investigating these agents should be encouraged on specific patient subsets including those with high PD-L1 expression, those with hypermutated or microsatellite-unstable tumors, and those enriched for germline and/or somatic DNA-repair gene mutations (e.g., intraductal/ductal histology, primary Gleason pattern 5, and perhaps AR-V7-positive tumors). Furthermore, neoadjuvant treatments which promote the development of an immunoreactive TIME could increase the sensitivity of CRPC patients to ICI and immunotherapy.

As the PCa TIME is usually non-inflamed and dominated by immunosuppressive cells, targeting or reprogramming these suppressive cell populations could skew the PCa TIME toward an inflamed phenotype and make PCa amenable for immunotherapy treatments. Accordingly, neoadjuvant administration of IFN1 agonists which activate cytosolic innate immune sensing pathways such as those mediated TLRs or STING, represents an area of unrealized potential in immunotherapy research for PCa. Preclinical findings have been promising; for example, the addition of intra-tumoral STING agonist injection to combination ICI treatment in a syngeneic mouse model of PCa increased overall survival by 35% compared to combination ICI alone (89). In this study, mice treated with both STING agonist and combination ICI had increased TIL: Treg and TIL: macrophage proportions, and decreased percentages of TAMs (89). Furthermore, it was demonstrated that this activation was not limited to STING agonists; poly I:C treatment in a syngeneic PCa mouse model has also shown to increase cellular differentiation and promote immunologically active lymphocyte infiltration (90). A more comprehensive understanding of the factors conferring sensitivity to IFN1 agonists is warranted as this approach moves forward. Discerning the immune pathways and mechanisms which significantly contribute to causing an inflamed and immunologically active TIME is required before these pathways can be therapeutically exploited. Finally, more trials, such as the recently initiated Quick efficacy seeking trial (Quest1) (86), are needed to determine precise immunotherapy combinations in PCa.

CONCLUSIONS AND FUTURE PERSPECTIVES

A detailed analysis of treatment naïve and treatment associated TIME is not currently available in PCa with reports to date mainly focusing on evaluation of limited phenotypes of activated or dysfunctional immune cell types. Sex-steroids, primarily androgens, play important roles in thymic involution or rejuvenation and thus therapeutic ablation of these could have significant impacts on the PCa TIME. The unique clinical and molecular features of each PCa case make it difficult to predict the status of the TIME, although some metrics such as TGFβ signaling and Treg infiltration may be useful. Importantly, use of genetic alterations such as PTEN loss and DDR status should be incorporated in trial design and accompany retrospective and prospective immune monitoring correlative studies.

AUTHOR CONTRIBUTIONS

MK and DS conceptualized the review. NV, SN, MK, and DS wrote and reviewed the review. NV and SN generated the figures.

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High-Throughput Microfluidic 3D Cytotoxicity Assay for Cancer Immunotherapy (CACI-IMPACT Platform)

Dohyun Park[1], *Kyungmin Son*[1], *Yunchan Hwang*[2], *Jihoon Ko*[1], *Younggyun Lee*[1], *Junsang Doh*[3]* *and Noo Li Jeon*[1,4,5,6]*

[1] Division of WCU Multiscale Mechanical Design, Seoul National University, Seoul, South Korea, [2] Department of Electrical Engineering and Computer Science, Seoul National University, Seoul, South Korea, [3] Department of Materials Science and Engineering, Seoul National University, Seoul, South Korea, [4] Department of Mechanical and Aerospace Engineering, Seoul National University, Seoul, South Korea, [5] Institute of Bioengineering, Seoul National University, Seoul, South Korea, [6] Institute of Advanced Machines and Design, Seoul, South Korea

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Edited by:
Leila Akkari,
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Medical College of Wisconsin, United States

***Correspondence:**
Junsang Doh
jsdoh@snu.ac.kr
Noo Li Jeon
njeon@snu.ac.kr

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Adoptive cell transfer against solid tumors faces challenges to overcome tumor microenvironment (TME), which plays as a physical barrier and provides immuno-suppressive conditions. Classical cytotoxicity assays are widely used to measure killing ability of the engineered cytotoxic lymphocytes as therapeutics, but the results cannot represent the performance in clinical application due to the absence of the TME. This paper describes a 3D cytotoxicity assay using an injection molded plastic array culture (CACI-IMPACT) device for 3D cytotoxicity assay to assess killing abilities of cytotoxic lymphocytes in 3D microenvironment through a spatiotemporal analysis of the lymphocytes and cancer cells embedded in 3D extra cellular matrix (ECM). Rail-based microfluidic design was integrated within a single 96-well and the wells were rectangularly arrayed in 2 × 6 to enhance the experimental throughput. The rail-based microstructures facilitate hydrogel patterning with simple pipetting so that hydrogel pre-solution aspirated with 10 μl pipette can be patterned in 10 wells within 30 s. To demonstrate 3D cytotoxicity assay, we patterned HeLa cells encapsulated by collagen gel and observed infiltration, migration and cytotoxic activity of NK-92 cells against HeLa cells in the collagen matrix. We found that 3D ECM significantly reduced migration of cytotoxic lymphocytes and access to cancer cells, resulting in lower cytotoxicity compared with 2D assays. In dense ECM, the physical barrier function of the 3D matrix was enhanced, but the cytotoxic lymphocytes effectively killed cancer cells once they contacted with cancer cells. The results implied ECM significantly influences migration and cytotoxicity of cytotoxic lymphocytes. Hence, the CACI-IMPACT platform, enabling high-throughput 3D co-culture of cytotoxic lymphocyte with cancer cells, has the potential to be used for pre-clinical evaluation of cytotoxic lymphocytes engineered for immunotherapy against solid tumors.

Keywords: cytotoxicity assay, microfluidics, cancer immunotherapy, cytotoxic lymphocytes, high-throughput screening

INTRODUCTION

Adoptive transfer of *ex vivo* cultured/engineered cytotoxic lymphocytes (CLs) is arising as a promising approach to treat cancers (1). In particular, T cells expressing chimeric antigen receptor (or CAR-T cells) have been extremely successful in the treatment of CD19 expressing leukemia and lymphoma (2–4). The success has led to FDA approval of two CAR-T cell-based therapies, Kymriah (Novartis) and Yescarta (Gilead), and new CAR engineering strategies have been studied to improve the performance, reduce toxicity, and broaden applications of CAR-T therapy (5, 6). In addition, NK cells and γδ T cells, which exhibit low cytotoxicity and minimum graft-vs. -host disease in allogeneic transfer compared with T cells, have been developed as alternatives of CAR-T cells as an off-the-shelf therapeutics (7, 8). In spite of these efforts, the performance of adoptive transferred CLs against solid tumors is still limited due to complex tumor microenvironment (TME) that limit trafficking and effector functions of CLs (9, 10). In addition to highly immuno-suppressive microenvironments caused by acidic and hypoxic conditions and enrichment of suppressive cells (11–13), fibrotic tumor stroma is an important factor limiting successes of cancer immunotherapy by acting as a physical barrier for CLs to access tumor cells (14, 15). Therefore, various factors comprising TME need to be considered for the development of engineered CLs for solid tumors.

Cytotoxicity assay measuring killing ability of CLs is one of the most critical assays for the development of CLs for cancer immunotherapy. Chromium or calcein release assay based on the measurement of released radioactive ^{51}Cr or fluorescence calcein from lysed cancer cells has been a standard method for assessing cell-mediated cytotoxicity (16, 17). These methods have been widely used because cytotoxicity can be assessed simply by co-culturing CLs with tumor cells loaded with ^{51}Cr or calcein. In addition, these assays are compatible with 96 well formats, thus can be performed in high-throughput fashions. However, in these assays, tumor cells are either adhered on flat surfaces or suspended in medium, thus complex TME in solid tumors limiting CL activity are lacking.

Microfluidic-based platforms, which allow the reconstitution of complex 3D microenvironments of human tissues in *in vitro* by compartmentalization of multiple cell types, applying chemical and mechanical stimulations, and controlling chemical gradient (18), can be a powerful method for the assessment of lymphocyte cytotoxicity for solid tumors (19). Recently, microfluidic chips based on poly(dimethyl siloxane) (PDMS), a common material for microfluidics, were developed as preclinical models to evaluate antitumor activities of engineered T cells expressing T cell receptors specific for tumor antigens (or TCR-T cells) (20, 21) or engineered NK cells expressing Fc receptors (22) in 3D microenvironments recapitulating various aspects of TME. Specifically, microfluidic chips compartmentalized with 3D ECM gels containing tumor cells and TCR-T cell loading zones were used to assess the roles of hypoxia, inflammatory cytokines, immunosuppressive conditions induced by mTOR inhibitors, and monocytes on the cytotoxicity of TCR-T cells (20, 21), and microchips filled with ECM gels containing tumor cell spheroids and perfusable tubular vasculatures were used to recapitulate TME for NK cell trafficking toward solid tumors and to test combination of immuno-stimulatory biologics with NK cell therapy (22). However, the devices aforementioned requires labor and time intensive batch fabrication processes because the device was made of PDMS (23).

In this study, we introduce a 3D cytotoxicity assay using an injection molded plastic array culture (CACI-IMPACT) platform with which we can monitor both migration and cytotoxic activity of CLs in 3D microenvironment, by customizing our previous IMPACT device (24). We adopted extracellular matrix (ECM), which is a basic component of TME and did not exist in the standard protocols of cytotoxicity assays. ECM acted as physical barrier to restrict CLs from access cancer cells embedded in it. The limited accessibility resulted in low cytotoxicity compared with 2D assay. In addition, fibrotic ECM of TME was reconstituted by using denser collagen which lowered migration and cytotoxicity in observation of large area, but induced faster lysis process than sparser ECM. Furthermore, we improved the assay throughput compared with PDMS devices due to enhanced productivity oriented by changing material and usability mediated by rail-based microstructures. This model allowed us to test the effect of the physical properties of the 3D microenvironment on cytotoxic activity and we expect that this model can be used for high-throughput screening platform for estimating the efficacy of engineered lymphocytes in more *in vivo* like environment than conventional assays.

MATERIALS AND METHODS

Cell Culture

HeLa cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% of fetal bovine serum (FBS) and 1% of penicillin–streptomycin (PS). NK-92 cells were cultured in minimum essential media alpha (MEM α) with 15% of FBS, 15% of horse serum (HS), and 1% of PS and other supplements, including myo-inositol (0.2 mM), 2-mercaptoethanol (0.1 mM), folic acid (0.02 mM). NK-92 cells were sub-cultured in every 2 days in 6 ml of the full medium and 1,200 units of Interleukin-2. Sub-culture was conducted in T25 flasks with cell concentration of 10^5 cells/ml.

Fluorescent Labeling of Live and Dead Cells

HeLa cells were labeled with CellTrace™ CFSE Cell Proliferation Kit (Thermo fisher, C34570) by incubating the cells in serum free DMEM with 2 μM of CFSE for 30 min. NK-92 cells were labeled with CellTrace™ Far Red Cell Proliferation Kit (Thermo fisher, C34572) by incubating the cells in serum free MEM α with 2 μM of the reagent for 20 min. To detect dead cells, propidium iodide (PI) was used. For live imaging, PI-containing medium was used from the beginning of imaging. For imaging after 24 h of NK-92/HeLa co-culture, the medium was replaced with PI-containing medium for 30 min prior to imaging.

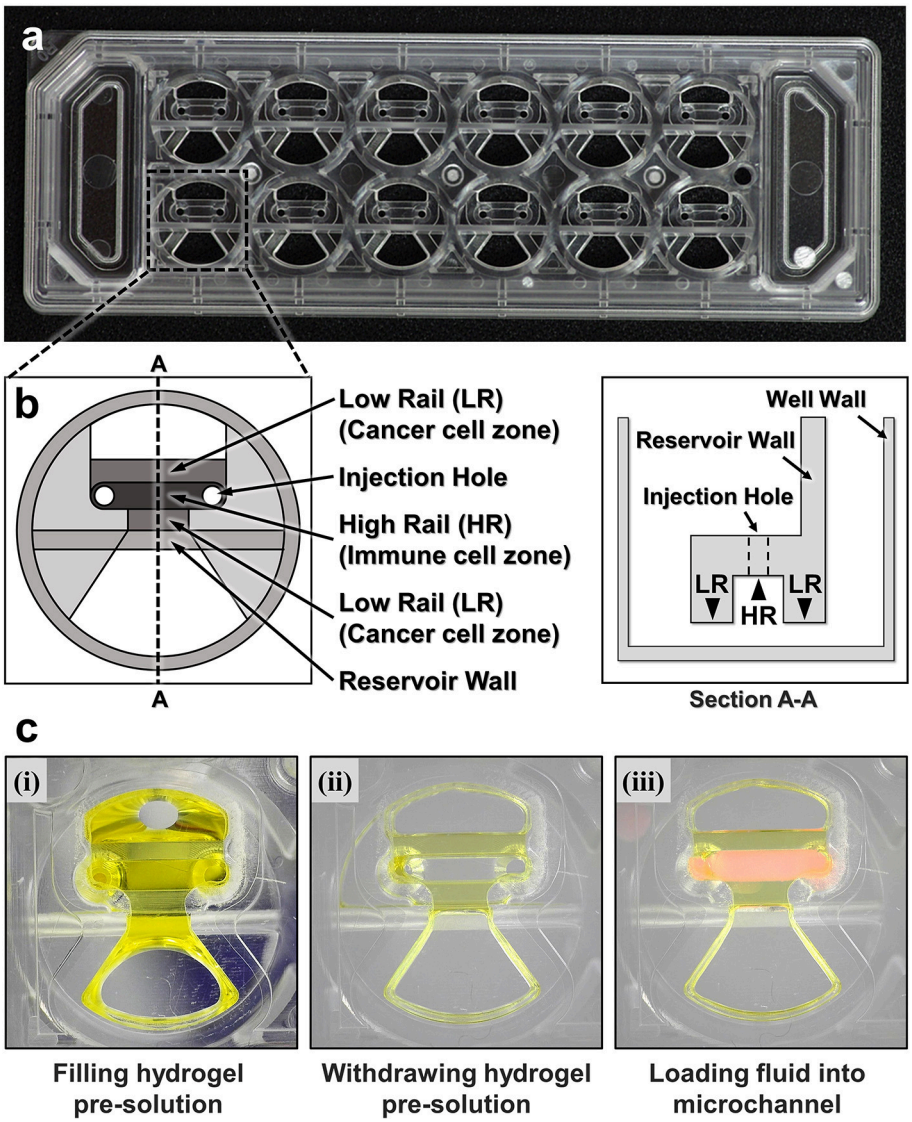

FIGURE 1 | A CACI-IMPACT platform and its working process. **(a)** Rail-based microstructures are embedded in microwells with 96 well plate format and the structures are integrated in a 2 × 6 rectangular array. Water tanks are allocated in both sides to maintain humidity in samples. **(b)** Schematic top and section view of a single well. The microstructure in a single well consists of two low rails (LRs) for primary hydrogel patterning and one high rail (HR) to form a channel for secondary fluid patterning after hydrogel cross-linking. **(c)** Procedure of using the device. Once a hydrogel pre-solution is filled and withdrawn through an injection hole, the solution remains only underneath LRs. When the hydrogel is cross-linked, a microfluidic channel is formed where another fluid can be loaded.

3D Cytotoxicity Assay Using Gel Patterned Device

CFSE-labeled HeLa cells were mixed with collagen gel pre-solution, which is a mixture of rat tail oriented collagen type I (Corning, 354249) with concentration of 9 mg/ml and 150 mM HEPES buffer at 2:1 ratio (v/v). The collagen pre-solution containing HeLa cells were patterned under the low rails of a CACI-IMPACT device (**Figure 2a**) following air plasma treatment with 70 W for 3 min. The device was incubated in a cell culture incubator with 37°C and 5% of CO_2 for 30 min to crosslink the collagen. The cross-linked collagen gel blocks were immersed in media and further incubated for 24 h in the cell culture incubator. NK-92 cell suspension (2×10^6 cells/ml, 2 μl) was loaded into the channel, and the device was stored in the incubator for 20 min at an angle of 90 degrees to let the NK-92 cells settle down on one side of the collagen block encapsulating HeLa cells. Then, the two medium reservoirs were filled with MEMα, and the devices were stored in the incubator for 24 h, or in a live imaging system. PI was added in the medium, and fluorescence images were acquired to assess cytotoxicity.

Image Analysis

Time-lapse images were acquired using an inverted microscope system (Nikon eclipse Ti-E). Endpoint images of NK-92/HeLa

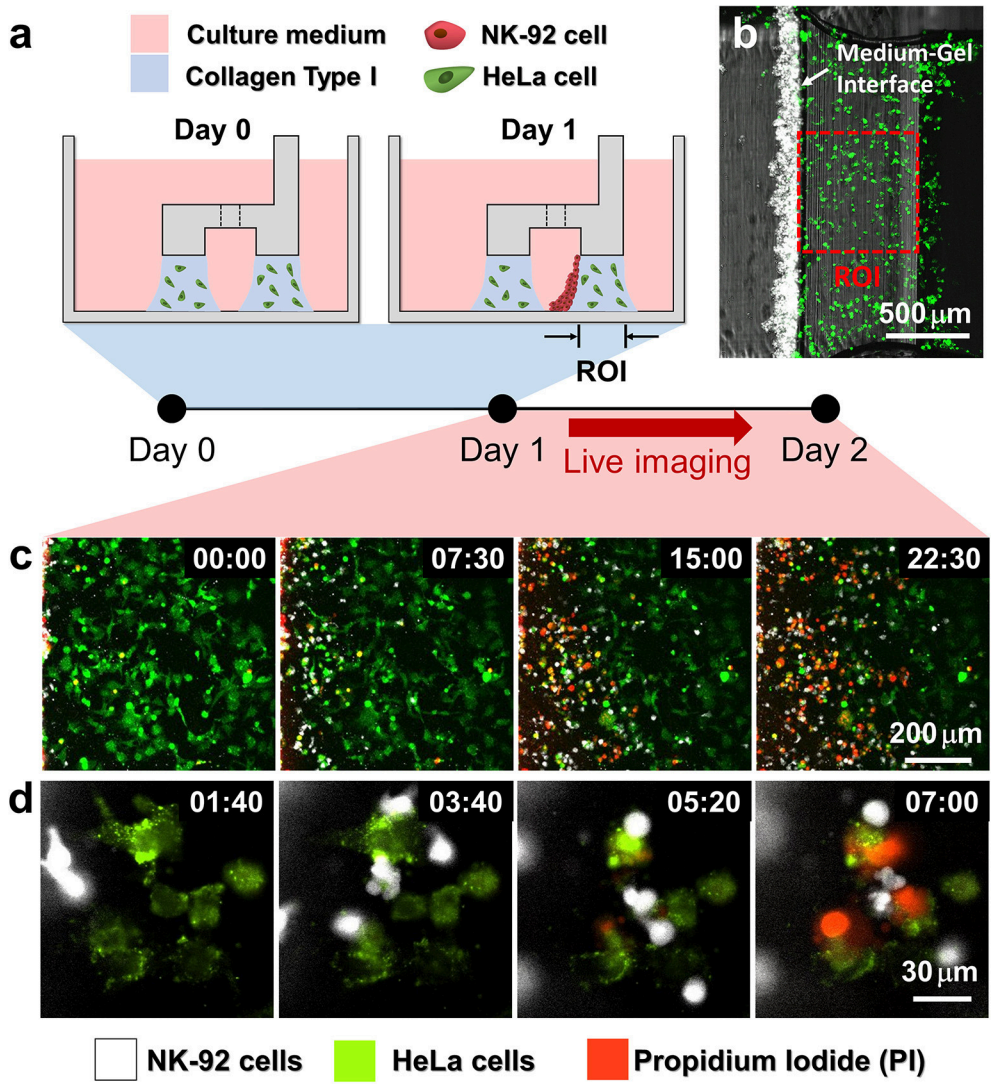

FIGURE 2 | Procedure of 3D cytotoxicity assay and its outputs. **(a)** Schematic process of the assay. HeLa cells embedded in collagen were patterned under low rails (Day 0). After 24 h of cultivation, NK-92 cells were loaded into a microchannel formed by the hydrogel. By tilting the device at an angle of 90°, NK-92 cells were deposited on a collagen block (Day 1) and cultured for additional 24 h to observe migration and cytotoxic activity of NK cells. **(b)** Initial state of the assay (Day 1). **(c,d)** Live monitoring of migration and cytotoxic activity of NK-92 cells. Time is indicated in HH:MM in the top right corner of each image. See also **Movies 2, 3**.

co-culture were acquired using a confocal microscope (Nikon Ti 2 A1) through optical z-sectioning (depth: 100 μm, interval: 4 μm). For image analysis, we used Fiji. Z-projected images were used for display and the images were converted into binary images using auto threshold ("Mean" method) in Fiji for quantitative analysis. The number of NK cells in each sub-region was estimated by dividing the total area of NK cells into the average single NK cell area. Similarly, the percentage of killed HeLa cells were estimated using HeLa cell areas. Since live HeLa cells exhibited extended morphology whereas dead HeLa cells were rounded, areas of dead HeLa cells were converted to those of equivalent number of live HeLa cells by multiplying an average ratio of single live and dead HeLa cell area. Finally, the percentage of killed HeLa cells were calculated by (converted dead HeLa cell area)/(live HeLa cell area + converted dead HeLa cell area). Five unbiased students manually selected 20 NK and HeLa cells, respectively, and the average areas of the selected 100 NK and HeLa cells were used for single cell area of each cell type.

2D Cytotoxicity Assay

CFSE-labeled HeLa cells were plated in each well of a 24 well-plate (6×10^4 cells/well) and cultured for 24 h. Then, various numbers of NK-92 cells were added to the well, and HeLa and NK-92 cells were co-cultured for another 24 h. PI was added to the media to have final concentration of 3 μM, and the percentage of dead HeLa cells were measured by fluorescence microscopy.

RESULTS

Design and Fabrication of CACI-IMPACT Devices for Compartmented Hydrogel Patterning

To fabricate microfluidic devices for cytotoxicity assays in 3D ECM gels, we first designed and fabricated injection molded microfluidic devices that enable facile hydrogel patterning (**Figure 1**). To efficiently observe cytotoxic activity of CLs through fluorescence microscopes equipped in typical biology labs, the device was designed to have the same dimension as a standard microscope slide (3" × 1"), and rail-based microstructures under which cytotoxicity assays would be conducted were embedded in 2 × 6 rectangular array of wells with the same pitches as the conventional 96 well-plate (**Figure 1a**). The rail-based microstructures for hydrogel patterning is composed of two primary patterning rails (low rail, or LR), which are 100 μm apart from the bottom surface, and one secondary patterning rail (high rail, or HR), 500 μm apart from the bottom surface (**Figure 1b**). The rail-based microstructures allowed spatially compartmented hydrogel patterning to be performed by a simple and fast patterning process (**Figure 1c**). First, the surfaces of the device are hydrophilically modified via air plasma treatment. Next, hydrogel pre-solution was injected through an injection hole to fill the entire microstructures (**Figure 1c**-(i)), and subsequently aspirated away by pipetting. Due to the hydrophilicity of the surfaces, only hydrogel pre-solution underneath LR regions remained (**Figure 1c,**ii). Importantly, this process can be performed for 10 wells in a slide within 30 s (**Movie 1**). After crosslinking the hydrogel underneath LR regions, the second solution was loaded underneath the HR region to form two separate compartments (**Figure 1c,**iii). Compared with the PDMS devices widely used in microfluidics that requires tedious batch processes for fabrication, this injection molding-based device can substantially enhance throughput of the assay because the devices can be massively produced. In addition, hydrophilic rail-based microstructures permit hydrogel patterning to be conducted by simple pipetting, thus entire devices can be readily fabricated without requiring any sophisticated equipment/techniques.

Cytotoxicity Assay in 3D ECM Environment

Using the hydrogel patterning technique, we first fabricated collagen gels encapsulating HeLa cells underneath LR regions (**Figure 2a** Day 0**)**, and cultured for 1 day. Then, NK-92 cell suspension was loaded next to the collagen gel, and the device was tilted to 90° for 20 min to accumulate NK-92 cells on one side of the collagen gel by sedimentation (**Figure 2a** Day 1). NK-92 cells attached on the collagen gel surfaces penetrated into collagen gel blocks and migrated toward HeLa cells to exert cytotoxicity.

HeLa cells and NK-92 cells were labeled with two distinct fluorophores, and propidium iodide (PI), a fluorescence dye labeling dead cells, was added in the media to visualize dead cells. In this way, we can simultaneously observe NK-92 cell migration and cytotoxicity along the collagen matrix containing HeLa cells by live imaging (**Figures 2b,c**). NK-92 cells uniformly deposited on collagen block at the beginning of imaging penetrated and migrated into collagen matrix as shown in time-lapse images acquired with a low magnification objective lens (**Figure 2b** and **Movie 2)**. As NK-92 cells propagated into collagen gels, PI-stained HeLa cells near NK-92 cells increased, meaning NK-92 cells exerted cytotoxicity against HeLa cells in collagen gels. Detailed procedures in CL-mediated cytotoxicity of cancer cells, rounding by detachment and subsequent membrane permeabilization allowing PI incorporation (25), can be visualized by time-lapse images using a high magnification objective lens: CFSE-labeled HeLa cells turned round after they made contact with NK-92 cells, followed by PI uptake (**Figure 2c** and **Movie 3**).

3D ECM Reduce Cytotoxicity by Limiting Cancer Cell Accessibility

With this experimental setting, we first investigated how the presence of ECM and the density of cancer cells influenced NK cell cytotoxicity. HeLa cells in two different cell densities (0.8×10^6 and 3.2×10^6 cells/ml) were encapsulated in collagen gels (3 mg/ml) while the NK-92 cell density added in the media was fixed (2.0×10^6 cells/ml), thus effectively total NK-92:HeLa were 5:1 and 20:1, typical ratio used for conventional cytotoxicity assays. Fluorescence images of square region of interest (ROI) with a side length of 700 μm, which is the dimension of the low rail width under which NK cells interact with HeLa cells, were acquired using a motorized stage 24 h after NK-92 cell seeding. The ROI was divided into seven sub-regions with a width of 100 μm, R0 to R6 (**Figure 3a**). The number of NK cells penetrated into the collagen gels and the percentage of killed HeLa cells, or PI-labeled HeLa cells, in each sub-region were measured and plotted (**Figures 3b,c**). R0 and R6 were not considered because they were located near the interface between collagen gel and liquid media where capillary force-mediated meniscus formed (**Figure 3a**), thus boundaries were not clearly defined in some cases.

NK-92 cell number was the highest in R1, and gradually decreased as the sub-region became deeper (**Figure 3b**). NK-92 cells exhibited significantly higher cell numbers in all sub-regions for the lower HeLa cell density, or 20:1, than the case of 5:1 except for R1 (**Figure 3b**). Similar trends were observed for HeLa cell killing, as HeLa cell killing requires close proximity of NK-92 cells and HeLa cells (**Figure 3c**). These results indicates that HeLa cells in collagen gels hold NK-92 cells nearby by forming dynamic immunological synapses (26), thus NK-92 cell migration toward deeper sub-regions gets delayed until they kill substantial fraction of HeLa cells. Percentages of killed HeLa cells in entire sub-regions R1-R5 were measured and compared with 2D cytotoxicity assays performed with equivalent NK-92:HeLa ratio. In 2D, NK-92 cells killed ∼90% of HeLa cells regardless of the ratio, whereas significantly lower percentage of HeLa cells were killed in 3D, and the higher HeLa cell killing occurred with the NK-92:HeLa ratio of 20:1. These results indicate that accessibility of cancer cells is a limiting factor, and migration of CLs is a rate limiting step in cytotoxicity in 3D ECM microenvironments.

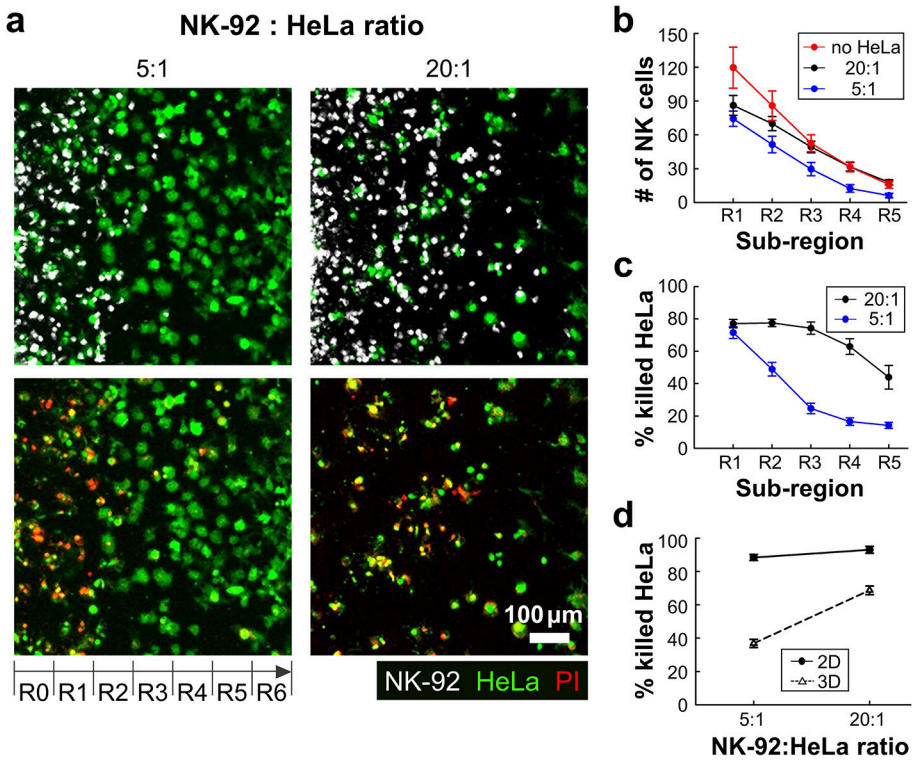

FIGURE 3 | 3D ECM reduces cytotoxicity by limiting cancer cell accessibility. **(a)** Images taken after 24 h of interaction of NK cells and HeLa cells in two NK-92:HeLa ratios. HeLa cells (green) and NK-92 cells (white) are displayed in upper images and lower images show live/dead HeLa cells at the same moment with the upper images. **(b)** The numbers of NK cells within the ROI sub-regions in collagens without HeLa cells (no HeLa), with 20:1 and 5:1 of NK-92:HeLa ratios ($n \geq 12$). **(c)** The percentage of killed HeLa cells within the ROI sub-regions in the two NK-92:HeLa ratios ($n \geq 18$). **(d)** The percentage of killed HeLa cells within the whole ROI from R1 to R5 in the two NK-92:HeLa ratios. ($n = 3$ for 2D assay, $n \geq 18$ for 3D assay). Dot plots in **(b–d)** show mean ± SEM.

Dense ECM Impede Migration of CLs but Facilitate Cancer Cell Lysis

In many solid tumors, fibrosis characterized by dense and stiff ECM generation occurs surrounding areas of tumor cells. Fibrosis not only affect cancer cells by triggering various mechanotransduction pathways by stiffening ECM (27), but also influence immunotherapy efficacy by limiting CL infiltration into tumors (9, 28). We sought to investigate the role of ECM density, a key component of fibrosis, on lymphocyte cytotoxicity in 3D by using the device patterned with various concentrations of collagen (2–4 mg/ml). Effective NK-92:HeLa ratio was fixed to 5:1. Representative still images of the patterned collagen gels with various collagen concentrations 24 h after NK-92 cell seeding are shown in **Figure 4a**. NK-92 cells distributed throughout the collagen gels in 2 mg/ml of collagen gel, whereas few NK-92 cells were observed in R4-R6 in 4 mg/ml of collagen gel. Overall, NK cell number was the highest in R1 and gradually decreased as the sub-region became deeper for all collagen concentrations (**Figure 2b**), and NK cell number was the highest for the lowest collagen concentration and gradually decreased as the collagen concentration increased for all sub-regions (**Figure 2b**). Similar trends in the percentage of killed HeLa cells were observed (**Figure 2c**). These results indicate that ECM density plays important role in NK cell migration in collagen gels, and consequently affect NK cell cytotoxicity.

Next, we investigated detailed interactions between NK-92 cells and HeLa cells encapsulated in different concentrations of collagen. By performing time-lapse imaging, we directly visualized NK-92 cell-HeLa cell interactions in a single cell level (**Figure 2d** and **Movie 3** in SI), and assessed how much time is needed for each NK-92 cell for successfully killing HeLa cells. Time for killing, which measures time from initial NK-92/HeLa contact to PI uptake in HeLa cells, was measured for each NK-92 cell successfully killed NK-92 cell and plotted for NK-92 cells in collagen gels with 2 and 4 mg/ml (**Figure 4d**). Interestingly, time for killing of NK-92 cells in 2 mg/ml collagen gels was significantly lower than that of NK-92 cells in 2 mg/ml collagen gels. This result indicate that cytotoxicity of individual NK-92 cells is higher in higher concentration of collagen gels. Taken together, ECM density can influence both migration and cytotoxicity of CLs.

DISCUSSION

Traditional *in vitro* 2D cytotoxicity assays against cancer cells have been widely used to evaluate *ex vivo* engineered or cultured CLs due to simplicity in assays, but the assay results may

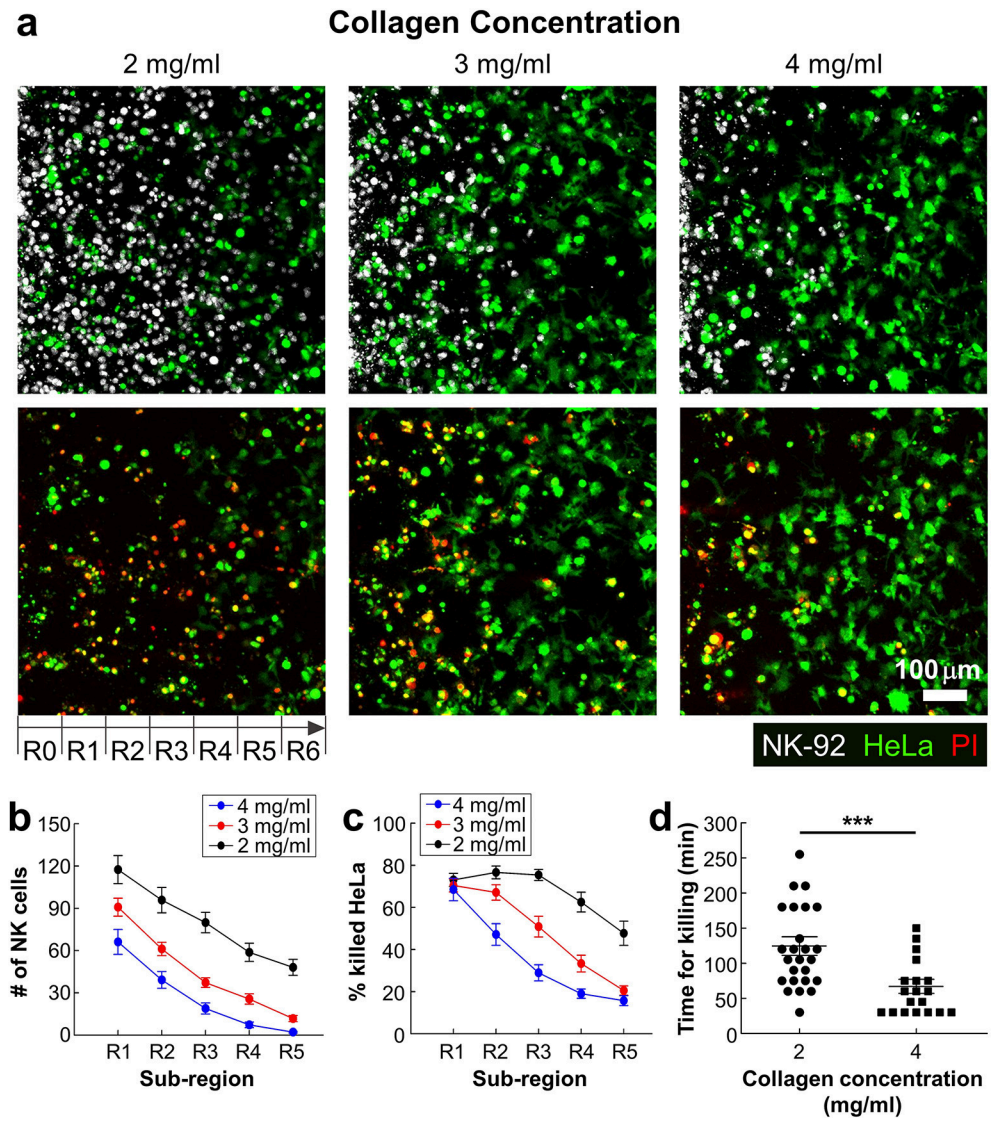

FIGURE 4 | Dense ECM impedes cytotoxic activity of NK cells. **(a)** Images taken after 24 h of interaction of NK cells and HeLa cells in three collagen concentrations. HeLa cells (green) and NK-92 cells (white) are displayed in upper images and lower images show live/dead HeLa cells at the same moment with upper images. **(b)** The numbers of NK cells and **(c)** the percentage of killed HeLa cells within the ROI sub-regions ($n \geq 16$). **(d)** PI uptake time from the moment that NK-92 cells contact with HeLa cells in 2 and 4 mg/ml of collagens. Each dot was obtained from a single HeLa cell killed by a single NK-92 cell. For statistical comparison, unpaired two-tailed Student's *t*-test was performed, and the statistical significance was $^{***}p < 0.001$.

not be consistent with *in vivo* results due to the absence of 3D tumor microenvironment (TME). PDMS-based microfluidic devices recapitulating various aspects of TME, including hypoxia, inflammatory cytokines, immunosuppressive conditions, and vasculatures, have been developed to evaluate CLs in 3D (20–22).

However, PDMS-based devices require labor and time intensive batch fabrication processes, thus device fabrication limits experimental throughputs (23). To overcome this limitation, the CACI-IMPACT devices used in this study were massively produced using injection molding with polystyrene (PS) by customizing the design of the IMPACT device (24), which was previously developed by our group for 3D compartmentalized cell culture. In our experience of conducting the same 3D cytotoxicity assays using our PDMS-based co-culture device (29), approximately 2 days of serial processes, including casting (7 h), punching (1 h), bonding (10 m), and surface hydrophobicity restoration (>1 d), were required. The series of manual processes can cause defects, resulting in lower yields or lower uniformity of the final devices to be used in experiments. Furthermore, pressure sensitive loading process in PDMS device reduces usability and experimental throughput. In sharp contrast, CACI-IMPACT device requires <10 min for device preparation including 3 min of hydrophilic surface modification. In case that the device was packed after

plasma treatment, no preparation is required except opening the packaging. Rail-based microstructures with hydrophilic surfaces further facilitated experiments by enabling simple and fast hydrogel patterning to be performed, and multi-well format further enhanced experimental throughputs by allowing multiple experiments to be performed simultaneously in a single device. In addition to improved device fabrication and experimental throughputs, long term monitoring of CL-cancer cell interactions is possible, as media change can be readily performed by aspirating media in the media reservoir and filling new media without perturbing hydrogels containing cells.

Using the injection molded devices, we performed 3D cytotoxicity assays with various density of cancer cells and various collagen concentrations. First, we found the presence of ECM and cancer cells could significantly reduce cytotoxicity of CLs by impeding migration and limiting accessibility of cancer cells compared with 2D cytotoxicity assays (**Figure 3**). Presence of cancer cells in ECM may play dual role in cytotoxicity: it may impede infiltration of CL by interacting with CLs as we have shown, but at the same time, cancer cells can promote CL migration by producing chemokines such as CXCL9, 10, and 11 (30). As shown in **Figure 3b**, NK-92 cell distribution in collagen gels lacking HeLa cells (no HeLa) was comparable to that in collagen gels with low density of HeLa cells (20:1) except for entry regions, where NK-92 cell numbers were slightly higher for collagen gels lacking HeLa cells, indicating chemotaxis-mediated NK-92 cell migration was minimal in our system. Second, we found ECM density played an important role in 3D cytotoxicity by independently regulating migration-mediated cancer cell contact and direct cancer cell killing (**Figure 4**). Indeed, collagen density can influence various physical properties of collagen gels such as pore size and stiffness (31). When collagen concentration was increased, NK cell infiltration into collagen gels was substantially reduced presumably due to reduced pore size that limits amoeboid-mode immune cell migration (32). Indeed, increased ECM density observed in fibrotic tumors reduced activity of T cells by limiting physical access of tumor cells (14), indicating our device may be a good model system to evaluate *ex vivo* engineered CLs for fibrotic tumors. Interestingly, cytotoxicity of individual NK cells were significantly enhanced when collagen concentration was increased (**Figure 4d**). While detailed mechanisms for enhanced cytotoxicity in collagen-dense environments need to be determined, it is possible that stiff ECM environments in high concentration of collagen facilitate tumor cell lysis by increasing tumor cell tension, which enhances perforin-mediated pore formation on tumor cell membrane (33).

Our preliminary 3D cytotoxicity assay using human primary NK cells revealed that CACI-IMPACT platform can be used for primary lymphocytes, while detailed assay conditions need to be adjusted depending on cell types (**Figure S1**). Primary NK cells exhibited much higher motility and cytotoxicity compared with NK-92 cells: they uniformly distributed in entire collagen gels (**Figure S1b**) and killed the majority of HeLa cells (**Figure S1c**) within 12 h in dense ECM (4 mg/ml of collagen), in which NK-92 cells killed only ∼40% of HeLa cells for 24 h (**Figure 4c**). Superior cytotoxicity of primary NK cells in our assay is partly due to smaller size of primary NK cells (diameter ∼8 μm) compared with that of NK-92 cells (diameter ∼ 14 μm), which plays important role in cell migration in dense ECM (31), further confirming importance of lymphocyte motility in 3D cytotoxicity.

To sum up, we introduced an injection molded microfluidic device for assessing cytotoxicity of CLs in 3D environment. The proposed device is characterized by (i) enhanced productivity via injection molding, (ii) enhanced experimental throughput mediated by multi-well format of the device, and (iii) hydrophilic rail-based microstructures facilitating hydrogel patterning with simple pipetting. Using the device, we found 3D ECM significantly reduce cytotoxicity of CLs by impeding migration and access to tumor cells compared with traditional 2D assays. We also found denser ECM impede migration of CLs but enable effective killing once CLs contact with tumor cells. The results show how important the presence of ECM is for accessing cytotoxicity of CLs against solid tumors. We think this injection molded 3D culture platform could be used to evaluate cytotoxicity of CLs in 3D environment and to identify new therapeutic approaches mediated by adoptive transferred CLs against solid tumors.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

DP designed the study, conducted experimental work, analyzed the data, and wrote the paper. KS conceptualized the work and conducted experimental work. YH conducted experimental work and analyzed the data. JK and YL designed the device used in the study. JD and NJ supervised experimental work, data analysis, and wrote the paper.

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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.2019.01133/full#supplementary-material

Movie S1 | Demonstration of high-throughput fluid patterning with green food dye mixed water.

Movie S2 | Live monitoring of cytotoxic activity of NK-92 cells against HeLa cells within CACI-IMPACT platform acquired with a low magnification objective lens.

Movie S3 | Live monitoring of single cell level cytotoxic activity of NK-92 cells against HeLa cells.

Figure S1 | 3D cytotoxicity assay performed with primary NK cells against HeLa cells. **(A)** Representative images taken after 6H (left) and 12H (right) of primary NK/HeLa cells co-culture. **(B)** The number of primary NK cells and **(C)** the percentage of killed HeLa cells within the ROI sub-regions after 6H (black) and 12H (red) of primary NK/HeLa cells co-culture.

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Conflict of Interest Statement: NJ is a founder of Curiochips inc., and he holds equity in this company. The CACI-IMPACT device is posted on the company website (https://www.curiochips.com/).

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

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IL-4/IL-13 Stimulated Macrophages Enhance Breast Cancer Invasion Via Rho-GTPase Regulation of Synergistic VEGF/CCL-18 Signaling

Andrew C. Little, Pragathi Pathanjeli, Zhifen Wu, Liwei Bao, Laura E. Goo, Joel A. Yates, C. Ryan Oliver, Matthew B. Soellner and Sofia D. Merajver**

Department of Internal Medicine, Hematology-Oncology, Rogel Cancer Center, University of Michigan, Ann Arbor, MI, United States

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Leila Akkari,
The Netherlands Cancer Institute (NKI), Netherlands

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Kawaljit Kaur,
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Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
Andrew C. Little
aclittle@med.umich.edu
Sofia D. Merajver
smerajve@med.umich.edu

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Tumor associated macrophages (TAMs) are increasingly recognized as major contributors to the metastatic progression of breast cancer and enriched levels of TAMs often correlate with poor prognosis. Despite our current advances it remains unclear which subset of M2-like macrophages have the highest capacity to enhance the metastatic program and which mechanisms regulate this process. Effective targeting of macrophages that aid cancer progression requires knowledge of the specific mechanisms underlying their pro-metastatic actions, as to avoid the anticipated toxicities from generalized targeting of macrophages. To this end, we set out to understand the relationship between the regulation of tumor secretions by Rho-GTPases, which were previously demonstrated to affect them, macrophage differentiation, and the converse influence of macrophages on cancer cell phenotype. Our data show that IL-4/IL-13 *in vitro* differentiated M2a macrophages significantly increase migratory and invasive potential of breast cancer cells at a greater rate than M2b or M2c macrophages. Our previous work demonstrated that the Rho-GTPases are potent regulators of macrophage-induced migratory responses; therefore, we examined M2a-mediated responses in RhoA or RhoC knockout breast cancer cell models. We find that both RhoA and RhoC regulate migration and invasion in MDA-MB-231 and SUM-149 cells following stimulation with M2a conditioned media. Secretome analysis of M2a conditioned media reveals high levels of vascular endothelial growth factor (VEGF) and chemokine (C-C motif) ligand 18 (CCL-18). Results from our functional assays reveal that M2a TAMs synergistically utilize VEGF and CCL-18 to promote migratory and invasive responses. Lastly, we show that pretreatment with ROCK inhibitors Y-276332 or GSK42986A attenuated VEGF/CCL-18 and M2a-induced migration and invasion. These results support Rho-GTPase signaling regulates downstream responses induced by TAMs, offering a novel approach for the prevention of breast cancer metastasis by anti-RhoA/C therapies.

Keywords: Rho (Rho GTPase), metastasis, invasion, breast cancer, migration

INTRODUCTION

Since the mid-1990s, strategies to detect and treat early breast tumors have greatly improved, reflected in improved survival rates (1). However, breast cancer remains a serious disease, projected to claim the lives of nearly 42,000 women in in the US 2018 (1). Of the many factors that contribute to breast cancer-related mortality, metastatic spread is the most important. While some patients can live for years with late-stage metastasis, early diagnosis of metastatic dissemination offers no improvement to 5-year survival rates over diagnosing metastases when symptoms occur, most likely due to our current lack of available therapies specifically designed to target metastases and/or inhibit widespread cancer cell dissemination from a micrometastatic disease stage. Therefore, it is paramount that we enhance our understanding of the molecular mechanisms which drive the early stages of metastasis to enable realistic strategies to attenuate metastatic spread.

Cell migration is critical for normal development and physiology, although it can be aberrant in chronic inflammation and cancer metastasis. Over the last 15 years, it has been shown that signals from non-cancer cells in the tumor microenvironment (TME) contribute to enhancing the invasive phenotype of cancerous cells (2). Intriguingly, tumor-associated macrophages (TAMs) are the most abundant immune cell population in mammary tumors and are associated in multivariate analyses with elevated proportions of invasive tumor cells, high vascular grade, and reduced overall survival, pinning them as an independent biomarker of cancer severity and a prognostic indicator of metastatic progression (3–5). Macrophages are an inherently plastic cell population, readily switching between pro- (M1) and anti-inflammatory (M2) phenotypes depending on their environment (6, 7). In cancer, an environment of chronic inflammation is presumed to direct macrophage polarization toward an anti-inflammatory, M2-like phenotype (7). Under normal circumstances, the physiological role of M2 macrophages is to diminish inflammation to aid in tissue and/or epithelial wound repair (8). However, the features acquired by M2 macrophages in the TME have effects that are paradoxically associated with tumor progression; for instance, they facilitate and/or enable angiogenic responses, promote tumor growth, and eventually lead to tumor metastasis. Despite the rapidly growing number of studies which have characterized TAMs, the vast number of secreted factors by cancer cells and other cells of the TME leads to a diverse and transient TAM population that can readily switch between polarization states. Thus, characterizing which M2 population of macrophages (e.g., M2a, M2b, M2c) are specifically responsible for promoting tumorigenic outcomes in breast cancer remains an important, but not yet achieved goal.

The Rho family of GTPases are recognized for their role in directing cell migration. Aberrant regulation of the Rho-GTPases has been identified as an important contributing factor in the acquisition of the metastatic phenotype (9–12). Starting with Rac1 and RhoA (13, 14), other Rho-GTPases, including RhoC (15, 16), have been described for their specific roles in cellular motility, invasion, metastases, and angiogenesis (12, 17, 18). Our previous work showed that RhoC regulates inflammatory breast cancer migratory responses to macrophage conditioned media (19). Therefore, we hypothesized that RhoC plays a regulatory role specifically in TAM-induced breast cancer cell migratory and invasive responses.

In this study, our data defines IL-4/IL-13 stimulated macrophages (M2a macrophages) as the strongest inducers of breast cancer cell migration and invasion. Intriguingly, we find that both the Rho-GTPases RhoA and RhoC regulate M2a-induced responses to varying degrees. Our analysis of the M2a macrophage secretome confirms high levels of CCL-18 (20, 21). Importantly, our results showed significantly higher levels VEGF in M2a conditioned media vs. their M2b or M2c macrophage counterparts. Intriguingly, we find that CCL-18 and VEGF synergistically promote breast cancer migration and invasion, and this response is diminished via treatment of cells with the Rho-associated kinase (ROCK) inhibitor(s) Y-27632 or GSK429286A, delineating a unique targetable regulatory role for the Rho-GTPases. Collectively, these findings suggest therapeutic targeting of the Rho-GTPases may offer a novel approach for the prevention of breast cancer metastases.

MATERIALS AND METHODS

Cell Models

Triple negative breast cancer (TNBC) MDA-MB-231 (MDA-231) cells were acquired from ATCC and maintained in Gibco RPMI-1640, 10% FBS, 5 μg/mL gentamycin, 2 mM L-glutamine, and 1X anti-anti. TNBC inflammatory cell model SUM-149 was kindly provided by Dr. Steve Ethier. SUM-149 cells were maintained in Gibco Ham's F12, 5% FBS, 0.5% penicillin-streptomycin, 2.5 μg/mL fungizone, 5 μg/mL gentamycin, 5 μg/mL insulin, 1 μg/mL hydrocortisone, and 2 mM L-glutamine. Randomized primary human female monocytes collected from whole blood were obtained from Astarte Biologics (Astarte, WA, USA). Human primary monocytes were cultured in X-VIVO 15 (Lonza, GA, USA), supplemented with 10% pooled human serum (Innovative Research, MI, USA), 50 ng/mL M-CSF, and 0.5% penicillin-streptomycin.

Monocyte Polarization/Characterization, Macrophage Propagation, and Isolation of Conditioned Media (C.M.)

U937 monocytes were matured to macrophages with 50 ng/mL macrophage colony stimulating factor (M-CSF) for 24 h. Adherent macrophages were then polarized for 24 h with either 50 ng/mL IL-4 and IL-13 (M2a), ovalbumin-ovalbumin antibody immune complex extracts (IC; M2b), or 50 ng/mL IL-10 (M2c). IL-4, IL13, and IL-10 were purchased from R and D Systems (R&D, MN, USA). Following polarization, conditioned media was collected and concentrated with Amicon 3K MWCO spin columns to a final 10X concentration. Macrophage total RNA was collected and isolated using Qiagen RNeasy Mini kit (Qiagen, MD, USA). Total RNA was converted to cDNA using Promega AMV reverse transcriptase kit (Promega, WI, USA) and gene expression was evaluated by RT-qPCR using an ABI

Quantstudio 3 (ABI, CA, USA). For RT-qPCR primers sequences and efficiencies, **Supplemental Table 1**. Human monocytes were differentiated to macrophages in the presence of 50 ng/mL M-CSF for 10–12 days until fully adherent and displayed proper macrophage morphology and size. Macrophages were then challenged with either 50 ng/mL IL-4 and IL-13 or vehicle control, daily for 4 days. Conditioned media was collected daily for 4 days and frozen at −80°C until concentration. Human macrophage conditioned media was concentrated in the exact fashion as described above for further use.

Migration and Invasion Assays

For 2D migration, we employed a wound closure assay using Ibidi wound closure inserts. Cells were seeded at 7×10^5 per mL and allowed to adhere overnight e.g., ∼16 h. Following insert removal, cells were supplemented with various treatments, and wound closure was imaged. For 3D invasion, cells were seeded at 3×10^4 in Corning ultra-low attachment spheroid round bottom 96-well plates and allowed to form spheroids for 3 days. Following spheroid formation, cells were embedded in an invasion matrix (Trevigen, CT, USA) and invasion was monitored over 6 days. For donut migration, technical details can be found here (22). For transwell migration assays, cells were seeded at 1×10^5 in the apical chamber of Corning BioCoat Matrigel Invasion Chambers (Corning #354480, USA) and allowed to invade for 24 h. Invaded cells were counted manually. All images were acquired on the BioTek Cytation 5 imaging station, in tandem with the BioTek BioSpa automated incubator and robotics system (BioTek, VT, USA). For each of the cell treatments, C.M. was supplemented at a final 1X concentration; 20 ng/mL VEGF, CCL-18, or IL-4; 1 μM Y-27632 or GSK429286A (Tocris, R&D, USA).

ELISA; Secreted Protein Evaluation, Cytokine Removal Assays

Aliquots of conditioned media isolates were submitted to the University of Michigan Immunology Core for pre-validated ELISA assays. Raw ELISA data was corrected for whole protein content determined by BCA (Pierce, Thermo Fisher). Corrected ELISA data was averaged across 4 independent experiments and plotted in a heat map using the free online matrix visualization software Morpheus (Broad Institute, USA). Statistical significance was determined by one-way ANOVA. For cytokine removal assays, we treated 10X M2a conditioned media with 10 μg/mL VEGF (R&D Systems #MAB293-100), CCL-18 (R&D #AF394), or a combination of VEGF and CCL-18 antibodies. Non-specific IgG_{2B} or IgG antibodies (10 μg/mL) were used as controls for VEGF or CCL-18, respectively.

Cell Proliferation

Cells were seeded in 96 well formats and supplemented with various treatment regiments. Cell proliferation was monitored every 12 h using Promega ATP Cell Titer Glo reagent, per manufacturers protocol (Promega, WI, USA). For label free cell growth, we utilized image-based cellular identification strategies on our BioTek BioSpa-Cytation 5 automated high throughput imaging station. Cell size thresholds and background removal were applied for either MDA-231 or SUM-149 cells, and cells were counted over the course of 4 days. Statistical significance was determined by one-way ANOVA.

RESULTS

IL-4/IL-13 Polarized Macrophages Are the Strongest Enhancers of Breast Cancer Cell Migration and Invasion

To examine the effects the diverse M2-like macrophage populations had on breast cancer cells, we first induced *in vitro* monocyte-to-macrophage polarization by the addition of M-CSF to U937 monocyte cells. Following macrophage differentiation and cell adhesion, we stimulated macrophages with either recombinant IL-4/IL-13 (to promote an M2a phenotype), ovalbumin-ovalbumin antibody conjugate (to promote the M2b phenotype), or recombinant IL-10 (to promote an M2c phenotype) (**Supplementary Figures S1A,B**). To confirm polarization, we surveyed each population's RNA expression profile using reverse transcriptase-quantitative PCR (RT-qPCR) (**Supplementary Figure S1C**). Primer efficiency for RT-qPCR primers utilized in this study were verified to ensure fidelity, and primer sequences are listed in **Supplementary Table 1.** To study the effects of the three M2-like macrophages on breast cancer cell motility, we collected conditioned media from the three populations, concentrated them 10-fold, and supplemented cancer cells with a 1X final dilution of TAM-conditioned media. Stimulation with each of the three M2-macrophage conditioned media enhanced migration in wound closure assays in the TNBC MDA-MB-231 cell model (MDA-231) (**Figure 1A**), as well as the inflammatory TNBC cell line SUM-149 (**Figure 1B**). Specifically, stimulation with conditioned media from M2a macrophages enhanced both MDA-231 and SUM-149 cell migration in wound closure assays greater than conditioned media from either M2b or M2c macrophages (**Figures 1A,B**).

To independently test our results from wound closure assays, we employed a modified donut assay to assess 2D migration (22, 23). Results from the donut assay support our findings from wound closure assays, whereas stimulation of MDA-231 cells (**Figure 1C**) or SUM-149 cells (**Figure 1D**) with macrophage conditioned media produces an enhanced migratory response to M2a macrophages. To confirm that we were observing migration and not just increased proliferation, we stimulated cells in the same fashion as described in the migration assays and surveyed cell viability with ATP Cell Titer Glo reagent. No significant changes in cell numbers or proliferation were observed upon comparing stimulation with conditioned media from the three M2 macrophage populations (**Figure 1E**).

As macrophages and TAMs alike are known to alter their metabolism depending on their microenvironment and functional phenotypic requirements (24, 25), next we explored their metabolic adaptations in response to cancer cells. Metabolic flux of innate immune cells in the TME, such as altered levels of secreted metabolites (e.g., lactate), has been shown to influence cancer cell behavior (26, 27). This is of particular interest in inflammatory breast cancer, as recent findings from our lab

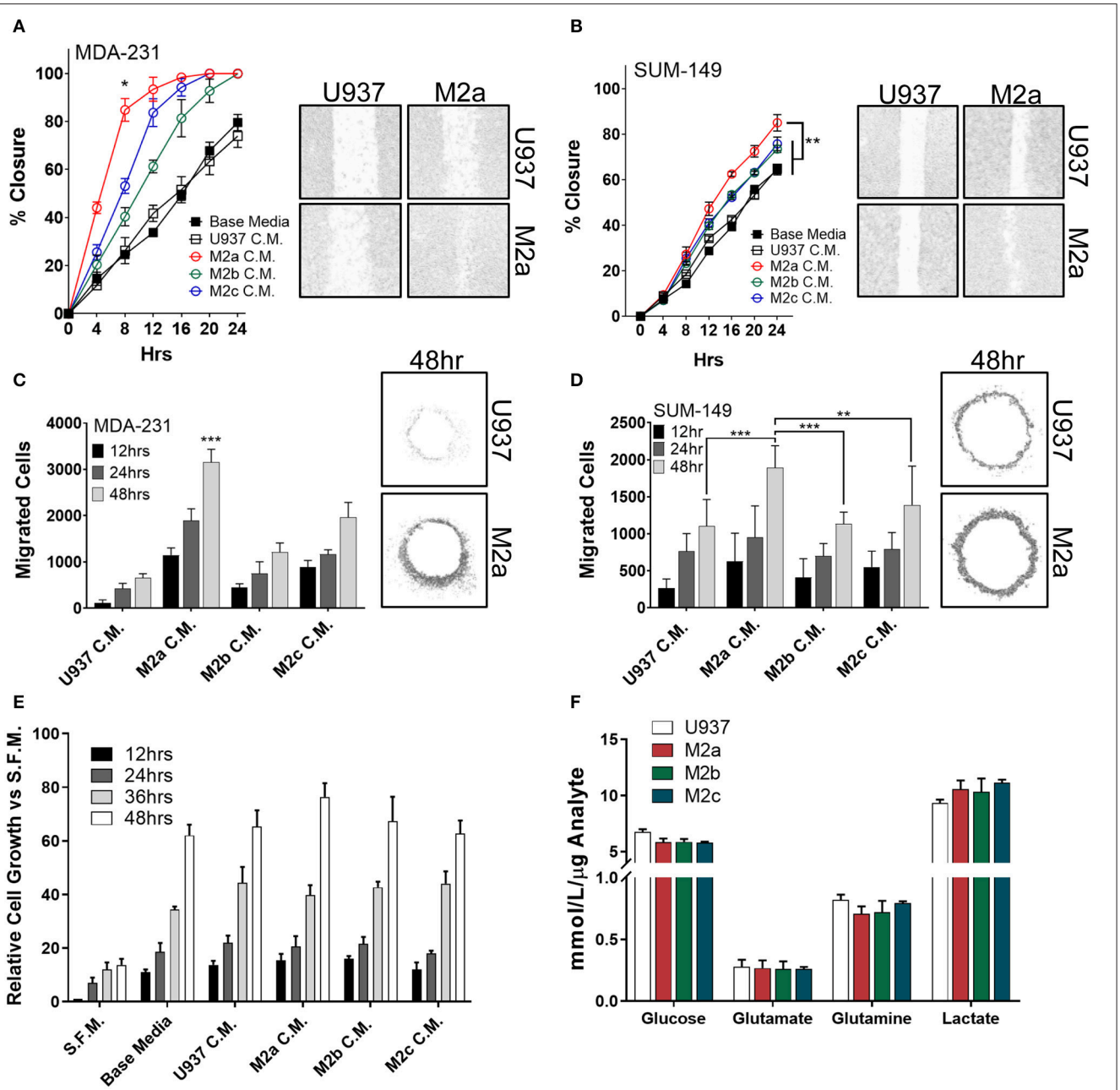

FIGURE 1 | M2a macrophage conditioned media is a potent inducer of breast cancer cell 2D migration. Results from scratch wound migration assays display M2a conditioned media elicits a significantly greater migratory response in MDA-231 cells **(A)** and SUM-149 cells **(B)**. Similar results observed in a modified donut cell migration assay in MDA-231 **(C)** and SUM-149 cells (**D**); 48 h post M2a C.M. addition in MDA-231 cells is statistically significant to all other 48 h time points, $p < 0.001$ **(C)**. The three M2 macrophage conditioned medias do not alter cellular growth rates in MDA-231 cells **(E)**. Results from YSI metabolite analysis display no changes in key metabolic analytes among the three M2 macrophages **(F)**. All results are compiled from ≥2 independent experiments; error bars represent ± standard deviation; Statistical significance determined by one-way ANOVA; *p*-value is displayed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

show SUM-149 cells are heavily glycolytic, heavily dependent on glutamine for survival, and SUM-149 metabolism is regulated by RhoC (28). Therefore, we surveyed levels of consumed glucose, glutamate, and glutamine, while simultaneously examining secreted levels of lactate in the cell culture medium of polarized M2a, M2b, or M2c macrophages. We did not observe any unique differences between the three M2 macrophages (**Figure 1F**), suggesting that their metabolic profiles are not significantly contributing to enhancing breast cancer cell migration.

Two-dimensional cell migration offers insight to unidirectional cellular motility, but it is a poor model for tumor cell invasion, as it does not recapitulate many of the

key features of an *in vivo* tumor, such as extracellular matrix (ECM) components and 3D sphere-like growth. Therefore, we next aimed to test M2 macrophage-induced effects on tumor cell invasion specifically in 3D formats. To this end, we employed a spheroid invasion assay. MDA-231 cells or SUM-149 cells were embedded in Trevigen ECM matrix, supplemented with M2a, M2b, or M2c conditioned media, and allowed to propagate and invade for 6 days. Over this period, MDA-231 cells had a significantly greater invasive response to M2a conditioned media than controls (**Figure 2A**). Surprisingly, M2b or M2c conditioned media had a suppressive effect on MDA-231 spheroid invasion, contrasting the results we observed in 2D migration assays (**Figure 2A**). Similarly, M2b and M2c were suppressive for 3D invasion in the SUM-149 cells, although to a lesser extent (**Figure 2B**). This difference could be accounted by SUM-149's slower migratory rates than MDA-231 cells. To examine if M2a-induced responses were sufficient to induce migration in normal breast cells, we performed the spheroid invasion assay in human mammary epithelial cells and stimulated them with the various M2 conditioned media. Importantly, no significant differences were observed in hME cells (**Supplementary Figure S2**), supporting that the response to macrophages may be particular to transformed breast cancer cells.

As an orthogonal approach to the spheroid invasion/growth assay, we utilized a transwell migration assay where cells seeded in an apical transwell insert must traverse through a collagen layer to seed and colonize the basolateral side of the insert. Again, M2a conditioned media induced MDA-231 and SUM-149 cell invasion at a significantly higher rate than M2b or M2c conditioned media (**Figures 2C,D**), further supporting our findings from the spheroid invasion experiments. These results show that M2a polarized macrophages strongly and specifically enhance breast cancer cell migration and invasion in 3D.

Rho-GTPases RhoA and RhoC Regulate M2a Induced Migratory Responses in Breast Cancer Cells

While an extensive body of research has characterized the Rho-GTPases role in the regulation of cancer cell motility, here we aim to understand the direct impact the Rho-GTPases have on communication signals from the tumor microenvironment to the cancer cell, a question that has remained largely unexplored. While RhoC is imperative for developmental processes (29–31), RhoC is also a crucial regulator of metastatic progression in various cancers likely due to its dysregulation (11, 32, 33). Thus, a detailed understanding of how it regulates the cancer cell responses to pro-tumorigenic macrophages would open new therapeutic strategies. Our previous report proved that the Rho-GTPase RhoC was necessary to regulate macrophage induced migration of the inflammatory breast cancer cell model SUM-149 (19). Therefore, we hypothesized that RhoC, and potentially RhoA, may both regulate TAM-induced migratory responses, particularly in response to M2a TAMs. To test the role of RhoA and RhoC in M2a-induced breast cancer cell migration, we stimulated MDA-231 or SUM-149 wild type (WT), RhoA CRISPR-Cas9 knockout (ΔRhoA), or RhoC CRISPR-Cas9 knockout (ΔRhoC) with either base media or M2a conditioned media and monitored wound closure over 24 h. Interestingly, both RhoA and RhoC knockout had significant and major impact on M2a-induced migration in both the MDA-231 cells (**Figure 3A**) and the SUM-149 cells (**Figure 3B**).

While both RhoA and RhoC appear to regulate M2a-induced responses, our data supports that RhoA is a quantitatively more critical mediator of TAM induced migration, as we observe significantly diminished migration in the RhoA knockout cell lines (**Figures 3A,B**). These results are supported in a 3D spheroid invasion assay, as the RhoC and RhoA knockout lines do not display a significant response to M2a-conditioned media in either MDA-231 (**Figure 3C**) or SUM-149 cells (**Figure 3D**). These data confirm that the Rho-GTPases, RhoA and RhoC, both regulate M2a-induced metastatic responses. Despite the overall diminished migratory/invasive responses in our RhoA or RhoC knockout cell lines, conditioned media from M2a macrophages is still able to elicit a pro-migratory effect. These results strongly support that RhoA and RhoC harbor unique and independent regulatory roles in breast cancer migration.

Stimulation of the Rho-GTPases activates their downstream kinase, Rho-associated protein kinase (ROCK), known to regulate cell motility (17). Our data suggest that RhoA and RhoC both harbor regulatory roles in mediating M2a macrophage induced responses; therefore, we hypothesized that responses may be directed through ROCK signaling. To test this, we pretreated MDA-231 cells or SUM-149 cells with the ROCK inhibitors Y-27632 or GSK429286A (GSK) and surveyed migratory responses to M2a conditioned media in a 2D migration assay. Indeed, ROCK inhibition slowed M2a-induced migration rates in both MDA-231 (**Figure 3E**) and SUM-149 cells (**Figure 3F**). We next aimed to determine if ROCK signaling regulated 3D invasion. We observe similar results in the 3D spheroid invasion assay in both the MDA-231 (**Figure 3G**) and SUM-149 cells (**Figure 3H**). Collectively, these findings show that Rho-GTPases regulate M2a-induced migratory and invasive responses in both MDA-231 and SUM-149 cells.

M2a Macrophage Derived CCL-18 and VEGF Synergistically Enhance the Breast Cancer Metastatic Phenotype, Regulated by ROCK Signaling

TAMs routinely secrete a large suite of cyto/chemokines, growth factors, and other components which can promote cancer cell extravasation, suppress innate immune function in the TME, and recruit other immune modulators to support cancer metastases (5, 34) by shielding cancer cells from immune mediated destruction. Therefore, it is critical to understand the mechanisms by which M2a TAMs promote breast cancer cell migration and invasion and how the Rho-GTPases regulate these processes. We surveyed conditioned media from U937 monocytes and the three M2-like macrophages for a large array of their potential secreted components. As others have reported (20, 21), we found significantly elevated levels of CCL-18 in M2a conditioned

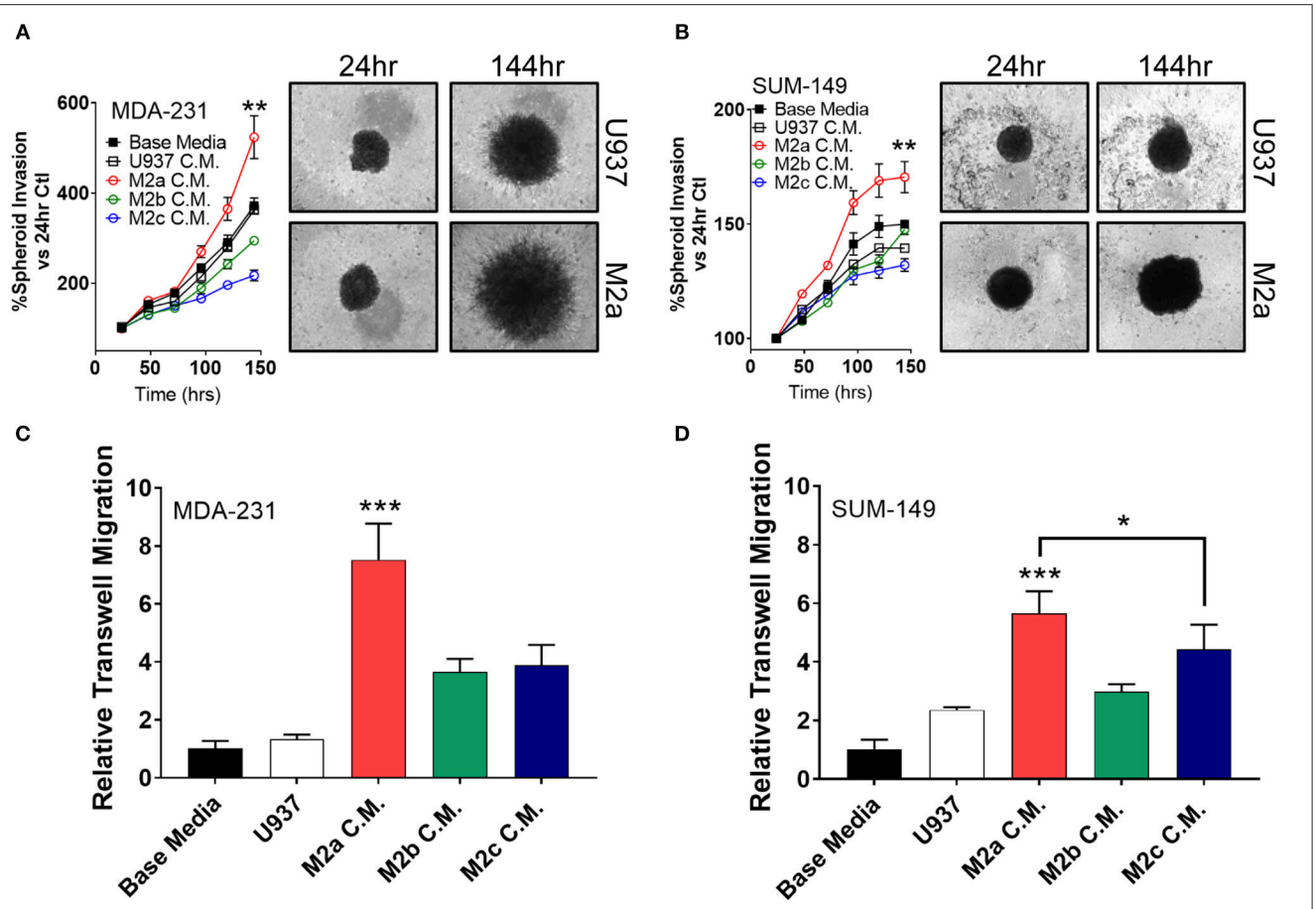

FIGURE 2 | 3D cell invasion assays confirm M2a TAMs as the greatest enhancer of breast cancer cell invasion. **(A)** Results from spheroid invasion assays in MDA-231 cells display a strong response to M2a conditioned media (C.M.). **(B)** Spheroid invasion results from the inflammatory TNBC cell line, SUM-149. Statistical significance in **(A,B)** is displayed as the 144 h time point compared against all other 144 h time points. **(C,D)** Results from 3D transwell migration confirm M2a conditioned media (red) enhances MDA-MB-231 **(C)** and SUM-149 **(D)** cancer cell invasion. All results are compiled from ≥2 independent experiments; Statistical significance was determined by one-way ANOVA; error bars represent ± standard deviation; *p*-value is displayed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

media vs. their other M2 macrophage counterparts (**Figure 4A**; **Supplementary Figure S3**). In parallel, we found significantly higher levels of IL-4 (data corrected to remove exogenously supplemented recombinant IL-4) and VEGF in M2a conditioned media (**Figure 4A**; **Supplementary Figure S3**). In contrast, we found significantly lower levels of chemokine (C-X-C motif) ligand 9 (CXCL-9) and chemokine (C-C motif) ligand 20 (CCL-20) in M2a conditioned media compared to the other M2 populations. We further aimed at understanding which of components were most salient in their influence on motility and invasion.

To evaluate which of these components were critically important in supporting breast cancer migration, we treated MDA-231 or SUM-149 cells with each of the individual proteins/cytokines and examined migratory responses. Our data exhibit strong migratory responses to recombinant CCL-18 (rCCL-18; 20 ng/mL) in both the MDA-231 and SUM-149 cells (**Figures 4B–D**). Exogenous addition of recombinant VEGF ($rVEGF_{165}$; 20 ng/mL) promoted cell migration, although to a lesser extent than rCCL-18. Interestingly, stimulation with both VEGF and CCL-18 promoted the fastest rates of migration, suggesting synergistic, or complementary mechanisms of action. Surprisingly, treatment with recombinant IL-4 (rIL-4; 20 ng/mL) had no effect on cell migration (**Figures 4B–D**). As mentioned previously, ELISA analysis of M2a macrophage conditioned cell medium revealed lower levels of CCL-20 and CXCL-9 vs. M2b or M2c conditioned medias. Therefore, we tested whether CCL-20 or CXCL-9 had an inhibitory effect on M2a-induced migratory responses, as some contradiction in the literature exists as to the role of each of these cytokines (35, 36). Stimulation of either MDA-231 cells or SUM-149 cells with recombinant CXCL9 (rCXCL9; 50 ng/mL), recombinant CCL-20 (rCCL-20; 50 ng/mL), or a combination of both cytokines each had no inhibitory effect on M2a-mediated migration in a wound closure assay (**Supplementary Figure S4**). Again, we employed the 3D spheroid invasion assay to examine the potential invasive effects of CCL-18, VEGF, and IL-4. Indeed, we find that rVEGF and rCCL-18 are sufficient to promote an

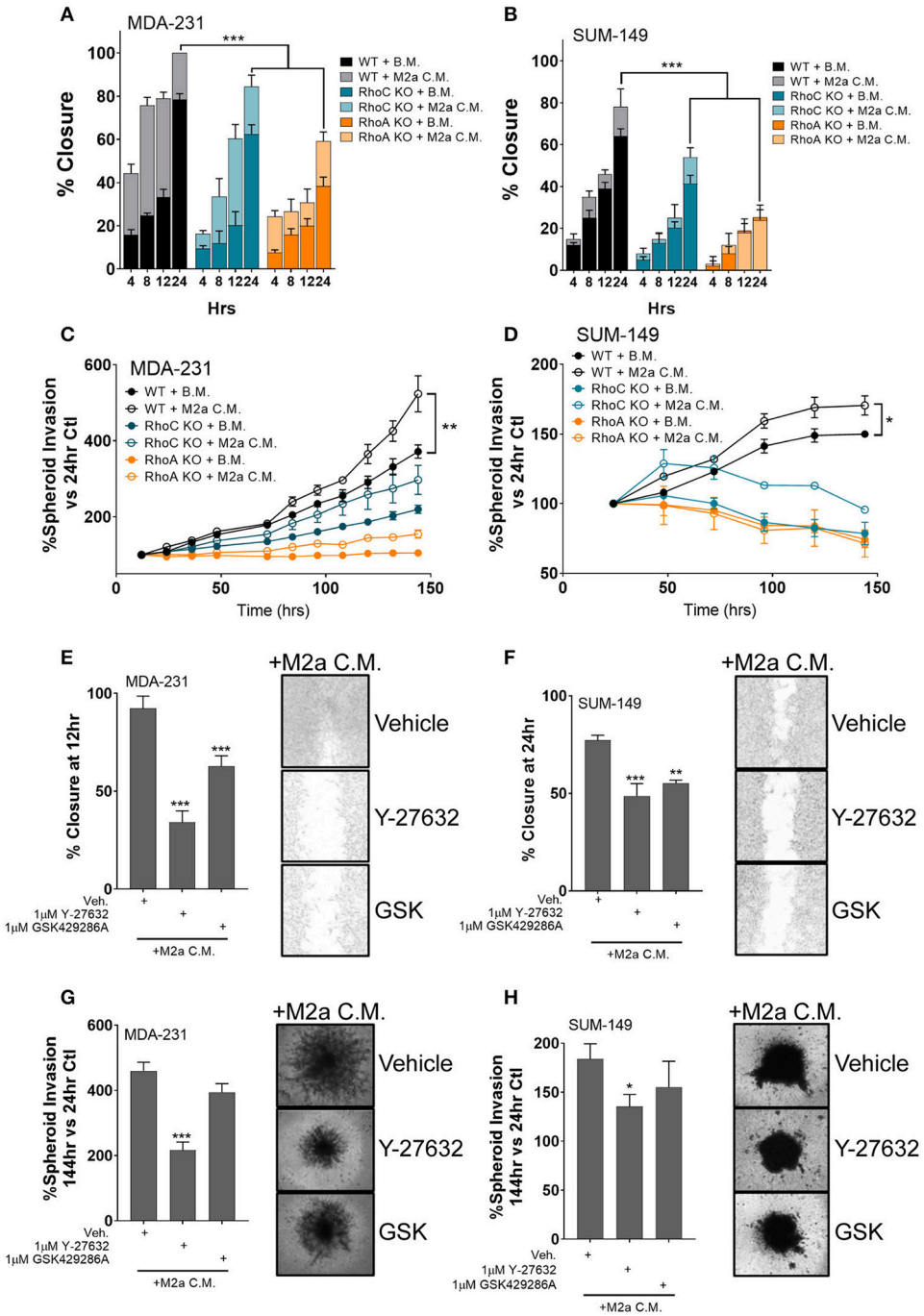

FIGURE 3 | The Rho-GTPases regulate migratory responses to M2a macrophage conditioned media. Scratch assays results display a reduction in M2a-induced migration in both MDA-MB-231 **(A)** and SUM-149 cells **(B)**. Similar results obtained from 3D spheroid invasion assays **(C: MDA-231; D: SUM-149)**. Pretreatment with 1μM ROCK inhibitor (either Y-27632 or GSK429286A e.g. "GSK") reduces M2a macrophage-induced 2D migration or 3D spheroid invasion in both MDA-231 **(E,G)** and SUM-149 cells **(F,H)**. All results are compiled from ≥2 independent experiments; error bars represent ± standard deviation; *p*-value is displayed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

invasive phenotype and stimulation with the combination of rVEGF/rCCL-18 had the strongest invasive response in both the MDA-231 and SUM-149 cells (**Figures 4E–G**). Addition of rIL-4 had no impact on spheroid growth or invasion in either cell model.

Based on the finding that recombinant CCL-18 and VEGF cause a strong migratory and invasive response in our aggressive breast cancer cells, we removed each of these components from M2a conditioned media and supplemented cancer cells with either VEGF-depleted media (ΔVEGF), CCL-18-depleted

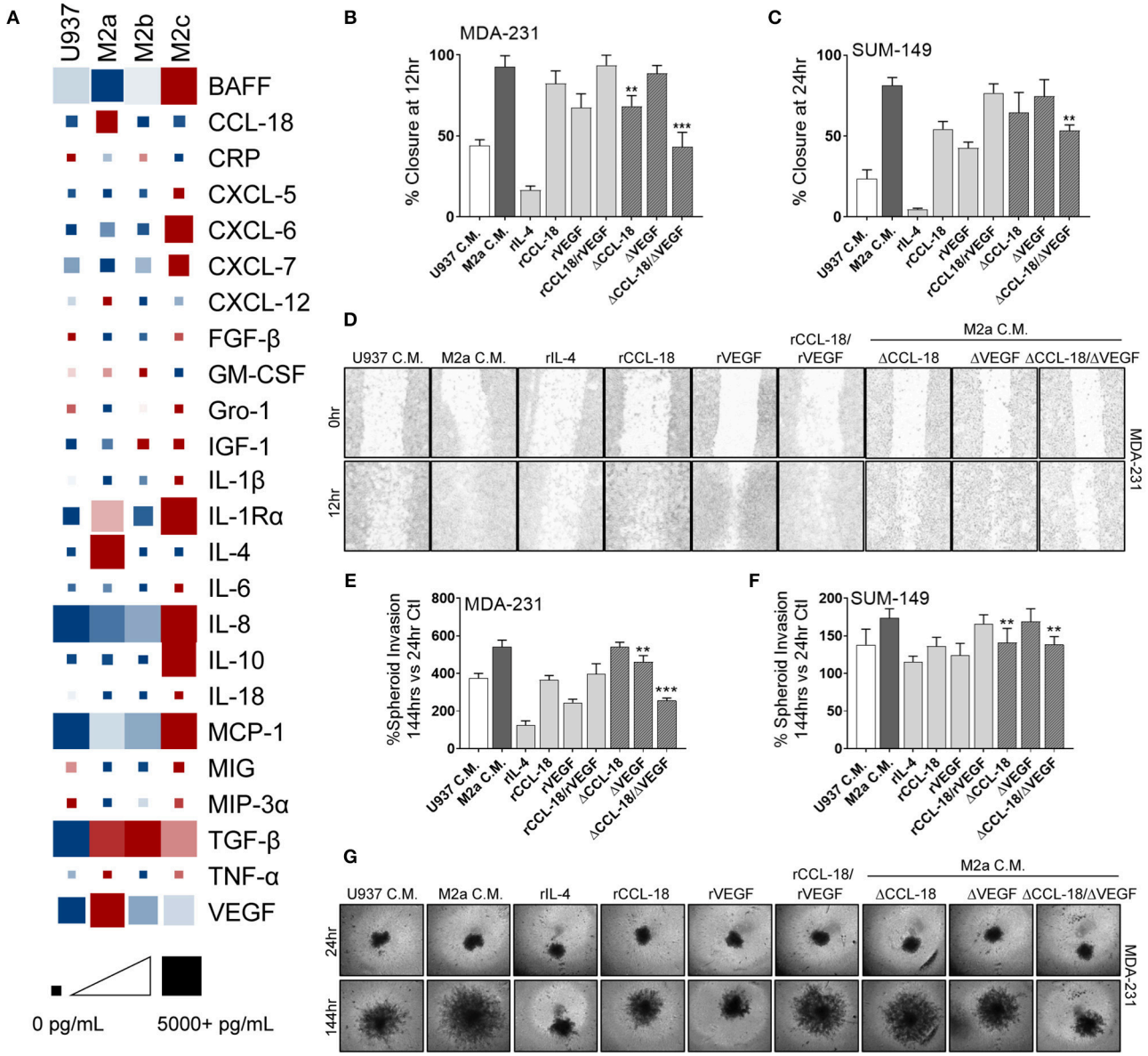

FIGURE 4 | Synergistic activity of CCL-18 and VEGF, secreted from M2a macrophages, enhance breast cancer cell migration. **(A)** ELISA results from concentrated cell supernatants indicate that M2a macrophages secrete higher levels of CCL-18, IL-4, and VEGF than their M2b or M2c counterparts. ELISA data is averaged across 4 independent experiments. Colors indicate row comparisons of a single analyte protein level [low levels (blue) vs. high levels (red)] compared among the macrophage populations. Square or box size is designed to provide quantitative global analysis of overall analyte protein levels e g., 1 pg/mL (small square) to 5,000 pg/mL (large square). Results from wound closure assay in MDA-MB-231 cells **(B)** and in SUM-149 cells **(C)** show removal of CCL-18 and VEGF from M2a C.M. significantly inhibits migration. **(D)** Representative images of MDA-MB-231 cells wound migration assays results. Similar results obtained from spheroid invasion assays in both MDA-231 **(E)** and SUM-149 cells **(F)**. Representative images from MDA-231 invasion assays shown in **(G)**. Data is reported as an average ±std. dev. of 3 independent experiments, analyzed by one-way ANOVA, $^{**}p > 0.01$, $^{***}p > 0.001$.

media (ΔCCL-18), or both VEGF- and CCL-18-depleted conditioned media (ΔCCL-18/ΔVEGF). We confirmed removal of VEGF and/or CCL-18 in our M2a conditioned media by ELISA (**Supplementary Figure S5**). Our data show that CCL-18-depleted M2a conditioned media slowed migratory responses in 2D migration in both MDA-231 and SUM-149 cells, with no observed differences following VEGF removal (**Figures 4B–D**). Intriguingly, we see significantly slower rates of migration following removal of both CCL-18 and VEGF from M2a conditioned media (**Figures 4B–D**). CCL-18 or VEGF depletion from M2a media had varying effects for spheroid invasion, although our results consistently show diminished invasion following removal of both proteins from the M2a media (**Figures 4E–G**). Taken together these data strongly

support that synergistic influence of VEGF and CCL-18 are critical for migratory/invasive responses of cancer cells to M2a macrophages.

Since our data shows that Rho-GTPase activation and downstream ROCK signaling regulates M2a-induced migration, we sought to understand whether ROCK signaling is also downstream of VEGF and/or CCL-18. To test this, we pretreated MDA-231 or SUM-149 cells with the ROCK inhibitors Y-27632 or GSK429286A and examined wound closure rates in the presence of rCCL-18 and rVEGF. As hypothesized, we find that pretreatment with the ROCK inhibitors significantly repress rCCL-18/rVEGF induced migratory responses in both MDA-231 and SUM-149 cells (**Figures 5A,B**). This result is recapitulated in MDA-231 invasion assays (**Figure 5C**) and a strong, but non-significant trend was observed in SUM-149 cells (**Figure 5D**). Again, to confirm migratory/invasive responses were not due to enhanced proliferation, we utilized label-free cell growth imaging assays. In parallel with our findings in **Figure 1E**, we did not observe any changes in proliferation following treatment with rVEGF, rCCL-18, or rVEGF and rCCL-18 combined (data not shown). Collectively, these data confirm that the Rho-GTPases and downstream ROCK signaling regulate CCL-18 and/or VEGF induced migratory responses.

Primary Human M2a Macrophages Enhance Breast Cancer Cell Migration and Invasion Through ROCK Signaling

It is well-recognized that results obtained from cultured cell models can widely vary from primary cells and may not entirely recapitulate responses observed *in vivo*. This is of particular concern in macrophage biology as they are an inherently plastic cell population, readily changing polarization, genotype, and phenotype, depending on their environment. Therefore, we aimed to determine if M2a macrophages derived from primary human monocytes displayed similar features and impact breast cancer cell phenotypes as the U937-derived M2a macrophages. Using primary human monocytes derived from whole blood, we initially differentiated them into unstimulated human macrophages by the addition of 20 ng/mL M-CSF to human monocyte culture medium (**Figure 6A**). Over the course of 4 days, we stimulated with IL-4/IL-13, collected and concentrated the conditioned media daily, as described previously. Initially, we surveyed the levels of CCL-18 and VEGF produced in human M2a (hM2a) macrophages and compared them to unstimulated human primary macrophages. We observed no change in VEGF production from hM2a human primary macrophages (**Figure 6B**), which was overall very low both in the primary and in the differentiated subpopulation. In contrast, we observed significantly enhanced levels of CCL-18 secreted by hM2a macrophages as compared to unstimulated human macrophages (**Figure 6B**). We predicted that hM2a macrophages would secrete VEGF and CCL-18 at differing concentrations as compared to U937-derived M2a macrophages; indeed, we observed different absolute levels of both VEGF and CCL-18 in the two complementary models (**Supplementary Figure 6**; **Supplementary Figure 3**), although the response trend for CCL-18 was equivalent in both models. These data highlight the significant heterogeneity that may be present between primary cells and cell culture models, further imparting the importance of experimental replication in various models to validate critical results. Despite the observed differences in secreted VEGF/CCL-18 levels, we still asked whether hM2a macrophages influence breast cancer cell migration and invasion through synergistic VEGF/CCL-18 signaling, and if this process proceeds through ROCK signaling. We generated VEGF depleted (ΔVEGF), CCL-18 depleted (ΔCCL-18), or VEGF and CCL-18 (ΔVEGF/CCL-18) depleted hM2a conditioned media (**Figure 6C**). Interestingly, removal of VEGF from hM2a media had little impact on either MDA-231 or SUM-149 wound closure rates or spheroid invasion (**Figures 6D,E**). Importantly however, removal of CCL-18 from hM2a conditioned media slowed 2D migration and 3D invasion (**Figures 6D,E**), but only significantly in MDA-231 wound closure (**Figure 6D**). Confirming our initial findings, removal of both VEGF and CCL-18 had significant impacts on MDA-231 2D migration and 3D invasion (**Figure 6D**), and in SUM-149 2D migration and 3D invasion (**Figure 6E**). Next, we examined if blocking Rho-GTPase signaling via ROCK inhibition would be sufficient to diminish hM2a macrophage-induced breast cancer cell migration and invasion. Both ROCK inhibitors, Y-27632 or GSK429286A, effectively diminished hM2a macrophage-induced metastatic phenotypes in both MDA-231 (**Figure 6D**) and SUM-149 (**Figure 6E**). These results suggest that targeting of the Rho/ROCK pathway is a promising and viable strategy for the potential management of metastatic progression, especially in patients who have elevated levels of M2a TAMs.

DISCUSSION

The roles of the Rho-GTPases in regulating cell migration and invasion were initially established roughly 20 years ago. These studies largely focused on the precise mechanisms by which Rho-GTPases harbor intrinsic regulatory roles as well as characterizing cofactors and surveying downstream effector responses. Recently, studies have begun to illustrate the complex regulation of the Rho-GTPases in response to stimulation or secreted signals from the cellular microenvironment. This is particularly relevant with the re-emergence of tumor immunology based anti-cancer strategies and the development of modern immunotherapies. Understanding how cancer cells respond to the multifaceted, dynamic signals that derive from infiltrated immune cells in the TME is an especially critical problem for aggressive breast cancers, such as triple negative and inflammatory phenotypes, for which there are at present no targeted therapies based on signaling (outside of PARP inhibitors for BRCA germline mutation carriers). Since macrophages are the most abundant population of immune cells that reside in the TME, we sought to better understand macrophage-breast cancer cell communication and how this process is regulated in triple negative and inflammatory breast cancer, both of which account for a disproportionate burden of morbidity and mortality from breast cancer.

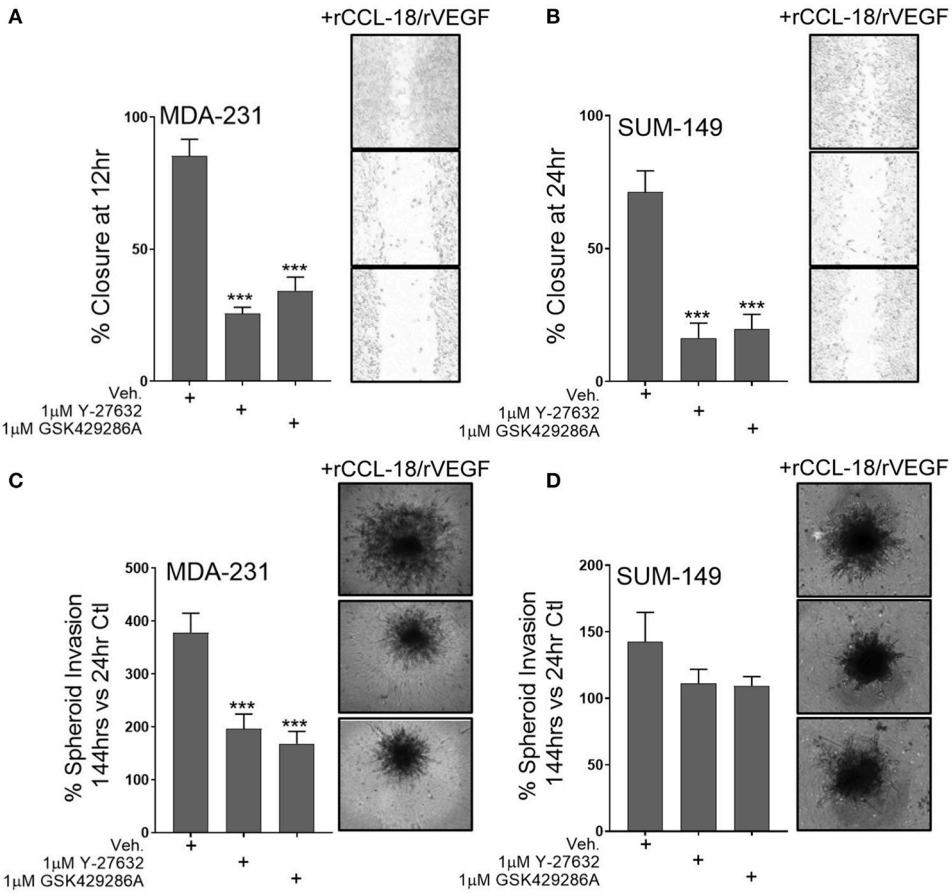

FIGURE 5 | ROCK inhibition significantly diminishes synergistic VEGF/CCL-18 included breast cancer cell migration and invasion. **(A)** Wound closure rates are dramatically slowed in MDA-231 cells pretreated with either 1 μM Y-27632 or 1 μM GSK429286A followed by VEGF and CCL-18 administration. **(B)** Wound closure rates in SUM-149 cells are slowed following pretreatment with the ROCK inhibitors, Y-27632 or GSK429286A followed by rVEGF/CCL-18 administration. **(C)** Results from spheroid invasion assays display VEGF/CCL-18-mediated invasion is reduced in MDA-231 cells pretreated with either ROCK inhibitor. **(D)** ROCK inhibition in SUM-149 cells does not significantly inhibit cellular invasion. Data is reported as an average ±std. dev. of at least 3 independent experiments, analyzed by one-way ANOVA, ***$p > 0.001$.

In our previous work, we observed that conditioned media extracts from *in vitro*, unpolarized macrophages significantly enhance migration in inflammatory breast cancer cells (such as SUM-149 cells) and that this process is regulated by RhoC (19). Macrophages are constantly adapting due to the variety of signals they encounter in the TME; therefore, in this study we aimed to understand their specific roles at the molecular level and better define the class of macrophages that is the main culprit in eliciting breast cancer metastatic progression. Here, we find that IL-4/IL-13 polarized M2a macrophages enhance breast cancer cell migration and invasion at a greater rate than either M2b or M2c macrophages and are thus an important subpopulation to target therapeutically. Moreover, we find that the Rho-GTPases RhoA and RhoC regulate M2a macrophage-induced responses through the synergistic effects of VEGF and CCL-18 signaling combined, and these effects can be attenuated by ROCK inhibition Collectively, our data strongly supports that use of ROCK inhibitors may be an effective strategy to diminish tumor invasion.

In a 2011 hallmark paper by Chen et al. CCL-18 from TAMs was found to promote breast cancer metastasis through the novel CCL-18 receptor, PITPNM3 (20). Since then, CCL-18 has been characterized as responsible for cancer progression in various cancer types (21, 37–41). Here, we provide evidence for a novel mechanism of Rho-GTPases regulating CCL-18-induced breast cancer migration. While direct targeting of CCL-18 or its receptor PITPNM3 or CCR8 (42, 43) seems like an attractive route for targeted therapy, this may have significant systemic side effects for patients, as CCL-18 signaling is critical for normal innate immune responses (43–45). As an alternative, our work suggests directing therapies toward Rho-GTPase signaling, for instance via ROCK inhibition, to diminish CCL-18 induced responses. Currently, ROCK inhibitors have been limited in their use in clinical trials as concerns over their potential systemic side effects are severe due to off-target effects of the existing compounds (46). Collectively, this work supports the need for better, more specific ROCK inhibitors. Overall, ROCK inhibition may be an efficacious, tolerable route for the management of

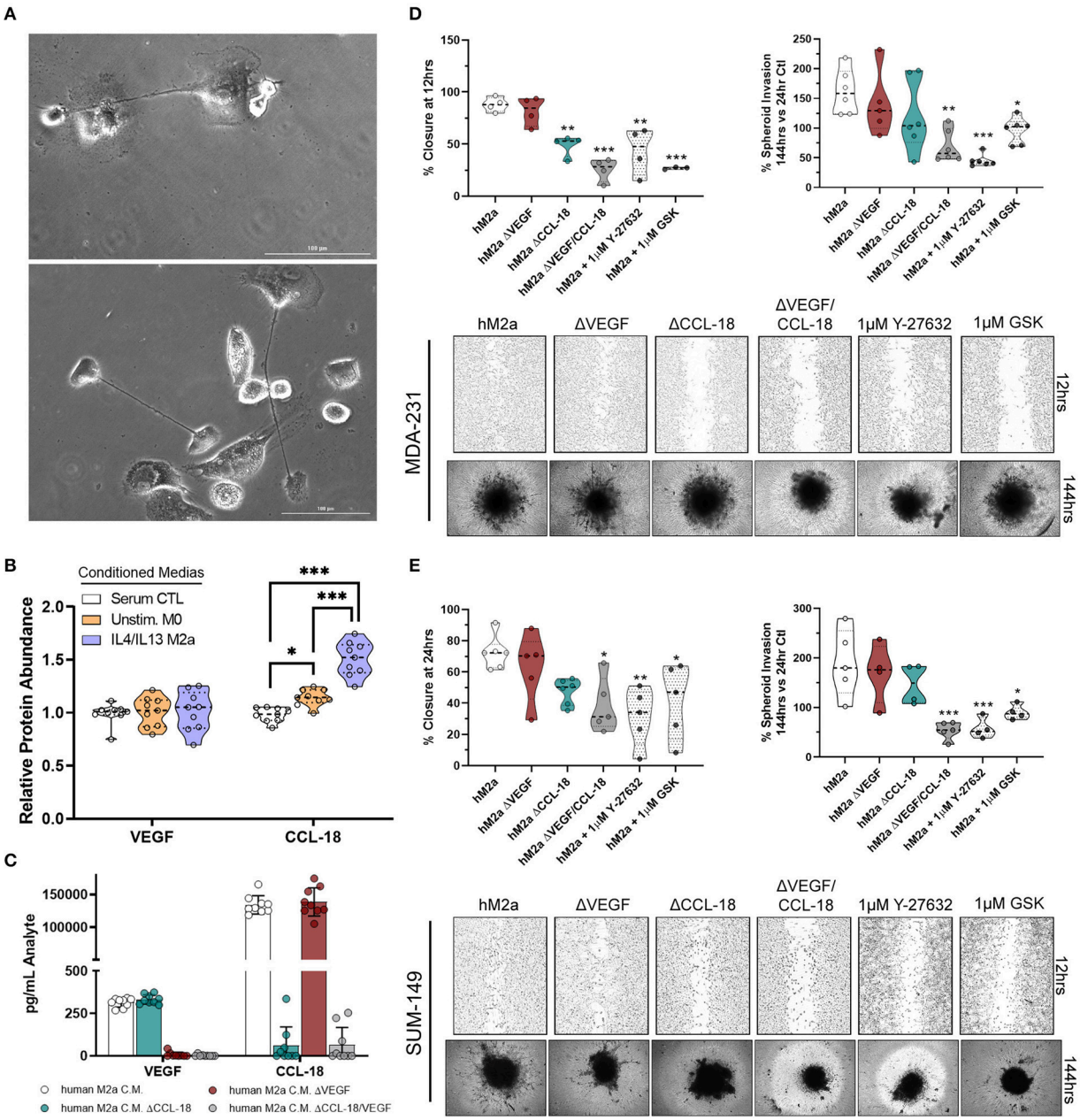

FIGURE 6 | Human M2a macrophages enhance breast cancer cell migration and invasion through VEGF/CCL-18 signaling regulated by Rho-GTPases. 40X phase contrast images of fully differentiated human macrophages **(A)**. ELISA results from concentrated conditioned media extracts from normal human macrophage growth media (serum containing; white), unstimulated M-CSF differentiated macrophages (orange), and IL-4/IL-13 polarized human M2a macrophages (purple/blue) **(B)**. ELISA results from analyte removal assays **(C)**. Results of wound closure 12 h post wound (left panel) or spheroid invasion 144 h post treatment/supplementation (right panel) experiments utilizing either human M2a conditioned media (M2a) analyte depleted hM2a media (e.g., hM2a ΔVEGF) or pretreatment with ROCK inhibitors with hM2a macrophage conditioned media in MDA-231 cells **(D)**. Representative images of either wound closure (top) or spheroid invasion (bottom) experiments in MDA-231 cells are shown below quantified data. Similar results displayed for wound closure (left panel) or spheroid invasion (right panel) with representative images below quantified data for SUM-149 cells **(E)**. All results are compiled from ≥2 independent experiments; Statistical significance was determined by one-way ANOVA; error bars represent ± standard deviation; *p*-value is displayed as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

metastatic breast cancer or other aggressive cancers and is worthy of further study.

Angiogenic factors are key contributors to cancer metastasis. VEGF is known to be secreted from a variety of cell types within the TME (e.g., cancer cells, TAMs, stromal cells, among others). Thus, our observation of enhanced secreted levels of VEGF from M2a macrophages is consistent with prior literature (21, 47). However, our data show the novel finding of synergy

between VEGF and CCL-18 signaling in their ability to enhance breast cancer motility and invasiveness. While VEGF has clearly defined roles for enhancing tumor cell migration via stimulation of angiogenesis, CCL-18 is a key factor in the chemotaxis of naïve T-cells and immune-suppression in the TME. However, these two signaling pathways do not have any previously described overlap or co-operativity regarding their regulation of breast cancer cell motility. Here we delineate that CCL-18 and VEGF enhance breast cancer migration and invasion, potentially as a pre-angiogenic step. These data confirm the importance of the CCL-18/VEGF axis in breast cancer metastasis, further supporting the need for future studies of their combined roles in priming the TME for angiogenesis and tumor progression. This work is potentially especially timely and relevant to efforts to enhance the efficacy of immune therapies in aggressive tumors such as triple negative and inflammatory breast cancer, where the performance of the latter has been modest.

In summary, this work shows that IL-4/IL-13 stimulated M2a macrophages are the most potent enhancers of breast cancer migratory and invasive phenotypes and thus the sub-population most likely to have anti-cancer effects if targeted as a single modality or in combination with anti-PD-1 or anti-PD-L1 antibodies, to help prime the TME for immune therapies. While *in vivo* polarized TAMs most likely experience a large diversity of cyto/chemokines and exist in a continuum of activation states, characterization of the various TAM polarization states and how they affect breast cancer cell behavior is critical in understanding the mechanisms which promote cancer outcomes. Synergistic utilization of CCL-18 and VEGF by M2a macrophages offers insight as to how these cells enhance metastatic outgrowth, most likely a precursor to angiogenesis, and supports their targeting for therapeutic intervention. Additionally, our results displaying that CCL-18 and VEGF signaling proceed through the Rho-GTPases, is a novel observation. This supports that combination targeting of the Rho-GTPases and M2 macrophage activation (e.g., CSF1R inhibition) would be an effective strategy to suppress breast cancer cell invasion for use for example in the adjuvant setting in high-risk lesions or in combination with other chemo or immune therapies, which elicit cancer cell death. Collectively, these data show that the Rho-GTPases are critical in the regulation of M2a macrophage-induced migratory and invasive responses in breast cancer cells and offers unique therapeutic opportunities for the suppression of breast cancer metastatic spread.

AUTHOR CONTRIBUTIONS

AL and SM conceived the study. AL and PP performed majority of experiments for the study. ZW, LB, JY, and LG assisted in preparing and/or performing experiments for the study. AL, PP, and SM analyzed data. AL, JY, CO, MS, and SM discussed analyzed results and contributed key intellectual input to the study. AL wrote the manuscript and prepared figures. AL, JY, MS, and SM assisted in drafting and editing the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00456/full#supplementary-material

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The Potential of Astrocytes as Immune Modulators in Brain Tumors

*Neibla Priego and Manuel Valiente**

Brain Metastasis Group, Molecular Oncology Programme, National Cancer Research Center (CNIO), Madrid, Spain

The neuro-immune axis has emerged as a key aspect to understand the normal function of the Central Nervous System (CNS) as well as the pathophysiology of many brain disorders. As such, it may represent a promising source for novel therapeutic targets. Glial cells, and in particular the extensively studied microglia, play important roles in brain disorders. Astrocytes, in their reactive state, have been shown to positively and negatively modulate the progression of multiple CNS disorders. These seemingly opposing effects, might stem from their underlying heterogeneity, an aspect that has recently come to light. In this article we will discuss the link between reactive astrocytes and the neuro-immune axis with a perspective on their potential importance in brain tumors. Based on the gained knowledge from studies in other CNS disorders, reactive astrocytes are undoubtfully emerging as a key component of the neuro-immune axis, with ability to modulate both the innate and adaptive branches of the immune system. Lastly, we will discuss how we can exploit our improved understanding of the basic biology of astrocytes to further enhance the efficacy of emerging immune-based therapies in primary brain tumors and brain metastasis.

Keywords: brain tumor, brain metastasis, astrocytes, immune system, cell-to-cell communication

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Tomasz Kordula,
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****Correspondence:***
Manuel Valiente
mvaliente@cnio.es

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ASTROCYTES IN HOMEOSTASIS AND DISEASE

Astrocytes are involved in a variety of phisiological functions including maintenance of the blood-brain barrier (BBB) and blood flow (1), modulation of synaptic plasticity (2), and regulation of energy homeostasis (3). All of these functions have a significant impact on many aspects of our daily life such as cognition (4), fear (4), sleep (5), and circadian rhythm (6). The heterogeneity of astrocytes might contribute to these pleiotropic functions. For instance, astrocytes from the hippocampus differ functionally in multiple aspects when compared to those from the striatum (7). But even within the same brain area, astrocytes have molecular differences that functionally correlate with their ability to interact with neurons (8). Single cell-RNA sequencing (scRNAseq) will undoubtedly help to clarify not only the diversity within what we call today astrocytes as a whole but also the origin of such heterogeneity. Sources of astrocyte heterogeneity might include different progenitors during development (9) or the ability to generate new astrocytes upon injury (10, 11). Especially interesting are novel technologies that allow mapping single cell transcriptomics within tissue sections (12). Given the link between location and astrocyte function, as shown by the different biology of juxtavascular astrocytes (13), having spatial resolution of transcriptomic profiles might be key to properly interpret the many flavors of astrocytes.

In addition to their homeostatic functions in the central nervous system (CNS), astrocytes are rapidly activated in response to various insults, including brain tumors (14, 15). The activation pattern of astrocytes and its consequences appear to be dependent on the nature of the initiating

pathogenic event. Moreover, this is a dynamic process that evolves throughout the course of the disease. While primarily limiting spread of the damage in the context of acute phase brain injury, astrocytes rather appear to worsen disease outcome in a chronic injury setting (16, 17). This also applies to brain metastasis, where reactive astrocytes play an anti-metastatic role that limits disease progression in early stages of brain colonization (18), while, later on, they become strongly pro-metastatic (19). Therefore, in order to fully comprehend the biological significance of astrocytes in brain physiology and pathology, we need to consider their highly plastic behavior and heterogeneous make up. These features allow astrocytes to trigger a remarkably fine-tuned response to adequately counteract a broad spectrum of injuries. Given the growing importance of the immune system and its therapeutic exploitation in many brain disorders, including cancer, addressing the biological significance of the cross-talk between immune cells and astrocytes might offer innovative means to challenge incurable CNS disorders, such as primary and secondary brain tumors.

INFLUENCE OF ASTROCYTES ON THE INNATE IMMUNE SYSTEM

Cross-Talk Between Astrocytes and Microglia

Microglia and astrocytes are resident glial cells that influence each other under homeostatic conditions (20) but also when the CNS is affected by pathology.

In vitro, the classical inducer of neuroinflammation LPS stimulates microglia to produce a secretome enriched in NFκβ-regulated molecules including IL-1, TNF and C1q. The microglia-conditioned medium was sufficient not only to turn non-activated into activated astrocytes, assessed by the gained expression of GFAP, but also to induce the production of an unidentified secreted factor/s by astrocytes that compromised the viability of neurons and oligodendrocytes (21) (**Figure 1A**). This particular behavior of activated astrocytes with neurotoxic properties has been suggested to be present in patients with neurodegenerative (Alzheimer, Huntington, Parkinson, amyotrophic lateral sclerosis) and autoimmune (multiple sclerosis) CNS disorders. This hypothesis was supported by the increased levels of three proteins (C3, CBF, and MX1) that were initially found to be upregulated in a transcriptional signature of microglia-stimulated astrocytes (21).

A similar crosstalk between astrocytes and microglia was probed to be involved in some rare forms of Parkinson with mutations in the orphan receptor NURR1. Under normal circumstances NURR1 blocks the activation of NFκβ-dependent genes, a function that is lost in these patients with Parkinson disease. The combination of engineered inactivating *Nurr1* mutations in glial cells with a background of increased inflammation (i.e., LPS treatment) lead to the death of dopaminergic neurons, which is a hallmark of Parkinson (22). The molecular analysis of this cross-talk probed that Nurr1-mutant astrocytes had an augmented response to microglia-derived TNFα and IL1β involving a sustained occupancy of the iNOS promoter by p65, thus secreting nitric oxide (NO) at levels that might be responsible for compromising neuronal viability (22) (**Figure 1A**).

The dependency of astrocyte activation on microglia behavior, was also validated *in vivo* in a mouse model of experimental autoimmune encephalomyelitis (EAE). Activation of the aryl hydrocarbon receptor (AhR) in microglia promotes the expression of TGFα. On the contrary, the absence of AhR signaling limits the expression of the NFκβ negative regulator *Socs2*, which increases the secretion of NFκβ-dependent molecules such as VEGFB (**Figure 1A**). Microglia secretomes enriched in either TGFα (when AhR is activated) and VEGFB (when AhR is inactivated) induced opposite transcriptomic responses when added to astrocytes by decreasing or inducing, respectively, the expression of *Ccl2, Nos2* and *IL1b*. Some of the deregulated genes were enriched in activated astrocytes with the ability to compromise the viability of neurons and oligodendrocytes (21). In fact, when *AhR* was targeted in the context of EAE, disease worsened. Furthermore, targeting *Ccl2, Nos2* and *IL1b* using cell-specific loss of function approaches either in microglia or astrocytes improved EAE outcome (23). AhR could be activated by tryptophan-derived metabolites (24). Since tryptophan is an essential amino acid provided by diet that is processed by the gut microbiome, this suggests the possibility that diet and the intestinal microbiota could have an impact on neuroinflammation. Interestingly, depleting tryptophan from the diet mimicked the phenotype of targeting *AhR* in microglia thus worsening EAE. Adding back the amino acid in the diet rescued the phenotype but only when the AhR receptor was present (23).

In summary, evidence exists about the critical influence of microglia on astrocytes in CNS disorders. The degree of activation of a NFκβ-dependent secretome in microglia defines the consequences on astrocytes. Microglia-activated astrocytes could worsen disease outcome by their negative influence on neuron and oligodendrocyte viability. Although the influence of microglia on astrocytes have been probed, whether astrocytes could influence microglia is less well-characterized (25).

Cross-Talk Between Astrocytes and Brain-Infiltrating Monocytes

Monocytes are excluded from the healthy brain. However, when the brain gets injured, CCR2+ circulating monocytes access the parenchyma (26, 27). As a key component of the BBB, astrocytes are one of the first cell types encountered by infiltrating peripheral immune cells, which provides the glial cell a strategic position to control this transit.

Traumatic brain injury has an impact in the viability of astrocytes located in the proximity of the damaged area. Simultaneously to the decrease in astrocytes, there is an increase in the infiltration of CCR2+ monocytes, which suggests that these cell types could influence each other. Juxtavascular astrocytes are a subpopulation that interacts physically with brain vessels and proliferation upon damage (13, 28). Although this subpopulation of astrocytes has been shown to correlate with

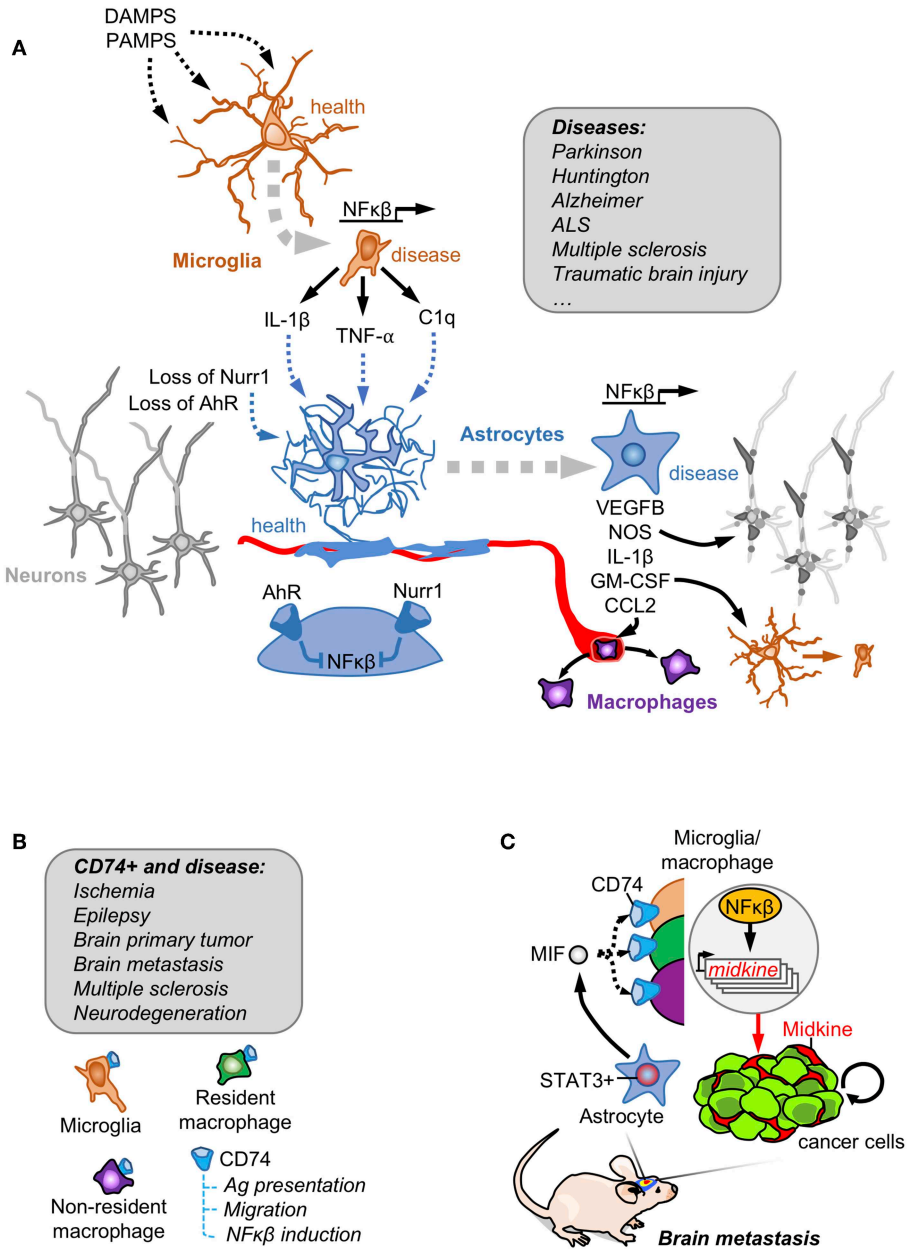

FIGURE 1 | Astrocytes and innate immunity. **(A)** Danger and pathogen associated molecular patterns (DAMPS and PAMPs, respectively) are detected by microglia that become activated secreting NFκβ downstream targets. Activated microglia crosstalk with astrocytes to induce a NFκβ-dependent program responsible for damaging neuronal components, favoring the access of peripheral macrophages and reinforcing the activation of the microglia. Under basal conditions, astrocytes have this NFκβ program shut-down by the action of Nurr1 and AhR receptors. This mechanism involving the crosstalk between reactive astrocytes and microglia has been described in several disorders affecting the Central Nervous System. **(B)** Brain macrophages in disease include microglia, non-parenchymal resident macrophages and infiltrating non-resident macrophages. A subpopulation expressing the CD74 receptor has been reported to be present in all of them, especially when the brain is affected by disease. **(C)** In brain metastasis, secretion of MIF by pSTAT3+ reactive astrocytes enrich CD74+ microglia/ macrophages in the tumor where they produce the NFκβ-dependent molecule midkine that promotes tumor cell survival.

a specific developmental origin, they were not characterized at the molecular level. Recently, juxtavascular astrocytes have been shown to preferencially activate AhR. Given that AhR blocks the production of CCL2, a strong chemokine for CCR2+ monocytes, this subpopulation of astrocytes acts as a selective barrier modulating the access of peripheral cells into the brain parenchyma (28).

Monocytes also influence astrocytes. If traumatic injury is generated in a mouse without CCR2+ monocytes, higher numbers of proliferative astrocytes are detected, suggesting a

deleterious influence of infiltrated monocytes on the proliferation of juxtavascular astrocytes (28). Interestingly, in spite of the increased proliferative rates of these astrocytes, the glia scar and extracellular matrix deposition surrounding the damage was reduced and consequently, better neuronal recovery was detected (28). This finding illustrates the importance of defining at the molecular level newly established cell-to-cell interactions that occur once peripheral cells from the innate immune system infiltrate the brain. It also illustrates the importance of characterizing astrocyte heterogeneity given the impact that specific astrocyte subtypes have on disease progression (28).

Cross-Talk Between Astrocytes and Macrophages in Brain Tumors

In spite of the evidences presented in other brain pathologies, the crosstalk between astrocytes and macrophages had been barely explored in brain tumors. This is surprising given that the majority of immune cells within brain tumors are macrophages either resident or infiltrated from the periphery (27, 29, 30). Recently, astrocytes have been proved to influence a subtype of microglia/ macrophage expressing CD74.

CD74 is among the most upregulated genes in human microglia in the context of brain tumors and other pathologies (31) (**Figure 1B**). The association of CD74 in microglia/macrophages and brain disorders have been recently extended and validated by scRNAseq approaches comparing healthy and brains affected by autoimmune disorders, neurodegeneration or ischemia. *Cd74* upregulation was consistently found in disease-associated macrophages including peripheral macrophages infiltrating the brain, non-parenchymal resident macrophages (meningeal, perivascular, and choroid plexus macrophages) as well as in one subclass of microglia (26, 32) (**Figure 1B**).

Funtionally, the CD74+ microglia/macrophages were shown to reduce the secretion of IFN-γ in the tumor microenvironment, which would contribute to established an immunosuppressed niche (33). More recently, the increase of CD74+ microglia/macrophages in the context of brain metastases was shown to be dependent on the presence of pSTAT3+ reactive astrocytes, describing a cross-talk between both cell types (19). The ligand of CD74 receptor, MIF, is highly enriched in the secretome of pSTAT3+ reactive astrocytes. CD74+ microglia/macrophages are preferentially located within the metastatic lesion. At this location CD74 could be found translocated in the nucleus where it promotes the expression of NFκβ downstream targets, such as midkine (19), a secreted molecule that accumulates in the extracellular space to promote cell viability (34) (**Figure 1C**). The importance of MIF binding to CD74+ microglia/macrophages was demonstrated by the reduction of brain metastasis upon treatment with the BBB-permeable MIF inhibitor ibudilast in organotypic cultures (19). Interestingly, ibudilast has been successfully used in patients with multiple sclerosis (35) and in experimental models of glioblastoma (36), which inspired a recently initiated clinical trial (NCT03782415). Although the biology of CD74+ microglia/macrophages remains poorly characterized, its strong association with different brain disorders and its diverse set of functions including the role as a chaperone for the MHCII complex (37), the modulation of migration by interacting with myosin (38) and the activation of NFκβ pathway (34) suggest relevant implications in disease.

INFLUENCE OF ASTROCYTES ON THE ACQUIRED IMMUNE SYSTEM

In contrast to the long-term dogma that defined the brain as an immune-privileged organ, the presence of primary or secondary brain tumors correlates with a significant infiltration of CD8+ and CD4+ T cells (39–41). Given that brain infiltrating T cells and reactive astrocytes co-exist in the same spatial location surrounding the tumor (19) and the strong secretory nature of astrocytes, it is quite likely that both cell types could influence each other. The molecular regulation of this cross-talk and its consequences are emerging linked to several brain disorders including cancer.

Cross-Talk Between T Regulatory Cells and Astrocytes

T regulatory cells have been described to actively modulate astrocyte behavior in ischemia (42). After stroke there is a massive accumulation of T regulatory cells in the brain that promotes neurological recovery. T regulatory cells are initially attracted to the ischemic brain by CCL1 and CCL20 produced by astrocytes and oligodendrocytes and later expanded by the combined action of IL-2 or IL-33 and T cell receptor recognition. Expanded T regs secrete the EGFR ligand amphiregulin (AREG) that decreases the expression of several astrocyte markers associated with potential negative effects on neuronal viability (42) (**Figure 2A**). In fact, intraventricular administration of AREG reduced neurological dysfunction associated with Treg-depleted-mice (42). Thus, T regulatory cells contribute to the control of brain damage by modulating astrocyte behavior.

Additional evidences of the crosstalk between T regulatory cells and astrocytes exist in EAE. Administration of an anti-CD3 antibody intranasally in this experimental model was shown to activate IL-10-producing T regulatory cells in the cervical lymph nodes (43). These T regulatory cells moved and became enriched in the CNS where they influence reactive astrocytes expressing the IL-10 receptor subunit alfa (*Il10ra*). Activation of IL10R1-dependent signaling in astrocytes decreased gene expression patterns typically associated with different aspects of EAE pathophysiology such as BBB degradation (*Mmp3, Mmp9*), monocyte recruitment (*Ccl2*), and microglial regulation (*Csf2*) (**Figure 2B**). Interestingly, astrocyte-specific downregulation of *Il10ra* fully impaired the clinical benefit provided by the administration of the anti-CD3 antibody in a pre-clinical model of multiple sclerosis (43).

Primary and secondary brain tumors are infiltrated with CD4+ CD25+ Foxp3+ T regulatory cells (44, 45). In addition, Treg signatures seem to predominate over those related to T-cell function involved in their activation or TCR antigen binding even when measured systemically (46). Consequently, dissecting

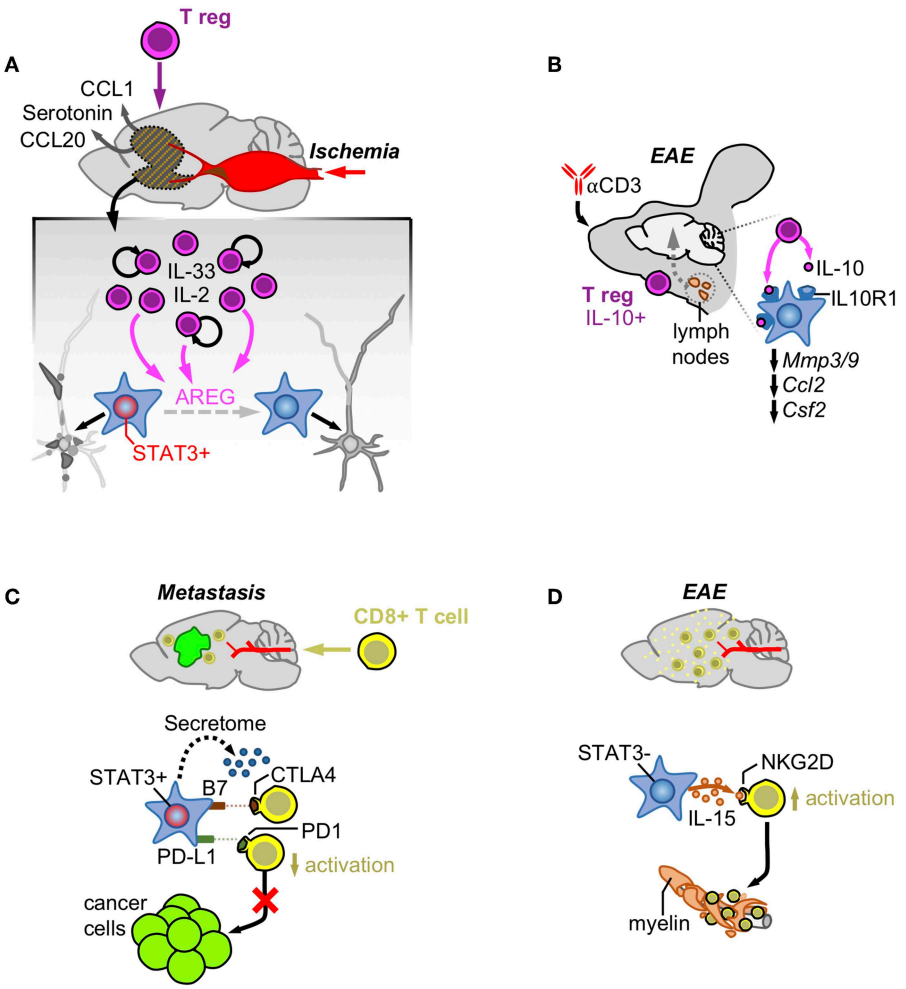

FIGURE 2 | Astrocytes and acquired immunity. **(A)** Ischemia induces the production of CCL1/CCL20/Serotonin that attracts T regulatory cells. Once in the brain, Tregs increase their numbers activated by IL-2 and IL-33 and produce AREG that reduces the neuronal damage by impairing STAT3 activity in astrocytes. **(B)** Treatment with an anti-CD3 blocking antibody intranasally stimulates the production of a subpopulation of T regulatory cells producers of IL-10 at the cervical lymph-nodes. After reaching the brain these cells are responsible for decreasing the expression of genes linked to the pathology by activating the IL-10 receptor in astrocytes. **(C)** Infiltrating CD8+ T cells are exposed to immune checkpoint ligands and an immunosuppressive secretome generated by pSTAT3+ reactive astrocytes that surround established metastasis. **(D)** In contrast, pSTAT3- reactive astrocytes produce IL-15 that binds to NKG2D leading to their increased activation and destruction of myelin.

the biology of the Treg compartment in brain tumors and its cross-talk with other components of the microenvironment including reactive astrocytes might help to develop novel strategies of immunotherapy.

Cross-Talk Between Astrocytes and CD8+ T Cells

Physical Interactions

Cell-to-cell contacts between astrocytes and T cells have been well-documented at the subcellular level (47). Virally infected astrocytes have been imaged *in vivo* when they initiate contacts with T cells in immunized animals (48). These contacts have all the components to be considered an immunological synapse including the central supramolecular activation complex (c-SMAC), composed of the TCR bound to the peptide within the MHC, which is surrounded by the peripheral supramolecular activation complex (p-SMAC), a ring of adhesion molecules including LFA-1 and ICAM-1 (48). The synapse between astrocytes and T cells activates in the later Talin, integrins, and the cytoskeleton that polarizes the T cell to secrete of IFN-γ, perforin, and granzyme-B on the virus-infected astrocyte (48). However, this does not only apply to virally infected astrocytes since the same behavior has been reported in models of multiple sclerosis targeting the gray matter (49) and between transformed glial cells and T cells (50).

However, the presence of immunological synapses between T cells and transformed glial cells do not necessarily correlate with anti-tumor effects, suggesting that astrocytes could negatively modulate T cell activity (51). As part of the neurovascular unit, astrocytes have the important role of blocking potential threats that might get access to a poorly regenerative organ such as the brain. Several mechanisms have proved the efficacy of

this natural defense such as the induction of FasL-dependent killing of T cells by reactive astrocytes (52). Interestingly, this mechanism also applies to the elimination of the majority of extravasated metastatic cells that are not adapted to the brain (18). In addition, reactive astrocytes have been shown to inhibit T cells by expressing B7, the ligand of the CTLA-4 checkpoint, whose activation is sufficient to trigger downstream inactivating signals (53). PD-L1 is also present in astrocytes of experimental models of viral encephalitis, where they contribute to limit the function of CD8+ T cells (54), as well as in brain metastases, where the known driver of *Cd274* expression, STAT3, has been shown to be enriched in a subpopulation of these glial cells (19) (**Figure 2C**). This last finding (the presence or absence of STAT3 in seemingly different astrocyte subpopulations) might underlie the different outcomes after astrocytes and T cells get in contact, emphasizing the importance of dissecting astrocyte heterogeneity in disease.

Paracrine Interactions

Astrocyte heterogeneity was initially detected regarding the ability of some of these glial cells to suppress the activation of T cells by unidentified secreted factors (55). More recently, this suppressive activity was linked to the subpopulation of reactive astrocytes activating STAT3 pathway (pSTAT3+) in the context of brain metastasis (19). Although the specific molecular mechanisms mediating these phenomena is still unknown, the secretome of pSTAT3+ reactive atrocytes contained known immunosuppressive molecules and, when evaluated functionally, it impaired the activated state of CD8+ T cells limiting their cytotoxic activity on brain metastatic cells *in vitro* (19) (**Figure 2C**). The accumulation of reactive astrocytes and CD8+ T cells within the same peri-tumoral area suggests that the paracrine crosstalk between these cell types might play a role *in vivo* (19). In fact, in the context of brain metastasis, where pSTAT3+ reactive astrocytes have been demonstrated to play a critical pro-tumor role, targeting STAT3 in astrocytes and blocking CD8+ T cells simultaneously reverted the decrease in metastasis derived from the loss of function of the transcription factor (19). This finding strongly suggests an important role of pSTAT3+ reactive astrocytes suppressing CD8+ T cells (19).

In contrast, reactive astrocytes in EAE have been shown to produce IL-15, which, upon binding to NKG2D in NK cells and CD8+ T cells (56), stimulates their cytotoxic behavior contributing to increase the damage associated with multiple sclerosis (**Figure 2D**). Interestingly CD8+ T cells in EAE infiltrate the damaged area leaving the glial cells behind (56), suggesting that the nature of astrocytes might be different to those present in brain metastasis, which retain T cells away from the cancer cells (19).

EXPLOITING THE INFLUENCE OF REACTIVE ASTROCYTES ON THE IMMUNE SYSTEM

The crosstalk between reactive astrocytes and different components of the immune system could have multiple and diverse consequences from neuronal viability to cancer cell proliferation. Thus, in order to target this complex cross-talk therapeutically, it is crucial to understand the role of reactive astrocytes in the specific pathology that is to be challenged. For instance, promoting the crosstalk between Tregs and astrocytes might be a valuable strategy in ischemia and autoimmune disorders but the benefit in the context of cancer is less predictable.

Thus, in primary and secondary tumors the priority is to challenge the survival of cancer cells, which usually hijack mechanisms that are also present in other pathologies and misuse them for their own benefit. There might be associated risks with strategies that look to boost anti-tumor responses by modulating the cross-talk between astrocytes and immune cells such as potential side effects regarding increased direct (due to astrocyte production of neurotoxic molecules) or indirect (due to an overactivation of CD8+ T cells) neuronal damage. Consequently, it is necessary to dissect in great detail the consequences of modulating this cross-talk in pre-clinical models to develop the best strategy for each brain disorder.

Clinical trials have used different strategies that modulate the immune system to treat brain tumors (57–59). Some efforts have reported encouraging results both with primary (58, 60–62) and metastatic tumors (63–65). Nevertheless, response rates remain modest and the question is whether taking into account the specific biology of the brain microenvironment could help to increase them. Given that reactive astrocytes have been proved to influence both branches of the immune system (see above), preclinical studies are needed to define the value of targeting astrocyte-derived local immunosuppression to boost intracranial efficacy of immunotherapies.

In the first place, limited efforts have been devoted to determine the amount of blocking antibodies against immune checkpoints that reach the brain parenchyma compared to extracranial locations (66). Given the presence of the BBB, it is expected that antibody concentrations, if any, will be lower in the brain than elsewhere. The still common argument that the mere presence of a tumor mass involves a disruption of the BBB, which would grant the immediate increase of drug permeability, is far from the reality as reported in exhaustive studies probing that this only affects 10% of fully established metastases (67). Rather than fully disrupted, the BBB seems to be modified into a brain-tumor barrier (BTB), whose biology has just started to be dissected (68).

Thus, if the levels of blocking antibodies reaching the brain parenchyma is a limiting factor, then the anti-tumor effects of such therapeutic approaches will depend on the ability of T cells, activated elsewhere by the action of immune checkpoint inhibitors, to first reach the brain and then get access to tumor cells to apply their cytotoxic activity. Two indirect findings argue in favor of this hypothesis. In experimental brain metastasis models, the presence of systemic disease favors the efficacy of immunotherapy in the brain (69) and, on the contrary, if there is only local disease in the brain, immune cells seem to be sequestered in the bone marrow (70). In other words, immunotherapy based on blocking antibodies solely is not optimized to the particular biology of the brain. Alternatively, the ability of astrocytes to negatively influence immune cells might

be exploited to develop novel strategies against brain tumors that could be combined with immune checkpoint blockade.

Reactive astrocytes with activated STAT3 pathway express PD-L1, which could contribute to the local immunosuppressive microenvironment present in brain metastasis (19) (**Figure 2B**). In fact, cancer cells with glial origin have been shown to induce T cell exhaustion partially due to their expression of PD-L1 (71). In addition, pSTAT3+ reactive astrocytes produce a secretome that impairs the activation state and the cytotoxic phenotype of CD8+ T cells *in vitro* while at the same time promotes the enrichment of pro-tumoral macrophages/microglia that favor the viability of tumor cells (19) (**Figures 1C**, **2B**). In fact, an enriched STAT3 signature brain tumor patients with partial responses to immunotherapy (61). This finding could be interpreted as an active cancer cell-induced mechanism to promote pSTAT3+ reactive astrocytes, which would be responsible for limiting the full potential of anti-tumor T cells thus preventing complete responses. Consequently, BBB-permeable inhibitors targeting STAT3 as well as other inhibitors targeting downstream mechanisms that negatively influence anti-tumor CD8+ T cells and/or impair pro-tumorigenic CD74+ microglia/ macrophages might be explored as a potential combination strategies with immune checkpoint blockade.

Studying the biology of the immune system in the CNS is fundamental to improve therapeutic strategies against brain tumors. The interaction between astrocytes and different branches of the immune system, as extensively proved in other CNS pathologies, suggests a potential avenue to increase the quantity and quality of anti-tumor approaches applied to the brain. The analysis of similar experimental therapeutic approaches across several brain disorders in pre-clinical models might also help to understand the role of astrocytes. For instance, pSTAT3+ reactive astrocytes have been described in brain tumors (19), traumatic injury (72), ischemia (73), neurodegenerative disorders (74, 75) as well as autoimmune diseases (76). However, inhibition of STAT3 in astrocytes is beneficial for some disorders (19, 77–79) while detrimental for others (72).

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NP and MV conceptualized and wrote the manuscript.

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REVIEW
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A Call for Epidemiological Research on Myeloid-Derived Suppressor Cells in Ovarian Cancer: A Review of the Existing Immunological Evidence and Suggestions for Moving Forward

Ashley E. Stenzel[1], *Scott I. Abrams*[2] *and Kirsten B. Moysich*[1]*

[1] Department of Cancer Prevention and Control, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States,
[2] Department of Immunology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States

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Leila Akkari,
The Netherlands Cancer Institute (NKI), Netherlands

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Anurag Singh,
University Children's Hospital Tübingen, Germany
Carlos Alfaro,
NavarraBiomed, Spain

***Correspondence:**
Kirsten B. Moysich
Moysich@roswellpark.org

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Recently, there have been encouraging findings suggesting that myeloid-derived suppressor cells (MDSCs) may be a good target for studying immune suppression in ovarian cancer. MDSCs are an abundance of immature myeloid cells that have demonstrated the ability to decrease tumor-infiltrating immune cells, increase the accrual of tumor-associated macrophages and regulatory T cells, as well as secrete various pro-inflammatory mediators and growth stimulating cytokines. Most studies on this topic utilized murine models, but there are limited reports in human subjects which have important limitations. With the majority of ovarian cancer patients presenting with distant metastases and a corresponding 5-year relative survival rate of $< 30\%$, continued efforts are obligatory toward identifying potential prognostic factors. Given the difficulty of studying exposures in this patient population, as well as the existing immunologic characteristics of this cancer, there is growing interest in further identifying genetic and immunologic associations with patient survival. Furthermore, prognostic factors that may necessitate therapeutic intervention may significantly alter disease outlook. In this review paper, we address the current literature on MDSCs and their immunosuppressive behavior in ovarian cancer patients. While the previous studies on these cells in ovarian cancer have demonstrated some potential prognostic significance, there are many limitations to such studies including small sample sizes, inconsistent staging and histology, as well as inconsistent surface markers for the identification of MDSCs. Additionally, such studies include minimal patient characteristics involved with the clinical course of ovarian cancer. Here, we have proposed improving on studies analyzing MDSCs as a potential prognostic factor in ovarian cancer patients, as well as further identifying the potential of this novel prognostic factor in future care, through the use of a comprehensive epidemiologic model.

Keywords: myeloid-derive suppressor cells (MDSCs), epidemiology, STAT (signal transducer and activator of transcription), IRF8 transcriptional coactivator, ovarian cancer

INTRODUCTION

Ovarian cancer is a rare, yet fatal disease. This cancer is found to be more prevalent in Caucasian women, with a median age at diagnosis of 63 (1). While it is not one of the higher incident cancers, it is the 5th leading cause of cancer-related deaths among women. Incidence has slightly decreased over time, however, mortality remains high. The 5-year survival rate for this malignancy is 46.5%, overall, but when broken down by stage, those with distant metastases have a 5-year survival rate of <30%. This is important to acknowledge as the majority of women with this disease (60%) have progressive disease with distant metastasis at initial presentation. This is largely due to the vague characteristics of symptoms for this disease, including, but not limited to: bloating, dyspepsia, early satiety, changes in urinary habits, and generalized pelvic pain and discomfort (2, 3). Such symptoms are frequently disregarded, as they can be explained by many non-malignant etiologies. Due to this, many women will not present for evaluation by their clinician until persistence of such symptoms, or may not experience such symptoms until late stage disease. Additionally, many women with ovarian cancer not only have widespread disease at diagnosis, but also present malignant ascites which is an indicator of poor prognosis.

The most common diagnosis for this disease is epithelial ovarian cancer (EOC), which can be further broken down by histotype to high grade serous, endometrioid, clear cell, mucinous, and low grade serous EOC. Of note, while many women will have a good initial response to tumor debulking and chemotherapy treatments, many women will have disease recurrence, develop treatment-resistant disease, and eventually succumb to their disease. For these reasons, it remains of high importance to continue exploring potential risk factors, as well as prognostic factors, for EOC.

At present, there are a handful of well-established risk factors for this malignancy. Reproductive risk factors such as early age at menarche, late age at menopause, post-menopausal hormone replacement therapy use, endometriosis, and nulliparous status have all been strongly supported in the literature (4–7). Other established and increasingly supported risk factors include smoking, physical inactivity, and BMI. There is currently a panel of pathogenic mutations with a significant association of risk with developing ovarian cancer, as well as known hereditary cancer syndromes, with the level of increased risk varying across specific mutations and syndromes (2, 8). An area of research with increased interest has been the role of immune suppression in EOC etiology and prognosis.

IMMUNOSUPPRESSION IN CANCER

Cells of the immune system are derived from various progenitor cells within the bone marrow that differentiate into a diverse range of mature cell types that ultimately comprise all lineages of the hematopoietic compartment. Such cell populations are programmed to provide effective host defense, which includes those with activating, as well as suppressive or phenotypes (9–11). In normal tissue, such suppressor cells are known to play key roles in regulating the immune response in response to pathogens or tissue repair following injury and damage. However, in several solid tumors as well as hematologic malignancies, there exist populations of suppressor cells that are thought to play major roles in creating a tumor-promoting or submissive microenvironment. Tumor-associated immune cells of myeloid origin (i.e., monocytic or granulocytic), for example, may occur from either an abundance of immature myeloid cells due to dysregulated myelopoiesis or myeloid cells that do not function properly (11). Examples of mature, dysfunctional myeloid populations include tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and tolerogenic dendritic cells (tolDCs). TAMs and TANs have been associated with tumor progression by promoting chronic inflammation in the tumor microenvironment, and have been found in abundance at multiple stages of cancer (11–13). TAMs are a subset of activated macrophages that become tumor-promoting in the tumor microenvironment (TME) via polarization from a functional, anti-tumor M1-like into an M2-like macrophage. Such macrophages have the ability to promote chronic inflammation via inflammatory cytokines, VEGF production for angiogenesis, as well as upregulation of insulin-like growth factor-1 (IGF-1) for invasion, and metastasis. These cells also express cytokines such as IL-10, subsequently aiding in suppression of tumor infiltrating lymphocytes (11). TANs are thought to play a role in secreting various chemokines that draw TAMs to the TME, as well as being associated with increased platelets which may play a role in the TME for maintenance of tumor health. TAMs and TANs have been associated with worse prognosis in EOC, noted to not only be associated with an increase in VEGF expression and tumor vascularization, signaling an increase in matrix metalloproteinases (MMPs), increasing tumor progression through aiding the disruption of the basement membrane and increasing the cellular mobility of ovarian tumor cells for metastasis (11, 12). It is thought that a portion of the aforementioned cell types are derived from immature myeloid-derived suppressor cells (MDSCs), or are stimulated by MDSCs (9, 10). Recently, there has been an increasing amount of evidence for the role of MDSCs in various cancers, including EOC.

MDSCs include both monocytic and granulocytic subtypes (9, 10). They have demonstrated potential to produce multiple chronic-inflammatory and mediators that support tumor growth, invasion, and metastasis. They have also been described as having the potential to suppress antigen-specific T cell responses through multiple mechanisms such as lacking MHC antigen expression, synthesis of chronic-inflammatory cytokines and mediators, including IL-10, arginase-1, transforming growth factor beta (TGF-β), and indoleamine 2,3-dioxygenase (IDO), all of which may play integral roles in tumor progression.

While MDSCs are thought to play a role in cytokine production, research has also suggested the role of cytokines in MDSC recruitment (14–16). For example, interleukin-8 (IL-8) has been implicated as a potential player in the accumulation of MDSCs in the tumor microenvironment. Such research has shown the CXCL/CXCR pathway and, IL-8 production in particular, suppresses immune infiltrating cells, and increases

MDSC activity, with high amounts of granulocytic MDSC activity noted in these studies. IL-8 can be produced by cancer cells within the tumor, demonstrating the cancer's ability to evade apoptosis through increasing these immunosuppressive MDSCs (14, 16). Specifically, IL-8 has been linked to neutrophil extracellular trap (NET) formation within granulocytic MDSC populations which may aide in the angiogenesis of a tumor promoting microenvironment (16).

In addition to suppressing T cell-mediated immune responses, these cells have also been associated with expanding the regulatory T cell population, which also act to suppress effector T cells (17–19). These above-mentioned mechanisms could explain the ability of MDSCs to suppress both the adaptive and innate immune responses in cancer, among other diseases. Lastly, MDSCs are noted to have increased expression of the programmed death ligand 1 (PD-L1), known to downregulate T cell function through engagement of cell surface PD-1 (17). Additionally; studies among other neoplastic disease have demonstrated an inverse relationship between MDSCs and tumor-infiltrating lymphocytes (TILs) (17). This suggests a complex relationship between TILs and MDSCs. These immunosuppressive pathways of MDSCs have led to considerable interest in measuring circulating MDSC levels as a potential prognostic factor in cancer. That is to say those individuals who have a higher accumulation of MDSCs are thought to have increased risk of progression of their malignancy, and worse overall survival. Additionally, targeting MDSCs in EOC may be a potential area for immunotherapeutic approaches in the future.

MDSC REGULATION IN NEOPLASTIC DISEASE

In an effort to understand MDSC accumulation in cancer, a number of studies have analyzed potential genetic and molecular factors. Several studies have reported interferon regulatory factor-8 (IRF-8), as well as the STAT family of transcription factors (STAT1, STAT3, STAT5, STAT6), as having potential roles in their development (11, 20–24). IRF-8 has been shown to be downregulated, resulting in increased levels of MDSCs (20–24). This is due to its presumed role in regulating the myeloid differentiation during hematopoiesis. Ordinarily, this particular transcription factor positively regulates progenitor differentiation to functional monocytes, macrophages, and dendritic cells, indicating that a loss of or a reduction in the expression of IRF8 may result in impaired myeloid differentiation and the production of aberrant, or immature myeloid cells with MDSC characteristics (20). IRF-8 can be induced by IFN-γ under pro-inflammatory conditions, which has an established role with activating antitumor immune responses.

Epigenetic silencing of IRF-8 in human tissue, as well as mouse models, was shown to increase the accumulation of MDSCs (25, 26). Lee et al. conducted a study assessing methylation of promotor CpG islands, resulting in the silencing of IRF-8 in human tissues of multiple carcinomas (25). Their results demonstrated that silencing IRF-8 led to the loss of IFN-γ stimulation, a known immune response-inducing cytokine. Waight et al. demonstrated that tumor-induced downregulation of IRF-8 led to an accumulation of MDSCs (26). Additionally, these investigators also noted a reduction of MDSCs when they utilized mouse models with IRF8 overexpression, indicating that not only does a loss of function of this transcription factor lead to the accumulation of such suppressive cells, but overexpression of IRF-8 as an interventional application may necessitate further research for potential clinical implication in reducing the amount of accumulated MDSCs. Lastly, these researchers addressed the role of the STAT family of transcription factors in increasing the accumulation of MDSCs by analyzing STAT3 and STAT5 activity in this process. Their results demonstrated that activation of STAT3 or STAT5 can downregulate IRF-8 expression, providing a molecular explanation for why such STATs influence the accrual of these suppressor cells. Multiple studies have demonstrated the association of the STAT family of transcription factors with the increased accumulation of MDSCs across many different malignancies and various disease models (27–30). Essentially, the aforementioned studies conclude that when the STAT3/5 pathway is upregulated and the expression of IRF-8 is downregulated, an increased accumulation of MDSCs is anticipated, demonstrating the strong role of these transcription factors in regulating the MDSC accumulation, and proper development of myeloid cells.

MDSC EXPRESSION AND MURINE OVARIAN CANCER

In murine models of ovarian cancer, results have demonstrated multiple potential factors influencing the expansion of MDSCs. In a study by Zhao et al. investigators evaluated depletion of SORBS2, a protein coding gene for sorbin and SH3, and its impact on the tumor microenvironment (31). When they utilized a knockdown murine model for SORBS2, they observed increased metastatic behaviors of the ovarian tumors, and noted increased MDSC levels and M2 (suppressive) polarization of TAMs. Subsequently, they reported decreased survival among the mice with SORBS2 knockdown, thus concluding that SORBS2 plays a role in suppressing the invasion of ovarian tumors. Interestingly, they did note the possibility for reversing such metastatic characteristics by forced expression of growth inhibitor protein coding gene WFDC1, and/or IL-17D, a gene that codes for cytokine production/stimulation, both of which bound to SORBS2 to decrease metastatic potential in this study.

Similarly, the EMT transcription factor, Snail, was evaluated in a knockdown model (32). By knocking down this transcription factor an increase in tumor infiltrating immune cells and a decrease in MDSCs were observed. These researchers speculated that this may be due in part to the relationship between Snail and the CXCL/CXCR pathway. This pathway is associated with cytokines that play a role in recruitment of MDSCs to the tumor site, and may be upregulated by Snail. Therefore, these investigators concluded that the promotional recruitment of MDSCs via the CXCL/CXCR pathway may be inhibited by Snail knockdown.

Previous studies have demonstrated that diminishing MDSC populations in ovarian tumor ascites was associated with

decreasing the levels of IL-10 (33). Further studies on this topic using mouse models have demonstrated the ability of IL-10 production by MDSCs, suggesting that IL-10 may be a product of these cells via changes in CD62L and lymphocyte acting gene, LAG-3. They also report that these processes aide in creating positive feedback by IL-10 stimulating further MDSC expansion and immune suppression.

Lastly, murine ovarian cancer models have led to the discovery that MDSCs may accumulate and develop in environments without NADPH oxidase, a component that was previously thought to be a factor in such cellular processes (34). This was previously thought to be due to the association of reactive oxygen and nitrogen species in environments with accumulating MDSCs. However, this was not the case in a study of NADPH defective mice, which still demonstrated the ability to accumulate suppression from the MDSCs, therefore was found to be independent of NADPH oxidase. Other murine studies on this topic have been conducted more closely related to therapeutic research on these suppressor cells, and as such are included in the later section on potential therapies. **Figure 1** includes an illustration of identified MDSC activity.

MDSC EXPRESSION AND HUMAN OVARIAN CANCER

As previously stated, various factors affecting MDSC expansion have been studied in multiple types of cancers and across various biological models. Like many other carcinomas, high levels of MDSCs in human EOC have also been associated with poor prognosis (13, 35–41). In human EOC models, various factors influencing the etiology of MDSC expansion in this disease are still under active investigation. Horikawa et al. studied the relationship between high levels of VEGF expression and accumulation of MDSCs in high grade serous ovarian cancer patients, as well as mouse models (36). Their results demonstrated a statistically significant increase of immune suppression (characterized as downregulation of lymphocytes) in patients with high levels of VEGF expression. Additionally, they reported significantly worse overall and progression free survival, among those with high VEGF compared to those categorized as having low VEGF expression. They then proceeded to test the correlation between VEGF expression and MDSC levels among their mouse models, finding that high expression of VEGF was significantly associated with MDSC expression, and inversely associated with lymphocytic expression. Additionally, the MDSCs were shown to have increased VEGFR2 expression. They also reported that VEGF–A appeared to be directly correlated to MDSC differentiation and migration, with VEGFR/VEGF-dependent recruitment to the tumor site.

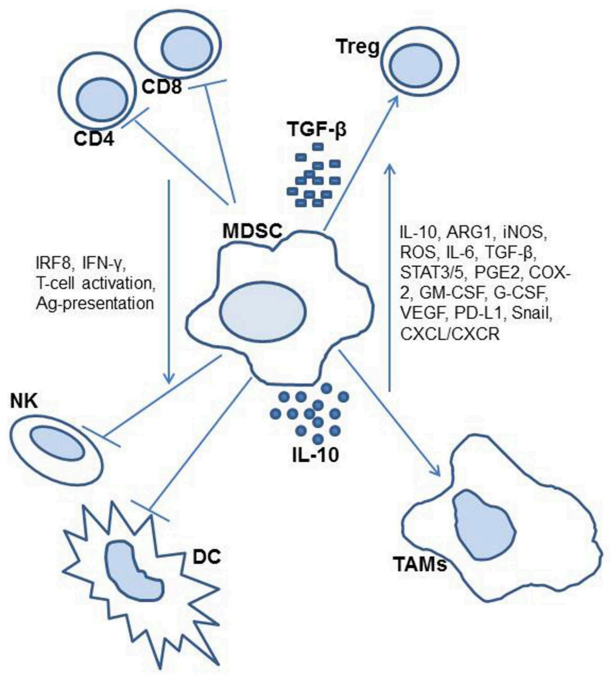

FIGURE 1 | Diagram of myeloid-derived suppressor cell activity in the cancer patient.

As stated above, recent studies in EOC have demonstrated the potential association between MDSCs and the upregulation of IGF1, which may promote both proliferative activity among the cancer cells, as well as migration for invasion and metastasis among these cells (13). Other studies have focused on ascites fluid and MDSCs, such as levels of interleukin-6 and-10 (IL-6, IL-10) in the ascites, suggesting that IL-6 and IL-10 in the ascites fluid may contribute to the expansion or function of MDSCs in EOC patients (37). Likewise, IL-1β has also been reported to have an association with increased levels of MDSCs in EOC patients when compared to healthy controls (39). It has been noted that in EOC, specifically, there appears to be inhibition of MDSCs recruitment to a tumor microenvironment lacking chemokine receptor CCR2. Additionally, TAM migration was explored in a population of patients with samples extracted from tumor or ascites fluid (40). The TAMs were then analyzed for CCR2 mRNA expression. Their results suggested that TAMs with defective CCR2 expression also demonstrated inhibited migration to the tumor, however TAMs were able to overcome this in the presence of complement component 5 (C5a).

If TAMs are in fact upregulated by and/or differentiating from MDSCs, then this would explain the potential role of CCR2 in both MDSC and TAM inhibition. Additionally, a tumor-associated inflammatory mediator, prostaglandin E2 (PGE2), has demonstrated a role in controlling the expression and interactions of CXCL12 and its respective receptor, CXCR4, which are implicated in the process of tumor progression (42). These interactions showed increased expression of CXCR4 on monocytic MDSCs, with PGE2 inducing CXCL12 in the tumor microenvironment, as well as CXCR4 on MDSC precursor cells. PGE2 was seen to induce COX2 expression, which further stimulates PGE2, thus having developed a positive feedback loop to continue the accumulation of MDSCs. Another study also identified that accrual of MDSCs may be associated with increased DNMT3A (involved in DNA methylation) in a PGE2 positive cellular environment (43). This suggests that

downregulating DNMT3A may improve prognosis with regard to MDSC activity. Lastly, a study by Santegoets et al. analyzed a monocytic MDSC to dendritic cell ratio to evaluate its usefulness as a prognostic factor among EOC patients after treatment (44). They reported this ratio as being an independent potential prognostic factor for EOC survival, with high levels of monocytic MDSCs being correlated with higher risk of mortality.

While the aforementioned studies have confirmed that MDSCs as a prognostic factor in EOC patients represent an area worth studying, the methodologies employed across studies demonstrate significant differences. As shown in **Table 1**, the research studies performed on human samples to date had several limitations including; small sample sizes, unspecified/diverse histotypes of EOC, variable staging, inconsistent source of collection (i.e., blood, tumor, ascites), and inconsistent use of surface markers for the identification of MDSCs. Many of the studies do not report on MDSC subset analysis within this cancer. Furthermore, to our knowledge, there are no studies published from an epidemiological/population science perspective. Implementing a study design with a well-defined patient population, clinical characteristics, consistent MDSC surface markers, and a larger sample size would allow us to draw more definitive conclusions on the value of MDSCs as a prognostic factor in this patient population. It would also be of great value to compare blood measurements to ascites fluid levels of MDSC activity in a larger sample size.

Additionally, as there is a growing interest in understanding the functionality of MDSCs and their mechanisms in

TABLE 1 | Summary of previous human studies on MDSC activity in EOC patients.

Author	Sample size	Histotype	Stage	MDSC collection	Controls	Surface markers
Horikawa et al. (36)	56	HGSOC	III, IV	Ascites fluid, pre-chemotherapy/ radiation	None	CD33+, CD11b+
Wu et al. (37)	31	Serous, mucinous, endometrioid, mixed	I–IV	Peripheral blood, sera, ascites, pre-chemotherapy/ radiation	31 age-matched, healthy peripheral blood donors	CD14+HLA-DR–/low
Huang et al. (40)	Not specified	Multiple solid tumor cancers, unspecified ovarian	Not specified	"Tumor and blood," "untreated"	None	Lin– HLA-DR–
Obermajer et al. (42)	24	Unspecified epithelial ovarian cancer	III, IV	Ascites and sera, prior to any adjuvant therapy	None	CD11b+
Rodriguez-Ubreva et al. (43)	22	Unspecified epithelial ovarian cancer	III, IV	Ascites and blood, untreated	10 healthy donors' blood	CD11b+CD33+ CD34+
Santegoets et al. (44)	36	Unspecified epithelial ovarian cancer	Not specified	After treatment with tocilizumab, carboplatin/doxorubicin or gemcitabine and interferon-α 2b	"Healthy donor blood"	CD14-CD15- double-negative (dn) CD33+CD11b+ and CD33-CD11b+

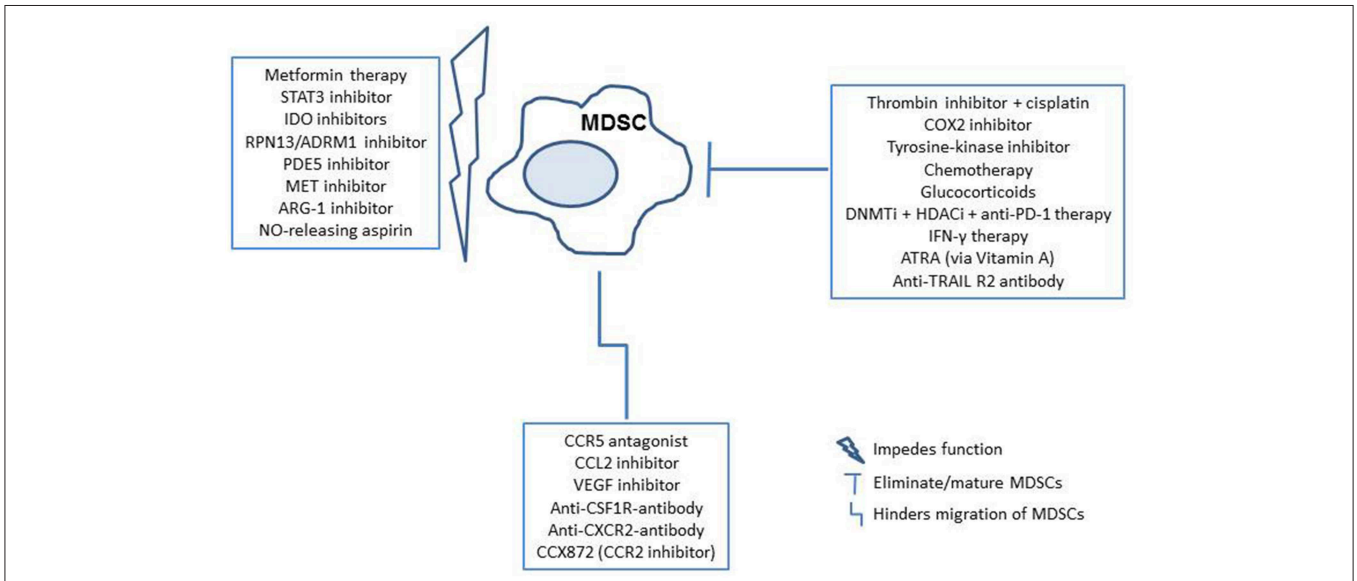

FIGURE 2 | Potential therapeutic implications for MDSC targeted therapy in the cancer patients. MDSCs can be targeted by elimination/maturation, impeding function, or hindering migration. Therapies are outlined in blue, with subsequent immune reactions in shapes that follow (35, 41, 46–55).

EOC progression, there have also been efforts put toward understanding potential genetic variation and MDSC activity. In a large consortium study, single nucleotide polymorphisms (SNPs) in 24 genes with presumed relationships to MDSC expansion were analyzed for their association with survival among ovarian cancer patients, which showed no significant associations for SNP variations (45). However, it is worth noting that many smaller studies, such as those previously mentioned (13, 36, 40, 42), have demonstrated the potential of genetic expression and interactions in the accumulation of MDSCs in the tumor microenvironment, therefore, further epidemiological research focused on gene-environment interactions may be warranted.

POTENTIAL FUTURE DIRECTIONS

In addition to studying the underlying characteristics of MDSCs in EOC, recent efforts have demonstrated the potential application of anti-PD-1 after anti-Gr-1 MDSC depletion therapy, as well as other immunotherapies that may effectively reduce MDSCs or interfere with their activity in ovarian cancer models to allow activation of a tumor-infiltrating immune response (35, 41). Interestingly, one group of researchers explored Metformin, a pharmaceutical commonly prescribed for diabetic patients, in EOC patients with high MDSC levels (46). They observed decreased levels in both granulocytic, as well as monocytic subsets of MDSCs, which was believed to have occurred due to restriction of adenosine generation. Other studies utilizing murine models have identified glucocorticoids, various checkpoint blockades, direct thrombin inhibitors, DNA methyltransferase (DNMTi)/histone deacytlase inhibitors (HDACi), and RPN13/ADRM1 inhibitors, all demonstrating their potential to alter the levels of MDSCs, or function (47–51). Such approaches have the general goal of enhancing overall survival, though are not yet demonstrated in human subjects. In addition to studies in ovarian cancer models, research on potential therapies to eliminate, impede function of, or inhibit MDSC migration to the tumor microenvironment include; PDE5 inhibitors, STAT3 inhibitors, tyrosine-kinase inhibitors, chemotherapies, CCL2 inhibitors, CCR5 antagonists, VEGF inhibitors, IDO inhibitors, COX2 inhibitors, MET inhibitors, ARG-1 inhibitors, NO-releasing aspirin, ATRA, CCR2 inhibitors, anti-CXCR2 antibodies, anti-TNF-related apoptosis-inducing ligand (TRAIL) death receptor R2 antibody, and with use of an anti-CSF1R antibody. (52–55) Furthermore, specific anti-MDSC antibodies that target surface MDSC proteins are of interest (53). All of these studies suggest that further clinical analysis of such drug applications and reduction of MDSCs in patients with EOC may improve outcomes, and are worth exploring. A brief overview of the mechanisms of MDSC interference by these potential therapies is demonstrated in **Figure 2**.

There is increasing interest in the role of MDSCs in the etiology and prognosis of cancers. While there is growing evidence building an association between MDSCs and EOC, there is still a wide range of unknown mechanisms and interactions necessitating further research on this topic. Given the above information, one can understand the intrigue in therapeutic intervention with regard to MDSCs in EOC. Developing an extensive understanding on this topic may allow further development of clinical interventions targeting such cellular involvement. However, the limited number of studies on these cells in human EOC has significant caveats. While a recent review paper on MDSCs in gynecologic malignancies was published, this paper was more so highlighting technical aspects of this topic and was not necessarily specific to ovarian carcinoma (56). While their review paper provides a thorough overview of the role of MDSCs as a whole, including examples from multiple malignancies, they do not provide detailed information on the various interactions of MDSC activity in ovarian cancer patients specifically. These authors focus on the overall evolution of MDSC data, and offer insight on potential targeting of MDSCs in general. Due to the minimal existing prognostic and therapeutic factors in this patient population, we feel it deserves special attention. Inclusion of characteristics such as histotypes sub-analysis, analysis of subsets of MDSCs, clinical and epidemiological patient characteristics, and a larger sample size would give rise to more conclusive data. An analysis of this topic utilizing a comprehensive epidemiological model would benefit the field of epidemiology, as well as clinical gynecologic oncology, to fully understand the value of collecting MDSC measurements for patient outcomes, and potential modifiable factors to reduce accumulation of MDSCs in EOC patients.

AUTHOR CONTRIBUTIONS

This review article was written by KM and AS, with substantial input from SA.

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REVIEW
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Microenvironmental Regulation of Tumor Progression and Therapeutic Response in Brain Metastasis

Michael Schulz[1,2†], *Anna Salamero-Boix*[1†], *Katja Niesel*[1†], *Tijna Alekseeva*[1†] *and Lisa Sevenich*[1,3,4*]

[1] Institute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Frankfurt, Germany, [2] Biological Sciences, Faculty 15, Goethe University, Frankfurt, Germany, [3] Frankfurt Cancer Institute, Goethe University, Frankfurt, Germany, [4] German Cancer Consortium (DKTK, Partner Site Frankfurt/Mainz) and German Cancer Research Center (DKFZ), Heidelberg, Germany

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***Correspondence:**
Lisa Sevenich
sevenich@gsh.uni-frankfurt.de

[†]These authors have contributed equally to this work

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Cellular and non-cellular components of the tumor microenvironment (TME) are emerging as key regulators of primary tumor progression, organ-specific metastasis, and therapeutic response. In the era of TME-targeted- and immunotherapies, cancer-associated inflammation has gained increasing attention. In this regard, the brain represents a unique and highly specialized organ. It has long been regarded as an immunological sanctuary site where the presence of the blood brain barrier (BBB) and blood cerebrospinal fluid barrier (BCB) restricts the entry of immune cells from the periphery. Consequently, tumor cells that metastasize to the brain were thought to be shielded from systemic immune surveillance and destruction. However, the detailed characterization of the immune landscape within border-associated areas of the central nervous system (CNS), such as the meninges and the choroid plexus, as well as the discovery of lymphatics and channels that connect the CNS with the periphery, have recently challenged the dogma of the immune privileged status of the brain. Moreover, the presence of brain metastases (BrM) disrupts the integrity of the BBB and BCB. Indeed, BrM induce the recruitment of different immune cells from the myeloid and lymphoid lineage to the CNS. Blood-borne immune cells together with brain-resident cell-types, such as astrocytes, microglia, and neurons, form a highly complex and dynamic TME that affects tumor cell survival and modulates the mode of immune responses that are elicited by brain metastatic tumor cells. In this review, we will summarize recent findings on heterotypic interactions within the brain metastatic TME and highlight specific functions of brain-resident and recruited cells at different rate-limiting steps of the metastatic cascade. Based on the insight from recent studies, we will discuss new opportunities and challenges for TME-targeted and immunotherapies for BrM.

Keywords: brain metastases, tumor microenviroment, microglia, astrocytes, immune system, immunotherapy, neurons

INTRODUCTION

The stepwise process in which cancer cells disseminate from the primary tumor site to colonize distant organs is biologically a highly inefficient process, yet metastasis accounts for 90% of cancer related deaths (1). In particular, metastasis to the brain represents a considerable burden and is associated with high morbidity and unfavorable prognosis for patients (2). A central question in the biology of metastasis remains the preference of certain tumor types to colonize individual organs, such as the brain. Gene signatures that mediate the preferential organ tropism have been identified (3). Differentially expressed genes in tumor cell variants with high tropism for a specific organ are often associated with factors that assist tumor cells to overcome tissue specific barriers, e.g., the blood brain barrier (BBB), or to generate a cancer permissive niche in potentially hostile environments (4, 5). In addition to tumor cell intrinsic traits, the ability of tumor cells to rapidly co-opt niche cells in foreign organs to exploit their functions and to block or evade anti-tumor activity is a key determinant for successful metastatic colonization (6, 7).

Upon entry into the central nervous system (CNS), tumor cells are confronted with the highly complex and specialized brain tissue environment that is fundamentally different from the primary site with respect to cellular constituents, matrix composition, metabolism, and immune landscape (6). The cellular composition of the brain is represented by the main functional cells, including neurons and auxiliary cell types, macroglia (astrocytes and oligodendrocytes), and microglia. In addition to brain resident cell types, blood-borne immune and inflammatory cells have recently gained attention as potent mediators of brain metastasis-associated inflammation. While the presence of tumor-infiltrating lymphocytes is often correlated with better prognosis and is indicative for higher response rates to immunotherapy, high content of myeloid cells is associated with immune suppression, tumor promotion, and therapy resistance (8). In this review, we highlight the complex interactions between tumor cells and tumor-associated niche cells and discuss current knowledge on cell type-specific pro- or anti-tumor functions of cells in the tumor microenvironment (TME) in brain metastases (BrM). Based on this knowledge, we will discuss opportunities and challenges for TME-targeted or immunotherapies against BrM.

Neurons in Brain Metastases—Innocent Victims or Critical Mediators?

Neurons, as highly specialized cells responsible for cell-to-cell signal propagation, are certainly one of the most critical and highly abundant cell types in the CNS (9). However, to date little is known about their contribution to BrM. Currently, astrocytes and microglia, as well as recruited peripheral immune cells, are within the main focus of research in the context of BrM. Neurons are mostly regarded as passive bystanders and neuronal cell death and dysfunction are rather thought to result from collateral damage in the process of BrM progression and/or treatment. Neuronal cell death results from persistent neuro-inflammation caused by reactive microglia and astrocytes in response to tumor cells. Myelinating glial cells and oligodendrocytes are also functionally compromised in this tumor-reactive milieu and thus further contribute to neuronal dysfunction (10). Interestingly, glial dysfunction and its effect on myelin sheath development are implicated in common side effects of chemotherapy. Those characteristic cognitive symptoms are collectively referred to as chemobrain (11). Moreover, a recent study by Seano et al. shed additional light on the cause of neuronal cell death in the presence of BrM. The authors demonstrated that mechanical compressive stress from a solid tumor leads to indirect neuronal malfunction and blood vessel degeneration in the peri-tumor area thereby causing neuronal cell death by critical deformation of the neuronal bodies (**Figure 1**; Boxes 6, 7). Intriguingly, the authors were able to show that common neuroprotective lithium medication was effective in preventing neuronal damage and alleviate in part negative cognitive symptoms (12).

While the niche cells in the CNS have to cope with the arrival and expansion of tumor cells, also metastatic cancer cells have to adapt to the brain microenvironment, which differs considerably from the tissue of origin (6). The extent of this adaptation has been demonstrated by Neman et al. (13). The authors show that breast cancer cells are capable to change their metabolic machinery and to mimic the reciprocal relationship between neurons and astrocytes by expressing all major genes of a GABAergic phenotype, a feature attributed to neurons (13) (**Figure 1**; Box 6). This adaptive mechanism allows cancer cells to utilize a novel energy source, glutamate, prevalent in the normal brain. A follow up study by Schnepp et al. has shown that this feature is not exclusive to breast cancer cells. The authors unveiled the mechanism of this genetic shift, implicating increased GABA synthesis by metastatic cancer cells via methylation-dependent upregulation of glutamate decarboxylase 1 (GAD1) expression (14). Interestingly, Schnepp et al. have shown that this precise feature can be used to explore novel treatment options, such as GABA antagonists, frequently used for seizure treatment. While it is increasingly recognized that tumor cells have to adopt to the unique metabolism of the brain in order to thrive, it is less well-characterized to which extent metastatic tumor cells that arise from epithelial origin can benefit from neuronal growth factors as previously demonstrated for primary brain cancers. Glioma, as primary brain cancer, arise from different neuronal or glial cell lineages (i.e., neural stem/progenitor cells or oligodendroglial lineage) (15) and hence originate from cells that are known to be influenced by neuronal activity (16, 17). Indeed, it has been shown that neuronal excitation and subsequent release of synaptic adhesion protein Neuroligin-3 (NLGN3), Brain Derived Neurotrophic Factor (BDNF), and neurotransmitter such as dopamine and serotonin are utilized by glioma cells to promote tumor growth (18–20). Moreover, it has been shown that glioma cells can influence neuronal excitation in the vicinity of tumors through secretion of glutamate, thus ensuring the supply of proliferative factors (18, 21). Interestingly, although breast-to-brain metastatic tumor cells are of epithelial origin, there is evidence that breast cancer cells express receptors for two major neurotrophic growth factors, neuronal growth factor (NGF) and BDNF (22). Moreover, a recent transcriptome analysis of

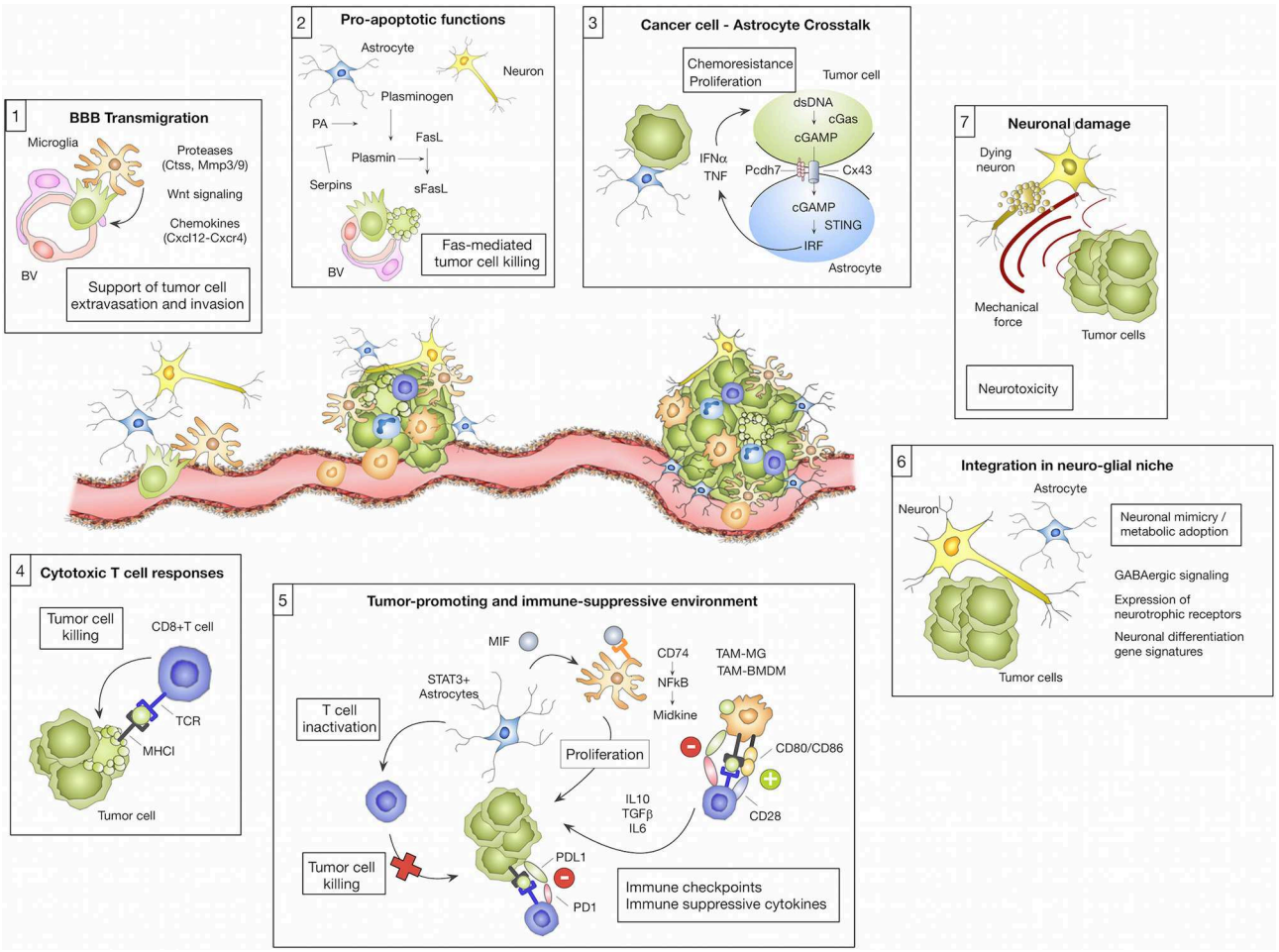

FIGURE 1 | Microenvironmental regulation of the metastatic cascade. The tumor microenvironment of brain metastasis comprises different brain-resident and recruited cell types with cell-type and/or stage-dependent pro- or anti-tumor functions. (1) Different microglial-derived factors including proteases (e.g., Ctss, Mmp3, and Mmp9), Wnt signaling components or chemokines (e.g., Cxcl12) have been implicated in assisting tumor cells to cross the blood brain barrier (BBB) and colonize the brain parenchyma. (2) In contrast, astrocytes were shown to prevent early stages of metastatic colonization by inducing soluble (s)-FasL-mediated tumor cell killing. Tumor cell-derived serpins can block this effect by inhibiting astrocyte-derived plasminogen activator (PA), therefore preventing the generation of active plasmin that converts FasL into sFasL. (3) While the initial tumor cell—astrocyte contact leads to tumor cell killing, close interactions between tumor cells and astrocytes via gap junctions foster tumor cell proliferation and protect tumor cells from chemotherapy. This process was linked to the transfer of cGAMP from tumor cells to astrocytes that triggers cGas-STING-mediated IRF activation leading to production of IFNα and TNF. (4) Cytotoxic T cells represent an important component of the adoptive immune response against brain metastasis by executing tumor cell killing. (5) However, T cell activity is efficiently blunted by the immune-suppressive milieu in brain metastasis. T cell activity is modulated through interaction with several cell types including tumor cells, tumor-associated macrophages/microglia (TAM-MG and TAM-BMDM) and astrocytes by expressing immune checkpoint molecules or by secreting immune-suppressive cytokines (e.g., IL10, TGFβ or IL6). Moreover, astrocytes with high STAT3 expression were shown to activate tumor-promoting TAMs via the MIF-CD74-NfkB-Midkine axis. (6) Tumor cells that colonize the brain were shown to adopt to the neuro-glial niche by acquiring neuronal gene signatures that induce specific metabolic programs (e.g., GABAergic signaling and the expression of neurotrophic factors). (7) Tumor expansion leads to neuronal damage by mechanical compression of neurons.

tumor- and stromal signatures in BrM revealed an enrichment of neuronal differentiation pathways in the tumor cell population (23). Further exploration of neuronal mimicry revealed that GABAergic signaling is not limited to the CNS, but has also emerged as a tumor signaling molecule in cancers of peripheral organs such as breast, liver, pancreas, and colon (24). Hence, it is possible that tumor cells are primed for GABAergic signaling already at the primary tumor site providing an advantage for rapid adaptation to metabolic conditions in the brain. Moreover, it was demonstrated that prostate cancer cells induce axonogensis and use growing axons as migratory tracts for cancer cell dissemination (19, 25).

To date it remains unknown to which extent neurons play an active role in BrM onset and progression. However, given the recently demonstrated role of neurons in glioma together with the observation that highly innervated tumors (i.e., prostate or head and neck cancer) are more aggressive than their less innervated counterparts (18, 19, 25), it might be premature to exclude neurons as active players in BrM. Future studies will hopefully provide more detailed insight into the role of neurons

in BrM and potentially open new therapeutic avenues against BrM by targeting interactions between tumor cells and neurons.

Astrocytes in Brain Metastases—Versatile Players in Mediating Distinct Steps Within the Brain Metastatic Cascade

Astrocytes belong to the glial cell types and represent the most abundant cell population within the CNS (26). Originally described as star-like "glue" cells of the CNS, the variety and complexity of astrocyte function in health and disease is increasingly recognized. Under normal conditions their role in tissue homeostasis includes maintenance of the blood brain-barrier (BBB), immune signaling, regulation of extracellular ion, and fluid homeostasis, as well as control and maintenance of a broad range of functions implicated in modulating neuronal networks, such as regulation of synaptogenesis, synaptic plasticity, and elimination, neurotransmitter clearance, and neurotrophin secretion (27–30). To fulfill this functional diversity, it is now widely accepted that astrocytes represent a highly heterogeneous cell population (28, 31–33). With the advent of high-throughput single cell sequencing and other "*omic*" approaches, the existence of several astrocyte subpopulations was revealed in rodents (31, 34, 35). An even higher heterogeneity was found within the human brain (36, 37). Interestingly, neuronal stimuli have been shown to determine distinct features of astrocytes (38). Moreover, it was shown that during aging, astrocytes change their transcriptomes in different regions of the murine brain (39), which is in part orchestrated by interacting with local microglia (40). Given the phenotypic and functional diversity of astrocytes, it is not surprising that astrocytes play a central role in maintaining tissue homeostasis and in regulating neuro-glial communication under physiological conditions. Consequently, astrocytes are also often found to be involved in disease progression of different CNS malignancies (30). Moreover, malignantly transformed astrocytes are the cell of origin for astrocytoma, the most common form of glioma (41). Astrocytes respond to disease-associated stimuli by undergoing morphological and functional changes, which are collectively referred to as reactive astrogliosis (30, 42, 43). A key feature of reactive astrocytes (RA) is the formation of a glial scar that confines pathological foci from the healthy parenchyma (27). Interactions between astrocytes and other brain-resident or recruited cells have been investigated in different disease models. It was shown that every cell type in the CNS can release factors that induce astrogliosis (27), and the outcome is regulated in a time- and context-dependent manner (44).

A particularly close connection between astrocytes and microglia was observed in the diseased CNS (10). Depending on the environmental stimuli, astrocytes acquire different activation states that are referred to as A1 and A2 following the previously defined nomenclature to classify macrophage polarization states into pro-inflammatory, anti-tumor M1 macrophages, and immune-suppressive, tumor-promoting M2 macrophages (45, 46). A1 astrocytes are regarded as neuro-inflammatory, while A2 astrocytes are associated with neuro-protective features by promoting survival and growth of neurons and by inducing repair mechanisms (43). However, it should be noted that the M1/M2 and A1/A2 nomenclature reflects functional and phenotypic extremes within a spectrum of activation states and that along the continuum of activation states mixed phenotypes have been reported (47). The high phenotypic plasticity of astrocytes was also reported in pre-clinical and clinical studies on different neurodegenerative disorders. For example, Liddelow et al. demonstrated that microglia induce A1 astrocytes with neurotoxic properties. The presence of A1 astrocytes was demonstrated in various human malignancies from the spectrum of neurodegenerative diseases (10). On the contrary, microglia were shown to exert crucial functions in activating neuroprotective astrocytes in a model of *Spinal Cord Injury* (SCI) (48), further underpinning the context-dependent outcome of cellular interactions. In line with this finding, microglia-mediated blockade of an A1 astrocyte conversion was shown to be neuro-protective in a mouse model of sporadic Parkinson's Disease (49). There is also evidence that RA are regulated by distinct T cell subsets in neuro-inflammatory conditions such as stroke, which then potentiates neurological recovery (50).

While our understanding of astrocyte function in neurodegenerative disorders is steadily increasing, we are just at the beginning to decipher the underlying mechanisms of pro- or anti-tumor functions of astrocytes in BrM (51, 52). Induction of astrogliosis is an early event during metastatic colonization and outgrowth. This early reaction is attributed to neuro-protection by delineating metastatic foci from the normal brain parenchyma. Valiente et al. proposed that early contacts between tumor cells and astrocytes lead to tumor cell death and clearance of the majority of tumor cells that enter the brain. In order to successfully colonize the brain, tumor cells have to acquire traits to block pro-apoptotic stimuli from astrocytes (53) (**Figure 1**; Box 2). On the other hand, there is accumulating evidence that astrocytes promote distinct steps of the metastatic cascade, including initial seeding and support of tumor outgrowth (54–56). Moreover, astrocytes have been shown to protect tumor cells from chemotherapy (57). This process was shown to be dependent on gap junction formation (57, 58). The importance of direct cellular connections between astrocytes and breast- or lung brain metastatic tumor cells via gap junctions was further demonstrated by Chen et al. (59). In this context, gap junction formation was mediated by connexin43 (Cx43) and protocadherin (Pcdh7) and activated the innate immune response pathway cGAS-Sting (Cyclic GMP-AMP synthase-stimulator of interferon genes) leading to secretion of tumor-supportive cytokines such as IFNα and TNF (**Figure 1**; Box 3). Functional co-option of RA by melanoma cells was further exemplified by Schwartz et al. (60). The authors demonstrated in a melanoma brain metastasis model that astrogliosis is exploited by the tumor cells to support their growth (60). Astrocytes are also emerging as critical modulators of immune responses in BrM by interacting with brain-resident and recruited inflammatory cells. Priego et al. recently proposed an important role of astrocytes in the modulation of innate and acquired immunity in BrM (61). The authors identified a subpopulation of RA with high STAT3 activation levels associated

with BrM of different primary origin. STAT3 activation was shown to affect microglia and T cell functions, likely leading to the establishment of an immunosuppressive microenvironment (**Figure 1**; Box 5). CD74+ TAMs were previously shown to generate an immunosuppressive milieu by reducing the secretion of IFNγ in glioma (62). More recently it was demonstrated in BrM that CD74+ TAMs depend on pSTAT3+ astrocytes that secrete macrophage migration inhibitory factor (MIF), the ligand for CD74. In response to ligand binding, CD74 acts as a transcription factor and promotes the expression of NFkB downstream targets, such as midkine, a factor that promotes cell viability (61). MIF inhibition by ibudilast led to a reduction of BrM in organotypic cultures (61). Moreover, genetic and pharmacological inhibition of STAT3 resulted in impaired viability of tumor cells and reduced outgrowth of brain metastasis (61). Heiland et al. recently confirmed the findings on STAT3+ astrocytes in primary brain tumors and demonstrated that astrocyte-microglia interactions generate a strong immune-suppressive environment due to up-regulation of PD-L1 on tumor-associated astrocytes and production of cytokines such as IL10 and TGFβ (63).

Taken together, astrocytes are emerging as one of the key regulators of brain metastatic colonization and outgrowth. Owing to their high phenotypic and functional heterogeneity, astrocytes exert pro-tumor as well as anti-tumor functions. Detailed insights into the existence of different astrocyte subpopulations or stimuli that polarize astrocytes at distinct stages of the brain metastatic cascade are required to develop astrocyte-targeted therapies.

Myeloid Cells in Brain Metastases—Origin and Location Matters

Myeloid cells in brain malignancies comprise a highly abundant and heterogeneous cell population and consist of brain resident myeloid cells as well as recruited cells including monocytes, bone marrow-derived macrophages (BMDM), and granulocytes (64). Brain-resident microglia are the major representatives of the innate immune system in the CNS and exert critical functions in immune surveillance and host defense. In addition to functions related to neuro-inflammation, microglia are also responsible for synapse pruning and remodeling (65). Microglia represent a unique cell type among the glial cells with respect to their ontological origin. In contrast to other glial cells, microglia are of mesodermal origin and arise from primitive hematopoietic progenitors (erythromyeloid progenitors) that are present in the yolk sac during embryonic development (66–68). In addition to parenchymal microglia, the CNS harbors myeloid cell populations that reside in specific regions of the CNS including the choroid plexus, the interphase between blood and meninges, and the perivascular space of vessels (69–71). Border-associated macrophages (BAMs) derive from erythro-myeloid precursors that arise from the yolk sac and the fetal liver. Interestingly, bone marrow-derived monocytes also contribute to the choroid plexus macrophage population (70–72). Moreover, monocytes have been shown to reside within the meninges (73). BAMs are believed to have a higher antigen presenting capacity than microglia, largely due to higher expression of MHCII (74), however their contribution to BrM progression remains to be elucidated. Detailed insight into transcriptional programs of microglia revealed a remarkable plasticity in response to a wide variety of stimuli, such as regional differences in the brain, aging, sex, or the composition of the microbiome and gene signatures reflect cellular functions during developmental stages (75–78). In addition to in depth analysis of microglial heterogeneity, dissecting gene signatures of disease-associated microglia provides detailed insight into lineage-dependent functions and cellular dynamics (79–82). In this regard, the identification of a disease-associated signature in microglia (DAM) by single cell sequencing in Alzheimer's disease, aging, multiple sclerosis, and amyotrophic lateral sclerosis models significantly contributed to our understanding how different pathological conditions shape the molecular identity of disease-associated cells and how the respective subpopulation might enhance or ameliorate disease progression. Upregulation of phagocytosis components and neurodegenerative markers such as Trem2 and ApoE and the downregulation of microglia homeostatic markers such as Cx3cr1 and Tmem119 were shown to be characteristic for the DAM signature (71, 79–82). Remarkably, single cell analysis of human microglia from multiple sclerosis patients revealed an even higher heterogeneity, as seven different populations of microglia were identified. Three of those populations represented homeostatic genes, one population showed an upregulation of chemokine and cytokine signaling, whereas the three other populations correlated with the clusters associated with demyelination and remyelination in mice (83). Although the BrM field currently lacks detailed insight into the molecular identity of disease-associated macrophages/microglia compared to neurodegenerative diseases or primary brain tumors, a series of pre-clinical studies shed light into tumor-associated macrophage (TAM) functions during distinct steps of the metastatic cascade. Invasion of metastasizing tumor cells is rapidly sensed by microglia and the presence of single tumor cells is sufficient to recruit and to activate microglia (84, 85). Given the role of microglia in immune surveillance and host defense, it is tempting to speculate that the initial contact between tumor cells and microglia at sites of extravasation leads to clearance of invading tumor cells. However, Chuang et al. demonstrated that tumor cells block pro-apoptotic functions of microglia and exploit tissue damage responses to increase their invasive capacity (86). The role of microglia in tumor cell extravasation was further confirmed by Qiao et al. using a CSF1R inhibitor to deplete microglia in prevention trial settings in a mouse model for melanoma BrM (84). The authors also found that Mmp3 expression by microglia was negatively correlated with ZO-1 expression on endothelial cells. Moreover, the incidence of melanoma BrM was decreased by Mmp3 inhibition (84). Co-option of microglial functions and adoption of leukocytic characteristics to increase the capacity of tumor cells to colonize the brain parenchyma was previously proposed (5) (**Figure 1**; Box 1). Interestingly, it was observed that tumor cells increase the expression of cathepsin S, a protease that is pre-dominantly expressed by leukocytes, to cleave junctional adhesion molecules that maintain the BBB integrity and thus

assist tumor cells to breach the BBB. Importantly, only the combined depletion of cathepsin S in the tumor and stroma compartment was efficient to reduce BrM burden (5). The co-option of leukocyte characteristics by tumor cells is also evident in the role of the C-X-C chemokine receptor type-4 (Cxcr4) along with its ligand Cxcl12 that are involved in lymphocyte chemotaxis. Cxcr4 expression has been detected in BrM tumor cells (87, 88). Remarkably, the inhibition of this pathway decreased breast cancer cell migration (89) and impaired BrM establishment (90).

In established BrM, tumor-associated macrophages and microglia are the most abundant non-cancerous cell type and constitute up to 30% of the total tumor mass (5). In primary brain cancer, TAMs tend to be pro-tumorigenic and accumulate with higher tumor grade (91, 92). As revealed by immunohistochemistry of BrM sections, microglia and macrophages showed signs of intratumoral activation and formed a boundary between the tumor mass and normal brain tissue (93–95). They were identified as foamy cytoplasmatic cells with shortened cell processes and immunoreactive to CD68. However, there is currently no clinical evidence in BrM for a correlation between microglia density and activation marker expression with treatment modality, anatomic brain regions or necrosis (96). Despite the lack of clinical correlation between TAM content and BrM patient prognosis, pre-clinical data indicate tumor-promoting functions of TAMs in BrM. The crosstalk between microglia and melanoma BrM is evident from the alteration of JNK and p38 components in microglia, which may attenuate their phagocytic response, as well as ERK and STAT3 in melanoma cells, which are linked to angiogenesis. The authors also provided evidence for a metastasis-supportive niche, as secretion of vascularization factors was reshaped and proliferation of both cell types was increased (97). Correlating with the latter finding, anti-inflammatory microglia depletion by mannosylated clodronate liposomes decreased the growth of intracranially implanted breast cancer cells (98). Another evidence from the interplay between cancer cells and microglia is that XIST-deficient-breast cancer cells led to an increased amount of M2-markers in microglia (99). However, the genetic programs that lead to an induction of tumor-promoting functions in TAMs in BrM are not well-characterized to date. Detailed analysis of signaling pathways and transcription factor activity is required to evaluate if similar mechanisms lead to the induction of a TAM gene signature in BrM as proposed for other tumor types including glioma (64, 100). For example, Blazquez et al. recently proposed the importance of PI3K signaling as a master regulator of tumor promoting activation states of macrophages/microglia (101).

Previous studies that interrogated the role of TAMs in BrM did not discriminate between cells originating from brain-resident microglia or from bone marrow-derived macrophages. As mentioned earlier, under steady-state conditions, bone marrow-derived myeloid precursors do not contribute to the microglia pool. However, damage to the blood-brain barrier as described for BrM (102–104) allows the recruitment of such progenitors that supplement the microglial population (105). In this context it is important to evaluate to which extent the integrity of the BBB has to be diminished in order for blood-borne cells to efficiently breach the BBB. It was shown that the BBB in BrM is not fully disrupted but rather remodeled into a blood-tumor-barrier (BTB) due to alterations in the pericyte subpopulation (106). While this is not sufficient to allow free penetration of therapeutic antibodies or chemical compounds that are not BBB permeable (107), it is possible that vessel structures of the BTB lose their capability of restricting the entry of blood-borne immune cells and at the same provide the necessary molecular structures such as adhesion molecules for efficient transmigration of peripheral leukocytes. Cell-tracing techniques based on the transplantation of genetically labeled HSCs into mice following whole-body irradiation or head protected irradiation have been used to decipher the origins of TAMs in primary brain tumors (108). By means of transplantation and lineage-tracing models, numbers for peripheral macrophages range between 25 and 75% in glioma and 25% in BrM (64, 92, 108). Similar to neurodegenerative disorders, the bulk FACS sorted TAMs showed a different expression profile compared to normal microglia and monocytes in a mouse glioma model (64). More importantly, the profile of tumor-associated microglia and macrophages was different, confirming the functional impact of their different ontological origin. While TAM-MG showed profiles rich in cytokines, chemokines, and complement components, TAM-BMDM signatures were associated with wound healing, antigen presentation and immune suppression (64, 92). Another evidence for the intrinsic differences within the macrophage/microglia population is the lack of impact of anti-CSF1 treatment on microglia compared to monocyte-derived cells, which may be due to the presence of the CSF1R alternate ligand IL34 (109). This observation is also supported by the fact that in multiple sclerosis dendritic cells (DC) and monocyte-derived cells are the major antigen presenting cells (APCs). Indeed interactions of microglia with infiltrating T cells were found to be transient (110). Importantly, in order to unravel the molecular pathways and functional predominance in every cell population, it is mandatory to properly distinguish them. Under physiological conditions, the different expression profiles of macrophages and microglia, residing in their respective environment enables their differentiation (69, 110–112). The identification of novel markers for microglia such as Tmem119, P2ry12, Sall1, SiglecH (113–116) is important to unravel their specific role in health and disease. However, these expression patterns are less well-defined in TAMs, as e.g., homeostatic Tmem119 is upregulated in TAM-BMDM, while it is downregulated in TAM-MG (64). Remarkably, CD49d has been described as a differential marker between blood-borne macrophages and microglia in brain malignancy (64). However, to date gene expression signatures of TAM-MG in comparison to TAM-BMDM have not been investigated.

In summary, within the myeloid compartment in BrM, TAMs constitute the most abundant cell population. Based on their ontological origin and localization within the brain parenchyma and border regions of the CNS, they represent a highly heterogeneous cell population. Until now, most pre-clinical studies that aimed to unravel the role of TAMs in BrM did not discriminate between different subpopulations.

The identification of lineage-restricted markers that allow to distinguish TAM-MG and TAM-BMDM as well as single cell sequencing approaches will help to unravel gene signatures of individual subpopulations and provide insight into their functional contribution in BrM.

Tumor-Infiltrating Lymphocytes in Brain Metastases—Can Activity be Unleashed Without Inducing Neurotoxicity?

Traditionally, the brain has been regarded as an immune privileged organ, with lack of peripheral immune surveillance through blood-borne immune cells such as T cells owing to the blood-brain-barrier (BBB) and the lack of effective lymphatic drainage (117, 118). However, this view has recently changed, as it is recognized that while the brain might be privileged to some extent, this does not mean total exclusion of blood-borne immune cells. Clearly, the entry to the parenchyma is strictly controlled to prevent fatal neurotoxicity, but patrolling leukocytes, such as bone marrow-derived antigen-presenting DC as well as CD4+ and CD8+ T cells, have been identified in the meninges and choroid plexus (70, 71, 77, 119, 120). DC have a higher capacity of antigen presentation and T cell stimulation than microglia (121). Furthermore, it has been demonstrated that afferent antigen sampling from the brain parenchyma does take place and CNS-derived antigens can lead to peripheral priming of T cells (122). Moreover, recent studies unveiled the existence of lymphatic vessels in the meninges, which can transport antigens, derived from the brain parenchyma via the cerebrospinal fluid/lymphatic system into the deep cervical lymph nodes (123–125), indicating that the brain has a functional draining lymphatic system. However, the details about anatomy and composition, as well as the efferent route of T cells into the brain, specifically in the scenario of BrM, require further investigation. As immunotherapies gain increasing attention, the infiltration of BrM with T cells and their function in the context of brain tumors comes into focus. By now, several studies demonstrated that tumor infiltrating T lymphocytes (TILs) are present in BrM of different primary cancers such as Non-Small Cell Lung Cancer (NSCLC), Small Cell Lung Cancer (SCLC), renal cell cancer (RCC), melanoma, or breast cancer. Of these RCC and melanoma show the highest CD3+ numbers and highest CD8+/CD3+ ratio. The infiltration patterns of TILs seem to be diverse, ranging from a diffuse spreading through the metastases to an accumulation in the stroma and around vessels, depending on primary tumor type (126). The prognostic value of TIL numbers is currently being disputed, with several studies indicating favorable outcome on survival (96, 127, 128), while Harter et al. could not find a significant correlation (126). Moreover, Mustafa et al. demonstrated that T cells promote breast cancer BrM. This is due to a direct interaction of T cells with tumor cells, leading to increased guanylate binding protein 1 (GBP)-1 expression by the latter, which in turn enables them to cross the BBB (129). These conflicting data indicate that further research is necessary to elucidate the complex function of T cells in BrM as well as possible influence of the TME on T cells. It is conceivable, that the latter might be polarized in the TME under certain conditions resulting in tumor promoting rather than anti-tumor functions, so they will not only be inhibited in their anti-tumor functions but promote tumor growth as discussed for macrophages and astrocytes in the sections above. Furthermore, it remains unclear what dictates the number of TILs in BrM. Generally, T cells are primed in peripheral lymph nodes (e.g., cervical lymph nodes). Extravasation and T cell homing to sites of inflammation or tissue injury is dependent on binding of VLA-1 and LFA-1 expressed by T cells to endothelial cellular adhesion molecules (CAMs) such as VCAM-1 or ICAM-1 (130–132). The exact entry route for T cells into BrM is not yet fully understood, however there is evidence that expression of CAMs on endothelial cells plays a central role in the homing process of T cells. For example, it was shown that vessels in the proximity of tumor lesions show expression of VCAM-1, ICAM-1, and other CAMs in different BrM models (133–136). Interestingly, it was proposed that tumor cells exploit this mechanism to breach the BBB and home to the brain parenchyma (134). Serres et al. could also detect VCAM-1 on human BrM samples, while healthy controls showed only minimal expression (133). Additionally, it was demonstrated that VCAM-1 expression increases with tumor progression in a BrM mouse model (133). Taggert et al. proposed that increased CD8+ T cell trafficking to BrM is dependent on VCAM-1 and ICAM-1 expression induced by IFNγ produced by BMDMs, microglia and NK cells (135). Hence, TIL numbers can at least in part be determined by IFNγ levels and CAM expression. However, other determinates of TIL numbers in BrM such as mutational load and presence of tumor antigens are expected to affect T cell infiltration. It was demonstrated for primary melanoma, a highly immunogenic tumor with high TIL content, that the density of antigens did not correlate with the presence or absence of TILs (137). Additionally, Mansfield et al. applied TCR profiling of patient derived samples and could show that the mutational burden is higher in BrM of NSCLC than in the respective primary tumor and is correlated with T cell richness (138). In this study the authors also observed a contraction of T cell clones compared to the primary site, with the 10 most abundant T cell clones being more heavily expanded compared to the primary tumor site (138). This expansion hints toward an immune response in BrM with the involvement of antigen specific T cells, even though in a more restricted manner than in primary tumors. The fact that BrM still represents a highly aggressive, fatal disease and monotherapies with immune modulatory agents only show modest effects indicates that this adaptive immune response is not strong enough to halt tumor growth. Comparison with the respective primary tumors led to the observation that BrM, e.g., derived from breast cancer, have a lower content of TILs than the primary counterpart, with 5 and 20%, respectively (139). While the extent of T cell exclusion is lower in BrM compared to many primary brain tumors, the TME is still highly immune suppressive. Current research investigates strategies to increase the inflammatory response against BrM, to render the tumors more prone to immune-modulating agents. Using adoptive T cell transfer is only one strategy, which has demonstrated encouraging results in melanoma BrM patients (140). However, not only the number of T cells plays an

important role in the immune response against brain metastatic cells, but also their activation status is relevant. The latter is dependent on many factors and the cell composition in the TME. The brain naturally constitutes an immune suppressive microenvironment to prevent fatal neurotoxicity, potentially resulting in the exhaustion and inactivation of T cells in primary brain tumors (141). Moreover, it has been demonstrated with different BrM mouse models, that the number of $FOXP3^+$ T regulatory cells (Tregs) is increased during BrM progression. These results have also been recapitulated on patient samples from melanoma and NSCLC BrM (142). Additionally, not only the tumors themselves are infiltrated with Tregs, but also the blood of patients bearing BrM contains an increased percentage of Tregs compared to healthy donors (143). Those inhibitory T cells can hypothetically contribute to the exhaustion of anti-tumor effector T cells.

Taken together, insights into the complex and dynamic interplay between different cell types of the TME in BrM, in which the activity of individual cell populations is tightly controlled by other cell types, underpins the challenges in developing effective therapies against BrM. However, a comprehensive view on the complex interactions provides opportunities for the development of improved therapeutic intervention strategies as discussed in the following paragraph.

Tumor Microenvironment-Targeted and Immunotherapies Against Brain Metastases

The development of effective therapies against BrM is one of the most challenging aspects of cancer research. Intervention strategies developed for extracranial tumors cannot easily be translated into effective therapeutic avenues for brain cancers. Instead, approaches have to be tailored to the unique brain environment to breach tissue-specific restrictions of therapeutic efficacy, but at the same time consider the protection of delicate anatomical structures that control higher cognitive functions. Detailed insights into the critical cellular and molecular drivers of BrM are necessary to provide a scientific rationale for the development of improved intervention strategies. Recent research efforts shed light on the complexity of the tumor-stroma crosstalk in BrM and indicate potential therapeutic targets for immune- or tumor microenvironment targeted therapies (**Figure 2**).

Immunotherapies Against Brain Metastases

The introduction of immunotherapy has recently revolutionized treatment options for a range of extracranial primary tumor types including melanoma and NSCLC that frequently metastasize to the brain. Hence, it appears logical to test the efficacy of immunotherapy against BrM, even though the brain tissue environment represents one of the most immune suppressed milieus. One arm of immunotherapy aims at re-activating T effector cells via immune checkpoint inhibition (**Figure 2**; Box 2). Indeed monoclonal antibodies, which block immune checkpoints (e.g., anti-CTLA4, anti-PD1, or anti-PDL1), demonstrate efficacy in individual BrM patients, but the overall response rates are modest, even in melanoma BrM, which is thought to be highly immunogenic (144–147). For example, a limited number of retrospective and prospective clinical trials indicate intracranial response rates of 16–25% following ipilimumab treatment in melanoma patients (148, 149) and 50–55% in trials combining ipilimumab and nivolumab (ABC trial and CheckMate 204) (150, 151). Therefore, other strategies are being explored to increase T cell immunity e.g., the combination of checkpoint inhibition with radiotherapy (RT). The latter has the potential to sensitize for immune modulation by inducing immunogenic cell death resulting in secretion of inflammatory cytokines, upregulation of MHCI and therefore increased trafficking of T cells to the BrM, as shown for other cancers (152, 153). Taggart et al. could show in a mouse model of melanoma BrM that successful immunotherapy depends on enhanced trafficking of CD8+ T cells, activated in peripheral lymphoid organs, to the brain parenchyma (135). Additionally, RT can potentially increase the tumor mutational load thereby broadening the immune response (154). Indeed, radio-immunotherapies show promising results and are currently being tested in clinical trials also for BrM (155). Nevertheless, T effector cell activity in the brain is not only dependent on Treg infiltration or immune suppressive cytokines in the TME, but also on the presence of APCs. As mentioned earlier, in the brain this role can be fulfilled by DC, BMDM, and to a lesser extent by microglia (100, 121). The presence of those cell types in the brain tumor context is not questioned anymore, but there are no detailed reports about specific interaction with APCs and TILs that lead to antigen-specific T cell activation in BrM. It is important to further investigate this in the future to improve response rates of patients to immunotherapy and to find new strategies against BrM by exploiting the full potential of T cell immunity in this context. Using DC vaccines to boost T cell responses is only one of many potential treatment possibilities, which could be explored in this context and is under current investigation in brain tumors (156). Another strategy of applying T cells for BrM treatment is the delivery of genetically engineered CAR T cells directed against known tumor antigens, which led to reduced tumor growth in a xenograft mouse model (157). Currently, this approach is investigated in a clinical trial for breast cancer patients with BrM (158). However, it remains questionable if T cell-directed therapies can be successful in the presence of a highly immune suppressive myeloid compartment. Alternatively, one could argue that myeloid-targeted therapies might be more promising.

Modulating the Myeloid Compartment in Brain Metastases

Cells of the myeloid compartment represent the most abundant non-malignant cell type in the BrM microenvironment. Pre-clinical data indicate a critical role in mediating distinct steps within the metastatic cascade leading to the generation of a cancer-permissive, immune suppressive environment (**Figure 1**). Different strategies have been employed to target TAMs in BrM to evaluate therapeutic efficacy. Blocking macrophage survival and differentiation by disrupting CSF1-CSF1R signaling represents one of the most promising strategies (**Figure 2**; Box 1). Since there are two cognate ligands that bind to CSF1R, targeting the receptor rather than the ligand, leads to efficient blockade of

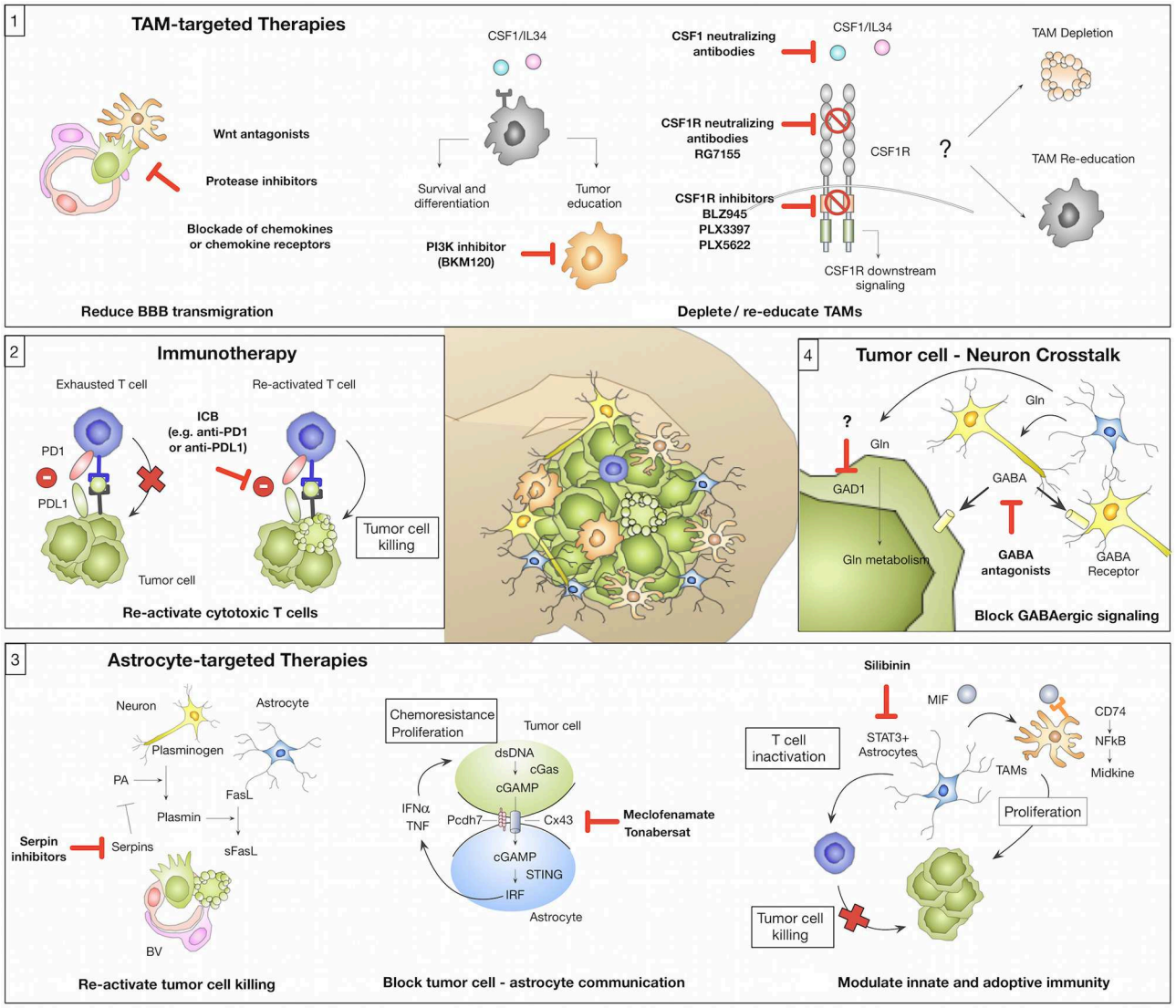

FIGURE 2 | Novel concepts of tumor microenvironment-targeted therapies or immunotherapies (1) Tumor-associated macrophages/microglia (TAMs) represent a highly abundant cell type in BrM with known roles in mediating tumor cell BBB transmigration and tumor-supportive functions that foster metastatic outgrowth. Strategies for TAM-targeted therapies include the reduction of tumor cell BBB transmigration (e.g., by Wnt antagonists, protease inhibitors, or blockade of chemokines/chemokine receptors). Blockade of CSF1-CSF1R signaling represents another strategy to target TAMs by inhibiting a central pathway for macrophage differentiation and survival. The CSF1-CSF1R signaling axis can be inhibited by (i) CSF1 blocking antibodies (with no effects on IL34 mediated CSF1R activation), (ii) CSF1R blocking antibodies, or (iii) ATP competitive small molecule inhibitors. Consequences of CSF1R inhibition on TAMs in established BrM (depletion vs. re-education) remain to be elucidated. An alternative strategy might be the inhibition of Pi3K by BKM130 to prevent the activation of pro-tumor TAMs. (2) Tumor-infiltrating T cells in BrM show signs of T cell exhaustion mediated by immune checkpoints (e.g., PD1-PDL1) or immune-suppressive cytokine milieus. Blockade of immune checkpoints e.g., by anti-PD1 or anti-PDL1 reactivates T cells and reinstates tumor cell killing by cytotoxic T cells. (3) Astrocytes represent a highly plastic cell type in BrM and their function was associated with pro- and anti-tumor activity. Inhibition of serpins could re-activate sFasL-mediated tumor cell killing and thereby prevent early metastatic colonization. Blockade of gap junctions by meclofenamate or tonabersat was shown to inhibit tumor cell-astrocyte crosstalk that supports proliferation and protects tumor cells from chemotherapy. Targeting of STAT3+ astrocytes by silibinin represents a strategy to block the induction of pro-proliferative functions of TAMs and reduce astrocyte-mediated inactivation of T cells. (4) Brain metastatic tumor cells adopt neuronal features to integrate into the neuro-glial niche and to exploit brain specific energy sources e.g., glutamate (Gln). GABA antagonists were shown to reduce GABAergic signaling in tumor cells. Furthermore, blockade of Gln influx into tumor cells by GAD1 inhibition could represent a promising therapeutic strategy.

CSF1R downstream signaling. CSF1R inhibition can be achieved by CSF1R blocking antibodies (e.g., RG7155) (159) or ATP competitive small molecule inhibitors (e.g., BLZ945, PLX3397, or PLX5622) (91, 160) (**Figure 2**; Box 1). Qiao et al. employed PLX3397 in a prevention trial setting and demonstrated that microglia depletion reduced tumor cell transmigration potential of melanoma brain metastatic cells (84). This is in line with previous findings that demonstrated that clodronate liposome

mediated microglia depletion resulted in a reduction of the BrM burden (98). Given the promising results of TAM-targeted therapies with the CSF1R inhibitor BLZ945 in a mouse model of pro-neural glioblastoma (91) it remains to be elucidated whether CSF1R inhibition in established BrM shows anti-tumor activity. Importantly, analyses in two independent glioblastoma models revealed that conditions in which CSF1R inhibition leads to TAM depolarization show higher efficacy compared to TAM depletion (91, 161). Consequently, research effort should be put on the identification of gene signatures that determine tumor-promoting vs. anti-tumor characteristics in TAMs to specifically target tumor supportive traits of TAMs but spare physiologically important functions. Blazquez et al. recently proposed Pi3K signaling as a master regulator of tumor-promoting functions of BrM-associated macrophages/microglia and demonstrated that BKM120, a pan-PI3K inhibitor, reduced tumor-promoting features of macrophages/microglia (101). However, it is important to note that clinical data revealed better overall survival for patients with high PI3K activity, while patients with moderate or low PI3K activity showed worse prognosis (101). Hence, inhibiting PI3K signaling in BrM might have opposing effects depending on which cell type is targeted.

Given the importance of the myeloid compartment to establish an immune suppressive environment to protect the CNS from neuro-inflammation, myeloid-targeted therapies should be taken into account carefully. Blocking an integral part of a tissue protective mechanism might unleash unwanted pro-inflammatory responses that lead to detrimental tissue damage. Detailed knowledge in disease-associated effector functions of different myeloid cell populations is therefore needed to block tumor-promoting functions but maintain critical functions in host defense and neuro-protection.

Astrocyte-Targeted Therapies

Astrocytes are emerging as one of the key regulators of BrM (51). However, pre-clinical studies revealed high functional heterogeneity with tumor-promoting and anti-tumor functions. Therefore, it will be critical to gain detailed mechanistic insight into functional subpopulations or conditions that favor the induction of anti- vs. pro-tumor functions. Pre-clinical studies provided critical insight into potential therapeutic targets for astrocyte-targeted therapies. Valiente at al. demonstrated that tumor cells successfully block Fas- mediated cell killing by blocking the activity of plasminogen activator via serpins (53). Neutralizing tumor-derived serpins could therefore reinstate tumor cell killing during early metastatic colonization (**Figure 2**; Box 3). However, from a clinical perspective, strategies that control established disease are more urgently needed. One possibility is the blockade of astrocyte-tumor cell crosstalk via gap junctions to block tumor promotion. Chen et al. demonstrated that shRNA-mediated knockdown of Cx43 or Pcdh7 reduced the tumor burden and pharmacological intervention with the gap junction inhibitors meclofenamate and tonabersat decreased growth kinetics of BrM in pre-clinical trials (59) (**Figure 2**; Box 1). Although targeting of gap junctions shows promising results in pre-clinical disease models, the applicability of this approach in the clinic has to be carefully evaluated. Given the physiological importance of gap junctions for tissue integrity as well as normal brain function (162), potential adverse effects have to be taken into account. Moreover, approaches that target the formation of gap junctions between astrocytes and tumor cells are expected to be most efficient at initial stages of brain colonization, when the majority of tumor cells is in direct contact with astrocytes, while at later stages only tumor cells at the tumor-stroma interface are in close vicinity to astrocytes (30, 38, 51). Indeed, the formation of gap junctions between tumor cells and astrocytes was detected in subpopulations but not ubiquitously (59).

Another promising approach was recently described by targeting STAT3 signaling in RAs via the inhibitor Silibinin (61) (**Figure 2**; Box 3). Clinical data from lung cancer BrM patients treated with Silibinin showed significantly increased overall survival in response to STAT3 inhibition (61). However, some patients did not respond and the progression of extra-cranial disease was not affected, providing the possibility for BrM relapse. It remains to be shown how patients with BrM derived from other primary tumor entities respond to this treatment approach, and if variability of the outcome is due to tumor heterogeneity, differences in the TME and/or different patient histories. It is also unclear why only a subset of astrocytes activates STAT3 signaling, which requires deeper understanding, especially with respect to other immune cells (e.g., macrophages, microglia) and how different cellular and also molecular (e.g., different cytokine milieus) microenvironments influence the outcome of impaired STAT3 signaling.

Targeting astrocytes in the context of BrM is a promising approach, since these cells are highly susceptible to tumor cell-mediated education within the brain, thus promoting BrM. However, it remains to be investigated how distinct astrocyte subpopulations support BrM formation to develop strategies that block tumor-promoting or enhance anti-tumor functions of astrocytes.

Prevention of Neuronal Mimicry of Tumor Cells

Tumor cells that successfully colonize the brain fulfill certain criteria that allow them to integrate into the neuronal niche to evade immune destruction and to exploit brain specific energy sources to propagate their growth (**Figure 1**; Box 6). Strategies that prevent tumor cells from functionally integrating into the neural niche and to exclude them from important energy sources are expected to have critical clinical impact. For example, blockade of GABAergic signaling with GABA antagonists was proposed as a promising strategy to block the availability of glutamate as an energy source (13, 14) (**Figure 2**; Box 4). However, strategies that target traits that tumor cells acquire to hijack the tissue environment bear the risk of adverse effects by targeting physiologically highly relevant pathways. Future studies are therefore needed to understand mechanisms used by tumor cells to adopt to the neuronal-glial niche to interfere with the acquisition of neuronal-like features, rather than blocking cell-cell communication or metabolic pathways within the CNS.

CONCLUDING REMARKS

The importance of the TME in BrM is increasingly recognized. In particular tumor immunology in BrM is an emerging field. While the brain was traditionally regarded as an immunological sanctuary site, it is now evident that BrM induce the recruitment of immune and inflammatory cells from the periphery and that routes for CNS-derived antigen presentation to peripheral immune cells exist. The presence of different brain-resident and recruited cell types in BrM opens new opportunities for TME-targeted interventions or immunotherapies. Recent studies that sought to unravel the functional contribution of different BrM-associated stromal cell types provide first insight into the complexity of tumor-stroma interactions as well as heterotypic signaling between niche cells that mutually modulate effector functions. Given the important role of the brain in controlling higher cognitive functions, it is particularly critical to consider a balance between the induction of anti-tumor responses and the maintenance of tissue protective mechanisms that prevent neurotoxicity. While we are just at the beginning to understand the complex interplay between different cells of the TME, more detailed insight is necessary to develop effective treatment strategies and to evaluate consequences of therapies that modulate effector functions within the BrM microenvironment.

AUTHOR CONTRIBUTIONS

MS, AS-B, KN, TA, and LS conceptualized and wrote the manuscript.

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REVIEW
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The Dark Side of Fibroblasts: Cancer-Associated Fibroblasts as Mediators of Immunosuppression in the Tumor Microenvironment

*Lea Monteran and Neta Erez**

Department of Pathology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

Cancer-associated fibroblasts (CAFs) are prominent components of the microenvironment in most types of solid tumors, and were shown to facilitate cancer progression by supporting tumor cell growth, extracellular matrix remodeling, promoting angiogenesis, and by mediating tumor-promoting inflammation. In addition to an inflammatory microenvironment, tumors are characterized by immune evasion and an immunosuppressive milieu. In recent years, CAFs are emerging as central players in immune regulation that shapes the tumor microenvironment. CAFs contribute to immune escape of tumors via multiple mechanisms, including secretion of multiple cytokines and chemokines and reciprocal interactions that mediate the recruitment and functional differentiation of innate and adaptive immune cells. Moreover, CAFs directly abrogate the function of cytotoxic lymphocytes, thus inhibiting killing of tumor cells. In this review, we focus on recent advancements in our understanding of how CAFs drive the recruitment and functional fate of tumor-infiltrating immune cells toward an immunosuppressive microenvironment, and provide outlook on future therapeutic implications that may lead to integration of preclinical findings into the design of novel combination strategies, aimed at impairing the tumor-supportive function of CAFs.

Keywords: CAFs, immunosuppression, immune modulation, inflammation, tumor microenvironment

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Leila Akkari,
The Netherlands Cancer Institute (NKI), Netherlands

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Claudia Cantoni,
University of Genoa, Italy
Kawaljit Kaur,
University of California, Los Angeles, United States

****Correspondence:***
Neta Erez
netaerez@tauex.tau.ac.il

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INTRODUCTION

Tumors are complex multicellular systems, characterized by reciprocal interactions between cancer cells and the tumor microenvironment (TME). The non-cancerous components that comprise the TME are central to all stages of tumorigenesis, progression, and metastasis (1). The TME is composed of the extracellular matrix (ECM), as well as various cell types including immune cells, endothelial cells, pericytes, and fibroblasts.

In non-cancerous homeostatic conditions, resident tissue fibroblasts are important sentinels of tissue integrity (2). Fibroblasts can sense and respond to mechanical changes, as well as to various tissue damage signals and react by differentiating to myofibroblasts that orchestrate tissue repair and wound healing, mediated by their ECM synthesis and remodeling and by their crosstalk with innate immune cells (3–5). Dysregulation of the physiological wound healing response and chronicity of inflammatory responses lead to fibrosis and scarring, characterized by excess ECM production and deposition by activated fibroblasts. These physiological functions of tissue fibroblasts are hijacked in cancer-associated fibroblasts (CAFs) in the microenvironment of tumors, consistent with the description of tumors as "wounds that do not heal" (6).

Cancer-associated fibroblasts (CAFs) are a vastly heterogeneous stromal cell population and are prominent components of the microenvironment in solid tumors. In some cancer types, including breast and pancreatic carcinomas, CAFs are the most prominent stromal cell type. The presence and function of activated CAFs in the microenvironment are associated with worse prognosis in multiple cancers (7). Moreover, tumors with high stromal signatures have been found to be associated with therapy resistance and disease relapse (8, 9).

CAFs are composed of multiple subpopulations that were shown to have diverse origins, including reprogrammed resident tissue fibroblasts (10, 11), bone marrow-derived mesenchymal cells (MSCs) (12, 13), adipocytes (14), and endothelial cells (15). Functionally, CAFs were shown to enhance tumor growth by several mechanisms: directly promoting cancer cell proliferation via secretion of growth factors, by inducing angiogenesis and by remodeling the ECM, which supports tumor cell invasion (5, 10, 16–18). Importantly, CAFs were also implicated in mediating tumor-promoting inflammation in various cancer types via secretion of cytokines and chemokines that mediate the recruitment and activation of immune cells, and by their reciprocal interactions with immune cells in the TME (2, 19). Studies in recent years have elucidated that this plethora of tumor-promoting activities of CAFs is mediated by functionally distinct subpopulations of fibroblasts (12, 20–22). Analysis of CAFs at the single cell level in the coming years will undoubtedly add complexity to the emerging landscape of CAF functional heterogeneity.

The role of the immune system in cancer is multi-faceted: In addition to an inflammatory microenvironment, tumors are also characterized by immune evasion and an immunosuppressive milieu, that were acknowledged as hallmarks of cancer (23). In order to survive and proliferate in the primary tumor site and in distant organs, which may be initially hostile, tumor cells must escape immune surveillance and avoid killing by cytotoxic lymphocytes. This is achieved by shaping the immune microenvironment toward a tolerant and immunosuppressive milieu, characterized by the presence of immature myeloid cells, T regulatory cells, decreased levels of infiltrating killer cells (T cells and NK cells), and dysfunction of their cytotoxic activity (24, 25). These mechanisms of immune escape and suppression are achieved by tumor cell downregulation of antigen presentation, elevated expression of surface inhibitory molecules, and secretion of immunosuppressive factors (24).

In addition to tumor cell-mediated signaling that drives immune suppression, fibroblasts are emerging as central players in shaping the TME toward an immunosuppressive and growth-promoting phenotype (26). CAFs contribute to immune escape via upregulation of immunosuppressive cytokine production and immune checkpoint ligands, exclusion of anti-tumor $CD8^+$ T cells from cancer cells, and by affecting the functional differentiation of tumor infiltrating inflammatory cells.

In this review, we focus on recent advancements in our understanding of how CAFs affect the recruitment and functional fate of tumor-infiltrating immune cells toward shaping an immunosuppressive tumor microenvironment, and examine future therapeutic implications.

CAFs ORCHESTRATE RECRUITMENT OF IMMUNE CELLS

Recruitment of Myeloid Cells

Myeloid cells are the most abundant hematopoietic cells in the body and are critical components of the tumor microenvironment, that contribute to all aspects of tumor progression (1). Myeloid cells in the TME include various populations of tumor-associated macrophages (TAMs), neutrophils, eosinophils, basophils, dendritic cells (DC) and mast cells (1, 27, 28). In addition, immature myeloid cells that express CD11b, Ly6G and/or Ly6C are sometimes referred to as myeloid-derived suppressor cells (MDSCs), based on their ability to functionally suppress the proliferation and activity of T cells. MDSCs are characterized by their expression of Arginase (ARG1), TGF-β, Programmed death-ligand 1 (PD-L1)/ 2, IL-10, Prostaglandin E2 (PGE2), S100A8/A9 and Indoleamine-pyrrole 2,3-dioxygenase (IDO), and by their capacity to regulate dendritic and T cell functions. MDSCs are commonly divided into two subsets based on their expression of surface markers: monocytic MDSCs ($CD11b^+Ly6C^{high}Ly6G^-$), and granulocytic (or polymorphonuclear) MDSCs ($CD11b^+Ly6C^{int}Ly6G^{high}$) (29, 30). Notably, these surface markers are used to identify mouse MDSCs. Human tumor-associated MDSCs are identified by their expression of $CD33^+CD14^+HLA\text{-}DR^{low/-}$ (monocytic MDSCs) or $CD11b^+CD14^-CD15^+/CD66b^+$ (29).

CAFs were shown to recruit macrophages into the TME in multiple mouse models of cancer, including squamous cell, prostate and breast carcinomas (19, 31, 32). In a mouse model of spontaneous lymphoma, tumor-educated CAFs (derived from MSCs) recruited $CD11b^+Ly6C^+$ monocytes, $F4/80^+$ macrophages, and $CD11b^+Ly6G^+$ neutrophils via the CCL2–CCR2 axis, thus enhancing tumor growth (33). Moreover, in a mouse model of breast cancer lung metastasis, MSCs that were pre-conditioned with TNFα and co-injected with tumor cells were shown to recruit $CXCR2^+$ neutrophils by secreting CXCR2 ligands (CXCL1, CXCL2, and CXCL5), resulting in enhanced lung metastasis (34). Expression of chemoattractants for myeloid cells was suggested to be mediated by enhanced expression of miR-1246 in breast cancer CAFs, in an NF-κB dependent manner (35). Notably, recent understanding of CAF heterogeneity implicates a distinct subpopulation of inflammatory CAFs (iCAFs) rather than myofibroblast-like CAFs (myCAFs) in the induction and maintenance of an inflammatory milieu via their expression of inflammatory mediators (IL-6, IL-11, CXCL1, CXCL2) (21, 36). While these findings were described in a mouse model of pancreatic cancer, they likely represent a general phenomenon whereby specific functions of CAFs in affecting immune cells are mediated by distinct subpopulations (12, 37).

Recruitment of tumor-promoting myeloid cells by CAFs is associated with shaping their functional differentiation toward an immunosuppressive phenotype: Secretion of Chitinase-like protein 3 (Chi3L1) by mammary CAFs was shown to drive an M2-like phenotype in recruited macrophages, associated with reduced infiltration of $CD8^+$ T lymphocytes (19). Interestingly, the expression of fibroblast activation protein (FAP) in CAFs

in multiple cancer types was shown to be associated with recruitment of immunosuppressive cells: In a mouse model of hepatic cancer, a subset of FAP^+ fibroblasts had an inflammatory phenotype directed by STAT3 activation and increased CCL2 expression, resulting in enhanced recruitment of CCR2-expressing circulating MDSCs and enhanced tumor growth (38).

Importantly, recruitment of myeloid cells by CAFs was also shown to be associated with resistance to therapy: In a mouse model of transplantable colorectal carcinoma (CRC), FAP^{high} fibroblasts were found to recruit myeloid cells via CCL2, leading to resistance to anti-PD-1 immune checkpoint therapy which was abrogated by targeting FAP. These findings were validated in human CRC tissue sections, where the abundance of FAP^{high} fibroblasts was in correlation with increased infiltration of myeloid cells and inversely correlated with infiltrated T cells (39). Similarly, pharmacological targeting of FAP in a transplantable model of pancreatic cancer resulted in decreased macrophage recruitment and enhanced T cell infiltration (40). Moreover, targeting of FAP^+ fibroblasts by immunization with an adenoviral vector in both transgenic and transplantable mouse models of melanoma abrogated the recruitment and function of immunosuppressive cells including monocytic and polymorphonuclear MDSCs within the TME (41).

CCL2-mediated recruitment of circulating monocytes by CAFs was also demonstrated in models of breast cancer, *in vivo* and in a 3D *ex-vivo* model (42, 43). Notably, while recruitment of macrophages into tumors by CAFs is operative in various cancer types, the molecular pathways are distinct: In primary *in vitro* cultures, CAFs isolated from human prostate tumors were found to recruit monocytes by secreting stromal cell-derived factor 1 (SDF1)/CXCL12. Moreover, these SDF1-producing CAFs enhanced M2-like polarization of circulating monocytes, reflected by high production of the immune suppressive cytokine IL-10 (44). These findings agree with the demonstrated functional role of CAF-derived SDF1 in promoting tumor growth and immunosuppression (45, 46).

Recruitment of myeloid cells into tumors by CAFs is not limited to monocytes: platelet-derived growth factor receptor A $(PDGFR\alpha)^+$ CAFs isolated from murine tumors were shown to be a major source of the granulocytic chemoattractant CXCL1, and to mediate the accumulation of $Ly6C^-Ly6G^+$ granulocytic cells (granulocytic MDSCs) with potent immune-suppressive activity, assessed by their ability to suppress T cell proliferation. Interestingly, this pathway may be an adaptive response to anti-CSF1R therapy, as it was induced in CAFs following treatment with CSF1R inhibitor in models of colon, lung, breast carcinomas and melanoma (47). These findings instructed the design of combination therapy, to block CSF1R signaling as well as CAFs: Combining CSF1R inhibitor with a CXCR2 antagonist blocked granulocyte infiltration and resulted in strong delay in tumor growth in models of lung carcinoma and melanoma (47). Interestingly, mast cells were also shown to be recruited by CAFs: CAFs isolated from hormone-dependent prostate tumors mediated the recruitment of CXCR4-expressing mast cells by secreting CXCL12 (48).

One of the suggested mechanisms for CAF-mediated recruitment of myeloid cells to the TME is the expression of a senescence-associated secretory phenotype (SASP) gene signature. Cellular senescence was originally thought to be a tumor-suppressive mechanism that limits malignant transformation by arresting cell proliferation. However, studies in recent years have shown that senescent fibroblasts acquire a senescence-associated secretory phenotype (SASP) that supports their pro-inflammatory and tumor-promoting functions (49, 50). Moreover, the acquisition of a senescent phenotype by CAFs was shown to contribute to recruitment of immunosuppressive cells: In a mouse model of stromal-specific induced senescence, senescent dermal fibroblasts were shown to mediate the formation of an immunosuppressive microenvironment by enhancing the recruitment of $CD11b^+Ly6C^-Ly6G^{high}$ cells and T regulatory ($CD3^+CD4^+FOXP3^+$) cells, and enhanced ECM deposition. Co-injection of senescent dermal fibroblasts with squamous cell carcinoma cells demonstrated that SASP-induced shaping of the immune microenvironment promotes tumor growth. SASP-mediated tumor promotion was inhibited by targeting SASP-derived IL-6 or by depleting $Ly6G^+$ cells (51).

Thus, by employing multiple molecular pathways, CAFs recruit myeloid cells into tumors, that contribute to the formation of an immunosuppressive immune milieu (**Figure 1**).

Recruitment of Regulatory T Cells

CAFs were found to potentiate the recruitment, differentiation and survival of T regulatory cells, contributing to the formation and maintenance of an immunosuppressive microenvironment. Treg cells are immunosuppressive T lymphocytes characterized by their expression of the IL-2 receptor α-chain (CD25) and the transcription factor forkhead box P3 (FOXP3). The mechanisms by which Treg mediate immunosuppressive function at tumor sites are not fully elucidated, but increased infiltration of Tregs within the tumor was shown to correlate with worse prognosis in multiple studies (52–55).

As the complexity of CAF populations is being gradually revealed, it is increasingly appreciated that mediating immunosuppression may be operative in a distinct subpopulation of CAFs: FACS-based analysis of CAFs in human breast tumors by using six surface markers identified four distinct CAF subsets which accumulated differently in different subtypes of human breast cancer (luminal A, $Her2^+$, and triple-negative). Of these CAF populations, the subtype designated CAF-S1, characterized by expression of FAP, smooth muscle actin α (αSMA), PDGFRβ, and CD29, was found to be associated with recruitment, retention and differentiation of Treg cells: By secreting CXCL12, CAF-S1 promoted the attraction of $CD4^+CD25^+$ T cells, and mediated their retention via expression of OX40L, PD-L2, and Junctional adhesion molecule B (JAM2). Moreover, CAF-S1 were able to increase T cell survival, induce their differentiation to $CD4^+CD25^+FOXP3^+$ regulatory lymphocytes and to enhance the capacity of Treg cells to inhibit the proliferation of $CD4^+CD25^+$ T cells *in vitro* (20). This mechanism is not restricted to breast cancer, as the presence and function of CAF-S1 in attraction, survival, and differentiation of $CD25^+FOXP3^+$ T lymphocytes was also demonstrated in human

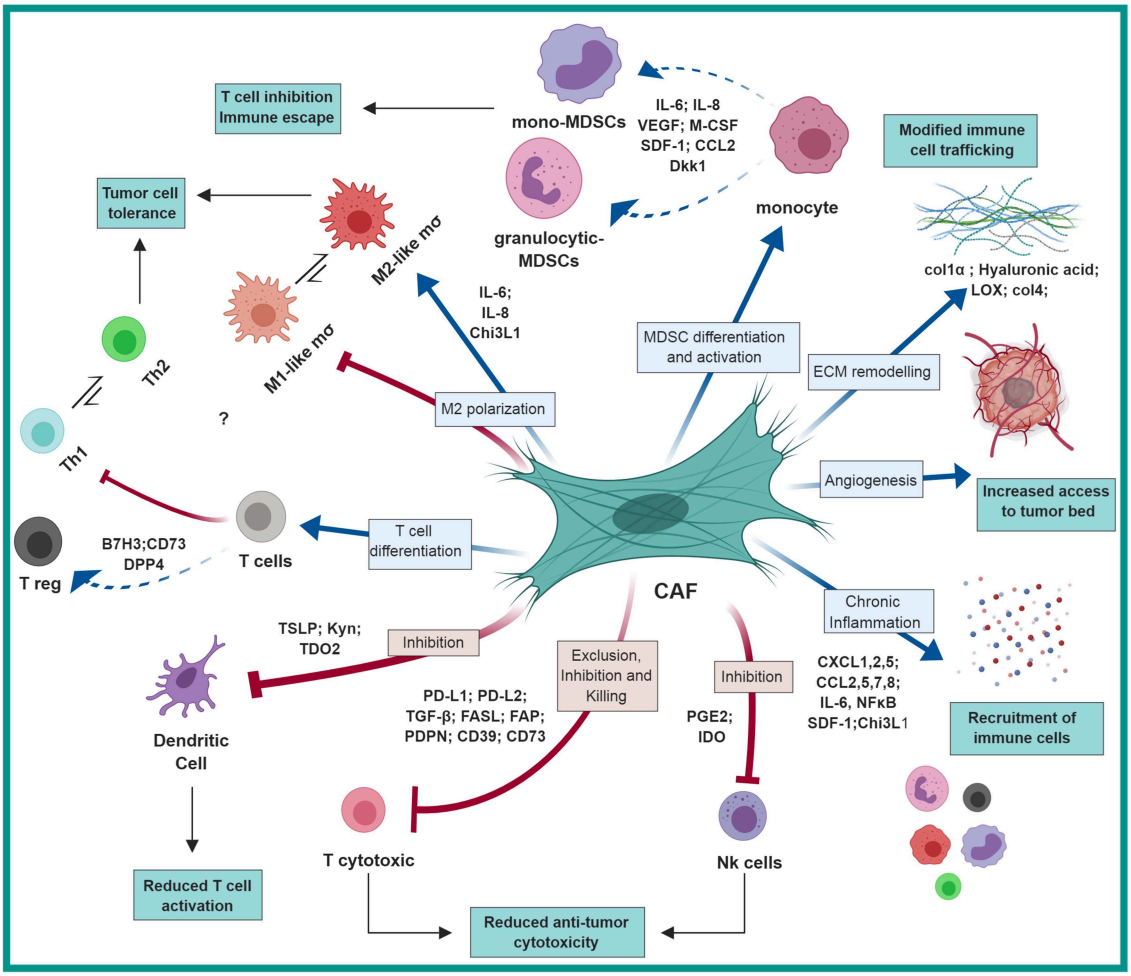

FIGURE 1 | CAF-mediated immunosuppression: CAFs shape the immune microenvironment in tumors toward a pro-tumorigenic and immunosuppressive milieu by affecting the recruitment and function of various innate and adaptive immune cells. Red arrows represent negative regulation/inhibition and blue arrows represent positive regulation/induction. This figure was designed by using graphical elements from BioRender.

high-grade serous ovarian cancers (HGSOC), where it was shown to depend on differential regulation of CXCL12 by miR-200/141. Interestingly, upregulation of FOXP3 required direct cell-cell contact between CAF-S1 and $CD4^+$ T cells (56). These findings are consistent with findings from immune competent mouse models which showed that targeting the CXCL12/CXCR4 axis inhibited the recruitment of Treg lymphocytes in ovarian and pancreatic cancer (45, 57).

Thus, by mediating the recruitment of various innate and adaptive immune cells, CAFs shape the immune composition of the TME and support tumor growth (**Table 1**; **Figure 1**).

CAFs DRIVE AN IMMUNOSUPPRESSIVE FUNCTION IN IMMUNE CELLS

In addition to their capacity to recruit immune cells that foster tumor growth, CAFs were also implicated in affecting the function of various immune cells toward an immunosuppressive phenotype by multiple mechanisms. This may resonate the role of fibroblasts in wound healing, where their function favors M2-like and Th2 type immune reactions (59, 60). Notably, our recent understanding of CAF heterogeneity in origin and function suggests that driving immune suppression may be mediated by distinct subpopulations of CAFs. For example, bone marrow-derived mesenchymal stromal cells (MSCs) were shown to be a significant source of CAFs in breast cancer (12). Interestingly, in physiological wound healing MSCs were shown to mediate immunosuppression: In models of acute liver injury, the presence of pro-inflammatory cytokines (IFNγ, TNFα, or IL-1) induced MSCs to produce iNOS, which in turn suppressed the function of T cells (61, 62). These physiological functions of MSCs and fibroblasts may be hijacked in tumors, to elicit the formation of an immunosuppressive TME via the effect of CAFs on specific immune cell populations (**Figure 1**).

Tumor-Associated Macrophages

Tumor-associated macrophages (TAMs) are a heterogeneous cell population arising from circulating monocytes or from

TABLE 1 | Recruitment or exclusion of immune cells.

Effect on immune cells	Type of immune cells	Cancer type	Tumor site	Molecule produced by CAFs	Targeted	References
Recruitment of myeloid cells	F4/80$^+$ macrophages	Breast	Primary	Chi3L1	No	(19)
	THP-1 monocytes	Breast	Primary	IL-6, CCL5 and CCL2	No	(35)
	CD11b$^+$Gr1$^+$ MDSCs	CRC	Primary	CCL2	No	(39)
	CXCR2$^+$ neutrophils	Breast	Primary	CXCL1; CXCL2 and CXCL5	Inhibition of one of CXCR2 ligands	(34)
	CD11b$^+$Ly6C$^+$ monocytes, and F4/80$^+$ macrophages	Lymphoma	Primary	CCL2	No	(33)
	Granulocytic MDSCs (Ly6C$^-$Ly6G$^+$)	Squamous cell carcinoma	Primary	SASP (CCL8; CXCL5; CCL2; CCL7; IL-6; CXCL1; CXCL14; CCL5)	Depletion of Ly6G$^+$ cells	(51)
	CCR2$^+$ circulating MDSCs	Hepatic	Primary	CCL2	No	(38)
	Granulocytic MDSCs (Ly6C$^-$Ly6G$^+$)	Colon, lung, breast, and melanoma	Primary	CXCL1	FAP-CAR T cells	(47)
	CD11b$^+$Gr1^{int} F4/80$^+$macrophages	PDAC	Primary		FAP Inhibition (UAMC-1110)	(40)
	Monocytes	Prostate	Primary	SDF-1	No	(44)
	CXCR4$^+$ mast cells	Prostate	Primary	SDF-1	No	(48)
	Monocytes	Breast	Ex-vivo	CCL2	No	(43)
	CD206$^+$ TAMs	Breast		CCL2	Zoledronic acid	(42)
Inhibition of T cell infiltration	CD8$^+$ T cells	Breast	Primary	Chi3L1	No	(19)
Inhibition of T cell infiltration & activation	CD3$^+$ T cells	Squamous cell carcinoma	Primary	SASP (CCL8; CXCL5; CCL2; CCL7; IL-6; CXCL1; CXCL14; CCL5)	Depletion of Ly6G$^+$ cells	(51)
Recruitment and retention of Treg cells	Treg (CD3$^+$CD4$^+$ FOXP3$^+$)	Squamous cell carcinoma	Primary	SASP (CCL8; CXCL5; CCL2; CCL7; IL-6; CXCL1; CXCL14; CCL5)	Depletion of Ly6G$^+$ cells	(51)
	CD4$^+$CD25$^+$ T cells	Breast and HGSOC	Primary	SDF-1; OX40L, PD-L2, and JAM2.	No	(20, 56)
Recruitment of neutrophils	Peripheral blood neutrophils	Hepatocellular carcinoma	Primary	SDF-1	No	(58)

tissue resident macrophages, and were implicated in various tumor-promoting tasks including pro-inflammatory signaling, enhancement of angiogenesis, invasion, metastasis and therapy resistance (63, 64). Macrophages can be classified according to their functional differentiation state and immunological responses: M1-like macrophages are involved in the response of type I T helper cells (Th1), they are activated by interferon gamma (IFNγ) and engagement of Toll-like receptors (TLRs) and characterized by production of pro-inflammatory molecules, nitric oxide (NO) and reactive oxygen species (ROS). M2-like macrophages, which are in general pro-tumorigenic, are involved in Th2-type immune responses, wound healing and tissue repair, activated by IL-4 and IL-13, and characterized by promotion of angiogenesis and secretion of immune suppressive factors that inhibit killing by cytotoxic T cells (63). This classification however, is not dichotomous, and different macrophage subtypes may share multiple features.

Functional differentiation of TAMs in the tumor microenvironment is affected by many factors. Recently, CAFs are emerging as novel effector cells in TAM differentiation toward an immunosuppressive phenotype, in addition to their role in monocyte recruitment. CAF-derived Chi3L1 was shown to be important for both recruitment and functional differentiation of bone marrow-derived macrophages in a mouse model of breast cancer: Genetic targeting of Chi3L1 expression in fibroblasts attenuated macrophage recruitment and their reprogramming to an M2-like phenotype, and promoted a Th1 phenotype in the tumor microenvironment (19). Prostate CAFs were shown to mediate both the recruitment and the M2-like differentiation of monocytes via SDF1 (44). A similar finding was demonstrated in an *ex-vivo* model of oral squamous cell carcinoma. CAFs isolated from human tumors, instigated an M2-like phenotype in patient-derived CD14$^+$ myeloid cells (manifested by production of ARG1, IL-10 and TGF-β), which in turn potently suppressed the proliferation of autologous T

cells (65). However, the underlying CAF-derived factors that mediated M2-like differentiation were not identified.

In this context, it is important to note that while TAMs and CAFs are both central players in the tumor microenvironment, their reciprocal interactions in cancer are not well characterized, and the main focus in the literature is on the effects of macrophages on fibroblasts. Future studies are required to further elucidate the contribution of CAF-derived signaling to the diverse functions of macrophages in the TME (**Figure 1**).

MDSCs

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature immune-suppressive myeloid cells. MDSCs are not found in healthy tissues and appear in pathologic conditions associated with chronic inflammation or stress, as well as in the microenvironment of tumors (29). The functional and phenotypic heterogeneity of MDSCs has been a source of confusion in their definition and terminology (66). Moreover, mouse and human MDSCs may be different in both surface markers (as detailed above) and in their immunosuppressive capacity: while in mice both granulocytic and monocytic MDSCs were shown to inhibit T cells *in vitro*, in human studies findings on the function of granulocytic vs. monocytic MDSCs depend on cancer type, underlying their diversity (30, 67). These differences should be considered when assessing murine studies.

Nevertheless, several studies in mouse and in human experimental systems demonstrated that CAFs are capable of reprogramming an immunosuppressive function in immature myeloid cells, typical of MDSCs. Primary pancreatic stellate cells (PSCs) isolated from human pancreatic tumors, but not normal PSCs, were demonstrated to induce an MDSC phenotype in peripheral blood mononuclear cells (PBMCs), manifested by inhibition of T cell proliferation *in vitro*. This reprogramming was dependent on IL-6 and STAT3 as their inhibition attenuated the induced immunosuppressive function (68). Moreover, IL-6 was found to be predominantly expressed in the stroma of human pancreatic tumors, and targeting it in transplantable and transgenic mouse models of pancreatic ductal adenocarcinoma (PDAC) in combination with PD-L1 blockade resulted in attenuated tumor growth, prolonged survival in a transgenic model of PDAC, and increased presence of intratumoral T cells (69). Importantly, this pathway may not be specific to pancreatic cancer: CAFs isolated from human hepatocellular carcinomas were capable of driving an immunosuppressive gene signature and functions in monocytes and in neutrophils via IL-6 mediated activation of STAT3 signaling (58, 70). Taken together, these findings suggest that combination of immune checkpoint therapeutics with targeting of stromal signaling may be beneficial in treatment of pancreatic and liver carcinomas.

Interestingly, stromal signaling that drives differentiation of peripheral MDSCs may be bone marrow-derived: In transplantable models of lung carcinoma and melanoma (Lewis lung carcinoma and B16 melanoma cell lines), tumor-bearing mice had elevated systemic levels of Dickkopf-related protein 1 (Dkk1), which were found to originate in the bone stromal compartment (osteoblasts and osteocytes) (71). Dkk1 effect on MDSCs was via inhibition of β-catenin, previously shown to be essential in mediating immunosuppressive functions of MDSCs (72). Thus, stromal signaling is central to shaping the functional differentiation of immature myeloid cells toward an immunosuppressive phenotype both locally and systemically in a variety of tumor types.

Importantly, the interactions of CAFs with recruited myeloid cells are reciprocal: Once monocytic and granulocytic cells are recruited to the TME they affect the activation of fibroblasts. For example, activated neutrophils secrete large amounts of reactive oxygen species which are known pro-fibrotic mediators (4, 18). Moreover, activated neutrophils release granules containing multiple proteases including MMPs, elastase, and cathepsins, capable of cleaving collagenous and non-collagenous connective tissue components. This release of ECM breakdown products further activates stromal fibroblasts, physiologically programmed to facilitate matrix remodeling during tissue repair. Similarly, macrophage secreted factors were demonstrated to facilitate reprogramming of resident dermal fibroblasts, or of mesenchymal stromal cells in an NF-κB dependent manner in skin and gastric carcinomas (32, 73).

T Cells

T cell-mediated immune response can be classified into Th1 or Th2-type immunity, based on their profile of cytokine production. In general, Th2-mediated immunity is considered tumor promoting, as it entails pro-angiogenic signaling, activation of M2-like macrophage function and inhibition of cell-mediated tumor cell killing (74).

Accumulating evidence suggest that signaling by CAFs may shape the T cell milieu in the TME toward a tumor-promoting function, either directly or via innate immune cells. Many of the findings emerge from murine models of cancer in which targeting of specific signaling molecules in CAFs resulted in attenuation of tumor growth and metastasis, accompanied by a shift in the T cell responses: *In vivo* elimination of FAP^+ CAFs by vaccination lead to a switch from Th2 to Th1-type immunity, characterized by increased expression of the cytotoxic cytokines IL-2 and IL-7, increased $CD8^+$ T cell tumor infiltration, and diminished recruitment of macrophages, MDSCs and T regulatory cells in a transplantable model of triple-negative breast cancer (75). Targeting of CAF-derived Chi3L1 had a similar effect in another transplantable model of breast cancer, and resulted in enhanced infiltration of $CD8^+$ T cells and a shift in the tumor cytokine profile toward a Th1-type phenotype. However, in both studies these effects of CAFs on T cells may be indirect.

A direct effect of CAFs on T cell function was demonstrated in an *in vitro* study that utilized fibroblasts and tumor-infiltrating T lymphocytes (TILs) isolated from human lung tumors: CAF-derived IL-6 enhanced production of IFNγ and IL-17A in activated TILs, suggesting that fibroblasts may also have an immunostimulatory effect on T cells (76).

Notably, the crosstalk between CAFs and T cells is reciprocal. Secreted factors from activated T cells enhanced the production of IL-6 by lung CAFs (76). Activated lymphocytes were also shown to induce the expression of cyclooxygenase-2 (COX-2) and intercellular adhesion molecule-1 (ICAM-1) in normal human lung fibroblasts. These activated fibroblasts then induced

a reduction in the expression of T cell activation/co-stimulation markers (CD69, LFA-1; CD3 and CD28) suggesting that fibroblasts are able to modulate effector functions of T cells recruited into sites of inflammation (77) (**Figure 1**).

Many of the pathways that are operative in tumors represent "hijacking" of physiological pathways. Indeed, the interactions between fibroblasts and T cells are probably not restricted to tumors and represent a physiological role of fibroblasts, as normal skin fibroblasts and autologous T cells showed similar interactions *in vitro* (76). The ability of fibroblasts to drive type-2 immunity may also be a physiological capability of fibroblasts: A recent study in normal lung tissue suggested that fibroblast-like adventitial stromal cells (ASCs) support the accumulation and activation of group 2 innate lymphoid cells (ILC2s), which are important mediators of type 2 immunity. Accumulation of ILC2s resulted in a formation of a tissue niche with Treg and dendritic cells, and depletion of ASCs abrogated these functions, suggesting that subpopulations of fibroblasts are required for optimal accumulation of ILC2s during type 2 immune responses (78). Future studies are required to demonstrate whether these fibroblast-mediated functions are also operative in tumors.

Dendritic Cells

Another mechanism by which CAFs hinder anti-tumor immune responses and impede on the function of T cells in the TME is by affecting the function of DCs, the most important population of antigen-presenting cells. Activated fibroblasts are a major source of transforming growth factor b (TGF-β), a pleotropic and immunosuppressive cytokine that functions in wound healing, ECM remodeling, and can affect the functional differentiation of multiple types of immune cells (79). TGF-β was shown to mediate downregulation of MHC class II molecules and the co-stimulatory molecules CD40, CD80, and CD86 in dendritic cells, thus inhibiting their antigen presentation capacity and their capability to activate cytotoxic T cell responses (80). CAF-mediated modulation of DC function was also shown to be mediated by their secretion of pro-inflammatory cytokines (**Figure 1**). CAFs isolated from human hepatic carcinomas were shown to secrete IL-6, which activated STAT3 in DCs, resulting in generation of regulatory DCs. These CAF-educated DCs exhibited lower expression of antigen presenting molecules and co-stimulatory molecules (CD1a, HLA-DR, CD80, CD86), and elevated expression of immunosuppressive cytokines (such as IL-10 and TGF-β). Moreover, hepatic CAF-educated DCs could affect T cells toward a suppressive phenotype, including induction of $CD4^+CD25^+Foxp3^+$ Tregs, and decreased production of IFN-γ in $CD8^+$ T cells (81). In lung cancer, inhibition of DCs differentiation and function was shown to be mediated via CAF-secreted tryptophan 2,3-dioxygenase (TDO2). Analysis of lung cancer surgical specimens revealed increased TDO2 expression in the fibroblasts adjacent to the cancer, and inhibition of TDO2 in a transplantable model of lung carcinoma resulted in improved DC function and T cell response, and decreased experimental metastasis (82).

The crosstalk between CAFs and dendritic cells was also shown to affect the ability of DCs to polarize the differentiation of T cells toward a Th2 phenotype in pancreatic cancer, via CAF secretion of Thymic stromal lymphopoietin (TSLP). CAFs that were activated by tumor-derived pro-inflammatory signaling (TNFα and IL-1β) secreted TSLP, which endowed them with the ability to drive the differentiation of naïve $CD4^+CD45RA^+$ T cells toward a Th2 phenotype. Human data from pancreatic cancer patients indicated that DCs with features of TSLP-treated DCs and Th2-attracting chemokines were present in pancreatic tumors, and the Th2/Th1 ratio in pancreatic tumors was an independent marker of poor survival (83).

Taken together, these studies demonstrate multiple mechanisms by which CAFs modulate the functional differentiation of immune cells in the TME toward an immunosuppressive function (**Table 2**, **Figure 1**).

CAF-mediated ECM Remodeling and Fibrosis Drives an Immunosuppressive Microenvironment

Fibrosis is a scarring process, characterized by excess deposition of collagenous and non-collagenous extracellular matrix (ECM) due to the accumulation, proliferation, and activation of fibroblasts. One of the hallmarks of CAFs is the excessive production/deposition of extracellular matrix components and degradation enzymes. This CAF-mediated deregulation of the ECM results in biomechanical and biochemical changes that facilitate tumor growth, invasion, and metastasis (84, 85). In addition to their effect on cancer cells, CAF-mediated deregulation of the ECM protein network modulates immune cells trafficking. Aberrant ECM protein composition and fragments of the ECM that are derived from tissue-remodeling processes can influence immune cell activation and survival, thereby actively contributing to immune responses at these sites (86). Various ECM components were shown to modulate macrophage polarization (toward an M2-like signature) and mediate the migration and maturation of monocytes and MDSCs (87). Moreover, stiffed collagen-rich matrix was found to induce CAFs production of monocytic chemoattractants like CCL2 and M-CSF (87), and CAFs in tumor-associated fibrosis produce high levels of cytokines and chemokines that favor tumor-promoting Th2 and Th17 responses (88). One example for an ECM component demonstrated to affect macrophage trafficking to tumors is hyaluronan (HA). Genetic targeting of the HA synthase gene in fibroblasts in a transplantable model of mammary carcinoma, leading to HA deficiency in the stroma, resulted in impaired macrophage recruitment and attenuated tumor angiogenesis and lymphangiogenesis (89).

While ECM components promote immune cell recruitment and activation, excessive deposition of collagen by fibroblasts in the TME leading to formation of scar-like tissue, was shown in pancreatic cancer to form a physical barrier that prevented cytotoxic T cell infiltration into the tumor, thus contributing to immune escape in pancreatic cancer. *In vitro* experiments demonstrated that while activated T cells migrated in low-density collagen matrices, migration was inhibited in dense collagen (90). Real-time imaging in viable slices of human lung tumors revealed that antigen-specific T cells within the tumor accumulate more in the stromal rich area than in

TABLE 2 | CAF-mediated modulation of immune cell differentiation.

Effect on immune cells	Type of immune cells	Cancer type	Tumor site	Molecule produced by CAFs	Targeted	References
M2-like differentiation	Circulating monocytes	Prostate	Primary	SDF-1	No	(44)
	TAMs	Breast	Primary	Chi3L1	No	(19)
Inhibition of Th1 immunity	Th1/Th2 cells	Breast	Primary and lung metastases	Not specified	Elimination of CAFs via pFAP vaccination	(75)
Th17 Differentiation	T cells (Th17 polarization)	Lung	Primary	IL-6	No	(76)
Shaping the activity of dendritic cells	Th2 polarization via DC conditioning	Pancreatic	Primary	TSLP	No	(83)
	DC	Hepatocellular carcinoma	Primary	IL-6	No	(81)
	DC	Lung	Primary	Kyn	TDO2 inhibitor	(82)
MDSCs differentiation & Activation	Monocytes	Hepatocellular carcinoma	Primary	SDF-1	No	(70)
	MDSCs	Melanoma and lung adeno-carcinoma	Primary	Dkk1	Inhibition of Dkk1	(71)
	Peripheral blood mononuclear cells	Pancreatic	Primary	IL-6, VEGF, M-CSF, SDF-1, MCP-1	IL-6 neutralization	(68, 69)
Treg cell Differentiation	$CD4^+CD25^+$ $FOXP3^+$ Treg	Breast and HGSOC	Primary	B7H3, CD73, DPP4	No	(20, 56)

the tumor islets. The density and the orientation of collagen and fibronectin fibers were suggested to play key roles in controlling T cells trafficking, as matrix loosening induced by collagenase treatment increased the ability of T cells to contact tumor cells (91, 92). Indeed, highly desmoplastic stroma, associated with activation of focal adhesion kinase (FAK) in pancreatic tumors, was shown to correlate with poor $CD8^+$ cytotoxic T cell infiltration. FAK inhibition in a transgenic mouse model of PDAC resulted in attenuated tumor fibrosis, improved response to immune checkpoint therapy and prolonged survival. These findings suggest that targeting fibrosis may be beneficial for overcoming CAF-mediated immune suppression (93).

The composition of the ECM is an important factor in enabling tumor metastasis (94). CAF-mediated remodeling of the ECM was recently shown to have an important role in enabling melanoma metastasis, in association with aging. While young skin fibroblasts produced abundant ECM components, aged fibroblasts were shown to lose the expression of the hyaluronic and proteoglycan link protein (HAPLN1), resulting in enhanced alignment of ECM matrices that promoted metastasis of melanoma cells in a mouse model of transplantable melanoma. However, the effect of matrices produced by aged fibroblasts was inhibitory on the migration of T cells, which may contribute to impaired immune response in the TME (95). Interestingly, age-related changes in HAPLN1 were also shown to increase lymphatic permeability, which affected melanoma lymph node metastasis. Age-related loss of HAPLN1 was shown to be associated with loss of integrity in the lymphatic vasculature and with enhanced lymphatic endothelial permeability, which enabled the escape of melanoma cells from the lymphatic system to distant metastatic sites (96).

Thus, CAF-mediated ECM remodeling and fibrosis contribute to the formation of an immunosuppressed and growth promoting microenvironment by multiple mechanisms (**Figure 1**).

DIRECT INHIBITION OF CELL MEDIATED KILLING: CAFs ABROGATE THE FUNCTION OF CYTOTOXIC LYMPHOCYTES

In addition to mediating the recruitment and functional differentiation of immune cells in the TME, findings from multiple studies implicate CAFs in affecting killing of tumor cells by cytotoxic lymphocytes.

CAFs isolated from human metastatic melanoma lesions or from hepatocellular carcinomas interfered in co-culture experiments with NK ability to kill melanoma cells. This inhibition was mediated by CAF-derived PGE2 and IDO, which abrogated NK cells expression of cytotoxic molecules (granzyme B and perforin) and cytokines, and impaired their cytotoxic activation and surface expression of NKp44 and NKp30 (97, 98). Another mechanism by which CAFs abrogate NK killing of tumor cells was demonstrated in melanoma-associated fibroblasts: CAFs isolated from tumors of melanoma patients decreased *in vitro* susceptibility of cancer cells to NK-mediated lysis. Mechanistically, CAFs secreted active matrix-metalloproteinases (MMPs) that were able to degrade two NKG2D ligands (MICA/B) on the surface of melanoma cells, resulting in an inhibition of NKG2-dependent cytotoxic activity of NK cells (99).

CAFs were also shown to directly abrogate the function of cytotoxic T cells by multiple mechanisms: Expression of immune

checkpoint molecules is emerging as an important process by which CAFs directly suppress the function of cytotoxic T lymphocytes (CTL). Fibroblasts isolated from melanoma patient biopsies could directly suppress $CD8^+$ T cells proliferation and function, via upregulating their expression of the PD-1 ligand PD-L1, mediated by IL-1α/β. These finding suggest that blockade of IL-1 may benefit melanoma patients and potentially synergize with immunotherapeutic interventions (100). CAFs isolated from resected human pancreatic tumors were recently shown to express PD-L1 and PD-L2. Furthermore, these CAFs were shown *in vitro* to inhibit the proliferation of T cells and to stimulate their expression of the inhibitory molecules TIM-3, PD-1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and LAG-3, possibly via the activity of CAF-derived PGE2 (101).

Expression of immune checkpoint molecules is likely a physiological pathway in fibroblasts during inflammation: Normal colon fibroblasts expressing PD-L1 and/or PD-L2 were shown to be involved in the regulation of mucosal $CD4^+$ T cell response (102). Moreover, expression of PD-L1 is upregulated in human dermal fibroblasts and in mesenchymal stromal cells via IFNγ, highly secreted by activated T cells (103, 104). Future studies are required to assess whether IFNγ also mediates the expression of PD-L1/2 in CAFs in tumors.

Another suggested mechanism for abrogating the function of cytotoxic T cells is by CAF-mediated metabolic effects. MSCs isolated from cervical tumors had elevated expression levels of CD39 and CD73 as compared with normal tissue fibroblasts. This feature was associated with the ability to strongly suppress the proliferation, activation and effector functions of cytotoxic T cells through generation of large amounts of adenosine from the hydrolysis of ATP, ADP, and AMP nucleotides (105). Similarly, glycolytic CAFs in prostate cancer were found to affect the polarization and function of effector T cells via their release of lactate (106).

Immunotherapy approaches are designed to unleash the cytotoxic T cell function of "dysfunctional" $CD8^+$ T cells by blocking the immunosuppressive signaling restraining these T cells. This requires not only that activated cancer-specific T cells be present in the TME, but also that their location allows physical contact with tumor cells. Many tumors exhibit an "immune excluded" phenotype, in which T cells are restricted to a peritumoral zone rich in fibroblasts, with few lymphocytes within the epithelial tumor mass itself (107). In a murine model of pancreatic cancer, CAF-derived CXCL12 protected tumor cells from T cell accumulation. Depletion of FAP^+ CAFs resulted in enhanced T cell infiltration and better response to anti-CTLA4 and anti-PD-L1 treatment in mice (45, 92, 108).

Indeed, CAFs were suggested to be important in affecting the non-effectiveness of immune therapy in multiple cancer types, partially via activation of TGF-β signaling. TGF-β signature in fibroblasts was shown to be associated with poor response to anti-PD-L1 treatment in metastatic urothelial cancer. Moreover, TGF-β signaling in fibroblasts was correlated with exclusion of $CD8^+$ T cells within the tumor, which were instead found in the fibroblasts and collagen-rich peritumoral stroma. Therapeutic co-administration of TGF-β-blocking reagents and anti-PD-L1 antibodies reduced TGF-β signaling in stromal cells, facilitated T-cell tumor penetration, and promoted tumor regression (109).

Similarly, targeting of TGF-β in a transgenic mouse model of metastatic colorectal cancer unleashed a potent and enduring cytotoxic T cell response. Tumors in these mice were characterized by T cell exclusion, highly activated stromal TGF-β, and a limited response to anti-PD-1 and anti-PD-L1 treatment. Inhibition of TGF-β enabled T cell infiltration to tumors and metastatic lesions, facilitated the response to anti-PD-1 and anti-PD-L1 therapy, diminished tumor growth and reduced liver metastasis (110). Taken together, these studies suggest that CAFs play a central role in inhibiting tumor cell killing by T cells, and advocate the use of combination therapies that target immune checkpoint inhibitors together with abrogating the immunosuppressive ability of CAFs (111).

Strikingly, recent evidence suggested that CAFs are capable of antigen presentation, leading to antigen-specific deletion of $CD8^+$ T cells to protect tumor cells. CAFs isolated from lung adenocarcinomas and melanoma tumors were shown to process and present antigens, and directly interact with activated $CD8^+$ T cells, thus inducing T cell death via PD-L2 and FAS ligand (FASL) engagement. Moreover, the capacity of antigen-specific T cells to kill their target tumor cells was dramatically impaired when conditioned by antigen-loaded CAFs, indicating that CAFs are capable of driving dysfunction and death of tumor-specific T cells, leading to enhanced tumor cell survival (112).

Interestingly, this function of CAFs is similar to the physiological role of lymph node fibroblastic reticular cells (FRCs) that display specific immunological properties to maintain peripheral tolerance. Lymph node structure consists of defined niches for B and T lymphocytes. This structure is provided by FRCs, which also provide the lymphocytes with a scaffold upon which to migrate. FRCs produce collagen rich reticular fibers that form a dense network within the lymphoid tissue. The network of fibers supports and guides the movement of dendritic cells (DCs), T lymphocytes and B lymphocytes (113). Moreover, FRCs express and present peripheral tissue antigens to T cells in lymph nodes (LNs), receive peptide-MHC II loaded exosomes from DCs, induce $CD4^+$ T cell hyporesponsiveness, and dampen T cell proliferation through the production of nitric oxide (114). These physiological functions may be hijacked in tumors, granting CAFs the capacity to actively regulate T cell function within the TME. For example, in lymph nodes, T cell migration is guided by FRCs that secrete the CCR7 ligands CCL21 and CCL19, which guide the interactions between $CCR7^+$ T cells and antigen-presenting cells (APCs) needed for T cell education and priming (115). These events are central for maintaining peripheral tolerance, as Treg cells require LN occupancy and CCR7 signaling for their activation and function, and the loss of CCR7 signaling is associated with spontaneous autoimmunity. Interestingly, similar pathways of immune tolerance and T cell exclusion are operative in tumors: Expression of CCL21 in melanoma tumors in mice was associated with the induction of stromal zones that were reminiscent of lymph node paracortex stroma, recruitment of regulatory immune cells, an altered cytokine milieu, and an

immunotolerant microenvironment, which depended on host expression of CCR7 (116).

In human and mouse breast tumors, this direct immunosuppressive capacity of CAFs was attributed to a distinct subpopulation of fibroblasts expressing FAP and Podoplanin (PDPN). FAP^+PDPN^+ CAFs expressed a TGF-β and fibrosis-related gene signature, and were in direct contact with T cells in the peritumoral dense ECM of mammary tumors. Moreover, FAP^+PDPN^+ CAFs were shown to suppress T cell proliferation in a nitric oxide-dependent manner (117). This function is reminiscent of the immune suppressive function of FRCs in the lymph nodes: Under inflammatory conditions, FRCs acquire immunosuppressive potential, and attenuate T cell expansion by producing nitric oxide (118, 119).

Thus, tumor cells co-opt tissue fibroblasts to generate stromal architecture and function that restrains tumor-infiltrating immune cells and impedes proper function of cytotoxic lymphocytes (**Table 3**, **Figure 1**). Taken together, these studies suggest that better understanding of CAF interactions with T cells and with regulation of immune checkpoint pathways may be beneficial for better design of immunotherapy treatments.

THERAPEUTIC PERSPECTIVES: CAF TARGETING APPROACHES

Therapeutic approaches of treating cancer are increasingly moving toward combinatorial strategies that target key operative pathways and mediators in the TME, rather than solely targeting cancer cell-intrinsic pathways. This is a result of improved understanding of the complexity of tumor eco-systems, as well as improved capacity of precision diagnostics that enable tailored therapeutic approaches. As our understanding of the important role of CAFs in mediating multiple tumor-promoting functions increases, it becomes clear that targeting CAFs in combination with other therapeutics may be beneficial. Based on the role of CAFs in mediating an immunosuppressed microenvironment that was reviewed herein, co-targeting of CAFs in combination with immunotherapeutics is an attractive option.

Pre-clinical trials targeting CAFs indicated that targeting a subpopulation of FAP^+ CAFs was beneficial in transplantable models of Lewis lung carcinoma and pancreatic adenocarcinomas. Depletion of FAP^+ CAFs using transgenic mice with FAP promoter- driven diphtheria toxin receptor (DTR) resulted in tumor regression in an IFNγ and TNFα dependent manner (120). Moreover, depletion of FAP^+ CAFs in a mouse model of PDAC enabled the therapeutic effects of anti-CTLA4 and anti-PD-1 (45). In an effort to design more applicable ways to target FAP, multiple other approaches were developed for the targeting of FAP^+ CAFs, including pharmacological inhibitors (e.g., PT630) (121), monoclonal antibodies (FAP5-DM1) (122), a FAP- targeting immunotoxin (αFAP- PE38) (123), and an oral DNA FAP vaccine (124, 125), which showed efficacy in mouse models of breast, pancreatic, lung and colon carcinomas. Moreover, a chimeric antigen receptor (CAR) T-cell specific for FAP was demonstrated to inhibit the growth of various subcutaneously transplanted tumors in mice by augmenting $CD8^+$ T cell antitumor responses (126). However, depletion of FAP^+ cells using the DTR system had severe systemic toxicity, including cachexia and anemia in mouse models of transgenic PDAC (KPC mice) and in transplantable colon carcinoma (C26 cells), likely reflecting the importance of FAP^+ stromal cells in maintaining normal muscle mass and hematopoiesis (127). Thus, caution should be taken when designing FAP-targeting approaches for clinical testing in cancer patients.

Depletion of αSMA^+ myofibroblasts in a mouse model of PDAC, utilizing thymidine kinase-Ganciclovir-mediated ablation, resulted unexpectedly in more invasive tumors, increased presence of $CD4^+Foxp3^+$ Treg cells and reduced survival (128). These findings suggest that the effect of targeting CAFs may depend on tumor type and on the experimental systems that were used, and requires careful consideration. In this context, it is important to note that depleting entire

TABLE 3 | CAF-mediated inhibition of anti-tumor cytotoxicity.

Effect on immune cells	Type of immune cell	Cancer type	Tumor site	Molecules produced by CAFs	Targeted	References
Inhibiting NK cytotoxic activities	NK cells	Melanoma	Metastatic lesions	PGE2	No	(97)
		Hepatocellular carcinoma	Primary	PGE2 and IDO	No	(98)
Exclusion of $CD8^+$ T cells	T cells	Pancreatic	Primary	CXCL12	Depletion of FAP^+ cells	(45, 92, 108)
		Urothelial cancer	Metastatic lesions	TGF-β	No	(109)
Inhibition of T cell activity		Pancreatic	Primary	PD-L1,2 and COX-2	PGE2 inhibitor	(110)
		Colorectal	Primary	TGF-β	TGF-β inhibitor	(110)
Exclusion and killing of $CD8^+$ T cells		Lung adeno-carcinoma and melanoma	Primary	FAS-L and PD-L2	No	(112)
Suppression of proliferation and activation		Breast	Primary	FAP and PDPN; TGF-β	No	(117)
		Cervical cancer	Primary	CD39 and CD73	No	(105)

fibroblast populations is highly problematic in human patients, as fibroblasts have many critical physiologic functions. Moreover, both αSMA and FAP are not expressed exclusively by CAFs, which adds to the complexity of targeting cell populations based on these markers.

Therefore, targeting of molecules or pathways that are essential for the tumor-promoting functions of CAFs is likely a more clinically relevant approach. For example, targeting the pro-fibrotic function of CAFs with Pirfenidone (PFD—an anti-fibrotic agent as well as a TGF–β antagonist) was shown to be efficient in combination with doxorubicin in a mouse model of triple-negative breast cancer (4T1) (129). Similarly, targeting the fibrotic activity of CAFs with tranilast, an anti-fibrotic agent, in transplantable tumor models (lymphoma, Lewis lung carcinomas and melanoma) resulted in decreased presence of Treg cells and MDSCs, and enhanced cytotoxic $CD8^+$ T cell response. These beneficial tumor-inhibitory effects were enhanced when CAFs were targeted in combination with effector-stimulatory immunotherapy such as dendritic cell-based vaccines (130). Importantly, CAF-mediated fibrosis contributes not only to enhanced tumor growth and invasiveness, but also to the immunosuppressive role of CAFs, as the increased matrix stiffness forms a physical barrier that limits the access of anti-tumor immune cells (87). Thus, targeting the pro-fibrotic functions of CAFs is beneficial for multiple reasons.

Another attractive option is targeting the transcriptional reprogramming of CAFs, which contributes to their activation. Vitamin D receptor (VDR) in pancreatic stellate cells was shown to be a central transcriptional repressor of their inflammatory and fibrotic functions, and treatment of mice with the VDR ligand calcipotriol induced stromal reprogramming that inhibited inflammation and fibrosis, enabled gemcitabine delivery into tumors, and improved survival in a PDAC model, suggesting that vitamin D may be utilized therapeutically in the treatment of pancreatic cancer (131). In addition, targeting central cytokines and chemokines that contribute to the pro-inflammatory, immunosuppressive and matrix remodeling function of CAFs may also be beneficial. For example, targeting IL-6 was suggested as a stromal-targeting therapeutic approach in cancer (132). In addition, CAFs are the main source of SDF-1/CXCL12 and blockade of the SDF-1/CXCL12-CXCR4 signaling pathway was shown to be beneficial in alleviating CAF-mediated immunosuppression (45, 92).

Importantly, the functional complexity and heterogeneity of CAF populations that may be specific to tumor type, specific organ and physiological context warrants careful consideration of CAF-targeted therapeutic strategies in patients.

FUTURE CHALLENGES AND OUTLOOK

The central role of fibroblasts in all stages of tumorigenesis and metastasis has emerged in recent years, as part of our growing understanding of tumors as multicellular organs. In addition to their "classical" functions in matrix remodeling and secretion of ECM components, accumulating evidence from many studies implicate CAFs in immunoregulatory functions that shape the immune milieu of tumors toward a pro-inflammatory and immunosuppressive function. These functions are mediated by CAF secretion of multiple cytokines and chemokines, and by reciprocal interactions with innate and adaptive immune cells. However, the heterogeneity and plasticity of CAFs are still poorly understood. This is partially a result of limited experimental tools: much of our knowledge relies on *in vitro* studies, or studies of CAF co-injection with tumor cells, which may not faithfully recapitulate the physiological function of CAFs. A major limitation to our ability to elucidate the role of specific CAF-derived factors is the sparsity of reliable CAF-specific Cre mice which will enable conditional ablation of candidate factors in CAFs, in order to identify potential therapeutic targets.

Another future challenge is the use of reliable pre-clinical models of spontaneous metastasis (133) that will enable better understanding of the role of CAFs in the formation of a pre-metastatic niche, and in facilitating the early stages of metastasis.

In the coming years, we will likely see multiple studies that will profile CAF populations at the single cell level, enabling better identification of their functional heterogeneity. Such understanding will provide us with both context-specific understanding of unique CAF functions, and unifying mechanisms that are common to CAF tasks in various cancer types. While knowledge from preclinical studies on immunoregulatory functions of CAFs is emerging, clinical data on CAF targeting is still limited. Hopefully, we will see in the future integration of the preclinical findings described in this review (**Figure 1**) into the design of novel therapeutic combination strategies aimed at impairing the tumor-supportive and immunosuppressive responses of CAFs.

AUTHOR CONTRIBUTIONS

LM analyzed the relevant literature, prepared the tables and figures, and participated in writing the manuscript. NE wrote the manuscript.

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Metastatic Latency, a Veiled Threat

Kangsan Kim[1,2], *Mauricio Marquez-Palencia*[1,2] *and Srinivas Malladi*[1,2]*

[1] Department of Pathology, UT Southwestern Medical Center, Dallas, TX, United States, [2] Harold C. Simmons Comprehensive Cancer Center, UT Southwestern Medical Center, Dallas, TX, United States

Metastatic relapse is observed in cancer patients with no clinical evidence of disease for months to decades after initial diagnosis and treatment. Disseminated cancer cells that are capable of entering reversible cell cycle arrest are believed to be responsible for these late metastatic relapses. Dynamic interactions between the latent disseminated tumor cells and their surrounding microenvironment aid cancer cell survival and facilitate escape from immune surveillance. Here, we highlight findings from preclinical models that provide a conceptual framework to define and target the latent metastatic phase of tumor progression. The hope is by identifying patients harboring latent metastatic cells and providing therapeutic options to eliminate metastatic seeds prior to their emergence will result in long lasting cures.

Keywords: metastasis, dormancy, immune-surveillance, microenvironment, minimal residual disease, latency

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Melanie Werner-Klein, Universität Regensburg, Germany

***Correspondence:**
Srinivas Malladi
srinivas.malladi@utsouthwestern.edu

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INTRODUCTION

In a significant number of cancer patients considered disease free, metastatic relapses occur. If and when relapse will occur is a question that is both indeterminate and unanswerable. Depending on the tumor type, these relapses might occur within a few months or decades after initial diagnosis and treatment (1–3). Very late recurrences are reported in a subset of breast cancer, head and neck squamous cell carcinoma (HNSCC), prostate cancer, melanoma and renal cell carcinoma patients considered disease free, presenting a major treatment follow-up challenge (4–18) (**Table 1**). In comparison, small cell lung cancer patients with aggressive disease have no reported latency as they are diagnosed with metastatic disease and have very poor survival rate (19). Lung cancers have short metastatic latency spans, with majority of relapses occurring within a year (20). Breast cancers with high proliferative index, triple negative breast cancers (TNBCs), tend to have shorter latency periods compared to estrogen receptor (ER) positive breast cancer (21). The frequency of late recurrence after 5 years is greatly reduced in TNBCs compared to ER^+ tumors, where disease recurrences have been reported in a significant number of patients as late as 20 years after primary diagnosis (22–24). Human autopsy and transplant studies report existence of disseminated tumor cells (DTCs; tumor cells that extravasate and reside in secondary organs) or metastatic lesions that persist as occult disease, highlighting the role of host immune system in limiting metastatic outgrowth (25, 26). Latency competent cancer cells (LCCs) are slow cycling or quiescent DTCs that persist in organs after surgery and initial therapy, and are the major source of disease relapse (2, 3, 27). LCCs reside next to the vasculature and are surrounded by extracellular matrix (ECM), soluble factors, stromal, and immune cells. LCCs remain unscathed in these sanctuaries, undergoing genetic/epigenomic adaptations that augment their ability to initiate metastasis and impede immune surveillance. Metastatic latency therefore depends on the oncogenomic status of the disseminated tumor cells, their proliferative capacity and the surrounding microenvironment.

TABLE 1 | Metastatic relapse rate and latency span in cancer patients.

Cancer type	Late recurrence rate	Late relapse span	References
Breast	∼15–20%	1–22 years	(4, 13, 14, 21, 23, 24)
Prostate	∼9.7–44%	1–20 years	(9, 13, 15)
Melanoma	∼6.8–11.3%	15–20 years	(7, 12, 18)
Renal	∼11–40%	1–25 years	(8, 13)
Lung	∼10–24%	Months–5 years	(13, 20)
Head and neck	∼24–33%	1–4 years	(13, 17)

Given that metastasis is the major cause for mortality in cancer patients, understanding how DTCs stay quiescent and remain viable for years before initiating metastasis is very critical (28). Assays to monitor the elusive LCCs and treatment strategies to effectively restrain or eliminate residual cancer cells is an unmet clinical need. Incorporating oncogenomic features of these cells along with tumor staging, presence of circulating/disseminated tumor cells or cell-free tumor DNA, will lead to better prediction of disease relapse in cancer patients with occult disease. Here, we summarize key determinants of metastatic latency, current concepts and proposed strategies to target and eliminate residual disease.

DISSEMINATION: GET OUT OF DODGE

As solid tumors grow, tissue constraints and cellular energy needs drive genetic or epigenetic changes in cancer cells that facilitate epithelial to mesenchymal transition and acquisition of invasive and stem-cell like characteristics (29–32). Key concepts discussing dormancy and epithelial mesenchymal transition (EMT) have been recently reviewed (33). Invasive cancer cells within the primary tumors breach the basement membrane, permeate the surrounding tissue as single cells or clumps and migrate into vasculature or lymphatics (31, 34). How and when tumors become invasive in patients? Are the early or late disseminated tumor cells from the primary tumor responsible for initiating metastasis in patients (35, 36)? Do the early disseminators aid in developing pre-metastatic niche (37, 38)? And the role of stromal cells in driving invasion (39), are some of the open questions actively investigated.

Although cancer cells intravasate in large numbers, very few survive in circulation. Given their prognostic value, circulating tumor cell (CTC) counts have been used to predict relapse or metastatic disease in breast, colorectal, small cell lung, and prostate cancer patients (40–43). Efforts from several labs have been directed toward improving CTC capture and enrichment protocols to define surface biomarkers on these potential metastatic seeds and to predict metastatic incidence (44, 45). Many ultrasensitive devices that are able to segregate CTCs from patient blood using size, density, electrical and compressibility differences have been developed to address this clinical need (46–50). However, isolating viable CTCs and performing functional experiments has been a challenge. With improved protocols and devices, several groups are now able to isolate, culture and characterize CTCs from patients (51–53). Such models are indispensable to study and advance concepts in metastatic evolution (44). CTC clusters or aggregates have also been isolated from blood stream and are reported to have greater predisposition to form metastasis than single cells in animal models (54). How CTC clusters survive the shear stress in circulation and avoid entrapment in lung capillaries is unclear (55). Further research is needed to determine how CTC clusters are dispersed or assembled in circulation (56, 57); what are they composed of; and what aspect of clustering aids metastatic competence. It should be noted that reliable CTC isolation and characterization is feasible only in metastatic disease and may not be able to identify patients with minimal residual disease. Nonetheless, diagnostic leukapheresis may enable reliable detection of CTCs in non-metastatic patients (58, 59).

METASTATIC LATENCY: IT'S NOT KANSAS ANYMORE TO MAKE ONESELF AT HOME

Metastatic latency span is both variable and indeterminate as it is a function of the rate at which the disseminated cancer cells adapt to and alter the surrounding microenvironment to initiate a metastatic lesion that impairs organ function. The composition and architecture of metastatic microenvironment determines the likelihood of DTC colonization (60). Majority of CTCs that extravasate into the new cellular milieu face resistance and perish upon extravasation (61). Cancer cells therefore have the propensity to reside in precincts that resemble stroma of the primary tumor (62). Depending on the robustness of the perceived cues, cancer cells are likely to proliferate, apoptose, or enter into a quiescent-slow cycling state (**Figure 1**). Proliferating DTCs are also more likely to be eliminated by chemotherapies and adjuvant therapies (22, 63). Slow cycling and quiescent LCCs, that are adapting to the new microenvironment remain unaffected by therapies targeting dividing cells and are enriched for stem cell like characteristics, that are critical to initiate secondary or metastatic tumors (27, 64, 65). Hypoxic microenvironments in the primary tumors promotes activation of dormancy programs and DTCs with these features are likely to survive better post-extravasation (66). Overall, absence of proliferating signals or a self-imposed block to these cues may result in activation of dormancy programs (27).

Extracellular Matrix

The distribution and availability of growth factors and cytokines is tightly regulated by the ECM surrounding DTCs (37, 67). Non-structural matrix protein such as Thrombospondin-1 (TSP-1) and Periostin through direct interaction with membrane receptors and fibrous ECM molecules modulate cancer cell proliferation status (68). Collagen enriched fibrotic environment leads to activation of myosin light chain kinase through integrin β1 signaling and promotes proliferation in cancer cells, while failure to engage proliferative signals results in dormancy (69, 70). Mitogen activated protein kinase (MAPK) activity has been clearly demonstrated to regulate proliferation status of human squamous carcinoma, melanoma, breast, and prostate

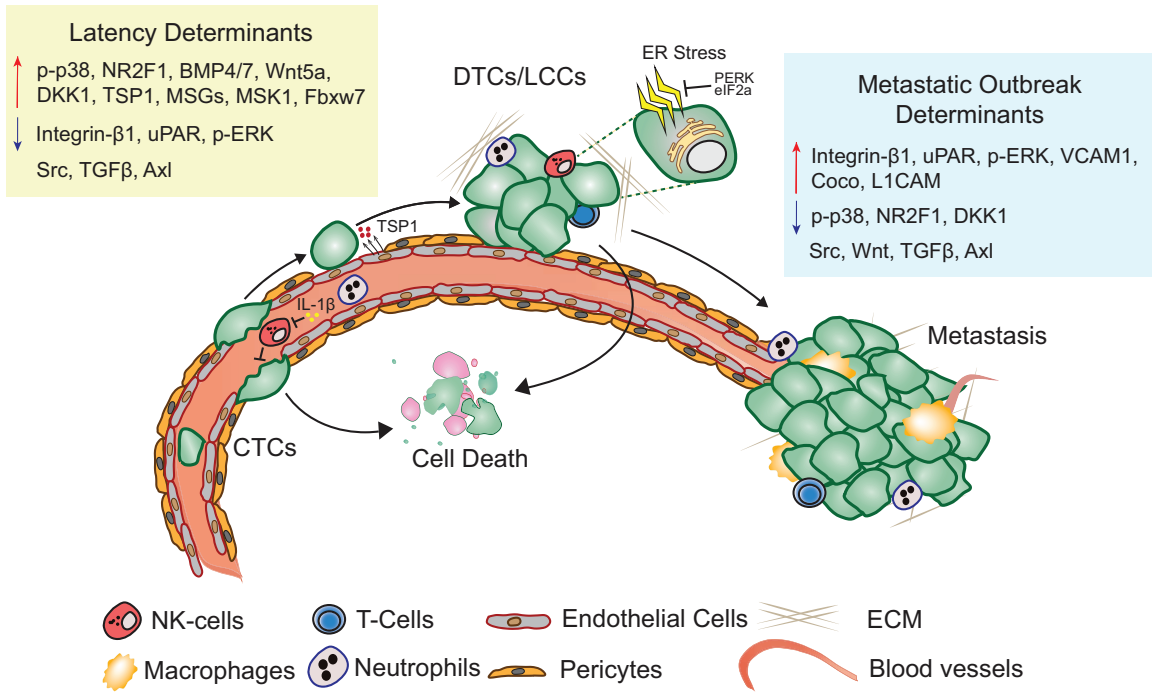

FIGURE 1 | Metastatic latency. Upon extravasation, many DTCs perish, few surviving LCCs adapt to and modify the surrounding microenvironment, eventually giving rise to metastasis. Key molecular determinants of latency and metastatic outbreak are highlighted (Refer text for more details).

cancer cells (71). Increased p38 and decreased ERK activity is observed in dormant cancer cells. Urokinase-type plasminogen activator receptor (uPAR) drives activation of ERK through FAK and Src kinases and promotes proliferation, while loss of either uPAR expression or FAK/Src activity leads to increased p38 kinase activity and unleashes downstream quiescence effectors DEC2, NR2F1, and CDK inhibitors (60, 64, 72–74). Src activation in response to CXCL12 and IGF1, potentiates PI3K/AKT activation, and aids survival of latent breast cancer DTCs independent of their hormone receptor status or cancer subtype (62). Expression of metastatic suppressor genes (MSGs: KISS1, KAI1, MKK4/6/7, and NM23) has also been reported to limit metastasis initiating capacity of DTCs by modulating the activity of MAPKs through G-protein coupled receptors and tyrosine receptor kinases (75, 76). Over-expression of KISS1 results in limiting metastatic outgrowth of aggressive human melanoma cell line (77). Similarly, NM23 and MKK4/6 activate p38 and inhibit ERK to induce dormancy in ovarian and breast cancer cells (76). Mitogen and stress-activated kinase 1 (MSK1) functions downstream of p38 and restrains breast DTCs into a steady micro-metastatic state by promoting luminal differentiation through GATA3 and FOXA1 in ER^+ breast cancer (78). Along these lines, GATA6 and HOPX have been reported to limit lung adenocarcinoma metastasis by promoting alveolar differentiation (79). Integrin α5β3 signaling response can also promote differentiation of luminal A breast cancer cells and limit tumor progression (80). L1CAM and YAP signaling enable the outgrowth of metastasis-initiating cells through integrin-ILK both immediately following their infiltration of target organs and as they exit metastatic latency (81). Taken together, altered ECM and MAPK activity in response to microenvironmental cues influences the proliferation status of latent DTCs.

Endoplasmic Reticulum (ER) Stress

Transducers of unfolded protein response (PERK and eIF2α) are also activated in p38 active dormant cells and have been shown to be essential for cancer cell survival under chemotherapy induced genotoxic stress (74, 82). CK19 and MHC class I negative dormant pancreatic DTCs activate PERK and relieving ER stress pharmacologically or by expression of XBP1 in combination with T-cell depletion resulted in metastatic outgrowth (83). Administration of chemical chaperone 4-PBA to relieve ER stress in DTCs preoperatively has been proposed to drive DTCs out of quiescence and be cleared by active adaptive immune surveillance (83). Likewise, Fbxw7, a component of SCF-E3 ubiquitin ligase complex has been reported to maintain dormancy in breast DTCs and its ablation led to increased proliferation in this model system. A combination of depleting Fbxw7 and chemotherapy has been proposed to limit residual disease (84). Will these approaches result in reduced metastatic incidence or worsen survival outcome in patients by unleashing restrained heterogeneous metastatic clones needs to be further explored.

Supportive Niches

Specialized microenvironments surrounding LCCs limit proliferation and facilitate cancer cell survival and quiescence. For example, perivascular niche (PVN) supports survival of hematopoietic stem cells (HSCs) as well as disseminated lung, melanoma, breast and prostate cancer cells in the bone marrow

(85–87). TSP1 secreted by the microvascular endothelium in the bone and lung induce growth arrest in breast DTCs, while high Periostin and TGF-β1 expression in the neovascular tip cells triggers metastatic relapse (68). Inhibition of integrin-mediated interactions between DTCs (either quiescent or proliferating) and PVN sensitizes them to chemotherapy (88). Several stromal derived factors have inhibitory effect on LCC proliferation. For example, leukemia inhibitory factor (LIF) secreted by osteoblasts and bone marrow stromal cells limit growth of breast cancer DTCs in bone by activating LIFR: STAT3 signaling (89). Prostate cancer DTCs and drug resistant dormant myeloma cells in the bone marrow respond to osteoblast derived growth arrest specific 6 (GAS6), through Axl, a receptor tyrosine kinase and remain dormant (73, 90, 91). HSC driving factors such as osteoblast secreted stromal cell-derived factor 1 (SDF-1/CXCL12) binds to CXCR4 on cancer cells and retains them in the HSC niche (87). SDF-1 CXCR4 interaction plays an important role in keeping chronic myeloid leukemia stem cells dormant. Depletion of CXCL12 in mesenchymal stromal cells led to increased proliferation of these dormant cells while deletion of CXCL12 in endothelial cells resulted in reduced proliferation (92). Dormant breast cancer cells are predominantly found in the E-selectin and SDF-1 rich perisinusoidal vascular regions. Simultaneous blockade of CXCR4 and E-selectin in patients could release dormant micro metastases from the protective bone microenvironment and also prevent adhesion in the first place (93).

TGF-β2 rich bone microenvironment promotes quiescence in HNSCC DTCs by inducing cell cycle inhibitor p27, metastatic suppressor DEC2 and SMAD1/5 activation, while the TGF-β2 low lung microenvironment permits metastatic outgrowth. Removing this break by inhibiting TGF-βRIII increased metastatic burden in mice (94). BMP7, another TGF-β family member secreted by bone stromal cells induces senescence in prostate DTCs through BMPR2 dependent activation of p38 and p21. Withdrawal of BMP7 in this mouse model of prostate cancer induces recurrent metastatic growth in the bone (95). Similarly, BMP4 supports breast cancer dormancy in the lung, while its antagonist, Coco, drives metastatic outgrowth (96). Cancer cells have also been reported to either cannibalize bone marrow derived mesenchymal stem cells or prime them to secrete microRNA packed exosomes that promote quiescence (97, 98). WNT5a from the osteoblastic niche induces dormancy in prostate cancer cells by activating non-canonical ROR/SIAH2 signaling and repressing canonical WNT/β-catenin signaling (99). In an autocrine fashion, breast and lung cancer DTCs can also enforce a slow cycling state by inhibiting WNT/β-catenin signaling (27).

Innate and Adaptive Leukocytes

Host immunity plays an important role in shaping and limiting tumor growth and progression (100–106). Neutrophils are the most abundant circulating immune cells and among the first ones to infiltrate the lung metastatic niche. Their role in either promoting or inhibiting metastasis is highly debated (107). MET expressing neutrophils secrete reactive oxygen species and are reported to be anti-metastatic (108, 109). In contrast, several studies identify a pro-metastatic function for neutrophils (110). Neutrophils inhibit natural killer (NK) cell function and facilitate extravasation of tumor cells by secreting IL-1β and matrix metalloproteinases (111). Neutrophil derived leukotrienes further support early colonization of breast cancer cells (112, 113). Depletion of neutrophils or genetic ablation of CXCR2, suppressed metastasis in pancreatic cancer models and lead to increased T-cell infiltration and extended survival (114). Recent reports highlight the role of neutrophils in metastatic outbreaks induced by sustained lung inflammation caused by tobacco smoke or bacterial derived lipopolysaccharide (115). Systemic inflammatory response induced after surgery can also promote the re-emergence of tumors that were kept in check by a tumor-specific T-cell response (116). Inflammation in lung, induced formation of neutrophil extracellular traps (NET) that resulted in cleavage and remodeling of laminin. Remodeled laminin activated integrin signaling and induced proliferation in otherwise dormant lung DTCs. This escape from latency is reported to be dependent on expression of Zeb1, a key modulator of EMT (117). Antibodies against NET-remodeled laminin prevented awakening of dormant cells and has been proposed as an approach to prevent metastatic outbreaks and prolong survival of cancer patients (115). Of note, obesity causes lung neutrophilia and the increase in neutrophils favors breast cancer metastasis to lung (118, 119).

Tissue resident macrophages or infiltrating monocytes are also reported to play an important role in either limiting or promoting early colonization of DTCs post extravasation (105, 120). Monocyte chemotactic and activating factor (CCL2) secreted by cancer cells and stroma recruits $CXCR2^{+}$ positive monocytes and macrophages to enable seeding, colonization and outgrowth (121, 122). VCAM1 on breast cancer cells in leukocyte rich lung microenvironment binds to α4β1 integrin on macrophages and activates Ezrin-AKT survival pathway in cancer cells (123). In the bone, aberrant expression of VCAM1 promotes transition from indolent to overt metastasis in breast DTCs. VCAM1 expressing DTCs attract and tether to integrin α4β1 expressing osteoclast progenitors and give rise to osteolytic metastasis. Antibodies against α4 integrin block this prosurvival function of VCAM1 and metastatic burden (124). NR4A1 positive patrolling monocytes that are enriched in the microvasculature of the lung, engulf melanoma, and breast tumor cells and reduce lung colonization and metastasis (125, 126). They also promote recruitment and activation of NK cells. Administration of selective class IIa histone deacetylate (HDAC) inhibitor, in MMTV-pyMT mouse model, resulted in reduced tumor burden and spontaneous pulmonary metastasis. HDAC inhibition reverts the pro-tumorigenic phenotype of tumor associated macrophages, recruits anti-tumor phagocytic macrophages and stimulates the adaptive immune response (127). Selective inhibition of histone deacetylase may unleash the antitumor potential of macrophages and keep DTCs in check.

NK cells play an important role in surveilling and eradicating cancer cells in circulation and upon extravasation (106, 128). By releasing cytolytic granules and pro-apoptotic factors or cytokines, NK cells kill tumor cells. They also release chemokines that attract T-cells, dendritic cells, and monocytes promoting

TABLE 2 | Metastatic latency preclinical models.

Cancer type	Preclinical model	Mechanistic insights into DTC biology	References
Breast	HCC1954	NK cell mediated immune evasion, self-imposed quiescence, SOX9, DKK1, p-p38	(27)
	4T07	BMP signaling	(96)
	MDA-MB-231	VCAM-1 mediated osteoclastogenesis, chemoresistance by Fbxw7	(84, 124)
	MMTV-HER2	Early dissemination of DTCs, parallel evolution of metastatic cancer	(35, 36)
	PDX	Stem cell program - OCT4, SOX2, DKK1	(27, 65)
	BT549	p-p38, relieving ER stress by PERK and EIF2a	(82)
	D2A1	anti-inflammation, integrin signaling	(69, 70, 117)
Prostate	PC3	GAS6/Axl, Wnt5a signaling, BMP7	(90, 95, 99)
	DU145	GAS6/Axl	(90)
Melanoma	RET.AAD	Early dissemination of DTCs, restrained outgrowth by CD8 T-cell	(137)
Lung	H2087	NK cell mediated immune evasion, self-imposed quiescence, SOX2, DKK1	(27)
Head and neck	HEp-3	Epigenetic repression by NR2F1, low p-ERK, high p-p53/p-p38, SOX9, TGFβ2	(64, 72, 94)
Pancreas	mM1	Immune evasion by relieving ER stress	(83)
Fibrosarcoma	GR9	Low MHC-I	(138, 139)

adaptive immune response (129, 130). NK cell cytotoxicity has been negatively correlated with metastatic burden in several cancer types (131, 132). Depletion of NK cells aid metastatic outbreaks in disseminated cancer cells from breast and lung cancers (27, 111, 133). As tumors become invasive and acquire mesenchymal traits, they upregulate expression of cell surface NK cell activating ligands and are more susceptible to elimination by NK cells (134). DTCs are therefore more susceptible to immune recognition in circulation and upon extravasation (106). Nonetheless, cancer cells evade NK mediated immune surveillance by either down regulating NK cell activating ligands and death inducing receptors (135, 136). For example, extravasated breast and lung cancer DTCs in lung, brain, liver, and kidneys evade immune attack by NK cells by entering into a slow cycling or quiescent state enforced by autocrine inhibition of WNT signaling pathway (27, 133). Through mechanisms yet to be defined, these slow cycling DTCs downregulate expression of several NK cell activating sensors (27).

In a spontaneous mouse model of melanoma, early dissemination of tumor cells to the lung was observed and the DTCs remained dormant for varying periods of time. Depletion of $CD8^+$ T cells in these metastasis models resulted to increased metastatic out breaks (137). Similarly, depletion of $CD4^+$ and $CD8^+$ T cells 5 months after surgical removal of methylcholanthrene-induced fibro sarcoma tumor results in lung metastasis, highlighting the role of T cells in eliminating proliferative DTCs (138). In this model, intratumoral MHC-I heterogeneity dictates metastatic capacity and is proposed to predict response to immunotherapy (139). It is possible that the immune equilibrium at the metastatic site is maintained by the immune suppressive (MDSCs, Treg) and tumor inhibiting (T cells, NK cells) cells. Taken together, all these studies reinforce the role of innate and adaptive immune system in either delaying or limiting metastatic incidence. They also provide a framework to investigate the effect of host microenvironment on metastatic latency. Given that mouse and human immune systems are different, development of reliable preclinical models that replicate human immune surveillance are desired.

TARGET RESIDUAL DISEASE: HOW TO ELIMINATE THE VEILED THREAT?

Tracking residual disease in patients with no obvious symptoms is challenging. In order to accurately predict relapse, genomic and epigenomic characteristics of divergent disseminated cancer cells at the metastatic site and their associated phenotypic information is needed. Disease predictions depend on preclinical models, that are imperfect as they are based on assumptions that change with novel insights and discoveries. Nonetheless, every model, in spite of its limitations, has advanced our understanding of this phase of tumor progression (140, 141) (**Table 2**).

Keeping DTCs in a quiescent non-proliferative state is an attractive viable approach to limit delayed metastatic incidence (115, 142, 143). Adjuvant anti-estrogen therapy with the ER antagonists is a standard of care for patients with ER^+ breast cancers for years after initial diagnosis and this approach has significantly improved survival outcomes (22, 63). FDA approved CDK4/6 inhibitors for ER^+ breast cancers, block cancer cell proliferation and induce dormancy or senescence in various models (144). Such inhibitors have potential to limit relapse in cancers with prolonged metastatic latency phase. Inhibition of integrin β1, uPAR, ERK, and Src driven signaling might prevent metastatic breakouts. Activation of p38, NR2F1, or administration of GAS6, BMP4/7, WNT inhibitors, and TGF-β2 might be effective in limiting relapse. The major challenge for this approach is identifying enforcers of quiescence that are effective in all tissues and specific for cancers with distinct oncogenomic features. Also unknown is how well tolerated these extended therapies will be in patients and how effective this approach will be on slow cycling DTCs. Nevertheless, the threat of disease relapse will still remain.

Removing the proliferative break or mobilizing DTCs from their niches and allowing anti-proliferative drugs or immune surveillance to target DTCs is an alternative strategy (83, 84, 94, 95, 145, 146). Unleashing the proliferative potential of quiescent population has disease management concerns. In order to be effective, this approach would have to drive all

DTCs out of quiescence and the subsequent treatment has to effectively eliminate all proliferating cancer cells, which is unlikely. Moreover, this approach may result in selection of clones that don't respond to available therapies and can be detrimental to patient health. Eliminating quiescent DTCs by targeting intrinsic or extrinsic enforcers of this state is an attractive approach that needs to be further explored in clinic (142, 143).

CONCLUDING REMARKS

Early detection of disseminated disease with improved understanding of cellular and molecular mechanisms driving metastatic latency in an organ with distinct tissue architecture is critical to provide effective therapeutic interventions. Designing a clinical trial to assess the benefit of proposed strategies is a major challenge. Some obvious questions apart from the cost being: how to define patients with likelihood of disease relapse, trial duration and endpoint criteria. Further research with preclinical models that faithfully represent this phase of tumor progression will provide risk prognostication tools, novel targets and treatment strategies to eliminate minimal residual disease.

AUTHOR CONTRIBUTIONS

SM conception, design, and wrote the manuscript. KK, MM-P, and SM literature search. All authors contributed to manuscript revision, read, and approved the submitted version.

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Lactate Induces Pro-tumor Reprogramming in Intratumoral Plasmacytoid Dendritic Cells

*Deblina Raychaudhuri[1], Roopkatha Bhattacharya[1], Bishnu Prasad Sinha[1], Chinky Shiu Chen Liu[1], Amrit Raj Ghosh[1], Oindrila Rahaman[1], Purbita Bandopadhyay[1,2], Jafar Sarif[1], Ranit D'Rozario[1], Santu Paul[3], Anirban Das[4], Diptendra K. Sarkar[4], Samit Chattopadhyay[1] and Dipyaman Ganguly[1]**

[1] IICB-Translational Research Unit of Excellence, Division of Cancer Biology and Inflammatory Disorders, CSIR-Indian Institute of Chemical Biology, Kolkata, India, [2] Academy of Scientific and Innovative Research, Kolkata, India, [3] Mass Spectrometry Core Facility, IICB-Translational Research Unit of Excellence, CSIR-Indian Institute of Chemical Biology, Kolkata, India, [4] Department of General Surgery, Institute of Postgraduate Medical Education and Research, Kolkata, India

Plasmacytoid dendritic cells are the most efficient producers of type I interferons, viz. IFNα, in the body and thus have the ability to influence anti-tumor immune responses. But repression of effective intra-tumoral pDC activation is a key immuno-evasion strategy exhibited in tumors—tumor-recruited pDCs are rendered "tolerogenic," characterized by deficiency in IFNα induction and ability to expand regulatory T cells *in situ*. But the tumor-derived factors that drive this functional reprogramming of intra-tumoral pDCs are not established. In this study we aimed at exploring if intra-tumoral abundance of the oncometabolite lactate influences intra-tumoral pDC function. We found that lactate attenuates IFNα induction by pDCs mediated by intracellular Ca^{2+} mobilization triggered by cell surface GPR81 receptor as well as directly by cytosolic import of lactate in pDCs through the cell surface monocarboxylate transporters, affecting cellular metabolism needed for effective pDC activation. We also found that lactate enhances tryptophan metabolism and kynurenine production by pDCs which contribute to induction of $FoxP3^+$ $CD4^+$ regulatory T cells, the major immunosuppressive immune cell subset in tumor microenvironment. We validated these mechanisms of lactate-driven pDC reprogramming by looking into tumor recruited pDCs isolated from patients with breast cancers as well as in a preclinical model of breast cancer in mice. Thus, we discovered a hitherto unknown link between intra-tumoral abundance of an oncometabolite resulting from metabolic adaptation in cancer cells and the pro-tumor tolerogenic function of tumor-recruited pDCs, revealing new therapeutic targets for potentiating anti-cancer immune responses.

Keywords: breast cancer, $FoxP3^+$ T cells, lactate, plasmacytoid dendritic cells, type I IFN

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Stephanie Hugues,
Université de Genève, Switzerland
Miriam B. F. Werneck,
Federal University of Rio de Janeiro, Brazil

****Correspondence:***
Dipyaman Ganguly
dipyaman@iicb.res.in

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INTRODUCTION

Cancer immunosurveillance mechanisms recognize transformed cells in the body to prevent neoplastic growth (1). Importance of type II interferon (IFN) in mediating efficient anti-tumor immune response is well-established (2, 3). But in addition, an essential role of type I IFNs has also been described in driving tumor rejection via both cancer cell intrinsic and extrinsic effects (3–8).

Given the importance of type I IFNs in anti-tumor immune response, it is imperative for cancer cells to adopt strategies to evade either induction or function of these cytokines in the tumor bed.

Plasmacytoid dendritic cells (pDCs) are the major producers of type I IFNs (9). Activation of endosomal toll-like receptors (TLRs), in human pDCs, in response to foreign nucleic acids is crucial for anti-viral immunity (10). A critical role of pDC-derived type I IFNs is also established in different clinical contexts of autoreactive inflammation (11–18). Although type I IFNs play a critical role in anti-tumor immune response, previous studies have reported pDC dysfunction and acquisition of tolerogenic function in the tumor bed (19–21), thus providing evidence that cancer cells do adopt immunoregulatory strategies to evade intra-tumoral activation of pDCs. Tumor-recruited pDCs have been shown to lack IFNα induction and drive expansion of regulatory T cells (Tregs) in different cancers (19, 20). Tregs (characterized by the master regulator transcription factor FoxP3) prevent aberrant activation of the immune system against self-antigens thus preventing autoimmunity (22). But, presence of Tregs in tumor bed is associated with poor prognosis in various types of cancers (23–25). The immune-regulatory mechanisms operative in the tumor bed that inhibit induction of type I IFNs by recruited pDCs and augment their ability to induce Tregs remain elusive.

To support the proliferative phenotype, cancer cells adapt metabolic changes such as the Warburg effect (26). The major oncometabolite resulting from this glycolytic switch is lactate, which is abundant in tumor microenvironment (26, 27). Interestingly, an immunoregulatory role of lactate abundance is evident from its effect on expansion of myeloid derived suppressor cells, inhibition of NK cell and T cell mediated cytotoxicity, anti-inflammatory M2 polarization of tumor associated macrophages in tumor microenvironment as well as suppression of pro-inflammatory cytokine production by dendritic cells (28–31). In the present study we explored if this major oncometabolite also regulated intra-tumor pDC function in breast cancer. We found that cancer cell-derived lactate attenuates activation of human pDCs in response to TLR9 ligand and consequent type I IFN induction. On mechanistic exploration we attributed this to intracellular Ca^{2+} mobilization driven by cell surface lactate receptor GPR81, the lactate receptor on pDC surface, as well as cytosolic import of lactate itself via monocarboxylate transporters. We also explored the impact of lactate on the ability of pDCs to induce Tregs and implicated lactate-induced modulation of tryptophan metabolism in pDCs in the expansion of $CD4^+$ $FoxP3^+$ Tregs in breast cancer. These findings were validated by looking at tumor-recruited pDCs from breast cancer patients as well as in a murine syngeneic model of breast cancer. Thus, we discovered a link between a major metabolic adaptation of cancer cells and a critical immune-evasion mechanism driven by them by modulating the functional phenotype of tumor-recruited pDCs.

MATERIALS AND METHODS

Plasmacytoid Dendritic Cell Culture

PBMCs were isolated using density gradient centrifugation (Histopaque, Ficol) from blood drawn from healthy donors (after obtaining informed consent and approval by the Institutional Ethics Committee and in accordance with the Declaration of Helsinki). For some experiments, human pDCs were also isolated from buffy coats collected from Tata Medical Centre Blood Bank, Kolkata, through an approved material transfer agreement, in concurrence with the institutional human ethics committee. PDCs were sorted from whole PBMCs by magnetic immunoselection, using anti-BDCA4 microbeads (Miltenyi Biotec, Germany) and cultured in 100 μl of complete RPMI media (GIBCO), at 37°C and 5% CO_2. pDCs in culture were treated as mentioned in the figure legends. CpG-ODN (Invivogen, USA), Potassium lactate, EGTA, Cyclosporin A (Sigma-Aldrich, St. Louis, MO, USA), Gallein, 8-Bromo cAMP sodium salt, AR-C155858 (Tocris Biosciences, Bristol, UK), and CAMKII Inhibitor (Calbiochem, USA) were used for treating pDCs as indicated.

Enzyme-Linked Immunosorbent Assay

Concentration of IFNα in pDC culture supernatants was determined using sandwich ELISA (Mabtech, Sweden) according to manufacturer's protocol. Concentration of IFNα in peritoneal fluid of BALB/c mice was determined by sandwich ELISA using 1:1,000 dilution of RMMA-1 primary antibody (pbl interferonsource,USA), 1:500 dilution of polyclonal rabbit antibody to Mouse IFN Alpha (pbl interferonsource) and 1:10,000 dilution of anti-rabbit IgG, HRP conjugated tertiary antibody (Cell Signaling, USA).

Gene Knockdown Experiments

Freshly isolated pDCs were allowed to recover in complete media in the incubator for 1 h followed by a PBS wash. Then cells were resuspended in 100 μl of supplemented P3 buffer (Amaxa Lonza 4D nucleofector kit, Koln, Germany) and either control siRNA (esiRNA targeting EGFP, Sigma-Aldrich) or GPR81 target siRNA (sequence: GUUGCAUCAGUGUGGCAAAdTdT, Eurogentec, Belgium) was added following which cells were nucleofected using the preset FF168 protocol in an Amaxa Lonza 4D nucleofector. Nucleofected cells were kept in culture for 16 h following which they were collected, counted, plated and treated as indicated.

Calcium Mobilization Assay

Isolated primary pDCs were stained with calcium binding dye, Fluo 3-AM (1.5 μM), for 30 min in PBS containing 1.2 mM $CaCl_2$ and 2% FBS (GIBCO) at 37°C. Following incubation, the cells were washed twice in the same buffer and allowed to rest for 30 min at room temperature to allow efflux of excess dye. Stained cells were then acquired on a BD Fortessa flow cytometer for indicated time periods before and after the addition of specified treatments. The change in MFI in the FITC channel (Fluo 3-AM) was indicative of the difference in intracellular calcium mobilization upon addition of treatment.

RNA Isolation and Real Time PCR

Total RNA was isolated from nucleofected pDCs, from pDCs isolated from tumor tissue, blood as well as mouse peritoneal cells using the TriZol method according to the manufacturer's protocol and reverse transcribed to form cDNA

(using Superscript III cDNA kit from Invitrogen, USA). The cDNA was used for various gene expression studies via Real Time PCR (Applied Biosystems 7500 Fast, USA). Primers used for real time PCR are listed in **Supplementary Table S1**.

Measurement of Extracellular Acidification Rate (ECAR)

Glycolysis Stress Test was done to measure the ECAR values of non-transfected, EGFP siRNA transfected (control), and GPR81 siRNA transfected pDCs seeded on poly-L-Lysine (Sigma-Aldrich) coated wells of a 24 well-plate, and pre-treated with the reagents indicated in the figure legend for 4 h before being subjected to XF-24 Analyzer (Seahorse Biosciences). The experiments were carried out according to the Manufacturer's protocol, using the reagents (10 mM D-glucose, 1 mM oligomycin, 100 nM 2-Deoxy D-glucose) and XF media (supplemented with 4 mM L-glutamine) supplied by the manufacturer.

Patient Samples

Patients with breast cancer were recruited from Department of General Surgery, Institute of Postgraduate Medical Education & Research (IPGMER), Kolkata, India, as per recommendations of the Institutional Review Boards of IPGMER as well as CSIR-IICB. All recruited patients had invasive ductal carcinoma of the breast without any organ metastasis (**Supplementary Table S2**). A portion of tumor tissue samples resected during Modified Radical Mastectomy, were collected after obtaining informed consent from the donors as well as ethical clearance by the concerned institutions.

Sorting of pDCs From Human Breast Tumor Tissue

Collected tumor tissue was washed twice with PBS to remove contamination from exogenous blood, before being minced into tiny pieces and digested for 2 h at 37°C under shaking condition in a digestion buffer containing 1 mg/ml type I collagenase (Himedia) and 0.15 g/ml BSA (Himedia). Following digestion the mixture was centrifuged at 500 rpm for 5 min and the supernatant was collected and passed through a 70 μ strainer. Then the supernatant was spun at 1,500 rpm for 5 min to obtain the cell pellet which was stained with CD45 FITC, CD3 PerCP, CD19 APC, BDCA4 PE, CD123 BV421 (BD Biosciences), and pDCs were sorted in a moFLO cell sorter. The isolated pDCs were either subjected to gene expression studies or co-cultured with naïve $CD4^+$ T cells.

Naive $CD4^+$ T Cell Isolation and Co-culture With pDCs

$CD4^+$ T cells were isolated from PBMCs by magnetic immunoselection using anti-CD4 microbeads (Miltenyi Biotec, Germany). Similarly, naive T cells were sorted from the isolated $CD4^+$ T cells using anti-CD45RA microbeads before being co-cultured with autologous pDCs in a 5:1 ratio for 5 days in supplemented RPMI media.

Flow Cytometric Analysis of $FoxP3^+$ $CD4^+$ T Cells

T cells along with untreated or lactate treated pDCs from 5 day old co-cultures were either stained with BDCA2 APC or CD4 BUV395 to differentiate the T cells from pDCs during flow cytometry. This was followed either only by intracellular staining with FoxP3 PE (clone: 259D/C7, BD Biosciences) or in some cases by surface staining with CD25 APC followed by intracellular staining with FoxP3 PE (clone: PCH101, eBioscience) and acquisition in a flow cytometer.

Suppression of T Cell Proliferation Assay

Flow cytometry assisted cell sorting in a moFLO cell sorter was used to isolate $CD4^+CD25^{high}CD127^{low}$ cells from pDC T cell co-cultures. CD25 efluor450, CD127 APC-Cy7 and CD4 FITC (BD Biosciences) were used for staining. These were in turn co-cultured for 5 days with autologous Cell Trace Violet (Invitrogen) stained $CD45RA^+CD4^+$ T cells stimulated with CD3/CD28 (5 μg/ml) and subsequently subjected to flow cytometry to determine the degree of cell proliferation from the extent of CTV dilution.

L-Kynurenine and Tryptophan Quantitation by Mass Spectrometry

Cell culture supernatants were extracted in a 1:4 ratio with acetonitrile +0.1% formic acid (J.T. Baker), by intermittent vortexing and incubation on ice followed by centrifugation at 14,000 rpm for 10 min at 4°C. Then the supernatant was subjected to LC-MS/MS for L-kynurenine and tryptophan quantitation. Standard solutions of L-kynurenine (Sigma Aldrich) and tryptophan (Sigma) were prepared in the same acetonitrile- formic acid mixture. Standards having concentrations from 2.5 to 62.5 ng/ml were prepared for tryptophan and 2.5–125 ng/ml for were used for kynurenine. LTQ ORBITRAP XL and Hypersil Gold C18 column with a diameter of 100 × 2.1 mm, particle size 1.9 μ, was used for the mass spectrometry. The column (stationary phase) was maintained at 40°C. A mixture of Solution A (H_2O +0.1% formic acid) and solution B (acetonitrile + 0.1% formic acid) formed the mobile phase. Injection volume was 10 μl and the samples were run in an isocratic system (40% acetonitrile). The cut off for detection of intact L-kynurenine was (m/z) 209.09 and tryptophan was (m/z) 205.09. The retention time for L-kynurenine was 0.83 min and retention time for tryptophan was 0.87 min. The (m/z) for detection of fragmented kynurenine was 192.06 and for tryptophan was 188.07. Thermo Xcalibur software was used for analysis.

In vivo Efficacy Assay

Female BALB/c mice, 6–8 weeks old, were divided into 3 groups (1-Vehicle, 2-CpGA/CpGB and 3-CpGA/CpGB+0.5 g/kg sodium lactate), each having 3 mice. The 6 mice in groups 2 and 3 were intraperitoneally injected with 25 μg of CpGA+ 25 μg of CpGB, whereas the mice in the first group were i.p. administered with 1X PBS only. At indicated time points following CpG injection, the mice in the third group were i.p. injected with 0.5 g/kg lactate. After 14 h, the mice were sacrificed, peritoneal

fluid wash collected and centrifuged to separate the liquid and the cellular components. The liquid component was subjected to mouse IFNα ELISA and cellular component was subjected to both flow cytometric analysis (to measure pDC infiltration) and gene expression studies (to assay expression of ISGs). All animal experiments were done on approval of the Institutional Animal Ethics Committee of CSIR-IICB.

4T1 Tumor Mouse Models

Six to eight weeks old female BALB/c mice were used for generating the syngeneic tumor model with 4T1 cells. All animal experiments were done on approval of the Institutional Animal Ethics Committee of CSIR-IICB. The mice were injected subcutaneously in the right flank with 1.5×10^6 cells of the mouse breast cancer cell line 4T1. Once the tumors became visually apparent, the diameters of the tumors were measured at 3 different axes daily till the mice were sacrificed. When the tumors crossed an average diameter of 6.5 mm, the mice were assigned to 4 groups for daily intra-tumoral injections of PBS, gallein, ARC, or gallein+ARC. On the 5th day, the mice were sacrificed and tumors excised. The harvested tumor was partly collected in RNA Later (Qiagen) for subsequent RNA isolation and gene expression studies and washed, digested and stained for flow cytometry using the same protocol as described for human tumor tissue processing.

Statistics

Paired Student's *t*-test, unpaired *t*-test, Wilcoxon matched paired *t*-test, Mann-Whitney *t*-test or Spearman's correlation test was done, as indicated in the figure legends using the GraphPad Prism 5.0 software.

RESULTS

Lactate Inhibits Type I IFN Induction in pDCs

In order to study the effect of lactate on type I IFN induction in pDCs, potassium lactate (K^+-Lac) solution was added directly to the pDC cultures, revealing dose-dependent inhibition of type I IFN induction with significant reduction in IFNα at a concentration of 10 mM (**Figure 1A**). The extent of inhibition ranged from 40% to 100% in presence of 10 mM K^+-lactate (**Supplementary Figure S1A**). We used 10 mM K^+-Lac for further experiments, as this concentration of lactate did not affect the pH of the media significantly (**Supplementary Figure S1B**).

Role of GPR81 in Lactate-Driven pDC Dysfunction

Extracellular lactate can communicate with cells through either the cell surface G-protein coupled receptor 81 (GPR81), or via direct import into the cells through lactate transporters on the cell surface, the monocarboxylate transporters (MCT)-1 and MCT-2 (32). Moreover, GPR81 has been shown to regulate the production of both pro as well as anti-inflammatory cytokines by intestinal antigen presenting cells in mice (33). Hence, to explore the role of GPR81 in mediating the effect of lactate on human pDCs, first we knocked down GPR81 in primary human pDCs by RNA interference (**Supplementary Figure S2**). We found that GPR81-deficient pDCs showed partial but significant reversal of the inhibition of IFNα induction in presence of lactate (**Figure 1B**). GPR81 is a G_i protein coupled receptor thus presumably driving typical G_i signaling downstream (34), involving reduction in cAMP generation driven by the $G\alpha_i$ subunit and cytosolic Ca^{2+} mobilization driven by the $G\beta\gamma$ subunit. Addition of 8-bromo cAMP, a cell permeable cAMP analog, could not reverse the lactate-driven inhibition of pDC activation (**Figure 1C**), thus excluding the contribution from $G\alpha_i$ subunit-mediated cAMP depletion.

In order to assess the effect of the $G\beta\gamma$ subunit signaling we used gallein, an inhibitor for $G\beta\gamma$ subunit. To optimize the inhibitory concentration of gallein, we tested the efficacy of a range of doses of gallein in preventing GPCR-mediated calcium influx in response to lactate as well as chemerin, the pDC-specific chemokine, used as a positive control since it interacts with its receptor CMKLR1 on pDCs, which is also a GPCR with Gi signaling and reported to cause calcium influx (35). We found a dose dependent decrease in calcium mobilization, driven by both chemerin and lactate, which were completely abrogated at 1 μM gallein concentration (**Supplementary Figure S3**). In presence of gallein at 1 μM concentration, there was again a significant reversal of the inhibitory effect of lactate on pDC activation (**Figure 1D**).

GPR81 Activation Induced Ca^{2+} Mobilization Mediates Lactate-Induced pDC Dysfunction

The major outcome of $G\beta\gamma$ signaling is cytosolic mobilization of Ca^{2+}. On addition of lactate, pDCs showed instantaneous induction of cytosolic Ca^{2+} mobilization in a flow cytometry-based assay (**Figure 2A**). Also, in the presence of EGTA, the cell non-permeable Ca^{2+} chelator, the cytosolic free Ca^{2+} accumulation was not affected (**Figure 2A**) and inhibition of type I IFN induction by lactate could not be reversed (**Figure 2B**). These data indicated that lactate induces mobilization of Ca^{2+} from intracellular sources in pDCs rather than inducing influx of extracellular Ca^{2+}. Intracellular Ca^{2+} mobilization is known to regulate downstream gene expression by either or both of Ca^{2+}/calmodulin dependent protein kinase II (CAMKII), and calcineurin phosphatase (CALN) (36). We found that when CALN was inhibited, but not CAMKII, a significant reversal of the inhibitory effect of lactate was registered (**Figures 2C,D**). Thus, increased free cytosolic Ca^{2+}, resulting from lactate-GPR81 interaction, engages CALN signaling, and this has a partial but significant contribution to the inhibitory effect of lactate on pDC activation and type I IFN induction.

Role of Cytosolic Import of Lactate in pDC Dysfunction

As discussed earlier, extracellular lactate can also influence pDCs through intracellular import via the monocarboxylate transporters. Primary human pDCs show significantly higher expression of MCT1 (**Supplementary Figure S4**). In order to explore this possibility, we pretreated pDCs with AR-C155858

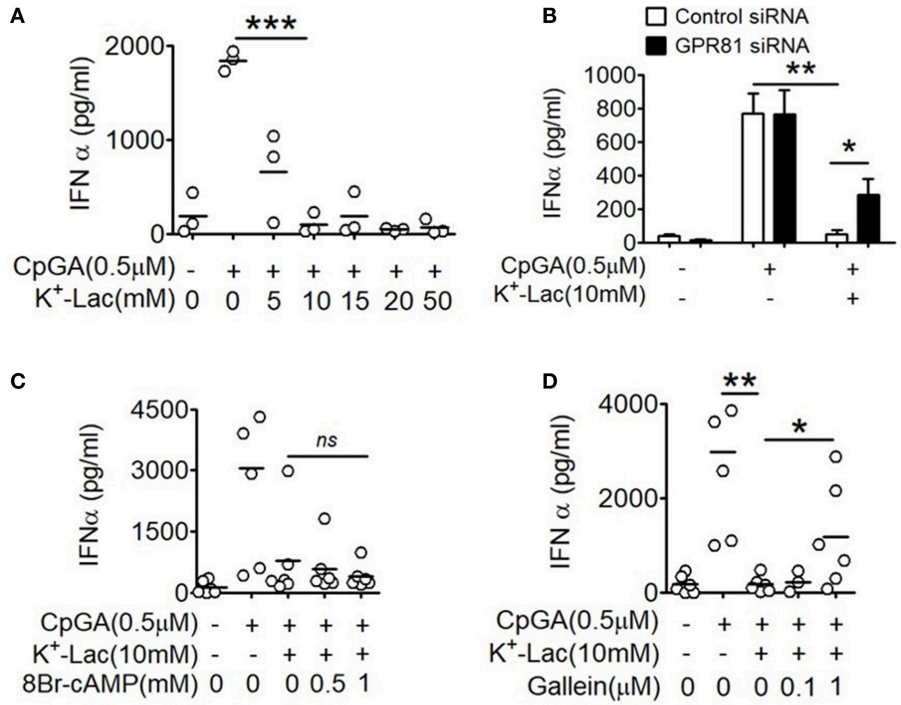

FIGURE 1 | Effect of GPR81 on lactate-induced inhibition of type I IFN induction in pDCs. **(A)** Indicated concentrations of K^+-lactate were added to overnight pDC cultures following which, IFNα concentration in the culture supernatants was estimated by ELISA. $n = 3$ from 2 independent experiments and two-tailed paired Student's *t*-test was done. **(B)** pDCs nucleofected with control siRNA or GPR81 siRNA, were treated with K^+-lactate and stimulated with CpGA. After 20 h, ELISA was done to measure IFNα concentration in the culture supernatants. $n = 6$ from 3 independent experiments and one-tailed paired Student's *t*-test was done. Data is represented as Mean ± SEM. **(C,D)** K^+-lactate was added to pDC cultures containing indicated concentrations of 8-Br cAMP **(C)** or gallein **(D)** and stimulated with CpGA. After 20 h of incubation IFNα concentration was measured in the culture supernatants. $n = 5$ from 2 to 3 independent experiments and one-tailed paired Student's *t*-test was done (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, and ns, not significant).

(ARC), an inhibitor of MCT-1 and MCT-2 (37). Interestingly, ARC also resulted in significant reversal of the lactate-mediated inhibition of IFNα (**Figure 3A**). In a recent study it was shown that a glycolytic switch in cellular respiration was characteristic and essential for type I IFN induction by pDCs in response to TLR stimulation (38). A negative feed-back effect of cytosolic import of extracellular lactate via MCTs on activation-induced glycolytic switch is also reported in immune cells, viz. in human monocytes (39). We looked into the effect of lactate on this TLR9-induced glycolytic switch in human pDCs, by measuring extracellular acidification on a metabolic flux analyzer. We found that presence of extracellular lactate indeed hinders the TLR9-induced glycolytic switch, with significant reduction in extracellular acidification rate (ECAR) (**Figure 3B**). The MCT transporters significantly add to the contribution from GPR81 triggering in lactate-mediated inhibition of type I IFN induction, as there was significant enhancement of type I IFN induction in GPR81-deficient pDCs when MCTs were also inhibited concomitantly (**Figure 3C**). Thus, the inhibitory effect of lactate on TLR-activation in pDCs is mediated by non-redundant contributions from cell surface GPR81 triggering as well as cytosolic import via the MCT transporters.

We found that treating healthy human pDCs with lactate in culture upregulates expression of both GPR81 and MCT1 genes (**Supplementary Figures S5A,B**). To validate if this is also true in the lactate-rich tumor microenvironment in human patients, we looked at breast cancer, as intra-tumoral pDC-dysfunction is well-documented in these patients (19, 20). We collected tumor tissues from breast cancer patients, isolated intratumoral pDCs as well as pDCs from peripheral blood of the same patients by flow cytometry assisted cell sorting and performed gene expression studies on them. We found intratumoral pDCs from these patients as well have enhanced expression of both GPR81 (**Supplementary Figure S5C**) and MCT1 (**Supplementary Figure S5D**) compared to their peripheral counterparts.

Lactate Enhances Regulatory T Cell Induction by pDCs

A major pro-tumorigenic property of tumor infiltrating pDCs is their ability to induce regulatory T cells (Tregs) thus adding to the immunosuppressive conditions in the tumor microenvironment (19, 20). Hence we explored if lactate also influences this aspect of intratumoral functional reprogramming of pDCs. We co-cultured lactate treated or untreated pDCs with autologous $CD45RA^+CD4^+$ naïve T cells and measured the abundance of $FoxP3^+$ $CD4^+$ T cells by flow cytometry (**Figure 4A**). We found that lactate-treated pDCs induced a higher percentage

of FoxP3$^+$ T cells, compared to untreated pDCs and this increased induction was abrogated in the presence of MCT inhibitor ARC (**Figure 4B**), thus implicating lactate transported into pDC cytosol, in this phenomenon. To further validate the identity of these FoxP3$^+$ T cell, we stained for CD25 (an established cell surface marker for Tregs) along with a different clone of anti-FoxP3 antibody and found that indeed the lactate-induced CD4$^+$FoxP3$^+$ cells are highly positive for CD25 (**Supplementary Figures S6A,B**).

In order to confirm the immunosuppressive nature of these T cells, we isolated them by flow-sorting, co-cultured them with dye-labeled autologous CD45RA$^+$ CD4$^+$ T cells on anti-CD3/anti-CD28 antibody-coated plates and assessed the degree of proliferation by dye dilution. We found that the Tregs induced by the lactate-treated pDCs were indeed capable of suppressing autologous naïve CD4$^+$ T cell proliferation (**Supplementary Figures S7A,B**).

Previous studies had reported that ability of pDCs to expand regulatory T cells may be mechanistically linked to either increased expression of ICOSL on pDCs enabling an ICOSL-ICOS mediated interaction with CD4 T cells (19, 40), or increased tryptophan metabolism leading to excessive production of kynurenines that in turn induces FoxP3 induction in T cells (41). We found that lactate fails to induce upregulation of ICOSL on human pDCs (**Supplementary Figures S7C,D**). On the other hand, we found that pDCs, cultured in the presence of lactate for 18 h, showed significantly higher tryptophan catabolism and secretion of kynurenine into the cell culture supernatants (detected by liquid chromatography of the supernatants followed by tandem mass spectrometry) (**Supplementary Figures S8**, **S9**). On inhibition of MCT-mediated lactate import into pDCs by adding ARC in the culture, the effect of lactate on pDC tryptophan metabolism was abolished (**Supplementary Figure S9**). This indicated that lactate transported into the cytosol via MCT drive the increase in kynurenine production. Furthermore, supernatant from 5 day co-culture of T cells with lactate treated pDCs had higher kynurenine: tryptophan ratio as opposed to supernatant from co-culture with either untreated pDCs or lactate-treated pDCs in presence of ARC (**Figure 4C**). As further validation for the link between lactate-induced kynurenine production by pDCs and generation of FoxP3$^+$ CD4$^+$ T cells, we explored the correlation between kynurenine abundance in pDC-T cell co-culture supernatants and the frequency of FoxP3$^+$ CD4$^+$ T cell generated after 5 days. Of note here, the correlation between

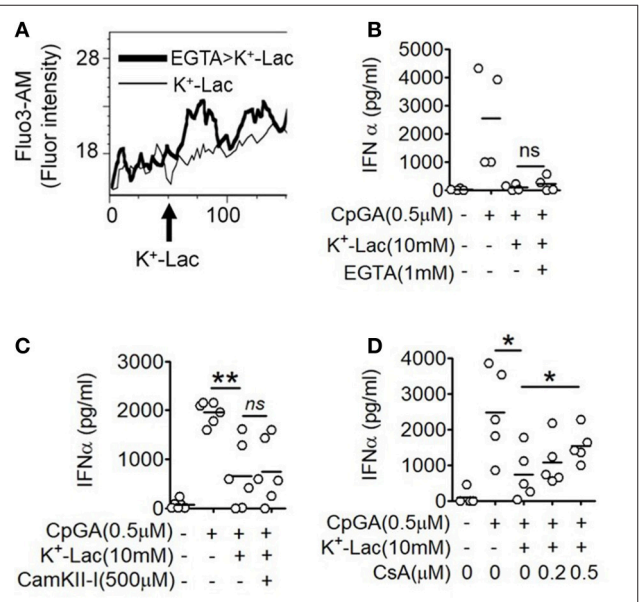

FIGURE 2 | Role of intracellular Ca^{2+} mobilization in lactate-induced GPR81 signaling in pDCs. **(A)** Primary pDCs were stained with calcium binding dye-Fluo-3AM and in the presence (thick line) or absence (thin line) of 1 mM EGTA acquired on a flow cytometer, before and after addition of K$^+$-lactate. The arrow represents the addition of 10 mM K$^+$-lactate and the Y-axis represents the fluorescence emitted by the dye. The figures are representative of 3 independent experiments. **(B,C)** K$^+$-lactate was added to pDC cultures preincubated with indicated concentrations of EGTA **(B)**, CamKII-I **(C)**, and CsA **(D)**, and then stimulated with CpGA. After 20 h, IFNα ELISA was done with the culture supernatants. $n = 4$–6 from 2 to 3 independent experiments and two-tailed paired Student's t-test (*$p < 0.05$, **$p < 0.005$ and ns, not significant).

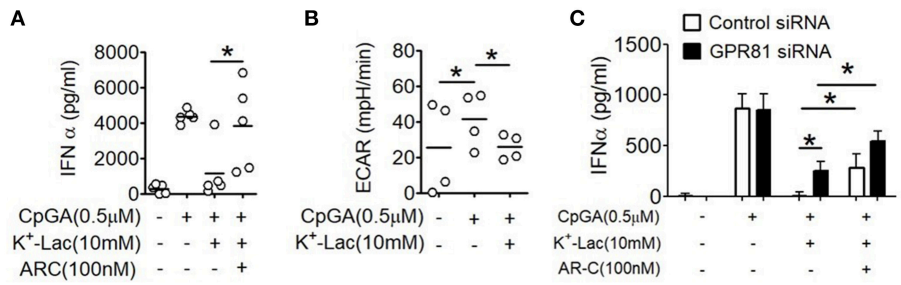

FIGURE 3 | Effect of MCTs on lactate-induced inhibition of type I IFN induction in pDCs. **(A)** K$^+$-lactate was added to pDC cultures preincubated with indicated concentrations of ARC, and then stimulated with CpGA. After 20 h, IFNα ELISA was done with the culture supernatants. $n = 5$ from 3 independent experiments and one-tailed Paired Student's t-test was done. **(B)** PDCs were subjected to the glycolysis stress test and ECAR values noted after the completion of the run. One-tailed Paired Student's t-test was done on the average ECAR values recorded over the three time points after oligomycin treatment. **(C)** pDCs nucleofected with control siRNA or GPR81 siRNA were incubated with ARC before addition of K$^+$-lactate and CpGA. Following 20 h of culture, supernatants were assayed for IFNα by ELISA. $n = 7$ from 3 independent experiments and one-tailed paired Student's t-test was done. Data is represented as Mean ± SEM (*$p < 0.05$).

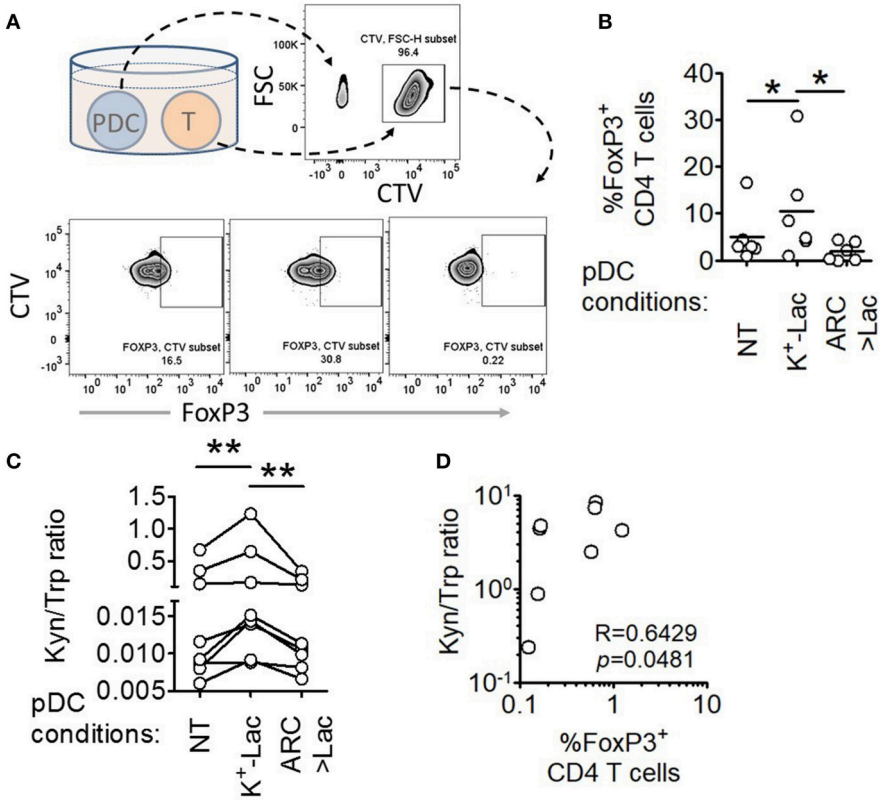

FIGURE 4 | Lactate drives human pDCs to generate FoxP3$^+$ T regulatory cells. **(A,B)** Autologous CD45RA$^+$CD4$^+$ T cells were co-cultured with untreated pDCs, K$^+$-Lac treated pDCs or lactate+ARC treated pDCs for 5 days before being intracellularly stained and acquired on a flow cytometer. $n = 6$ from 2 independent experiments and one-tailed Wilcoxon matched-pairs signed rank test was done. **(C)** Cell culture supernatants from pDC-T cell co-cultures, where pDCs were pre-treated as indicated, were subjected to acetonitrile extraction followed by LC-MS/MS to quantify the concentration of tryptophan and kynurenine in them. $n = 8$ from 3 independent experiments and two-tailed Wilcoxon matched-pairs signed rank test was done. **(D)** The ratio of kynurenine: tryptophan in the culture supernatants was correlated with the percentage of FoxP3$^+$ cells in the co-cultures by Spearman's correlation ($N = 4$ in duplicate) (*$p < 0.05$, **$p < 0.005$).

these two parameters had to be done from a single experiment with multiple donors ($N = 4$) as the kynurenine flux and Treg frequency in co-cultures varied from experiment to experiment. We found strong positive correlation between the kynurenine: tryptophan ratio in the pDC: T cell co-culture supernatants and the percentage of FoxP3$^+$ cells induced (**Figure 4D**). We concluded that lactate import into pDC cytosol via MCTs modulates pDC metabolism in a way that enables pDCs to induce FoxP3$^+$ CD4$^+$ regulatory T cells.

Intratumoral pDCs Show Enhanced Tryptophan Metabolism and Higher Capacity to Induce FoxP3$^+$CD4$^+$ T Regulatory Cells

In order to validate our findings in *ex-vivo* tumor tissue samples, we isolated intratumoral and peripheral pDCs from patients with breast cancer, co-cultured them with autologous peripheral CD4$^+$CD45RA$^+$ T cells and measured the fraction of FoxP3$^+$ cells by flow cytometric analysis (**Figure 5A**). Intratumoral pDCs (assumed to be exposed to higher concentrations of lactate) induced larger percentage of FoxP3$^+$ T cells compared to peripheral pDCs (**Figure 5B**). Thus, this corroborates our *in-vitro* results by showing that tumor infiltrating pDCs derived from human breast tumors are capable of inducing more FoxP3$^+$ CD4$^+$ T cells, as well. Next, we explored whether aberrant tryptophan metabolism and excessive production of kynurenine by tumor infiltrating pDCs is responsible for this phenomenon in breast tumors as well. We found significantly enhanced kynurenine:tryptophan ratio in supernatants of co-cultures involving intratumoral pDCs compared to those involving peripheral pDCs from the same patients (**Figure 5C**). Moreover, there was positive correlation between the kynurenine: tryptophan ratio and the percentage of FoxP3$^+$ cells induced (**Figure 5D**). Thus, tumor infiltrating pDCs from human breast cancer patients display enhanced tryptophan metabolism and are capable of inducing more FoxP3$^+$ T cells.

Lactate Abrogates IFNα Induction in Response to TLR9 Activation *in vivo*

Next, in order to decipher whether these oncometabolite-mediated immune evasion pathway are operative *in vivo* as well, we performed an *in vivo* efficacy assay wherein we injected CpG stimulated BALB/c mice with 0.5 g/kg lactate (**Figure 6A**), and

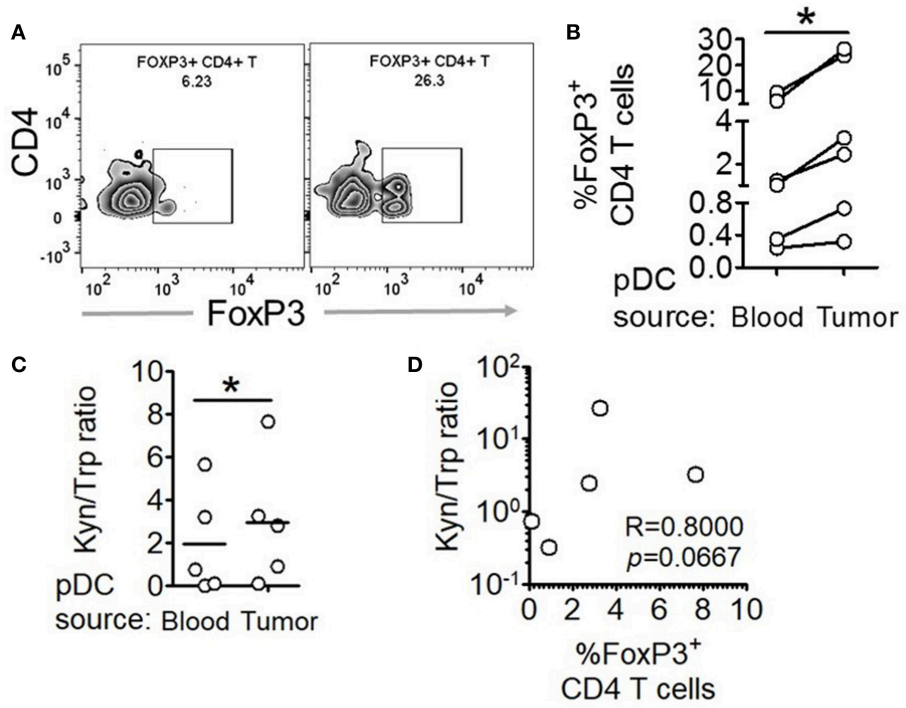

FIGURE 5 | Intratumoral human pDCs expand FoxP3$^+$ Tregs in breast cancer. **(A,B)** Blood and tumor tissue from patients with breast cancer was collected and processed to isolate pDCs, which were then co-cultured with autologous CD4$^+$CD45RA$^+$ T cells for 5 days, following which the cells were intracellularly stained for FoxP3 and subjected to flow cytometry. $n = 6$ from 6 independent experiments and two-tailed paired Student's t-test was done. **(C)** The culture supernatants from **(B)** were extracted and subjected to LC-MS/MS. Wilcoxon matched-pairs signed rank test was done. **(D)** The ratio of kynurenine: tryptophan in the culture supernatants was correlated with the percentage of FoxP3$^+$ cells in the co-cultures by Spearman's correlation (*$p < 0.05$).

found significantly lesser IFNα accumulation in peritoneal fluid (**Figure 6C**) as well as lesser expression of Interferon Signature Genes (IRF7 and IFIT) in peritoneal cells (**Figures 6D,E**) compared to mice which had been stimulated with CpG alone—though both groups had comparable pDC infiltration in peritoneum (**Figure 6B**). Thus, pathways responsible for inhibition of IFNα production pathways by lactate is operative *in vivo* as well.

Intratumoral Injection of Gallein and ARC Cause Significant Reduction in Tumor Burden

Next, in order to further explore the *in vivo* efficacy of inhibition of lactate signaling pathway in mouse preclinical breast cancer model, we developed 4T1 breast tumor model in BALB/c mice. We let the tumors grow to an average diameter of 6.5 mm before injecting them daily with the given doses of gallein (the inhibitor of GPR81 mediated pathway) and/or ARC (the MCT inhibitor) for 4 consecutive days (**Figure 7A**). Gallein alone, ARC alone as well as the two in combination, caused sharp retardation in tumor growth (**Figure 7B**). This was further validated by the weights of the excised tumors which were significantly less in the inhibitor treated groups compared to the vehicle treated group (**Figure 7C**). Thus, inhibition of lactate signaling pathways in 4T1 breast tumor containing BALB/c mice caused significant reduction in tumor load indicating both GPR81 and MCTs as potential targets for future chemotherapeutic drugs needing further investigation.

Inhibition of Pathways Involved in Lactate Signaling Enhances Intratumoral Interferon Signature and Reduces Expansion of FoxP3$^+$ Cells

It is well-known that lactate exerts both cancer cell intrinsic and extrinsic effects (27–31). Hence, inhibitors of lactate signaling pathways must also have both kinds of effects which together might be responsible for the reduction in tumor burden. But, our interest lay in the effect of these inhibitors, *in vivo,* on the intratumoral functional reprogramming of pDCs and expansion of regulatory T cells, as a proof of principle for our *in vitro* studies. Toward this, we compared the percentage of tumor infiltrating pDCs as well as the expression of Interferon Signature Genes (ISGs-representative of the extent of interferon signaling) in the tumor tissue upon injection of gallein alone, ARC alone or the two in combination, with vehicle treated control group mice. There was appreciable pDC infiltration in tumor tissues with no significant difference in terms of extent recruitment among the four group of mice (**Figure 7D**). Intratumoral injection of gallein caused a significant increase in the expression of ISGs such as MX1, IRF7, and IFIT, thus supporting our *in-vitro* data.

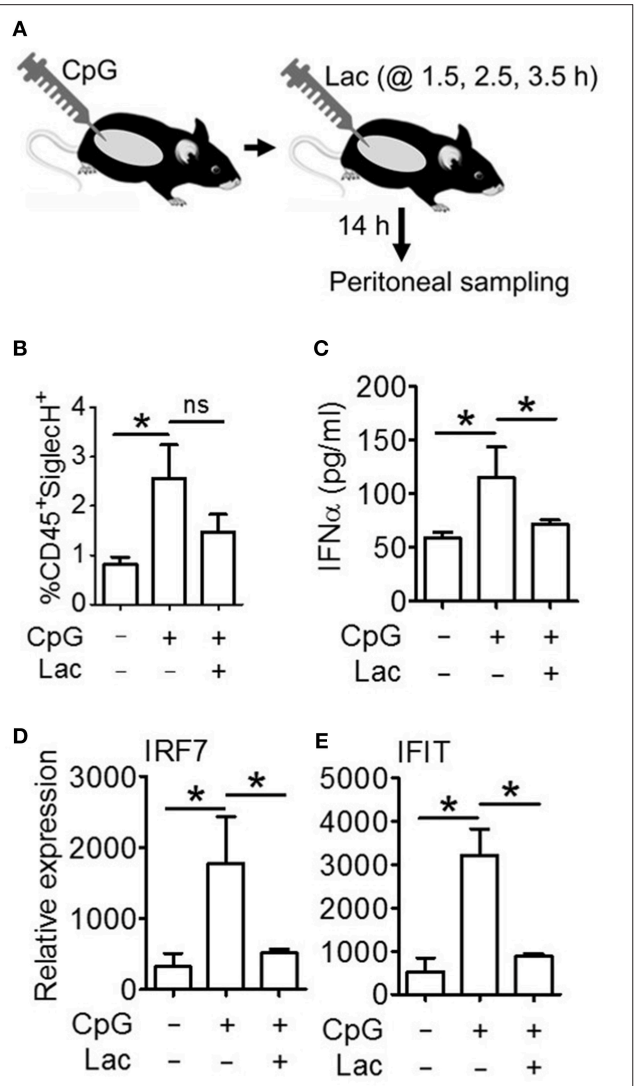

FIGURE 6 | Lactate inhibits IFNα induction *in vivo*. **(A)** BALB/c mice were divided into 3 groups (Vehicle, CpG, and CpG+Lac) each having 3 mice The mice belonging to CpG and CpG+Lac groups were injected with CpG intraperitoneally followed by three doses of lactate at the indicated time points. Mice were sacrificed after 14 h, following which peritoneal wash with 1X PBS was collected and centrifuged to separate the liquid portion from the cellular portion which were then subjected to further downstream processing. **(B)** Peritoneal cells were stained with CD45 PerCP and SiglecH BV421 before flow cytometric acquisition to determine the percentage of infiltrating pDCs. **(C)** Peritoneal wash was subjected to mouse IFNα ELISA to determine its concentration in peritoneal fluid. **(D,E)** Gene expression studies were done on the isolated peritoneal cells to measure the expression of ISGs- IRF7 **(D)** and IFIT **(E)** relative to the expression of the housekeeping gene 18S. $n = 3$ from 1 independent experiment and Mann-Whitney test was done. Data is represented as Mean ± SEM (*$p < 0.05$ and ns, non-significant).

This recovery of intratumoral IFN induction was more variable in response to concomitant MCT inhibition (**Figures 7E–G**). This can be explained by the fact that TLR stimulation in pDCs should lead to accumulation of lactate in the cytosol due to the glycolytic switch in cellular respiration and that should also have negative feed-back on glycolysis as well as type I IFN induction. As MCTs can mediate both passive extracellular export of lactate and intracellular import, inhibiting them in the presence of low levels or absence of extracellular lactate should actually inhibit type I IFN induction by helping in endogenous lactate accumulation. Accordingly, in the absence of extracellular lactate, ARC dampened type I IFN induction by human pDCs as well in response to TLR9 ligand *in vitro* (**Supplementary Figure S10A**). On the other hand gallein had no effect on IFNα induction by human pDCs in the absence of exogenous lactate (**Supplementary Figure S10B**), as expected, since it is known to inhibit the cell surface receptor mediated arm of lactate signaling and should not affect endogenous lactate-mediated signaling. Thus, it was evident that in 4T1 syngeneic breast tumor model in mice too, lactate mediated immunosuppression of intratumoral type I IFN induction do occur and inhibition of the two major arms of lactate signaling causes increased interferon signaling. When we compared the percentage of $FoxP3^+$ $CD4^+$ T cells between the control and the treated groups, we could see significant decrease in the percentage of $FoxP3^+$ $CD4^+$ T cells when both gallein and ARC were injected (**Figures 7H,I**). Thus, we found that the lactate-induced functional reprogramming of human pDCs was also operative in this syngeneic mouse tumor model *in vivo* and interference with the identified pathways affected tumor growth.

Hence, to summarize, lactate produced by cancer cells inhibits type I IFN induction by pDCs by binding to GPR81 on pDC surface or via transport into pDC cytosol via MCT. In addition, once transported into pDC cytosol, lactate enhances tryptophan catabolism and kynurenine production by pDCs thus enhancing Treg expansion (**Figure 8**).

DISCUSSION

Tumorigenesis does trigger anti-tumor immune activation, and the interaction evolves through a phase of relative equilibrium before finally getting subverted by the proliferating tumor cells (1). Gradual development of an immuno-suppressive milieu in the micro-environment in addition to immunoediting of the cancer cells contributes to cancer growth and eventual immune escape. Metabolic adaptation and a characteristic cellular respiration with glycolytic dominance play a crucial role in enabling cancer cells to balance between energy expenditure and macromolecular biosynthesis to support high rates of proliferation (21). Lactic acid is the major metabolite released by the cancer cells as a consequence of these metabolic adaptations (26). Several studies now show that this metabolite can actually link the pro-growth metabolic adaptations to immune-suppression in cancer (27), with evidence for suppressive effects against myeloid cells (28, 30), NK cells and cytotoxic T cells (29). Here we show that lactate also attenuates IFNα induction in response to TLR ligands by pDCs, the most important cellular producers, thus evading a critical component of anti-tumor immunity. Lactate also enhances the ability of pDCs to induce regulatory T cells which are

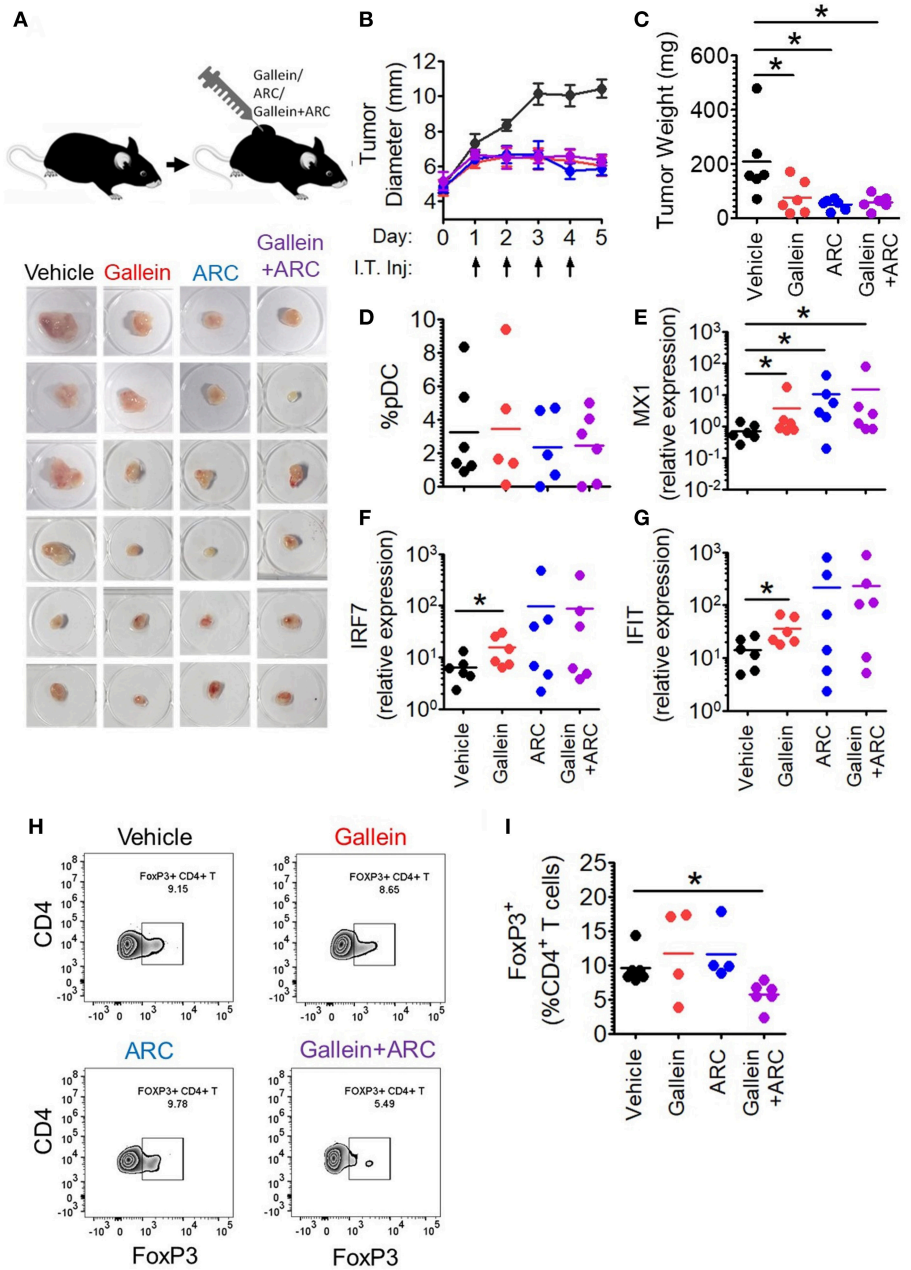

FIGURE 7 | Intratumoral gallein and ARC recovers type I IFN induction and reduces Treg expansion in syngeneic breast cancer model in mice. **(A)** 4T1 tumor bearing BALB/c mice were divided into 4 groups each having 6 mice. Daily intratumoral injection (5 μl) of either PBS, 160 ng gallein alone, 18.5 ng ARC alone or ARC in combination with gallein was done for each mouse for 4 consecutive days. **(B)** The diameter of the tumors was recorded each day with a vernier caliper and compared between the different groups. Data is from 3 independent experiments and represented as Mean ± SEM. **(C)** On the 5th day mice were sacrificed and tumors excised, weighed and imaged. Data is from 3 independent experiments. One-tailed unpaired *t*-test was done. **(D)** Tumors from each group of mice were digested and the cellular suspension was stained with anti-mouse CD45 PerCP, Siglec-H BV421, and PDC-TREM PE antibodies before being subjected to flow cytometry to determine the percentage of pDCs. Data is from 3 independent experiments and one-tailed unpaired *t*-test was done. **(E–G)** Real-time PCR was done to measure the expression levels of 3 ISGs-MX1 **(E)**, IRF7 **(F)**, and IFIT **(G)** in tumor tissue. Mann-Whitney test was done ($n = 6$ from 3 independent experiments). **(H,I)** Staining with anti-mouse CD45 PerCP, TCR-β APC-Cy7, CD4 APC, and FoxP3 V450 (intracellular staining) was done to measure the percentage of intratumoral $FoxP3^+$ $CD4^+$ T cells by flow cytometry. Data is from 3 independent experiments and two-tailed unpaired *t*-test was done (*$p < 0.05$).

well-known components of an immunosuppressive milieu in the TME of multiple types of solid tumors associated with a poor prognosis.

PDC infiltration into the tumor bed has been associated with poor prognosis in breast cancer and pDC-driven expansion of regulatory T cells has been implicated in this

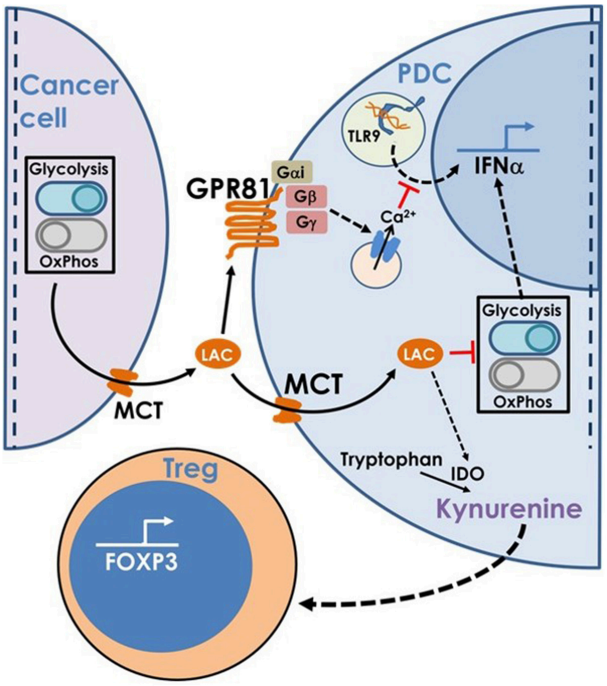

FIGURE 8 | This model is based on our findings that the excess lactate secreted by cancer cells as a result of characteristic adaptations in their metabolic profile, leads to suppression of type I IFN induction in pDCs, via two distinct cellular mechanisms. Binding of lactate to GPR81 receptor on pDC surface leads to intracellular calcium mobilization which inhibits IFNα production as well as influences the glycolytic switch. Import of lactate through monocarboxylate transporters and its subsequent accumulation in the pDC cytosol also inhibits the CpGA induced glycolytic switch essential for IFNα production. Apart from this, lactate transported into pDC cytosol also enhances tryptophan catabolism leading to excessive production of kynurenine which in turn leads to Treg expansion, thus adding to the overall immunosuppression in the tumor microenvironment.

immunosuppression (19, 20). But how do the tumors evade intra-tumoral type I IFN induction by pDCs, despite being rich sources of TLR9 and TLR7 ligands due to high cell turn over and cell death, is far from being clear. Suppressive role of tumor-derived TNFα and TGFβ on pDCs have been implicated in a previous study (20). We found here that cancer cell-derived lactate, through the cell surface lactate receptor GPR81 as well as via intracellular transport through MCTs, can potently inhibit TLR signaling in pDCs as depicted in the model. We presume that these two mechanisms either co-operate for this immune-evasion or one may become more dominant based on specific microenvironmental context (**Figure 8**). The GPR81-mediated inhibition was dependent upon Gβγ-dependent cytosolic free Ca^{2+} mobilization, without any effect of the Gαi signaling. This was not unexpected, as cAMP accumulation itself has previously been shown to inhibit type I IFN induction in intra-tumoral pDCs (42). On the other hand MCT-mediated cytosolic import of lactate interferes with the similar metabolic adaptation required by activated pDCs rendering them dysfunctional.

We also explored the effect of lactate on the ability of pDCs to induce Tregs since the presence of Tregs in the tumor microenvironment is strongly associated with poor prognosis. It has been proposed earlier that ICOSL overexpression on intratumoral pDCs enable them to expand Tregs through ICOSL-ICOS interaction (19, 40). But we found lactate does not drive ICOSL overexpression in human pDCs. Previous reports have also implicated tryptophan catabolism leading to the production of kynurenine as the causal factor for pDC mediated Treg induction (41) and kynurenine is known to induce Tregs via interaction with the aryl hydrocarbon receptor (43). We found that lactate enhances tryptophan catabolism and the production of kynurenine by pDCs which is strongly correlated with their increased ability to induce $FoxP3^+$ Tregs compared to untreated pDCs. Moreover, intratumoral pDCs showed the same characteristics of elevated tryptophan catabolism as well as FoxP3 induction, unlike peripheral pDCs of patients with breast cancer. Moreover, intraperitoneal injection of lactate abrogated IFNα induction thus confirming its inhibitory effect on IFNα production *in vivo*. Finally, intratumoral injection of a combination of lactate signaling pathway inhibitors gallein and ARC in 4T1 tumor mouse models, led to significant reduction in the percentage of intratumoral $FoxP3^+$ $CD4^+$ T cells, thus confirming the role of lactate in enhancing Treg induction.

We think that this oncometabolic-driven reprogramming of pDC function in a tumor is a critical immune-evasive mechanism, which in turn contributes to dysregulation of other innate and adaptive mechanisms of anti-tumor immunity. A critical role of type I IFNs in the regulation of NK cells is well-documented (44). Anti-tumor effects of NK cells have been shown to be critically regulated by type I IFNs (45). A potential role of type I IFNs in driving pro-inflammatory polarization of tissue-recruited macrophages has also been reported recently (16). Thus, cancer cell-derived lactate-driven attenuation of pDC function can actually result in multiple immune dysfunctions, contributing greatly to the immune escape of tumors. Same is true for intratumoral Treg expansion, for which intratumoral functional reprogramming of pDCs has been shown to be involved in previous reports as well (19, 20, 40). Administration of immune checkpoint blocking agents or TLR-agonists are promising immunotherapeutic strategies against cancer, but to circumvent issues with variability and multiplicity of immunosuppressive mechanisms more efficacious combinatorial strategies targeting microenvironmental factors are essential (46). The mechanistic insights into this lactate-driven attenuation of two critical components of anti-tumor immune response creates possibility of developing new generation therapies, targeting key events in this immuno-evasion pathway (e.g., the GPR81 receptor or the MCT transporters), that can further potentiate the usual anti-cancer immunotherapeutic strategies.

Thus, we could identify a hitherto unknown link between intra-tumoral abundance of the oncometabolite lactate, resulting from metabolic adaptation in cancer cells, and the pro-tumor tolerogenic reprogramming of plasmacytoid dendritic cells. We also could identify non-redundant role of the cell surface lactate receptor GPR81 and the monocarboxylate transporters in mediating the effects of lactate on pDCs, in terms of both

IFNα induction as well as ability to expand regulatory T cells, revealing new therapeutic targets for potentiating anti-cancer immune responses. We could validate our model by looking into tumor recruited pDCs from patients with breast cancer, as well as in a syngeneic model of breast cancer in mice.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

All experiments with patient samples were done as per recommendations of the Institutional Review Boards of Institute of Postgraduate Medical Education & Research, Kolkata, India as well as CSIR-Indian Institute of Chemical Biology (IICB), Kolkata, India, and after obtaining informed consent from the donors. The human study was conducted on de-identified samples. All animal experiments were done on approval of the Institutional Animal Ethics Committee of CSIR-IICB.

AUTHOR CONTRIBUTIONS

DG conceived and designed the study. DR did most of the experiments. RB, AG, and OR participated in the experiments with patient samples. CL, OR, and PB helped with the pDC functional assays and gene expression studies. SC helped in designing the *in vivo* experiments. BS, AG, JS, and RD'R helped with the *in vivo* experiments. SP helped with the LC-MS/MS studies. AD and DS recruited the patients. DG and DR wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01878/full#supplementary-material

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Immunological Regulation of Vascular Inflammation During Cancer Metastasis

Sheri A. C. McDowell[1,2] *and Daniela F. Quail*[1,2,3]*

[1] Department of Physiology, Faculty of Medicine, McGill University, Montreal, QC, Canada, [2] Rosalind and Morris Goodman Cancer Research Centre, McGill University, Montreal, QC, Canada, [3] Division of Experimental Medicine, Department of Medicine, McGill University, Montreal, QC, Canada

Metastasis is the predominant cause of cancer-related mortality, despite being a highly inefficient process overall. The vasculature is the gatekeeper for tumor cell seeding within the secondary tissue microenvironment—the rate limiting step of the metastatic cascade. Therefore, factors that regulate vascular physiology dramatically influence cancer outcomes. There are a myriad of physiologic circumstances that not only influence the intrinsic capacity of tumor cells to cross the endothelial barrier, but also that regulate vascular inflammation and barrier integrity to enable extravasation into the metastatic niche. These processes are highly dependent on inflammatory cues largely initiated by the innate immune compartment, that are meant to help re-establish tissue homeostasis, but instead become hijacked by cancer cells. Here, we discuss the scientific advances in understanding the interactions between innate immune cells and the endothelium, describe their influence on cancer metastasis, and evaluate potential therapeutic interventions for the alleviation of metastatic disease. By triangulating the relationship between immune cells, endothelial cells, and tumor cells, we will gain greater insight into how to impede the metastatic process by focusing on its most vulnerable phases, thereby reducing metastatic spread and cancer-related mortality.

Keywords: metastasis, microenvironment, vascular inflammation, innate immunity, endothelial adhesions

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***Correspondence:**
Daniela F. Quail
daniela.quail@mcgill.ca

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INTRODUCTION

Metastasis is a process through which primary tumor cells spread to secondary organs, and is the leading cause of cancer-related mortality. The metastatic process is composed of a number of sequential steps, each with varying levels of efficiency that together dictate whether successful metastases will form (1). Initially, cancer cells from a primary tumor acquire the capacity to invade into adjacent tissue and intravasate into the blood circulatory or lymphatic system. Within the circulation, cancer cells must survive in suspension and interact with the endothelium in order to extravasate into the secondary tissue parenchyma. In parallel, the endothelium becomes primed to allow cancer cells to transmigrate, and the pre-metastatic niche evolves into a fertile soil equipped to nurture metastatic cells. Upon arrival, cancer cells quickly adapt to the foreign niche, to enable their colonization and outgrowth within the new microenvironment. Each of these stages requires cancer cells to exhibit remarkable plasticity, allowing them to adapt to continuous changes and unfamiliar stimuli that are encountered within their surroundings.

The overall process of metastasis is highly inefficient, and early kinetic studies using experimental metastasis models have shown that the efficiency of each individual step of the metastatic cascade differs dramatically (2, 3). Early steps, such as local invasion and survival within the circulation, are very efficient; however, later stages that take place within the secondary niche are relatively inefficient. In cancer patients, although the frequency of circulating tumor cells is an independent predictor of overall survival (4), some patients with circulating tumor cells within their blood may never develop metastatic disease. This suggests that metastatic potential is partially influenced by the ability of circulating tumor cells to access the metastatic microenvironment (5–9). Therefore, understanding how the vasculature acts as the gatekeeper for metastatic disease is critical to limit disease progression.

The role of tumor cell-mediated mechanisms of extravasation during metastasis has been covered by several excellent reviews (10–12). Here, we discuss immune-mediated mechanisms of vascular physiology that influence extravasation efficiency, with a focus on innate immune mechanisms of vascular inflammation and metastasis. We first discuss how the structure of the endothelium mediates vascular inflammation (including permeability of the endothelium, and transmigration of leukocytes), and how chronic inflammatory conditions that have direct ties to cancer (e.g., obesity, smoking tobacco) can exacerbate these effects. We then discuss the role of vascular inflammation during cancer metastasis, and how cancer cells can hijack innate immune processes to enhance their metastatic behavior. Finally, we discuss how mechanisms of vascular inflammation can be targeted as a preventative approach for metastatic disease.

STRUCTURE AND FUNCTION OF THE ENDOTHELIAL BARRIER DURING VASCULAR INFLAMMATION

Blood vessels function as boundaries between blood and tissue, by regulating permeability, blood fluidity, and the flow of cells and other substances between the vascular system and tissues throughout the body. Generally, mature blood vessels consist of a monolayer of endothelial cells connected to one another through distinct junctional boundaries, which are further wrapped by pericytes or vascular smooth muscle cells that maintain structural support and integrity, and in most tissues, are enveloped by adipose tissue (**Figure 1A**). However, the intricacies of vascular endothelia architecture vary between different organs and vascular beds, resulting in differences in mechanisms of leukocyte trafficking during inflammation. For example, in the skin, muscle and mesentery leukocytes typically exit the blood in postcapillary venules, while in the lung and liver leukocytes exit the blood in the microvasculature, and in lymphoid organs leukocytes exit the blood in high endothelial venules (HEV); all of these endothelia have different structures, functions, and blood flow dynamics (13). Furthermore, there are differences in leukocyte trafficking between different leukocyte subsets. For example, innate immune

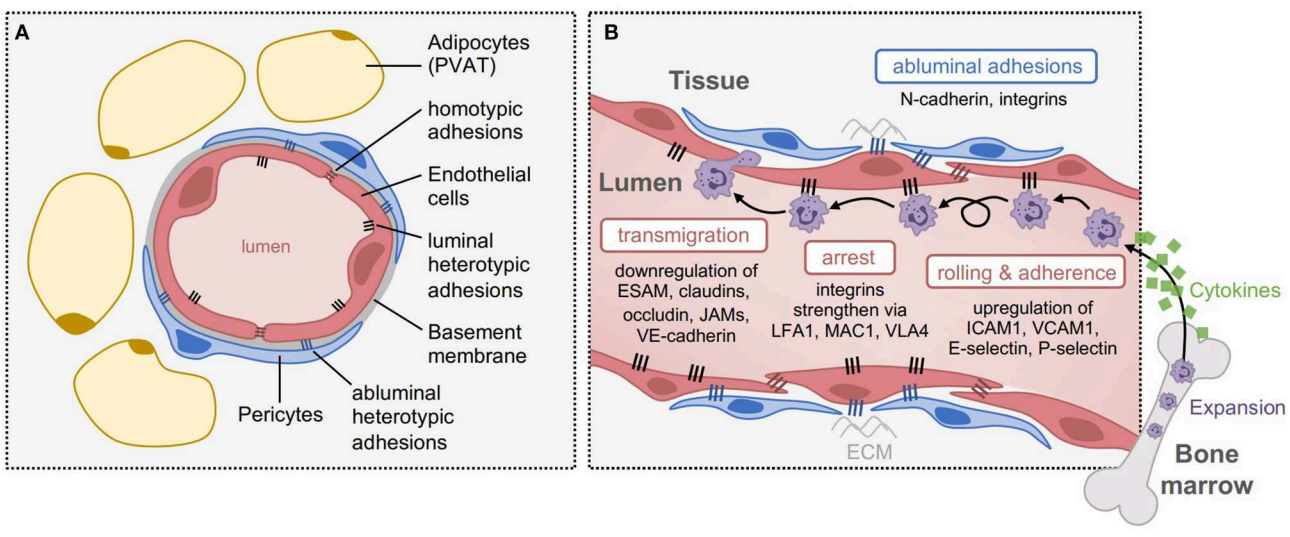

FIGURE 1 | Structure and function of the endothelium during vascular inflammation. **(A)** Structure of blood capillaries, with surrounding perivascular adipose tissue (PVAT). The lumen is formed by 1-2 endothelial cells that are sealed by homotypic junctional adhesions, including tight junctions and adherens junctions. The endothelial cells are bound to a specialized basement membrane, and enveloped with pericytes once mature. On their luminal side, endothelial cells express heterotypic adhesions that assist with cell attachment within the periphery, and on their abluminal side, they express distinct heterotypic adhesions (e.g., N-cadherin and integrins) that facilitate pericyte coverage and attachment to the extracellular matrix (ECM). **(B)** Adhesions involved in different steps of leukocyte transmigration. In response to inflammation, cytokines are released to cause expansion and recruitment of leukocytes from the periphery, such as neutrophils from the bone marrow. Upon arrival at the inflamed tissue, leukocytes roll and adhere to the endothelium through luminal adhesion proteins including upregulation of E-selectin, P-selectin, ICAM1, and VCAM1. To induce their arrest, integrins strengthen these interactions through interactions with LFA1, MAC1, and VLA4, which are expressed by leukocytes. Once arrested, transmigration can occur through endothelial junctions, by downregulating homotypic adhesions such as ESAM, claudins, occludin, JAMs, and VE-cadherin.

cells are structurally and functionally different from adaptive immune cells and thus the cellular and molecular mechanisms of recruitment and extravasation are distinct; this review will focus on the extravasation of innate immune cells—the first responders to inflammatory stimuli.

Under inflammatory contexts, the endothelium becomes activated to facilitate leukocyte recruitment into the affected tissue through a process called vascular inflammation (14, 15). During vascular inflammation, luminal endothelial adhesion proteins are upregulated to enhance leukocyte rolling, arrest and adherence to the endothelium, even when exposed to high shear stress (16), and in parallel, endothelial junctional adhesions are downregulated to enable leukocyte transmigration (17). It has been proposed that cancer cells mimic leukocyte transmigration to facilitate their extravasation into tissues, therefore, insights that are gained from leukocyte dynamics with the endothelium may be relevant to cancer metastasis.

Several canonical adhesion proteins regulate transmigration of cells across the endothelium (**Figure 1B**). Heterotypic endothelial adhesions regulate interactions between endothelial cells and their surrounding environment. On the luminal side, this includes interactions with circulating immune cells, which need to arrest and adhere to the endothelium prior to transmigration. These heterotypic interactions are mediated by a distinct set of luminal transmembrane adhesive proteins, such as selectins (e.g., E-selectin, P-selectin; leukocyte rolling) and Immunoglobulin (Ig)-like cell adhesion molecules (e.g., ICAM1, VCAM1; leukocyte arrest, firm adhesion, and crawling) (17). On the abluminal side, endothelial adhesions mediate interactions with pericytes and the extracellular matrix, such as neural (N)-cadherin, which regulates pericyte coverage and vessel maturity. By contrast, homotypic endothelial adhesions primarily function to regulate barrier integrity of the endothelium and vascular permeability, and are thus composed of proteins involved in tight junctions (e.g., junctional adhesion molecules (JAMs), claudins, and occludin) and adherens junctions (e.g., vascular endothelial (VE)-cadherin, which associates with the intracellular β-catenin protein) between endothelial cells (18). These adhesions play an important role specifically during the process of cellular transmigration. Collectively, both heterotypic and homotypic adhesion proteins act as gatekeepers of tissue homeostasis, and therefore, the plasticity of endothelial adhesion expression is essential to this phenotype.

Leukocyte Rolling, Adherence, and Transmigration Across the Activated Endothelium

Patrolling leukocytes move through blood vessels in a passive manner based on simple flow dynamics. However, under inflammatory conditions, leukocytes are attracted to specific tissues through cytokines that are produced in response to pathogen exposure and/or tissue damage (19). Once leukocytes arrive, infiltration into tissues is first initiated by rolling along on the activated endothelium, which is frequently mediated by selectin-based interactions between immune and endothelial cells. Endothelial cells express selectin proteins, such as P- and E-selectin, along with ligands for L-selectin (L-selectin is expressed on naïve leukocytes prior to activation), while leukocytes express glycosylated selectin ligands, such as P-selectin Glycoprotein Ligand-1 (PSGL-1; constitutively expressed by neutrophils) and E-selectin ligand-1 (ESL-1) (20, 21). Selectin-mediated rolling activates leukocytes by facilitating interactions with inflammatory chemokines from the activated endothelium such as interleukin-8 (IL8) (22) and platelet-activating factor (PAF) (23), which bind to chemokine receptors on leukocytes to initiate a signaling cascade resulting in the activation of integrins (20, 24, 25). Integrin-mediated signaling via lymphocyte function-associated antigen 1 (LFA1; expressed by all leukocytes), macrophage antigen 1 (MAC1; expressed by monocytes/macrophages), and very late antigen 4 (VLA4; expressed by lymphocytes and monocytes) increases the affinity of immune cells for the endothelium, allowing for more firm and stable adherence, in preparation for subsequent transmigration across the endothelial barrier (26). In addition, leukocytes may crawl toward suitable emigration sites prior to extravasation, depending on the inflammatory phenotype of the endothelium, as well as the activation state and type of leukocyte (27). For example, intravital videomicroscopy of murine postcapillary venules has shown that following adhesion to the endothelium, neutrophils crawl intraluminally to sites of extravasation prior to transmigration (27). Thus, luminal endothelial adhesion proteins are the first line of regulation of peripheral cell infiltration into tissues.

Of note, platelets (cell fragments derived from megakaryocytes from bone marrow) also play a role in the extravasation of leukocytes. They typically function to form blood clots, but more recently have been shown to play a role in vascular inflammation (28). In addition to being able to interact with both immune and endothelial cells, a novel role for platelets in efficiently directing neutrophils and inflammatory monocytes to sites of extravasation has been identified, whereby platelets interact with endothelial cells and arrest neutrophils upon initiation of an inflammatory stimulus (29). This interaction then mobilizes inflammatory monocytes to these specific locations, facilitating the successful extravasation of both neutrophils and inflammatory monocytes into the tissue parenchyma.

Once leukocytes establish tight adhesions at endothelial junctions, they begin the process of extravasation known as diapedesis (30). Diapedesis most often occurs through a paracellular pathway (i.e., in between cells of the endothelial barrier). This is mainly regulated by changes in vascular permeability via tight junctions, including downregulation of endothelial cell-selective adhesion molecule (ESAM) (31), and tight JAMs, JAMA, JAMB, and JAMC (30, 32, 33). Less frequently, leukocytes may also transmigrate through the transcellular pathway (i.e., through the endothelial cell body), which is dependent on the formation of transmigratory cup-like projections that are enriched for ICAM1 and VCAM1 (34). Given that vascular inflammation usually requires a more rapid response rate, regulation of adhesion molecule expression is usually done at the post-translational level. For example, this can be achieved via proteolytic cleavage induced by innate immune cells within the microenvironment, such as neutrophil-derived

neutrophil elastase (NE) (35). Other methods of regulation include the post-translational modification of integrins, along with changes in the storage and release of selectins to the cell membrane, specifically P-selectin. P-selectin, is stored in Weibel-Palade bodies (WPB) in endothelial cells and becomes recruited to the cell membrane upon inflammatory signals (36). Cell adhesion molecules, such as ICAM1 and VCAM1, can be regulated through changes in expression. For example IL1β-, tumor necrosis factor-α (TNFα)-, or lipopolysaccharide (LPS)-stimulated endothelial cells can induce expression of VCAM1 and enhance expression of ICAM1 (37). It is important to note that extravasation not only mediates leukocyte recruitment to sites of inflammation, but also regulates leukocyte phenotype. This enables leukocytes to be better equipped to pursue further migration and specific immune functions, for example, increased survival and pathogen-killing activities (38).

Once leukocytes permeate the endothelial cell barrier they encounter the endothelial basement membrane network made up of protein laminins (e.g., laminin-8 and laminin-10), collagen type IV, nidogens, and heparan sulfate proteoglycan perlecan (39). In the majority of venules, leukocytes will also encounter the pericyte sheath and perivascular tissue. Neutrophil migration through the basement membrane and pericyte sheath has been shown to occur at sites with low extracellular matrix protein accumulation, specifically laminin-10, collagen IV and nidogen-2, and between neighboring pericytes in murine cremasteric venules (40). Similarly, monocytes have been shown to use comparable methods to cross the basement membrane in CCL2-stimulated murine cremaster muscles (41).

Taken together, each of these factors that regulate transmigration of cells across the endothelium may have relevance to cancer, if similar mechanisms are used by tumor cells.

Factors That Regulate Vascular Inflammation and Barrier Integrity

There are numerous factors that regulate endothelial adhesions, and as a consequence influence vascular inflammation and permeability. Many of these factors play a crucial role in physiologic oxidative functions of innate immunity, to facilitate subsequent amplification of inflammatory safeguards when pathogens or tissue damage are detected. For example, activated neutrophils produce reactive oxygen species (ROS) during vascular inflammation which can have effects on the surrounding tissue microenvironment, notably endothelial junctional integrity (15). Activated porcine neutrophils cultured with endothelial monolayers have been shown to enhance endothelial permeability by altering phosphorylation of VE-cadherin and β-catenin, resulting in conformational changes to the adherens junctions that disrupt endothelial barrier function (42). Of note, the VE-cadherin-catenin complex in adherens junctions can be regulated by ROS via induction of phosphorylation which promotes junctional disassembly (43), and is required for neutrophil transendothelial migration (44), highlighting an important link between neutrophils, ROS, and vascular permeability. Other innate immune cells such as macrophages can be a major source of vascular endothelial growth factor A (VEGFA) within the microenvironment, which can also induce oxidative stress and vascular permeability by phosphorylating VE-cadherin (45) or causing its endocytosis (46). In mouse models of sterile injury, leukotrienes have also been shown to act on neutrophils to induce their release of NE to cleave JAM-C (35). Interestingly, intravital microscopy has shown that neutrophil communication with the endothelium in this manner can also enable reverse transmigration of neutrophils from local tissues back into the peripheral circulation (35, 47), however, it is unclear if this process serves to resolve local inflammation, or to amplify systemic immunological responses. Taken together, while these inflammatory responses function as an acute protective mechanism for the host, chronic vascular inflammation can be detrimental.

Indeed, there are numerous pathological conditions that can aberrantly weaken the endothelial barrier, which have strong ties to cancer. This can leave the host prone to immune exhaustion, disruption of tissue homeostasis, edema, or nutrient imbalance, and ultimately may modify the ability of cancer cells to extravasate into secondary tissues. For example, obesity is a chronic inflammatory condition that is linked to numerous co-morbidities that affect the vascular system, such as hypertension, coronary artery disease, and stroke, and is associated with enhanced cancer incidence (48) and mortality (49). In fact, obesity is thought to be responsible for up to 20% of cancer-related deaths in adults (49), making it a leading risk factor for cancer mortality. Indeed, there exists an intimate relationship between adipose tissue and the vascular system, both anatomically and functionally, as the majority of blood vessels are enclosed by perivascular adipose tissue (PVAT), which plays a role in guiding vascular function and homeostasis by releasing a myriad of bioactive adipokines and cytokines (50). Under normal physiologic conditions, PVAT secretes anti-inflammatory factors and hormones, such as adiponectin, which have protective effects on the cardiovascular system (51). However, during weight gain, adipocytes within PVAT exhibit impaired differentiation and increased expression of pro-inflammatory cytokines, such as interleukin-6 (IL6), IL8, and monocyte chemoattractant protein-1 (MCP1) (52), leptin production (53, 54), and oxidative stress (55) which lead to vascular dysfunction.

In addition to the direct effects of PVAT on adjacent endothelium, obesity-associated adipose tissue can also have systemic effects that influence vascular function. For example, in lung (one of the most frequent sites of cancer metastasis), mouse models have shown that obesity impairs vascular homeostasis when adiponectin levels drop, characterized by an increase in the expression of luminal adhesions including ICAM1, VCAM1, and E-selectin, and a decrease in endothelial adhesions such as VE-cadherin (56). These changes increase neutrophil influx into the lung parenchyma and enhance susceptibility to lung injury by LPS (a side effect of the leaky gut epithelium), which can be attenuated by hydrodynamic adiponectin gene delivery (56). In humans, obesity is similarly associated with oxidative stress and endothelial activation, as assessed by increased plasma levels of oxidized low-density lipoprotein, C-reactive protein, and soluble

forms of ICAM1 and E-selectin (57, 58). In porcine models of diet-induced obesity, high-fat diet is associated with elevated superoxide species, nitrotyrosine and NADPH-oxidase subunits in the coronary endothelium, in concordance with enhanced myocardial microvascular permeability prior to the development of insulin resistance (59). These data suggest that oxidative stress and vascular dysfunction may precede the chronic inflammatory effects of obesity that present with the onset of metabolic syndrome. Given the association between obesity and cancer mortality, these findings raise the possibility that obesity-associated vascular inflammation may facilitate tumor cell transendothelial migration, akin to its effects on leukocytes.

Surpassing the effects of obesity on cancer mortality risk, cigarette smoking remains the leading risk factor for lung cancer, and remarkably, is responsible for ∼22% of all cancer-related deaths (60). In addition to cancer, smoking tobacco is associated with numerous cardiovascular conditions including atherosclerosis, heart disease, and acute lung injury (61, 62), which is not surprising given the highly vascularized nature of lung tissue. Similar to obesity, smoking causes profound lung inflammation (e.g., increased IL10 and TNFα production, and accumulation of neutrophils and alternatively-activated macrophages), and susceptibility to LPS-induced acute lung injury (63), which together underlie vascular inflammation. In addition to direct effects on the lung capillaries, numerous studies have demonstrated that exposure to cigarette smoke is also associated with a reduction of vascular function in many tissues in the body, linked to aberrant nitric oxide (NO) production (64), an increase in inflammatory markers (e.g., TNFα) (65), and local recruitment of leukocytes to the endothelium (66). In fact, there is even evidence that certain chemical components of cigarettes can weaken endothelial junctions of the blood-brain barrier (67). This may in part explain the high propensity of lung cancer patients to exhibit metastatic disease to the brain compared to other primary malignancies, although this has not been formally tested. Given the causal connection between smoking tobacco and lung cancer incidence and mortality, the effects of smoking on vascular function may have multifaceted effects on cancer progression.

Taken together, chronic inflammatory conditions can mediate changes in endothelial cell homeostasis and alter vascular permeability, much in the same way that an acute inflammatory stimulus does. These conditions (and others) share a common theme of affecting vascular permeability through aberrant production of inflammatory mediators, and notably through enhanced oxidative stress. How these disease states and their corresponding effects on the vascular system affect metastatic efficiency, particularly during transmigration of tumor cells across the endothelial barrier, is a key question that remains largely unexplored.

VASCULAR INFLAMMATION DURING CANCER METASTASIS: INFLUENCE OF THE INNATE IMMUNE SYSTEM

It is well-accepted that inflammation can strongly impact tumor progression (68, 69). Similar to the consequences of vascular inflammation on permeability of the endothelium and leukocyte transmigration, it has been proposed that metastatic tumor cells can mimic leukocyte behavior and exploit the inflammatory effects of cancer to assist their spread to secondary organs (70) (**Figure 2**). This is achieved through upregulation of heterotypic adhesions on the endothelium to facilitate tumor cell rolling and transendothelial migration, and in parallel, weakening of vascular integrity to facilitate tumor cell crossing. Immune cells that are activated toward a pro-tumorigenic phenotype participate in both of these processes, by secreting pro-inflammatory factors that activate the endothelium. These immune cells are recruited to the perivascular microenvironment through tumor-derived factors, or in response to other underlying inflammatory conditions. Therefore, a comprehensive understanding of the mechanisms that mediate these processes may be useful to develop therapeutics to prevent metastatic progression.

Vascular Trapping, Luminal Adhesions and Tumor Cell Rolling

Numerous studies have investigated tumor cell extravasation using *in vivo* imaging techniques (71–73). As leukocytes are relatively small, they can comfortably roll along blood vessels during leukocyte trafficking. However, tumor cells can be much larger in diameter and may not be able to move through blood vessels as easily. Studies have investigated the relative contribution of physical trapping due to size constraints versus the distinct adhesion of tumor cells during shear-resistant arrest. Intravital videomicroscopy in mice has demonstrated that fluorescently labeled Chinese Hamster Ovary (CHO-K1) cells initially arrest in liver sinusoids following injection into the mesenteric vein due to mechanical trapping (72). Similarly, mechanical trapping and tumor cell arrest has been observed in melanoma and sarcoma models when vessel diameter was less than tumor cell diameter (71, 73). However, studies have also shown that tumor cells can arrest in capillaries in the absence of physical trapping by forming vascular adhesions. For example, colon cancer cells injected into rats were observed to arrest in microvessels even when vessel diameter was greater than tumor cell diameter (74). Similarly, both human HT-29 and murine CC531 colon cancer cells injected intraarterially into rats were shown to adhere to sinusoidal capillaries that were larger in diameter than the tumor cells themselves (75). Tumor cells may therefore become trapped in capillaries due to size-restriction, or form adhesions to the endothelium in the absence of mechanical trapping.

Once tumor cells are trapped in or adhere to blood capillaries, they must cross endothelial barriers. To achieve this, tumor cells utilize many of the same pathways that mediate leukocyte transmigration under inflammatory conditions, such as selectins and cell adhesion molecules (76, 77). Selectin-mediated rolling of tumor cells has been described, but appears to be less common than selectin-mediated leukocyte rolling prior to firm adhesion and extravasation. Nonetheless, rolling of human bone-metastatic prostate tumor cells has been reported, and relies on E-selectin expression on bone marrow endothelial cells and the complimentary expression of cognate ligands on the tumor cells (78). E-selectin-dependent tumor cell rolling on the endothelium

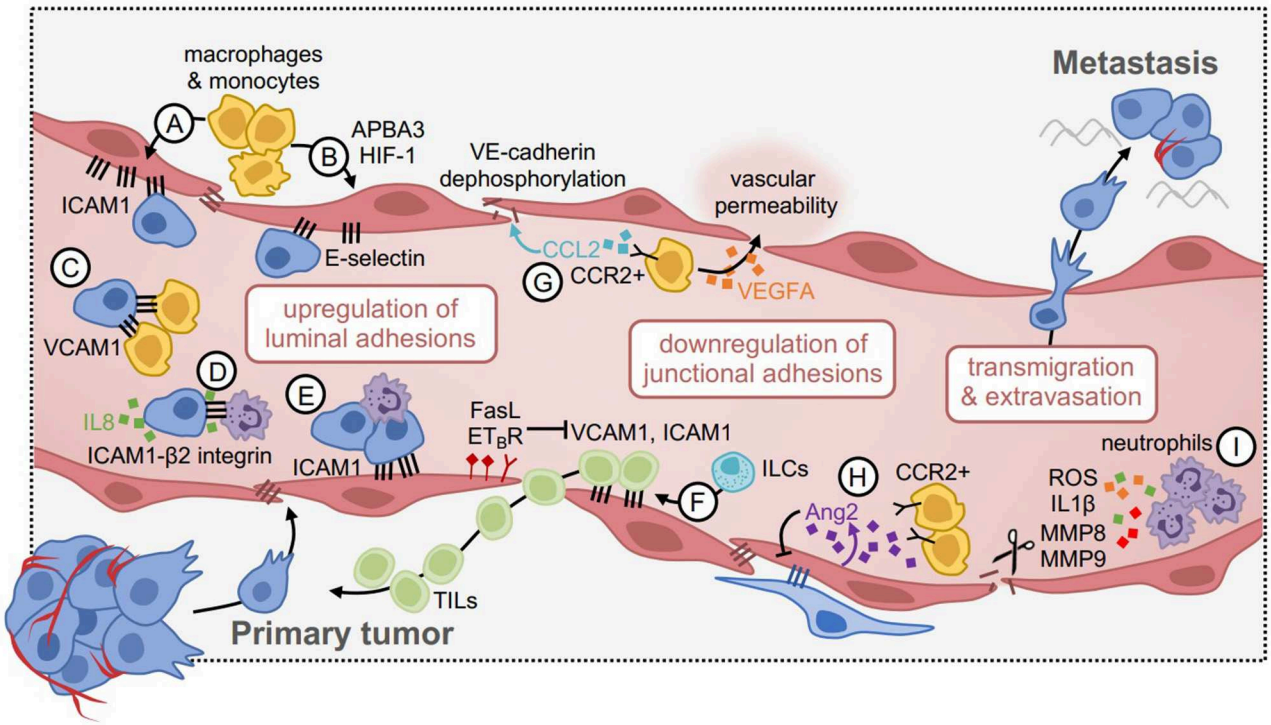

FIGURE 2 | Vascular inflammation during tumor cell extravasation and metastasis. **(A)** Inflammatory macrophages and monocytes induce the expression of ICAM1 and **(B)** E-selectin via APBA3-HIF1 signaling in endothelial cells. This enhances tumor cell attachment. **(C)** Tumor cells mimic the endothelium by upregulating VCAM1, which tethers them to β1 integrin-expressing macrophages and enhances metastatic seeding. **(D)** Similarly, tumor cells can upregulate ICAM1, and tethers to β2-integrin expressing neutrophils. This interaction is mediated by IL8 produced by tumor cells, which promotes neutrophil recruitment. **(E)** Neutrophil-tumor cell clusters enhance attachment to the endothelium via ICAM1 under flow conditions. **(F)** Some positive effects of vascular inflammation include enhanced recruitment of tumor-infiltrating lymphocytes (TILs), through upregulation of VCAM1 and ICAM1. This pathway can be negatively regulated, for example via FasL and ET_BR, such that blocking these factors can improve TIL delivery to tumors. **(G)** CCR2+ monocytes are attracted to the metastatic niche in response to CCL2 expressed by tumor cells, where they produce VEGFA to increase vascular permeability. CCL2 can also act directly on the endothelium by dephosphorylating VE-cadherin, disrupting junctional integrity, and increasing tumor cell transmigration. **(H)** Angiopoietin 2 (Ang2) expression by endothelial cells reduces pericyte coverage and promotes the recruitment of CCR2+ monocytes, which promote permeability. **(I)** Neutrophils produce proteolytic and inflammatory factors (e.g., ROS, IL1β, MMP8, MMP9) to disrupt endothelial junctions and increase vascular permeability in cancer models.

following TNF activation has also been described for breast and colon cancer cells (79). However, breast and prostate cancer cells have been shown to express Thomsen-Friedenreich antigen to mediate their arrest on the endothelium via interactions with galectin-3 (80). Furthermore, prostate cancer cell expression of selectin ligands does not correlate with selectin-mediated adhesion to the endothelium (81). This suggests that tumor cells may express selectin ligands, but may not necessarily use them for initial tethering and rolling on the endothelium. Thus, whether selectin-mediated adhesions are requisite for tumor cell binding to the endothelium and extravasation remains unclear.

Tumor cells may also utilize mechanisms initiated by innate immune cells within the microenvironment, which can activate vascular inflammation. For example, macrophages and monocytes have been shown to influence endothelial activation by regulating the expression of luminal adhesions such as ICAM1 (82, 83). In syngeneic melanoma models, glycolytic macrophages upregulate the expression of E-selectin on the endothelium through HIF-1 and its activator APBA3, such that APBA3 depletion in monocytes reverses this effect in association with reduced metastasis to lung (84). In breast cancer models, tumor cells mimic the inflammatory state of the endothelium via endogenous expression of VCAM1, which tethers them to macrophages expressing α4β1 integrin that promote metastasis to the lung (85). Surprisingly, VCAM1 depletion in tumor cells had no influence on the ability of cancer cells to cross the endothelium, rather, this vasculogenic mimicry phenotype enhanced the ability of cancer cells to colonize and remain viable within the secondary niche. This is consistent with reports that the perivascular space acts as a specialized reservoir for cancer stem cell viability (86), and also regulates dormancy in the metastatic setting (87). It is thus conceivable that the viability and/or growth of cancer cells could be influenced by adhesion factors expressed by the adjacent endothelium within this niche, in addition to the capacity for transmigration.

As with vascular permeability, neutrophil-supplied factors can also influence the expression of luminal adhesions that facilitate

tumor cell rolling and attachment to the endothelium as they travel through the circulation. For example, *in vitro* microfluidic models of the human microvasculature have shown that LPS-stimulated neutrophils and melanoma cells form aggregates under flow conditions, and arrest on the endothelium in part due to neutrophil-endothelial cell interactions via ICAM1. This heterotypic clustering mechanism could be reversed by blocking ICAM1 on vessels or tumor cells, however, endothelial-specific ICAM1 blockade was much more potent, suggesting that ICAM1 enables tumor cell attachment through both direct and indirect mechanisms (88). Similarly, in mouse models of melanoma, melanoma-specific expression of ICAM1 facilitated tumor cell-neutrophil interactions via β2 integrin on neutrophils, which facilitated attachment to the endothelium in the secondary lung microenvironment (89). This was dependent on IL8-secretion by melanoma cells, a potent neutrophil chemokine, indicating that tumor cells manipulate their environment to support their own progression. Taken together, heterotypic endothelial adhesions appear not only to enable tumor cell adherence to the endothelium during metastasis, but also enable tumor cell tethering to innate immune cells within the microenvironment which further support transmigration.

Although luminal adhesions can facilitate tumor cell extravasation during metastasis, they can also improve anti-tumor immunity by facilitating immune cell access to the tumor niche. For example, in a mouse model of melanoma, NKp46+ innate lymphoid cells (ILCs) upregulate vascular adhesions such as ICAM1 and VCAM1, which facilitate the infiltration of additional immune cells with anti-tumor functions (90). In mice lacking NKp46+ ILCs, this phenotype was reversed. In mouse models of ovarian cancer, overexpression of the endothelin B receptor (ET_BR) negatively regulates ICAM1 expression on the endothelium and limits the ability of T cells to access the tumor, such that inhibition of ET_BR improves T cell infiltration in an ICAM1-dependent manner (91). Others have shown that expression of Fas ligand (FasL) on the endothelium restricts leukocyte extravasation across the vascular barrier, including CD8+ T cells (92) and mononuclear cells (93), such that targeting FasL reverses this effect. These studies suggest that broadly targeting mechanisms of transmigration in the context of cancer would unlikely yield positive benefits; although this may reduce tumor cell extravasation, it may also restrict the infiltration of anti-tumor immune cells.

Vascular Integrity and Permeability

Enhanced vascular permeability through downregulation of endothelial adhesions has been shown to influence the ease of tumor cell transmigration. While this can be regulated by a number of different factors, innate immune cells that are upregulated in response to tumor progression appear to play an important role. During tumor progression, macrophages, neutrophils, and various other myeloid cell types accumulate in both the primary tumor microenvironment and secondary niche. These cells contribute to a pro-inflammatory milieu that mimics normal responses to pathogen exposure, however, in the context of cancer, they can inadvertently facilitate dissemination (69). For example, in mouse models of breast cancer metastasis, CCR2+ inflammatory monocytes are attracted to the metastatic microenvironment by CCL2-producing tumor cells, where they promote vascular permeability and extravasation in a VEGFA-dependent manner (94). Tumor-derived CCL2 has also been shown to act directly on the endothelium to promote its activation, resulting in enhanced monocyte recruitment, dephosphorylation of VE-Cadherin, reduced tight junction integrity, and a consequential increase in tumor cell transmigration (95). Consistently, others have shown in mouse models of breast and lung cancer that inhibition of angiopoietin-2 (Ang2) (which is produced by the activated endothelium) in the post-surgical adjuvant setting improves pericyte coverage of the endothelium and reduces CCR2+ macrophage accumulation within secondary sites, leading to reduced metastatic progression (83). Therefore, the accumulation of inflammatory monocytes/macrophages that coincides with metastatic progression may dually serve to weaken endothelial barriers and enable additional tumor cells to access the metastatic niche. This may be in part due to the armamentarium of proteases that macrophages produce, which can cleave adhesions between endothelial cells. This has even been shown in mouse models of breast cancer metastasis through the blood-brain barrier, which is weakened by Cathepsin S production even though it should otherwise be a tight barricade to exclude peripheral cells and inflammatory factors from being able to access the brain parenchyma (96).

Neutrophils are another potent source of cytokines and proteases (most notably MMPs, NE, and cathepsin G) that can trigger vascular inflammation. This is an essential function so that neutrophils can rapidly access tissues as the first line of defense in the innate immune system (17). In mouse models of breast cancer metastasis, neutrophils promote metastasis by impairing the tumor-clearance capacity of NK cells in the circulation, and by releasing elevated levels of IL1β, MMP8, and MMP9 into the microenvironment, which increase vessel permeability (97). Additionally, in mouse models of melanoma and Lewis lung carcinoma, lung metastasis is enhanced in LPS-instilled lungs through the local recruitment of neutrophils, and their subsequent degranulation to release NE and cathepsin G (98). This causes protease-mediated degradation of the adhesion protein thrombospondin-1 and results in enhanced lung metastasis. ROS production by neutrophils has also been shown to promote tumor metastasis, through induction of neutrophil extracellular traps (NETs) (99–102); NETs may promote metastasis by trapping tumor cells (99) and/or by remodeling the extracellular matrix to awaken dormant tumor cells (102). ROS production by neutrophils has also been shown to promote tumor metastasis through the suppression of T cell immunosurveillance (103, 104). However, the role of neutrophil-ROS in vascular permeability during tumor metastasis specifically is less understood, despite its known role during inflammation. Therefore, cytokines, proteases and ROS that are produced by neutrophils to facilitate peripheral recruitment of immune cells during normal inflammatory responses may similarly facilitate peripheral recruitment of tumor cells. Thus, the ability of tumor cells to stimulate the accumulation and activation of neutrophils within the

microenvironment (97, 98, 105–109) represents a critical way that tumors highjack and manipulate their niche to support their own progression.

Given the role of platelets during leukocyte extravasation, it is not surprising that they have similarly been shown to influence tumor cell extravasation. In murine models of experimental lung metastasis, platelet-tumor cell interactions promote tumor cell extravasation through the secretion of TGF-β from platelets and the subsequent activation of Smad and NFκB signaling within colon and breast carcinoma cells (110). This facilitates progression to an invasive mesenchymal-like phenotype and metastatic progression. Platelets have also been shown to recruit granulocytes to colon tumor cells within the lung in murine models of experimental lung metastasis, allowing for the formation of "early metastatic niches" in the lung microenvironment (111). Furthermore, using both *in vitro* Transwell assays and murine spontaneous lung metastasis assays, platelets activated by melanoma or lung tumor cells facilitated tumor cell transendothelial migration and extravasation via the secretion of adenine nucleotides (112). This promoted the opening of the endothelial barrier by acting on the endothelial $P2Y_2$ receptor, supporting metastasis. Thus, platelets can modulate tumor cells, innate immune cells and/or the endothelium to facilitate the metastatic process.

TARGETING INNATE IMMUNITY TO IMPROVE VASCULAR INTEGRITY AS CANCER THERAPY

There are several therapeutic approaches that may be useful to minimize chronic vascular inflammation and thus impede the ability of tumor cells to access the metastatic niche. One obvious approach is to target the vasculature directly, for example through anti-angiogenic strategies like bevacizumab (a VEGFA neutralizing antibody). However, while preclinical studies using anti-VEGFA antibodies showed great success leading to their clinical development (113), bevacizumab only improved progression-free survival, but not overall survival, in clinical trials for metastatic breast cancer (114–117). Although limiting nutrient delivery to tumors may seem logical to restrict viability and growth, crude attempts to broadly ablate the tumor vasculature may mitigate the beneficial effects of the blood vessels, such as leukocyte infiltration, oxygenation, and drug delivery. Vascular normalization strategies that aim to improve vascular maturation and integrity have been proposed as an alternative to anti-VEGFA treatments (118). For instance, preclinical studies have shown that VEGFR2 antibody blockade using DC101 can normalize the structure of the tumor-associated endothelium by improving the quality of the basement membrane and enabling improved pericyte coverage (119, 120). Whether these normalization strategies will be effective in the context of metastatic cancer, and how this will influence tumor cell interactions with the endothelium, have yet to be determined.

Given the potentially beneficial effects of luminal adhesions in bringing specific types of immune cells into tumors to enhance anti-tumor immunity, disrupting endothelial cells broadly may not be an optimal therapeutic approach. Several methods to enhance anti-tumor lymphocyte-specific recruitment have been proposed (121). For example, in ovarian cancer patients, ET_BR expression correlates with low tumor infiltrating lymphocytes, and experimental models have shown that pharmacological blockade of ET_BR with BQ-788 enhances T cell infiltration into tumors by modifying the endothelial barrier via a NO- and ICAM1-dependent mechanism (91). Importantly, rendering tumors "immune hot" through this method enhanced response to immunotherapy via cancer vaccination, whereas control tumors remained unresponsive (91). Interestingly, studies have also shown that VEGFA induces the expression of luminal adhesion proteins on endothelial cells, including ICAM1, VCAM1, and E-selectin, and that this can be blocked using an NFκB inhibitor, pyrrolidine dithiocarbamate (PDTC) (122), a chemical compound that dually serves as an oxygen radical scavenger. If these adhesions play a functional role in anti-tumor lymphocyte recruitment, this may partially contribute to the limited effects of anti-VEGFA therapies. Together these studies and others support the notion that endothelial barrier phenotypes and immune-surveillance are two intimately linked components of an immunoregulatory program in cancer, and that reprogramming the endothelium to enable leukocyte entry into tumors may have beneficial anti-tumor effects (123). This becomes particularly relevant in the context of cancer immunotherapy, as "immune-hot" tumors (i.e. those with high abundance of tumor-infiltrating lymphocytes) are more likely to respond to immune checkpoint blockade. Of note, the endothelium itself is capable of expressing checkpoint molecules that can negatively regulate T cell responses, including PDL1, PDL2, and TIM3 (124–126); whether endothelial-specific expression of these factors functionally influences response to immune checkpoint blockade remains uncertain.

Alternatively, there may be therapeutic opportunities to target innate immune cells in the microenvironment that both regulate vascular phenotypes, and dually act on tumor cells directly to promote progression. For example, several studies have shown that neutrophil depletion through antibody blockade can reverse metastasis of breast cancer (106, 108), including in the experimental setting where metastasis is assessed after 48 h following tail vein injection (potentially representative of extravasation) (97, 127). Alternatively, pharmacologic inhibition of CXCR2 has also been explored as a therapeutic approach to limit neutrophil infiltration and improve T cell infiltration in association with reduced metastatic progression in pancreatic models (128), which may help mitigate chronic oxidative and proteolytic effects on the endothelium. Indeed, pharmacologic agents targeting CXCR2 such as AZD5069 are now being explored in the clinical setting for metastatic cancer. In addition, Tie2-expressing monocytes/macrophages can trigger angiogenesis and vascular activation by inducing the expression of ICAM1 on the endothelium through interactions with its ligand, Ang2 (83, 129, 130), and several compounds that inhibit the Ang2-Tie2 axis are now being explored in the clinical setting for metastatic cancer including in the context of improving response to immune checkpoint inhibitors (131). Taken

together, these trials demonstrate the clinical relevance of targeting vascular inflammation in cancer patients to improve metastatic outcomes.

CONCLUSIONS AND FUTURE PERSPECTIVES

Innate immunity and vascular inflammation are two intimately connected biological processes that rely on one another to mediate physiologic responses to infection/inflammation. However, these intricate networks become undone in the context of cancer, and can be amplified by chronic inflammatory states. Given the complex nature of cell-cell interactions within the tumor microenvironment, consideration of all cellular players during different stages of the metastatic cascade is critical in order to optimize disease outcomes. Broadly inhibiting specific cell types is unlikely to yield favorable benefits; rather, reprogramming the microenvironment to work favorably and productively is key to improving survival. The endothelium in particular regulates multifaceted aspects of the microenvironmental landscape in all tissues throughout the body, as it is the gatekeeper of immune cell transmigration, nutrient and oxygen delivery, and a critical source of systemic soluble factors. Cancer hijacks these critical roles, and takes advantage of vascular plasticity to support disease progression. Therefore, by improving our understanding of normal physiologic functions of blood vessels and their interactions with regulatory cells within their environment, we will be able to improve our ability to target specific aspects of extravasation and metastasis by reprogramming the microenvironment to our advantage.

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SM and DQ reviewed the literature and wrote the manuscript.

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Regulatory T Cells Control the Switch From *in situ* to Invasive Breast Cancer

Leandro M. Martinez[1], *Valentina Robila*[2], *Nicholas M. Clark*[1,3], *Wei Du*[1], *Michael O. Idowu*[2], *Melanie R. Rutkowski*[4] *and Paula D. Bos*[1,5]*

[1] *Department of Pathology, Virginia Commonwealth University School of Medicine, Richmond, VA, United States,* [2] *Anatomic Pathology Service, Department of Pathology, Virginia Commonwealth University School of Medicine, Richmond, VA, United States,* [3] *Integrative Life Sciences Doctoral Program, Virginia Commonwealth University, Richmond, VA, United States,* [4] *Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, VA, United States,* [5] *Massey Cancer Center, Virginia Commonwealth University School of Medicine, Richmond, VA, United States*

Ductal carcinoma *in situ* (DCIS) is a non-obligate precursor of breast cancer, and it only progresses to invasive breast cancer in around 40% of patients. While immune infiltrates have been observed in these early cancer lesions, their potential prognostic value is still unclear. Regulatory T (Treg) cells accumulate in advanced breast cancers, and predict poor outcome. We have shown before that ablation of Treg cells in established tumors leads to significant decrease in primary and metastatic tumor burden. In this work, we sought to investigate Treg cell function in the progression from non-invasive to invasive breast cancer lesions. To this end, we used the murine mammary tumor virus polyoma middle T (MMTV-PyMT) murine model of spontaneous, stage-wise breast carcinogenesis crossed to Foxp3^{DTR} knock in mice, allowing Treg cell ablation by administration of diphtheria toxin. Transient targeting of Treg cells at the *in situ* carcinoma stage resulted in a significant increase in the number of tumor-bearing mammary glands and size of growing tumors compared with control mice. Whole mammary gland mounts and histological examination confirmed larger emergent tumor area in Treg cell-ablated mice, and revealed that these tumors were characterized by a more advanced tumor staging, with presence of early invasion, increased desmoplasia and collagen deposition. Furthermore, Treg cell ablation increased the percentage of cancer stem/progenitor cells in the mammary compartment. Interestingly, Treg cell ablation resulted in increased inflammatory cytokines IL-4 and IL-5 with a concomitant reduction in classically activated tumor associated macrophages. This TH2-biased immune regulatory mammary inflammation was consistent with the enhancement in tumor promotion that we observed. Overall, our study demonstrates that Treg cells oppose breast cancer progression at early stages, raising a cautionary note regarding the consideration of immune intervention targeted at boosting immune responses for DCIS.

Keywords: regulatory T cells, non-invasive carcinoma, early stage breast cancer, immunosurveillance, immunotherapy

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Bonnie F. Sloane,
Wayne State University, United States
Oakley C. Olson,
Columbia University Irving Medical Center, United States

***Correspondence:**
Paula D. Bos
paula.bos@vcuhealth.org

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INTRODUCTION

While death from breast cancer has slowly declined in the past few years, mammographic screening has led to a dramatic increase in the detection of pre-invasive breast lesions in women (1–3). This paradoxical observation can be explained by the fact that only a low percentage of early breast disease progresses to invasive, metastatic carcinomas. Ductal carcinoma *in situ* (DCIS) is a heterogenous group of neoplastic lesions confined to the breast ducts, and can remain indolent for life in up to 60% of cases (2). Patients diagnosed with DCIS undergo breast-conserving therapy or mastectomy, frequently accompanied by radiotherapy and in some cases, hormonal therapy (4). Thus far, there are no reliable parameters to distinguish those cases that will progress, resulting in significant overtreatment (5). Furthermore, our sparse understanding of the mechanisms leading to the transition from pre-invasive to invasive cancer deprives patients from targeted therapies that could improve outcomes (6, 7). Therefore, identifying cellular or molecular drivers of early tumor invasion may lead to the identification of biomarkers that can reduce the overtreatment in low-risk invasive breast cancer patients, or actionable targets that enable early management of the disease (5).

Evidence of tumor-infiltrating lymphocytes paralleling disease progression suggests that the interactions of immune cells and tumor cells are important for tumor evolution (8). T cell presence is a positive indicator of good prognosis, suggesting an active involvement in immunosurveillance (8). On the other hand, suppressive Foxp3$^+$ regulatory T (Treg) cells, which represent a significant proportion of the CD4$^+$ population in tumors, have been shown to increase with tumor stage and correlate with poor prognosis in invasive carcinomas (9). We have demonstrated that ablation of Treg cells in advanced primary tumors induces a strong anti-tumor response, which is dependent on CD4$^+$ T cells and IFNγ (10). However, the role of Treg cells during the initial stages of breast cancer tumorigenesis remains obscure. In the present work, we addressed the effect of transiently ablating Treg cells during the non-invasive stage, using a spontaneous model of breast carcinogenesis driven by expression of the polyoma middle T oncogene from the murine mammary tumor virus LTR (MMTV-PyMT). Our results indicate that transient Treg cell ablation in *in situ* breast lesions results in acceleration of progression to invasive carcinoma, suggesting that Treg cell presence may be a positive prognostic indicator for pre-invasive breast cancer.

MATERIALS AND METHODS

Mouse Models

Foxp3$^{DTR-GFP}$ mice were a gift from A. Rudensky (Memorial Sloan Kettering Cancer Center, New York, NY). C57BL/6 *MMTV-PyMT* mice were generously provided by M.O. Li (Memorial Sloan Kettering Cancer Center, New York, NY). All animal protocols were reviewed and approved by VCU Institutional Animal Care and Use Committee (IACUC #AD10001219).

Primary Tumor Growth Evaluation

Primary tumor incidence and growth was monitored weekly by palpation of all mammary glands, and caliper measurements of the length (L) and width (W) of each tumor. Individual tumor volume was calculated using the formula $\pi LW^2/6$. For Kaplan-Meier analysis of disease-free survival, a mouse was no longer considered disease-free when the first tumor reached a diameter of 2 mm.

Histology

We restricted all histological analysis to the fourth pair of mammary glands. Whole mounts were obtained as described Rasmussen et al. (11). Briefly, mammary glands were resected and spread onto a glass slide, fixed in Carnoy's fixative for 4 h at room temperature, and progressively hydrated. Glands were then rinsed in tap water, stained in carmin alum overnight, dehydrated, and cleared in xylene. Glands were mounted with Permount and scanned using an Olympus BX51 + CAST2 Stereology System microscope. Tumor area was calculated as a percentage of total area using Image J software. Whole mounts were subsequently embedded in paraffin and sectioned at 5–7 μm thickness. Hematoxylin and eosin (H&E) staining was carried out following standard protocols. All histological analysis and scoring were performed by a blinded expert breast pathologist. Tumor staging was scored as percentage of each stage out of the whole tumor area, as described by Lin et al. (12). Histological characteristics such as intra-tumor inflammatory infiltrate, desmoplasia, and collagen deposition were scored using a 0–3 scale as: absent (0); scanty (1); moderate (2); or extensive (3). Collagen deposition was evaluated by Masson Trichrome stain following specific manufacturing recommendations (NavaUltra Masson Trichrome Stain Kit, cat IW-3006). For alpha smooth muscle actin (α-sma) immunofluorescent staining, sections were deparaffinized, and rehydrated. Antigen retrieval was performed by incubation in citrate buffer (0.01 M, pH 6) at 60°C for 30 min. Tissues were then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h, and incubated with eFluor 660 conjugated primary antibodies anti-α-sma (1/100, 50-9760-82, eBioscience). Slides were then washed in PBS and mounted with DAPI containing Vectashield medium (H-1500, Vector). All images were acquired on a Microbrightfield-Neurolucida System microscope.

Flow Cytometry

Efficiency of systemic and local Treg cell ablation over time was evaluated in peripheral blood and mammary gland, respectively, by calculating the frequency of CD4$^+$ Foxp3$^+$ cells. For all flow cytometric analysis of mammary glands, the whole mammary gland was dissected, and central lymph node removed. Tissues were minced and enzymatically digested using 400 μg/ml Liberase TL (Roche) in a rotary shaker for 30 min at 37°C. Single cells were obtained by filtration through 100 μm cell strainers (Fisherbrand) and centrifugated at 300 × g for 5 min. Cells were incubated for 20 min in FC block (anti-CD16/32, Tonbo) on ice. Cells were then stained for 30 min on ice with specific antibody cocktails diluted in PBS with 0.5% BSA: violet Fluor 450-conjugated Ab anti-CD4 (1/500, 75-0042, TONBO), BUV395-conjugated

anti-CD45 (1/1000, 565967, BD Biosciences), eFluor 450-conjugated anti-CD24 (1/500, 75-0242, TONBO), red Fluor 710-conjugated anti-CD44 (1/500, 80-0441-U025, eBioscience), PE-Cy7-conjugated anti-CD49f (1/500, 25-0495-82, eBioscience), APC-conjugated anti-CD29 (1/500, 17-0291-82, eBioscience), FITC-conjugated anti-CD61 (1/500, 11-0611-82, eBioscience), APC-eFluor 780-conjugated anti-CD11b (1/1000, 47-0112-82, eBioscience), PE-Cy7-conjugated anti-Ly-6C (1/500, 25-5932-82, eBioscience), FITC-conjugated anti-Ly-6G (1/500, 127605, Biolegend), PerCP-Cy5.5 -conjugated anti-F4/80 (1/500, 45-4801-82, eBiosciences), redFluor™ 710-conjugated anti-MHC Class II (1/500, 80-5321-U025, TONBO), Alexa Fluor 647-conjugated anti-CD206 (1/500, MCA2235A647T, Serotec). Ghost Violet 510 viability dye (13-0870-T100, TONBO) was used to discriminate live/dead cells. For intracellular staining, cells were permeabilized using the FoxP3/Transcription Factor Staining Buffer Kit (TNB-0607-KIT, TONBO) according to the manufacturer's instructions, and stained using FITC-conjugated anti-FoxP3 antibody (11-5773-82, eBioscience). After staining, cells were washed and fixed in 2% paraformaldehyde. Flow cytometry was carried out using LSRFortessa-X20™ equipment (BD). Data analysis was performed using FlowJo 10.2 software.

In vivo Tumor Initiating Capacity

Single mammary cell suspensions from control and diphtheria toxin (DT)-treated mice were obtained from the mammary gland at 10 weeks of age, as previously described for flow cytometry. Briefly, 325,000 live cells were re-suspended in PBS and mixed at a 1:1 ratio in growth factor reduced Matrigel (BD). Cell suspensions were injected bilaterally into the fourth mammary gland of isoflurane-anesthetized C57BL/6 mice. Primary tumor growth was weekly monitored and tumors were harvested at the humane end-point.

In vitro Mammosphere Assay

Single mammary gland cell suspensions were depleted of hematopoietic cells by incubation with anti-CD45 (70-0451, TONBO), followed by Dynabeads® Sheep anti-Rat IgG according to the manufacturer's instructions (Invitrogen, 11035). Depletion was confirmed by flow cytometry using APC-conjugated anti-CD45.2 (20-0454-U025, TONBO). Mammosphere assay was performed as described by Boyle et al. (13), with a few modifications. Briefly, 2×10^4 freshly isolated $CD45^-$ mammary gland cells were seeded in triplicates into 96-well ultra-low attachment plates (Corning Inc.) pre-coated with poly(2-hydroxyethyl methacrylate) (P3932, Sigma) in a 1:1 mixture of DMEM and Ham's F12 medium (Sigma) supplemented with NeuroCult SM1 Neuronal Supplement (05711, StemCell Technologies), 20 ng/ml bFGF (78003.1, StemCell Technologies), 20 ng/ml EGF (78006, StemCell Technologies), 4 μg/ml heparin (07980, StemCell Technologies), penicillin-streptomycin and fungizone. Mammosphere cultures were incubated at 37°C for 7 days. At the end point, mammospheres of at least 40 μm diameter were counted under the microscope at 40X magnification. Digital images were used to calculate mammosphere size using Image J software.

Cytokine Analysis

Tumors were lysed in buffer containing 50 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM EDTA, and protease inhibitors. Cleared lysates were quantified and extracts bearing 20 μg of total protein were used to quantify specified cytokines using a Luminex bead assay (Millipore), according to the manufacturer's instructions.

Quantitative PCR Analysis

Frozen mammary glands were pulverized on a dry ice bed, and resuspended in TRIzol reagent (Invitrogen). RNA was extracted following standard protocols and reverse-transcribed using SuperScript III Reverse transcription kit (Invitrogen). Semi-quantitative PCR was performed using an ABI Prism 7900HT instrument (Applied Biosystems) and SybrGreen PCR master mix (Applied Biosystems). The indicated transcripts were assayed using the following primers:

β-actin forward, 5′-AAGGCCAACCGTGAAAAGAT-3′;
β-actin reverse, 5′-GTGGTACGACCAGAGGCATAC-3′;
F4/80 forward, 5′-GGAGGACTTCTCCAAGCCTATT-3′;
F4/80 reverse, 5′-AGGCCTCTCAGACTTCTGCTT-3′;
iNOS forward, 5′-CTTTGCCACGGACGAGAC-3′;
iNOS reverse, 5′-TCATTGTACTCTGAGGGCTGAC-3′;
Arg-1 forward, 5′-GAATCTGCATGGGCAACC-3′;
Arg-1 reverse, 5′-GAATCCTGGTACATCTGGGAAC-3′.

Statistical Analysis

Statistical analysis was performed with Prism software (GraphPad Software), using parametric and non-parametric tests, as indicated in each figure. Differences were considered statistically significant when $p < 0.05$ (two-tailed).

RESULTS

Treg Cell Ablation During the Non-invasive Stage Accelerates Breast Primary Tumor Growth

In order to investigate the potential role of regulatory T (Treg) cells in the transition from hyperplastic, benign lesions to cancerous lesions, we utilized the polyoma middle-T-driven model of murine breast carcinogenesis (*MMTV-PyMT*) in the C57BL/6 background (14) crossed to *Foxp3*^{DTR} mice (15) that we have previously generated (10), to allow for the specific and efficient ablation of Treg cells. This transgenic breast cancer model has been molecularly characterized as clustering with the luminal type of human breast cancer (16), and shows well-defined stages of tumor development that progress through hyperplasia/adenoma, early carcinoma and late carcinoma (**Supplementary Figure 1**) (12). First, we performed mammary gland whole mounts (**Figure 1A**) and histological examination of hematoxylin and eosin stained sections (**Figure 1B**) to identify the time point at which hyperplasia/adenoma was mostly found. Consistent with previous reports (17, 18), we identified the 8-week-old mammary gland as the one showing consistent benign lesions. At this early stage, frequency of Treg cells was similar to the naïve mammary gland (**Supplementary Figure 2A**). We performed ablation of $Foxp3^+$ Treg cells at this time point by

intravenous injection of diphtheria toxin (DT) at a 25 μg/kg dose on days 0, 2, 4, as depicted (**Supplementary Figure 2B**). Using this ablation schedule, Treg cells are almost completely lost from the peripheral blood lymphocyte population by 24 h after the first injection, and remain at low levels for about a week after that, followed by a slow recovery of initial circulating levels by 2 weeks after initial treatment (**Supplementary Figure 2C**). Importantly, analysis of the mammary gland 14 days after the first DT injection showed significantly reduced Treg cells, suggesting that tissue-specific ablation is more stable than in the periphery (**Supplementary Figure 2D**). Of note, with this schedule, there is low mouse morbidity, and after treatment is stopped, animals recover.

We then compared the effects of this treatment on the MMTV-PyMT mice. First, we evaluated tumor-free survival over time, and only found a slight acceleration of tumor initiation upon DT treatment (**Figure 1C**). However, when we counted the number of mammary glands developing tumors in each group, we found more tumor-bearing glands in the DT-treated mice (**Figure 1D**). There were no apparent differences in the pattern of tumor location between the two groups. Moreover, based on calculated tumor volume, there was a significant increase in the size of the tumors in those mice that underwent Treg cell ablation (**Figure 1E**). These results suggest that Treg cell presence in the breast environment represents a constrain on invasive progression during early stages of breast cancer.

Treg Cell Ablation Results in Progression to Early Invasive Carcinoma

The rapid growth of tumors in the 8-week-old, Treg cell ablated mice led us to examine their histopathological characteristics. To that end, we collected the abdominal mammary glands from both groups of mice 14 days after the first DT injection (10-week-old mice), and performed whole mounts followed by sectioning and H&E staining.

Corresponding with the increased tumor volumes measured in Treg cell ablated mice at similar temporal points, evaluation of the abdominal mammary gland whole mounts demonstrated significantly greater tumor areas within the gland in Treg cell ablated mice compared to control (**Figures 2A,B**). Examination of the histological sections by a blinded breast pathologist at 10 weeks, revealed that Treg cell ablation led to more advanced tumors, with increased proportion of early invasive carcinomas (**Figures 2C,D**). The presence of invasion was confirmed by immunofluorescent staining of alpha-smooth muscle actin (α-sma), which showed an evident disruption of the myoepithelial cell layer (**Figure 2E**). In addition, histological sections from Treg cell ablated tumors displayed a much higher degree of reactive stroma, characterized by increased desmoplasia (**Figures 2F,G**), collagen deposition (**Figures 2H,I**), and intra-tumor inflammatory infiltration (**Figures 2J,K**).

Together, these observations confirm that Treg cell ablation during non-invasive breast cancer stage induces histological changes associated with progression of the disease to early invasive carcinoma.

Ablation of Treg Cells Results in Expansion of the Mammary Cancer Stem/Progenitor Cell Pool

Treg cells have recently been recognized as critical regulators of stem cell homeostasis (19–21). Moreover, tumor initiation, progression, spread and resistance to therapy is dependent on the activity of a small population of cells with the ability to self-renew (22). Given the increased incidence and aggressiveness of tumors in mice that had been depleted of Treg cells, we wondered if this was due, at least in part, to the modification of the cancer stem cell niche. To explore the effect of Treg cell ablation on mammary cancer stem cell pool, we utilized previously defined flow cytometric staining to delineate mouse mammary cancer stem/progenitor cell population (13, 23–26). When we compared dissociated mammary glands from control and Treg cell-ablated mice, we found that treatment resulted in a significant expansion of $CD45^-$ $CD24^{-/lo}$ $CD44^+$ and $CD45^-$ $CD24^+$ $CD49f^+$ stem cell like-populations as well as, $CD45^-$ $CD24^+$ $CD29^{hi}$ basal stem cell- and $CD45^-$ $CD24^+$ $CD29^{lo}$ luminal progenitor-enriched population (**Figures 3A,B**). Furthermore, we observed that the increase of the luminal progenitor-enriched population was due to an expansion of an immature luminal progenitors ($CD45^-$ $CD24^+$ $CD29^{lo}$ $CD61^+$) over differentiated ones ($CD45^-$ $CD24^+$ $CD29^{lo}$ $CD61^-$) (**Figures 3A,B** bottom row).

To interrogate these differences functionally, we evaluated the tumor initiating capacity of the mammary cells through a transplantation experiment. We treated mice with DT as before, dissected the mammary glands at 10 weeks of age, and prepared single cell suspensions. We orthotopically transplanted 325,000 dissociated cells into a naïve host, and evaluated tumor appearance and volume over time. Consistent with our previous observations, tumors manifested earlier, and incidence was higher from the suspensions prepared from DT-treated mice (11 out of 18 transplants vs. 6 out of 14 from control mice) (**Figures 3C,D**).

Finally, in order to evaluate cancer stem cell activity more directly, we isolated CD45-negative mammary cells from Treg cell ablated and control mice, and seeded them in non-adherent, mammosphere-forming conditions during 7 days. We found that mammary gland cells from DT-treated mice grew a similar number, but bigger mammospheres at the end of the assay (**Figures 3E–G**).

Combined, these experiments highlight an important role for Treg cells in the homeostasis of the breast stem cell-like population.

Treg Cell Ablation Results in an Immune Microenvironment Associated With Tumor Progression

In order to shed light into the mechanisms by which Treg cell ablation promotes progression of non-invasive into invasive breast cancer, we evaluated changes in the cytokine milieu of the mammary gland upon DT treatment. For the most part, no changes were observed in TH1-related cytokines such as IFNγ, IL-12, or TNFα. However, we observed significant elevation of the TH2 cytokines IL-4 and IL-5 (**Figure 4A**). Furthermore, we observed significantly higher number of $F4/80^+$ macrophages in

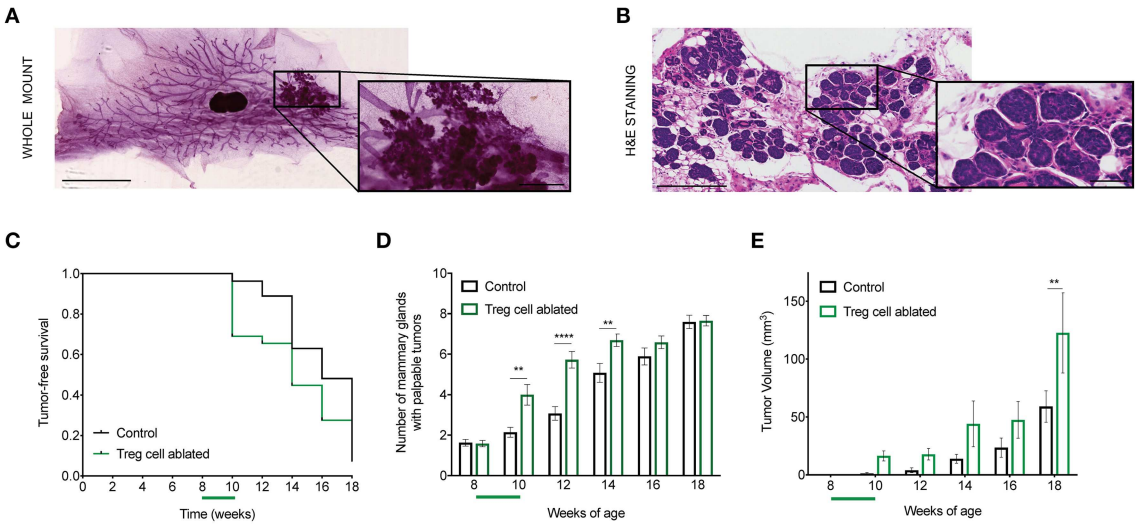

FIGURE 1 | Treg cell ablation accelerates primary tumor growth. **(A,B)** Representative mammary gland whole mount **(A)** and section stained with H&E **(B)** from 8-week-old MMTV-PyMT mouse. Amplified region depicts tumor area **(A)** and hyperplasia/adenoma pre-invasive stage **(B)**, respectively. Scale bars represent 5 mm and 1 mm (lower and higher magnification, respectively; whole mount) as well as 250 and 50 μm (lower and higher magnification, respectively; H&E stain). **(C)** Tumor-free survival curve **(D)**, number of mammary glands with palpable tumors, and **(E)** tumor growth kinetics in Treg cell-ablated mice compared with control mice (n = 30–33 mice). $^{**}p = 0.006$; $^{****}p < 0.0001$ **(D)** and $^{**}p = 0.0075$ **(E)**. Values are expressed as mean ± SEM and *p-values* were calculated using two-way ANOVA, followed by Bonferroni's *post-hoc* test. Green bar on the x axis indicates period of Treg cell ablation. Data were pooled from two independent experiments.

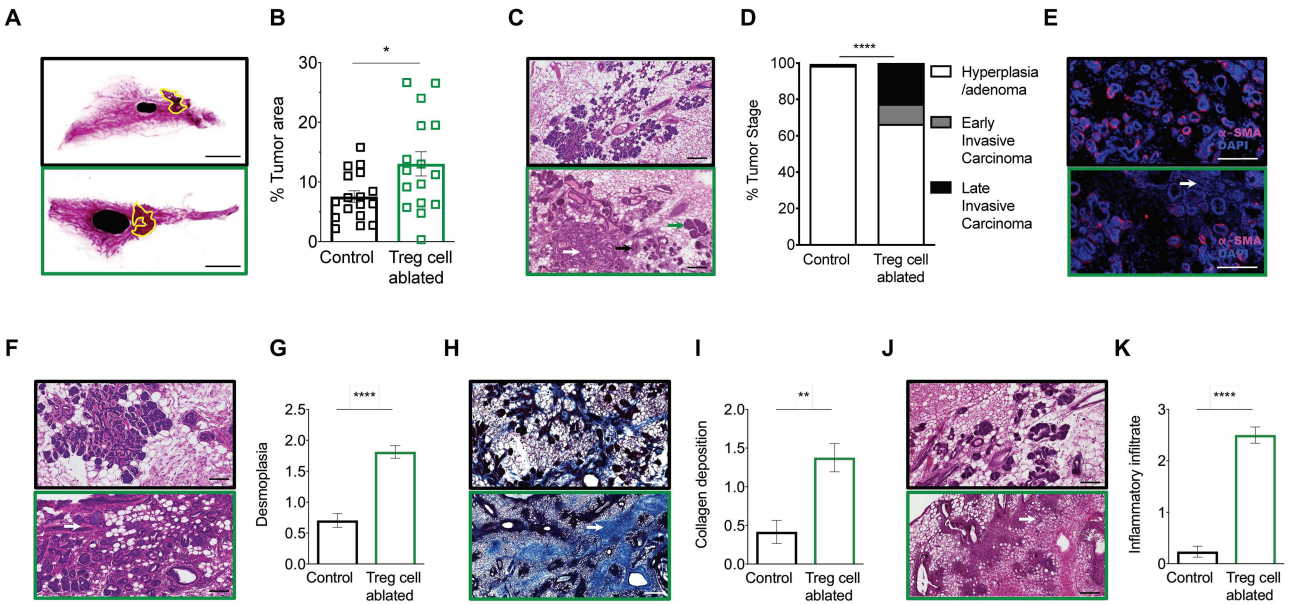

FIGURE 2 | Treg cell ablation results in advanced tumor staging. **(A,B)** 10-week-old mammary gland whole mount representative images **(A)** and quantification **(B)** of the percentage of tumor area of control (black) and Treg cell-ablated (green) mice (n = 16–17 individual mammary glands). Scale bars represent 5 mm, $^{*}p = 0.0405$ calculated by Mann-Whitney test. **(C,D)** Representative image **(C)** and quantification **(D)** of tumor stage. Arrows indicate examples of various tumor stages: hyperplasia/adenoma (green arrow), early invasive carcinoma (black arrow), and late invasive carcinoma (white arrow) **(E)** α-SMA myoepithelial staining confirming disruption of myoepithelial layer **(F,G)**, representative image **(F)** and quantification **(G)** of desmoplasia; **(H,I)** representative image of Masson trichome staining **(H)** and quantification **(I)** of collagen deposition; and **(J,K)** representative image **(J)** and quantification **(K)** of inflammatory infiltration. **(E,F,H,J)** White arrows indicate examples of histological observation. Values are expressed as mean ± SEM. Tumor staging was compared by two-way ANOVA, $^{****}p < 0.0001$; other comparisons were done by Mann-Whitney test. $^{****}p < 0.0001$ and $^{**}p = 0.0021$. Data were pooled from four independent experiments. **(C,E,F,H,J)** Scale bars represent 250 μm.

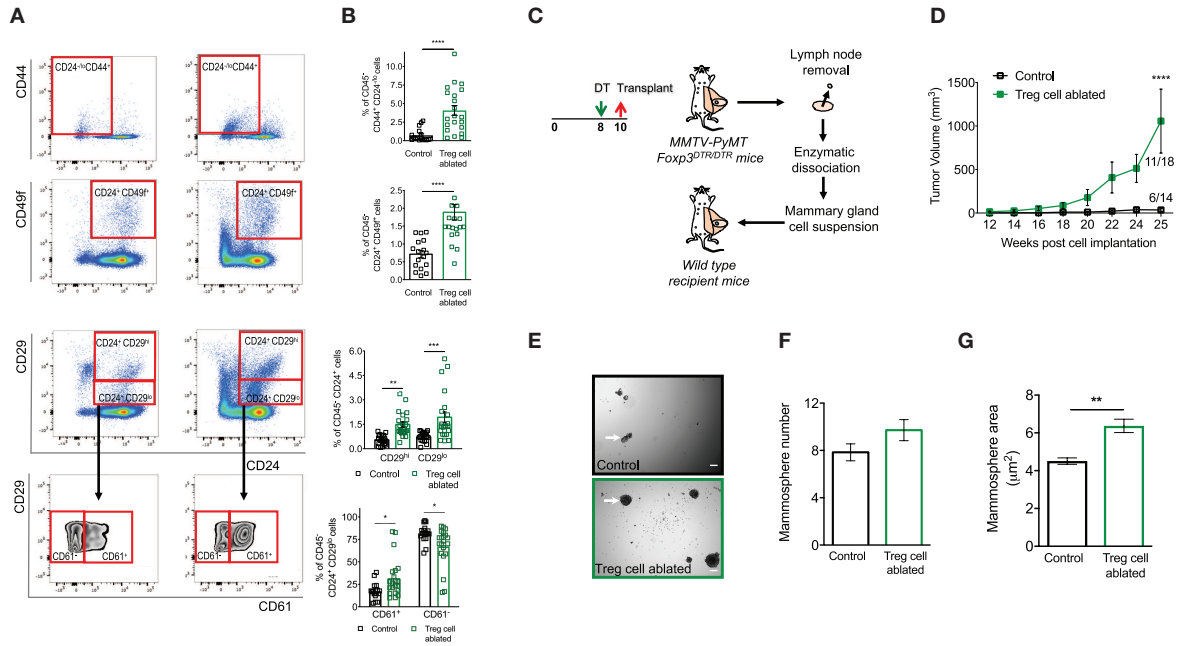

FIGURE 3 | Ablation of Treg cells results in expansion of the mammary cancer stem/progenitor cell pool. **(A,B)** Representative flow cytometric plots **(A)** and quantification **(B)** of stem cell like-populations ($CD24^{-/lo}$ $CD44^{+}$, $CD24^{+}$ $CD49f^{+}$, $CD24^{+}$ $CD29^{hi}$) and luminal progenitor-enriched population ($CD24^{+}$ $CD29^{lo}$) of Treg cell-ablated and control mice. Gating on the luminal progenitor-enriched population, we compared immature ($CD61^{+}$) and differentiated luminal progenitors ($CD61^{-}$) between both groups (bottom). Indicated cell populations highlighted with red frames. Values are expressed as mean ± SEM, control $n = 11$, Treg cell ablated $n = 12$, $^{****}p < 0.0001$, and was calculated by Mann-Whitney test. $^{**}p = 0.0045$; $^{***}p = 0.0003$; $^{*}p = 0.0237$; $^{*}p = 0.0219$ was calculated by two-way ANOVA, followed by Bonferroni's *post-hoc* test. Data were pooled from four independent experiments. **(C,D)** Schematics **(C)** and tumor growth kinetics **(D)** of mice orthotopically transplanted with dissociated mammary epithelial cells. Values expressed as mean ± SEM. $^{****}p < 0.0001$ by two-way ANOVA, followed by Bonferroni's *post-hoc* test **(D)**. **(E–G)** *In vitro* mammosphere forming capacity of CD45 depleted-mammary cells. Representative images **(E)**, mammosphere number **(F)** and area **(G)** of control (black) and Treg cell-ablated (green) conditions ($n = 16$ and $n = 13$, respectively). Arrow depicts a representative mammosphere in each image. Scale bar represents 200 μm. Values are expressed as mean ± SEM. $^{**}p = 0.0069$ was calculated by Mann-Whitney test. Data were pooled from five independent experiments.

the mammary gland tissue of Treg cell-ablated mice (**Figure 4B**). Alternative activation of macrophages mediated primarily by IL-4 leads to an array of pro-tumorigenic functions that promote tumor progression, dissemination, and inhibit response to therapy (27). To address this, we looked at the polarization status of $F4/80^{+}$ cells in the mammary gland by flow cytometry, evaluating the CD206/MHCII cell ratio. Consistent with the increase in TH2 cytokines, we observed a significant reduction in the $MHCII^{+}CD206^{-}$ macrophage subset, with a slight increase in the $CD206^{+}MHCII^{-}$ population (**Figure 4C**). Furthermore, we performed semi-quantitative real time PCR on RNA extracted from the mammary glands, and detected significantly less iNOS and more Arg1 in tissues (**Figure 4D**), after normalization for the macrophage marker F4/80 and the housekeeping gene beta-actin. Altogether, our observations suggest that Treg cells in the early breast cancer microenvironment function to prevent the establishment of a pro-tumorigenic microenvironment, which results in delayed tumor invasion.

DISCUSSION

The early events leading to progression of *in situ* breast lesions to invasive cancer are poorly understood (7). While all patients with DCIS are heavily treated with surgery and radiation at least due to the lack of biomarkers, for most of them this results in unnecessary morbidities and side effects (1). Moreover, early intervention with targeted therapies is not possible despite the fact that a subset of DCIS patients will go on to develop invasive cancer (4). Thus, understanding the cellular or molecular mechanisms that govern the transition from non-invasive to invasive cancer is critical.

Breast cancer accumulates $Foxp3^{+}$ Treg cells upon tumor progression, and we have demonstrated that transient ablation of Treg cells in established, highly immuno-suppressive breast tumors results in a significant increase in anti-tumor immunity in primary and metastatic tumors (10). In this context, while cytotoxic T and NK cell activity is dispensable for the antitumor effect, IFNγ-dependent reprogramming of the tumor microenvironment is required (10). In contrast, intraductal immune cell accumulation is rarely detected in early DCIS lesions (28), and Treg cell frequency in normal and neoplastic 8-weeks mammary gland is similar, suggesting a microenvironment more similar to the normal gland. In this study, we found that transient Treg cell ablation at this pre-invasive breast tumor stage accelerates the rate of tumor progression to invasive cancer, increasing the number of mammary glands harboring tumors and promoting the development of early invasive carcinoma. In addition, Treg cell ablation heightened mammary reactive

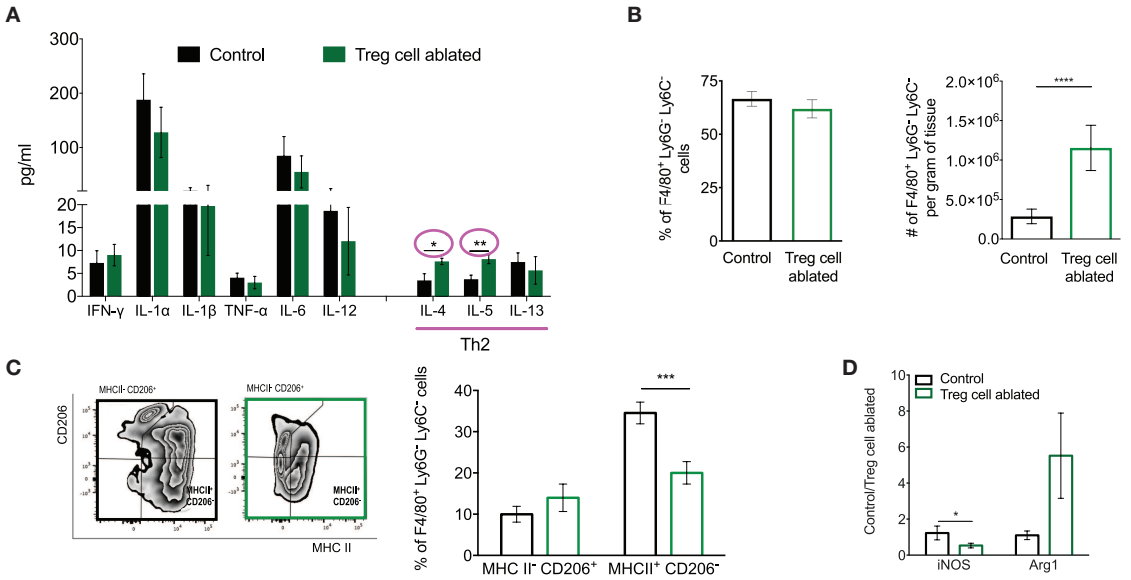

FIGURE 4 | Treg cell ablation results in an immune microenvironment associated with tumor progression. **(A)** Multiplex analysis of shown cytokines. Values are expressed as mean ± SEM, $n = 3$ mice per group. $^{*}p = 0.0278$ and $^{**}p = 0.0082$ were calculated by unpaired *t*-test. **(B)** Percentage (left) and absolute number (right) of tumor associated macrophages ($CD11b^{+}$ $LY6C^{-}$ $LY6G^{-}$ $F4/80^{+}$). $^{****}p < 0.0001$ was calculated by Mann–Whitney test. **(C)** Representative flow cytometric plot (left) and quantification (right) of TAM ($Ly6G^{-}$ $Ly6C^{-}$ $F4/80^{+}$) cell polarization as relative amounts of $MHCII^{-}$ $CD206^{+}$ and $MHCII^{+}$ $CD206^{-}$ cells. $^{***}p < 0.0007$ was calculated by two-way ANOVA, followed by Bonferroni's *post-hoc* test. **(D)** Mammary gland relative expression of iNOS and Arg-1 (M1 and M2 markers, respectively) quantified by qPCR. Values are expressed as mean ± SEM and were normalized by F4/80 and beta-actin levels. $n = 5$–6, $^{*}p = 0.0479$ was calculated by unpaired *t*-test. Data were pooled from two independent experiments.

stroma, characterized by a higher desmoplasia and collagen deposition. In line with our observations, this stromal change has been associated with the activation of angiogenic programs, recruitment of inflammatory cells, invasive phenotype, and metastatic progression (29).

It is now well-established that Treg cells play critical roles in maintaining non-lymphoid tissue homeostasis (30–32). More recently, a relationship between Treg cells and tissue-specific stem cells has been identified. In the bone marrow, Treg cells create an immune-privileged site enabling allo-hematopoietic stem/progenitor cell persistence and quiescence (19, 20). In addition, skin Treg cells play a major role in hair follicle biology by promoting the function of hair follicle stem cells (21). Cancer stem cells are required for the initiation, progression, metastatic dissemination and response to therapy in breast cancers (33, 34). Here, we describe a previously unrecognized effect of Treg cells on mammary cancer stem/progenitor cells during the early stages of tumorigenesis. Specifically, Treg cell ablation induced expansion of $CD45^{-}$ $CD24^{-/lo}$ $CD44^{+}$, $CD45^{-}$ $CD24^{+}$ $CD49f^{+}$, and $CD45^{-}$ $CD24^{+}$ $CD29^{hi}$ stem cell like-populations, as well as an immature luminal progenitor-enriched population ($CD45^{-}$ $CD24^{+}$ $CD29^{lo}$ $CD61^{+}$). The murine $CD44^{+}$ $CD24^{-}$ cancer stem cell population found in the primary tumors of MMTV-PyMT transgenic mice exhibits functional characteristics of human breast cancer stem cells (23), which highlights the clinical impact of our finding. Our data suggest that Treg cells negatively regulate the early cancer stem cell niche. Supporting this, we demonstrated that dissociated mammary gland from Treg cell ablated mice progressed into tumors faster and with increased penetrance after transplantation into naïve hosts. Additionally, mammospheres from Treg cell ablated mice were significantly larger when cultured under non-adherent conditions. Whether this is a direct effect of the Treg cell interaction with the stem cell niche or an indirect effect due to changes within the tumor microenvironment that occur after Treg cell depletion remains to be investigated. Furthermore, our unpublished observations suggest that similar expansion of normal mammary gland stem cell is observed when Treg cells are ablated in naïve mammary gland (data not shown).

Lastly, we found dysregulated amounts of IL-4 and IL-5 cytokines, and a concomitant increase in the number of tumor associated-macrophages (TAMs). Whether the increase in macrophages is due to expansion of tissue-resident populations or recruitment of inflammatory monocytes remains to be determined. Consistent with the increase in TH2-type cytokines, we observed increased frequencies of alternatively activated TAMs, as defined by their expression of CD206 and MHCII. Furthermore, semi-quantitative PCR to detect macrophage effectors Arg1 and iNOS from the mammary gland tissue after Treg cell ablation suggested qualitative changes in the macrophage infiltrate. Specifically, we observed lower levels of iNOS (classical activation marker), and higher levels of Arg1 (alternative activation marker). TH2 cytokines such as IL-4 have been shown to induced tumorigenic properties in TAMs (35), facilitating invasion and metastasis by regulating their phenotype and function (36), such as the production of cathepsins B and S (37). These results suggest that Treg cells regulate the immune environment of non-invasive breast cancer at least in

part by their effects on mammary gland macrophages. Future studies utilizing genetic or chemical deletion of macrophages will be necessary to evaluate this possibility. It is interesting to note that the changes observed upon Treg cell ablation in the hyperplastic mammary gland are similar to those taking place during the involution of the lactating mammary gland, a state that has been mechanistically linked to the increased chance of metastatic recurrence observed in pregnancy-associated breast cancer (38).

In summary, our study demonstrates that Treg cells prevent the transition of pre-invasive to invasive breast cancer by selectively suppressing pro-tumorigenic TH2 responses and restraining the cancer stem cell pool. Ongoing and future studies will shed light into the cellular mechanisms underlying this observation. Furthermore, validating whether increased numbers of Treg cells present within early *in situ* breast lesions associates with a more favorable outcome could justify future studies to investigate the potential of Treg cells, macrophage infiltrates, and stem cell profiles as biomarkers that accurately enable identification of the DCIS patients that will most likely benefit from receiving radiation therapy and surgery. These studies should help to contribute to the development of paradigm shifting standard of care treatment for DCIS patients.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

LM and PB designed the study, analyzed the data, and wrote the manuscript. VR performed histopathological analysis of mammary glands. NC and WD assisted with flow cytometry studies. MI supervised histological analysis. MR contributed to the interpretation of the studies. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01942/full#supplementary-material

Supplementary Figure 1 | Tumor staging **(A)** representative picture of an H&E stained MMTV-PyMT mammary gland exemplifying the different tumor stages **(A)** hyperplasia/adenoma **(B)**, early invasive carcinoma, and **(C)** late invasive carcinoma. Scale bars represent 500 and 100 μm (lower and higher magnifications, respectively).

Supplementary Figure 2 | Treg cell frequencies and ablation efficiency. **(A)** Frequency of Treg cells ($CD4^+$ $Foxp3^+$) in the mammary gland of naïve and MMTV-PyMT mice at 8 weeks of age. Representative of two independent experiments with similar results. **(B)** Schematic of Treg cell ablation. $Foxp3^+$ Treg cells were ablated by intravenous injection of DT on days 0, 2, and 4 in 8-week-old mice. **(C)** Kinetics of Treg cell frequency in peripheral blood in control (black) and DT-treated (green) mice ($n = 4$, respectively). Values are presented as percentage of day 0. **(D)** Frequency of Treg cells ($CD4^+$ $Foxp3^+$) in the mammary gland of control and DT-treated MMTV-PyMT mice at 10 weeks of age (2 weeks after initial treatment). $^*p < 0.05$ by two-tailed unpaired *t*-test. Data were pooled from three independent experiments.

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S100A9 Regulates MDSCs-Mediated Immune Suppression via the RAGE and TLR4 Signaling Pathways in Colorectal Carcinoma

Mao Huang[1†], Rui Wu[2†], Lu Chen[1], Qi Peng[1], Shue Li[3], Yan Zhang[1], Lan Zhou[1] and Liang Duan[4*]*

[1] Key Laboratory of Laboratory Medical Diagnostics, Ministry of Education, Department of Laboratory Medicine, Chongqing Medical University, Chongqing, China, [2] Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China, [3] Department of Academic Research, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China, [4] Department of Laboratory Medicine, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China

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***Correspondence:**
Liang Duan
duanliang@cqmu.edu.cn
Lan Zhou
zhoulan@cqmu.edu.cn

[†] These authors have contributed equally to this work

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Myeloid-derived suppressor cells (MDSCs) are a major component of the immunosuppressive tumor microenvironment (TME) and have been recognized as a contributing factor to inflammation-related cancers. However, the molecular mechanisms of MDSCs accumulation and activation remain elusive. We previously showed that the proinflammatory molecule S100A9 in TME exerts a tumor-promoting effect in colorectal carcinoma (CRC). In this report, we investigated the effect and molecular mechanisms of S100A9 on the accumulation and immunosuppressive function of MDSCs in CRC. Elevated S100A9 and MDSCs were found in tumor tissue and peripheral blood from CRC patients. Circulating S100A9 and MDSCs were positively associated to each other, and both S100A9 and MDSCs were correlated to neoplastic progression. Using a CRC cell line LoVo-induced MDSCs model, we found that S100A9 stimulated chemotaxis and activation but not viability of MDSCs. Mechanistic studies demonstrated that activation of RAGE-mediated p38 MAPK and TLR4-mediated NF-κB signaling pathways were involved in S100A9-induced chemotaxis and MDSCs activation, respectively. Furthermore, ROC analysis showed that combination detection of S100A9 and MDSCs was superior to individual detection of these two factors for diagnosing CRC patients with advanced staging and lymphatic metastasis, which yielded an area under the ROC curve (AUC) of 0.92 with 86.7% sensitivity and 86.4% specificity, and an AUC of 0.82 with 75% sensitivity and 77.1% specificity, respectively. Collectively, our study suggests that the S100A9 plays a pivotal role in immunosuppressive TME by stimulating MDSCs chemotaxis and activation, and combination detection of S100A9 and MDSCs may serve as a potential marker for diagnosis of CRC progression.

Keywords: CRC, S100A9, MDSCs, migration, activation

INTRODUCTION

Colorectal carcinoma (CRC) is the third most common cancer and the fourth leading cause of cancer death worldwide (1, 2). Inflammatory bowel disease (IBD) is associated with an increased risk of CRC. A growing body of studies has suggested that inflammation plays an important role in initiation of colitis-associated CRC (3). However, the immune status in the inflammatory microenvironment and the underlying mechanisms related to immune escape in CRC remain unknown.

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of immature myeloid cells consisting of precursors for granulocytes, macrophage, and dendritic cells that are major components of the immunosuppressive TME (4). Accumulation of MDSCs has been thought to be a significant factor linking inflammation and cancer (5). Human MDSCs are characterized by the myeloid marker HLA-DR$^-$CD33$^+$CD11b$^+$ (6). This tolerogenic appearance of MDSCs represents a common trait of cancer and other non-cancerous diseases such as sepsis, bacterial, viral, and parasitical infections, autoimmune diseases, and aging (5–7). As one type of the most potent immunosuppressive cells, MDSCs facilitate tumor progression by suppressing T cell response, blocking natural killer cell activation, limiting dendritic cell maturation, and inducing regulatory T (Treg) cell generation (5), which are associated with high expression and secretion of immunosuppressive molecules such as interleukin (IL)-10, arginase-1(Arg-1), inducible nitric oxide synthase (iNOS) and ROS (5–7). The accumulation of MDSCs in TME involves multifarious mechanisms including trafficking, expansion and activation in different types of cancer (3, 8–13). Increased circulating and tumor-infiltrating MDSCs are also found in CRC, which correlates to cancer progression (14, 15). However, the exact molecular mechanism of MDSCs accumulation and activation in CRC remains elusive.

S100A9 belongs to a family of intracellular EF-hand motif calcium-binding proteins found exclusively in vertebrates. S100A9 is constitutively expressed in myeloid cells including granulocytes, monocytes, early-differentiation cells of the myeloid lineage, and cancer cells (16). It has been shown that S100A9 overexpression is correlated to invasion and metastasis in various cancers, and S100A9 directly enhances tumor cell malignancy by activating TLR4-mediated or RAGE-mediated signaling cascades (17–19). Our previous study, consistent with others, showed that S100A9 in the CRC microenvironment directly contributes to malignancy in CRC cancer cells (20, 21). Recently, S100A9 was shown to modulate inflammatory or immune cell migration and activation through TLR4-mediated or RAGE-mediated signaling pathways (22–25). Given that MDSCs are precursors for inflammatory and immune cells, we hypothesized that S100A9 regulates trafficking, expansion and activation of MDSCs to establish an immunosuppressive microenvironment to potentiate CRC progression.

In this study, we investigated the association between MDSCs and S100A9 in CRC tumor tissues and peripheral blood, and the effect of molecular mechanisms of S100A9 on trafficking, cell vitality, and activation of MDSCs in CRC. We found that both MDSCs and S100A9 are correlated to Dukes staging and lymph node metastasis, and activation of the RAGE-mediated p38 MAPK and TLR4-mediated NF-κB signaling pathways are involved in S100A9-induced trafficking and activation of MDSCs, respectively. Combination detection of S100A9 and MDSCs may be a serum marker for CRC diagnosis, particularly for CRC staging and metastasis. Our results highlight the significance of S100A9 in regulating MDSCs in the immunosuppressive microenvironment and implicates that S100A9 could be a potential therapeutic target for CRC.

MATERIALS AND METHODS

Patient and Sample Collection

Whole blood samples from CRC patients ($n = 52$) and healthy controls ($n = 30$) were collected from the Second Affiliated Hospital of Chongqing Medical University from September 2015 to August 2017. The clinicopathological data of the subjects including gender, age, tumor location, Dukes staging, cell differentiation, and metastasis at initial diagnosis are shown in **Table 1**. Serum samples from 4 ml of coagulated blood by centrifugation were immediately separated and frozen at −80°C until use. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood via Ficoll-Hypaque gradient centrifugation. Clinical histological proven CRC tissue samples ($n = 16$) and the matched distal normal tissues (n=16) were collected from patients who underwent surgical resection at the Second Affiliated Hospital of Chongqing Medicine University. All of the patients and healthy donors provided written informed consent before sampling.

TABLE 1 | The characteristics of enrolled individuals.

Parameters	CRC ($n = 52$) n, %	HC ($n = 30$) n, %
Gender		
Male (n, %)	34 (65.38%)	15 (50%)
Female (n, %)	18 (34.62%)	15 (50%)
Age		
<60 (n, %)	30 (57.69%)	20 (66.6%)
≥60 (n, %)	22 (42.31%)	10 (33.3%)
Location		
Colon (n, %)	25 (48.08%)	NA
Rectum (n, %)	27 (51.92%)	NA
Tumor differentiation		
Low (n, %)	30 (57.69%)	NA
High/middle (n, %)	22 (42.31%)	NA
Dukes staging		
A/B (n, %)	24 (46.15%)	NA
C/D (n, %)	28 (53.85%)	NA
Lymphatic metastasis		
Absent (n, %)	36 (69.23%)	NA
Present (n, %)	16 (30.77%)	NA

NA, not available.

Antibodies, Inhibitors, and Preparation of the Recombinant Proteins

The antibodies included anti-S100A9 (Cat no. ab92507; Abcam), anti-TLR4 (Cat no. sc-293072; Santa Cruz Biotechnology), anti-RAGE (Cat no. sc-80653; Santa Cruz Biotechnology), anti-p38 (Cat no. 9212; Cell Signaling Technology), anti-p65 (Cat no. 3034; Cell Signaling Technology), anti-ERK1/2 (Cat no. 4695; Cell Signaling Technology), anti-JNK (Cat no. 9253; Cell Signaling Technology), anti-AKT (Cat no. 8596; Cell Signaling Technology), anti-phospho(p)-p38 (Cat no. 4511; Cell Signaling Technology), anti-p-p65 (Cat no. 3033; Cell Signaling Technology), anti-p-ERK1/2 (Cat no. 3510; Cell Signaling Technology), anti-p-JNK (Cat no. 4668; Cell Signaling Technology), anti-p-AKT (Cat no. 9271; Cell Signaling Technology), anti-CD8 (Cat no. 340046, BD), anti-HLA-DR (Cat no. 4310370, eBioscience), anti-CD33 (Cat no. 4296343, eBioscience) and CD11b (Cat no. 4291932, eBioscience), and horseradish peroxidase-conjugated anti-mouse, anti-rabbit IgG antibodies. The inhibitors contained TAK-242 (MedChemExpress, New Jersey), FPS-ZM1 (MedChemExpress, New Jersey), SB203580 (Beyotime) and BAY 11-7082 (Beyotime). The preparation of the recombination GST-S100A9 protein, as well as its control protein GST, have previously been described (21).

Cells Culture

The human CRC LoVo cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The LoVo-induced MDSCs were cultured in 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin 1640 medium (Gibco, Life Technologies). The cell culture was maintained at 37°C in a humid atmosphere containing 5% CO_2.

ELISA Assay

Serum levels of S100A9, Arg-1, and iNOS were measured using a commercially available enzyme-linked immunosorbent assay by ELISA kit (ELISA LAB, Wuhan, China) according to the manufacturer's instructions.

Single Cell Suspension

The collected CRC and paraneoplastic tissues were washed with PBS three times and then cut into pieces, and enzymatically digested with type I collagenase (Sigma, USA) for 1∼2 h at 37°C with mixing every 20 min. The resulted single cell samples were used for flow cytometry (FCM) analysis.

Immunohistochemistry (IHC)

Immunohistochemical staining for CD33 and S100A9 was performed using anti-human CD33 (Cat no. ab92507, eBioscience) and an anti-S100A9 (Cat no. ab92507, Abcam) antibodies following the manufacturer's instructions. Briefy, the deparaffinized and dehydrated sections were boiled for 10 min in 0.01 M citrate buffer and incubated with 0.3% hydrogen peroxide (H_2O_2) in methanol for 15 min to block endogenous peroxidase, incubated with primary and peroxidase-tagged secondary antibodies sequentially, and colorized with 0.05% 3,3-diaminobenzidine tetrachloride (DAB). The sections were counterstained with hematoxylin, and observed, and representative images were captured under an inverted phase contrast microscope (Olympus B640, Japan).

Western Blot

The cells were collected and washed with ice-cold PBS and lysed on ice in radio immunoprecipitation assay (RIPA) buffer. An equal amount of proteins of the samples was separated in 10% SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked with 5% bovine serum albumin (BSA) and incubated with the primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The proteins of interest were detected using the SuperSignal West Pico Chemiluminescent Substrate kit. The results were recorded using the Bio-Rad Electrophoresis Documentation (Gel Doc 1000) and Image Lab version software.

Flow Cytometry (FCM) Analysis

Human monoclonal Abs against HLA-DR-PE (Cat no. 4310370, eBioscience), CD33-FITC (Cat no. 4296343, eBioscience), and CD11b-APC (Cat no. 4291932, eBioscience) conjugated with different fluorescent dyes were used for FCM analysis. Immunophenotyping of circulating or tumor-infiltrating MDSCs were classified as HLA-DR$^-$CD33$^+$CD11b$^+$ cells via FCM staining using the multiplex gating strategy.

Human monoclonal Abs against Arg1-Alexa Fluor 488 (Cat no. 53369782, invitrogen) and iNOS (Cat no. MA517139, Invitrogen) were used for FCM analysis for Arg1 and iNOS expression in MDSCs. Samples were analyzed on a BECKMAN COULTER Navios FCM, and the data were analyzed using the Flowjo software.

Induction of Tumor-Associated MDSCs *in vitro*

CD33$^+$ cells were separated from mixed PBMCs from different CRC patients using human CD33 MicroBeads (Cat no. 18257, STEMCELL Technologies Inc) according to the manufacturer's instructions. Isolated CD33$^+$ cells were co-cultured with LoVo cells in 6-well plates in a Transwell System (0.4 μm pore, Corning) at a ratio of 1:3 for 48 h. CD33$^+$ cells cultured in medium alone were included as a control. The LoVo-induced MDSCs markers in the resulted cells were analyzed by FCM.

CD8$^+$ T Cells Proliferation Suppression Assay

For the analysis of suppressing CD8$^+$ T cells proliferation by MDSCs, PBMCs from healthy donors were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 10 μM), seeded in OKT3-coated 96-well plates and co-cultured with LoVo-induced MDSCs at a 1:2 ratio for 3 days, stained with an anti-human CD8 mAb followed by FCM analysis. Individually cultured CFSE-labeled PBMCs were used as a control.

Chemotaxis Assay

Chemotaxis assay was performed using 24-well plates with 5-μm-pore size inserts (Corning) according to the manufacturer's instructions. A total of 1×10^6 MDSCs in serum-free medium were loaded into the upper chamber and GST and GST-S100A9 proteins were in the lower chamber in the presence or absence of specific inhibitors. After incubation for 24 h, migrated cells were counted in the upper chamber.

RNA Isolation and Real-Time PCR Analysis

The LoVo-induced MDSCs were stimulated with GST and GST-S100A9 proteins for 24 h and total RNA was extracted from cells using Trizol (Invitrogen) in accordance with the manufacturer's instructions. Reverse transcription-PCR was done using the PrimeScript™ RT Reagent Kit (Takara, Japan) and TB Green™ Premix Ex Taq™ II (Takara, Japan). The sequences of primers were: GAPDH primers: (forward) 5′-CAGCGACACCCACTCCTC-3′ and (reverse) 5′-TGAGGTCCACCACCCTGT-3′; Arg-1 primers: (forward) 5′-GTTTCTCAAGCAGACCAGCC-3′ and (reverse) 5′-GCTCAAGTGCAGCAAAGAGA-3′; iNOS pimers: (forward) 5′-CAGCGGGATGACTTTCCAA-3′ and (reverse) 5′-AGGCAAGATTTGGACCTGCA-3′; IL-10 primers: (forward) 5′-GGCTTCCTAACTGCTACA-3′ and (reverse) 5′-CTCCTGACCTCAAGTGAT-3′; TLR4 primers: (forward) 5′-AGAATGCTAAGGTTGCCGCT-3′ and (reverse) 5′-CTATCACCGTCTGACCGAGC-3′; RAGE primers: (forward) 5′-ACTACCGAGTCCGTGTCTACC-3′ and (reverse) 5′-GGAACACCAGCCGTGAGTT-3′. Reactions were performed in triplicate using SYBR Green master mix (Takara, Japan) and normalized to GAPDH using the ΔΔCt method.

ROS Detection

ROS was measured with the Reactive oxygen detection kit (Beyotime, Jiangsu, China). LoVo-induced MDSCs (1×10^5) were seeded in 96-well plates and the probe (DCFH-DA) was loaded into the cells according to the manufacturer's instructions. Then the DCFH-DA loaded MDSCs were cultured in the cell incubator for 30 min and washed 3 times with serum-free medium to eliminate the residual probe. The recombination proteins and specific inhibitors were added to the labeled MDSCs for 30 min, then the fluorescence was measured by a fluorescence microplate reader.

Cell Viability Analysis

To detect the effect of S100A9 on viability of LoVo-induced MDSCs, the cells (1×10^5 cells/well) were grown in triplicates in 96-well plates and treated with or without GST or GST-S100A9 for 12, 24, and 36 h and cell viability was analyzed by CCK8 assay according to the manufacture's instruction. CCK8 reagent was added into the medium at the indicated time. After a 1 h incubation under the culture condition, absorbance at 450 nm was measured on a microplate reader.

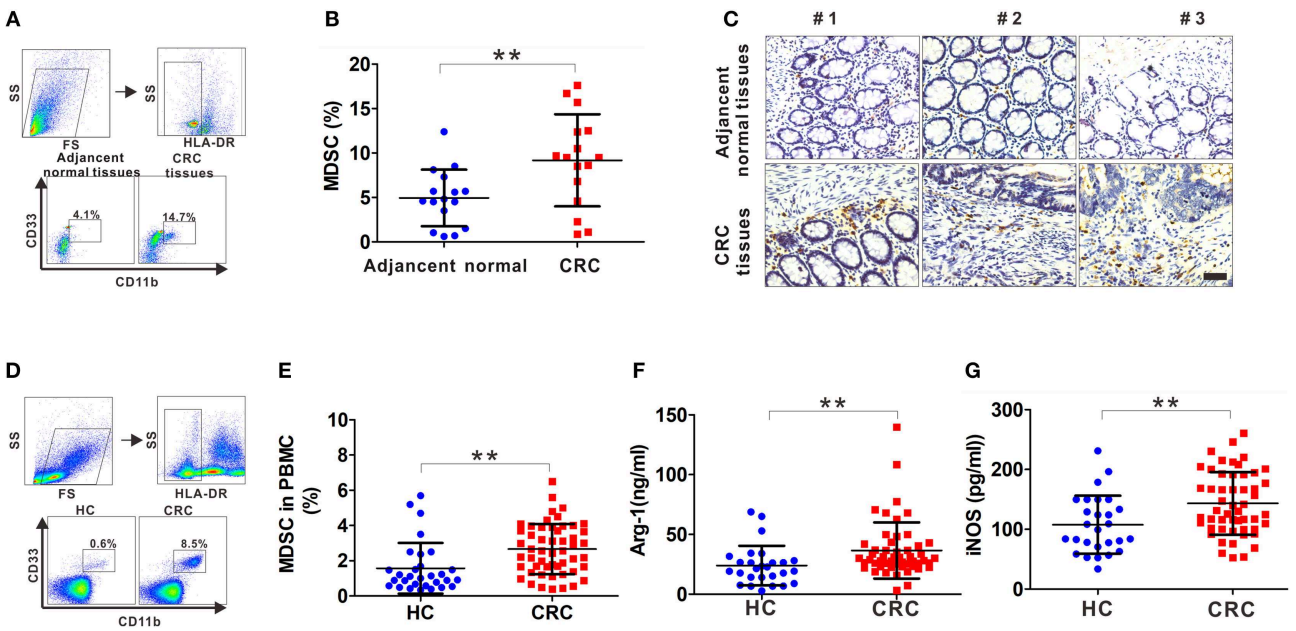

FIGURE 1 | The frequency of MDSCs in CRC patients. **(A)** The gating strategy for HLA-DR$^-$CD33$^+$CD11b$^+$ cells from tissue samples of CRC patients. **(B)** Statistical analysis for the percentage of infiltrating MDSCs in 16 paired CRC samples including tumor tissues and adjacent normal tissues. **(C)** Representative IHC staining for CD33 in tumor tissues and adjacent normal tissues from three CRC patients. Blank scale bars = 100 μm. **(D)** The gating strategy for HLA-DR$^-$CD33$^+$CD11b$^+$ cells from the peripheral blood of CRC patients. **(E)** Statistical analysis for the percentage of circulatory MDSCs in PBMCs from healthy controls (HC, $n = 30$) and CRC patients ($n = 52$) by FCM. **(F–G)** ELISA analysis of serum Arg1 and iNOS levels from HC ($n = 30$) and CRC patients ($n = 52$). Data represents the mean±SD. $^{**}p < 0.01$.

Statistical Analysis

All the numerical data were presented as means ± standard deviation. All the statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). One-way ANOVA followed by the S-N-K test, was used for the analyses of quantitative RT-PCR, cell viability, and transwell migration. The Mann-Whitney test was used for clinical data, such as the expression of S100A9, Arg1, and iNOS in serum. The Spearman test was used to analyze the relationship of S100A9 with MDSCs, Arg-1, and iNOS. The ROC analysis was used to prove the diagnostic power for S100A9, MDSCs, and their combination in CRC progression. Significant probability values were indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

Increased Number of Tumor-Infiltrating and Circulating MDSCs in CRC Patients

Fresh tumor tissues and adjacent normal tissues collected from CRC patients were used to generate single cell samples, and MDSCs were detected by FCM using the myeloid marker HLA-DR^{-}CD33^{+}CD11b^{+}. There was a significant increase in MDSCs in CRC compared with that in the adjacent tissues (**Figures 1A,B**). Also, the infiltrating CD33^{+} cells were prominently increased in the CRC tissues (**Figure 1C**). Next, we determined the number of MDSCs in peripheral blood. As expected, there was increased MDSCs circulating in the CRC compared with the healthy controls (**Figures 1D,E**). Furthermore, the serum levels of Arg-1 and iNOS, the immunosuppressive molecules mainly expressed and secreted by MDSCs for suppressing T cell function, were measured. Arg-1 and iNOS were at high levels in the serum of CRC patients compared to that in healthy controls (**Figures 1F,G**), indicating that the accumulated MDSCs were in an activated state. Altogether, these results suggest that both infiltrating MDSCs in tumor tissues and circulating MDSCs are increased in CRC patients.

Elevated S100A9 Expression in CRC

S100A9 expression in tumor and adjacent normal tissues from CRC patients was detected by IHC. We observed increased S100A9 expression, which was mainly localized to the cytoplasm, in CRC tissues compared with adjacent normal tissues (**Figure 2A**). Additionally, increased S100A9 expression in CRC was detected in five randomly selected CRC patients' tumor tissues and adjacent normal tissues by Western blot (**Figure 2B**). Similarly, serum S100A9 levels in CRC patients were markedly higher than that in healthy controls (**Figure 2C**). All these data suggest that S100A9 expression is elevated in CRC tissues and sera.

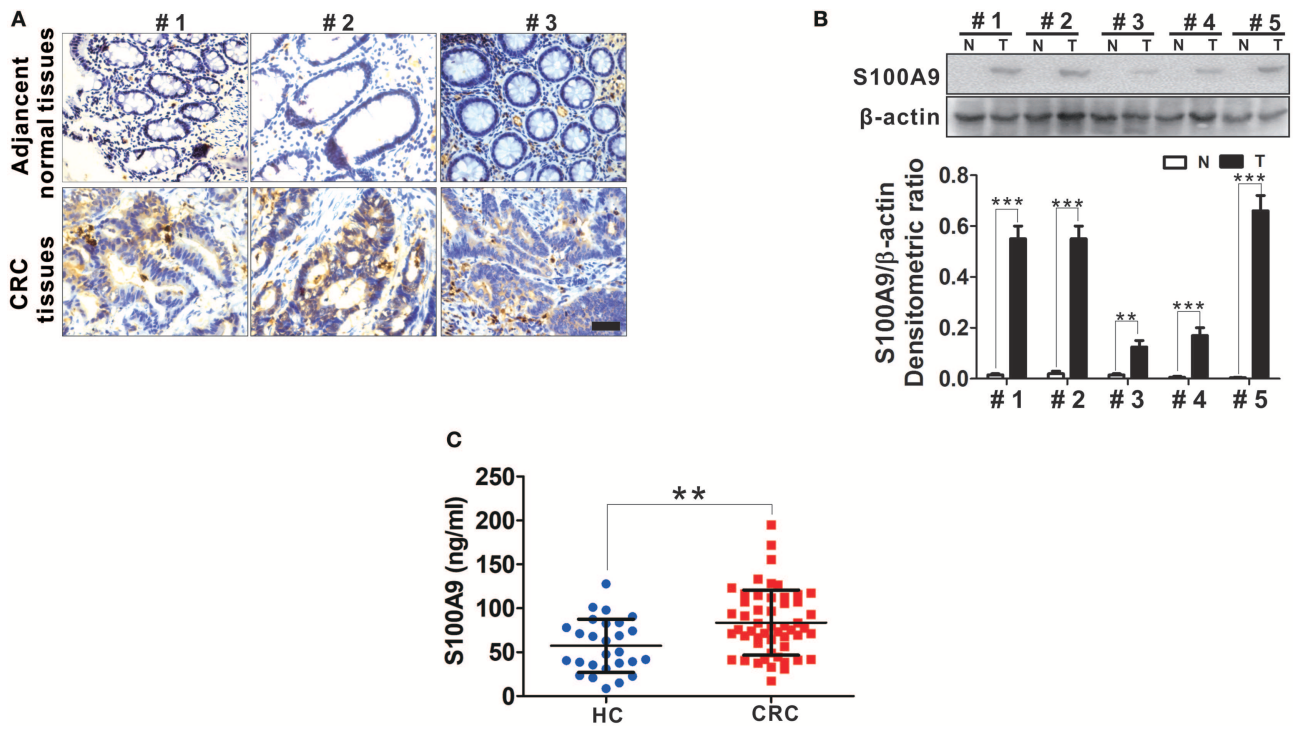

FIGURE 2 | The expression of S100A9 protein in CRC. **(A)** Representative IHC staining for S100A9 expression in tumor tissues and adjacent normal tissues from three CRC patients. Blank scale bars = 100 μm. **(B)** Western blot analysis of S100A9 expression in five randomly selected CRC patients' tumor tissues and adjacent normal tissues. N, adjacent normal tissues; T, tumor tissues. β-actin served as a loading control. **(C)** ELISA analysis of serum S100A9 level from HC ($n = 30$) and CRC ($n = 52$) patients. Data represents the mean±SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

Increased S100A9 Expression and MDSCs Number Are Associated With Neoplastic Progression of CRC

Although one previous report showed high levels of serum S100A9 in CRC (26), the association of S100A9 levels in the neoplastic properties of CRC has not been studied. We found that S100A9 expression was correlated to Dukes staging and metastasis status but not to tumor location and histological differentiation. Moreover, there was a similar result in the relationship between MDSC numbers and Dukes staging and metastasis status but not tumor location and histological differentiation (**Table 2**). These findings imply that the increased S100A9 and MDSCs are closely related to Dukes staging and the metastasis of CRC. Additionally, serum S100A9 levels and MDSC numbers were positively correlated to each other in CRC patients (**Figure 3A**), and there was also a positive correlation of serum S100A9 levels with Arg-1 and iNOS levels, two immunosuppressive molecules mainly expressed and secreted by MDSCs, in CRC (**Figures 3B,C**).

S100A9 Effectively Stimulates CRC-Associated MDSC Chemotaxis and Activation *in vitro*

$CD33^+$ cells from peripheral blood could be differentiated into MDSCs by co-culture with cancer cells including CRC cell lines HCT116 and SW480 *in vitro* (8, 14). Here, $CD33^+$ cells were co-cultured with another CRC cell line, LoVo (**Figure 4A**), which had a stronger metastasis potential for 48 h and the cell phenotypes were further examined by FCM, showing that the proportion of $HLA\text{-}DR^-CD33^+CD11b^+$ MDSCs was markedly higher when $CD33^+$ cells were co-cultured with LoVo compared with $CD33^+$ cells cultured in medium alone (**Figure 4B**). We then investigated the possible regulatory effects of S100A9 on cell vitality, chemotaxis and activation of MDSCs. There was no detectable effect of recombinant GST-S100A9 protein at different concentrations on the vitality of LoVo-induced MDSCs (**Figure 4C**). In contrast, GST-S100A9 significantly elevated MDSCs migration index in a concentration-dependent manner, suggesting that S100A9 promotes MDSCs chemotaxis (**Figure 4D**). GST-S100A9 (20 μg/ml) also remarkably up-regulated mRNA levels of the immunosuppressive molecules *Arg-1, iNOS,* and *IL-10* increased ROS production (**Figures 4E–H**). S100A9-induced increased protein levels of the Arg-1 and iNOS were also confirmed (**Figure S1**). In addition, PBMCs isolated from the peripheral blood of healthy donors were labeled with CFSE and co-cultured with S100A9-stimulated MDSCs, and T cell proliferation was examined. The results showed that S100A9 (20 μg/ml) potentiated the suppressing effect of MDSCs on T cell proliferation (**Figures 4I,J**). Altogether, these results imply that S100A9 can stimulate traffic and activate MDSCs but not cell vitality, and enhances the suppressing effect of MDSCs on T cell proliferation.

TABLE 2 | Relationship between MDSCs frequency or serum S100A9 levels and clinicopathological parameters of CRC patients.

Variables	MDSCs		S100A9	
	Mean ± SD (%)	*p*-value	Mean ± SD (ng/ml)	*p*-value
Location				
Colon	2.851 ± 1.607	0.498	87.58 ± 37.034	0.621
Rectum	2.488 ± 1.245		79.981 ± 37.589	
Differentiation				
Low	2.806 ± 1.283	0.744	82.826 ± 33.713	0.807
High	2.557 ± 1.625		84.139 ± 43.010	
Dukes staging				
A/B	1.887 ± 1.127	0.000	66.734 ± 30.764	0.002
C/D	3.327 ± 1.332		96.028 ± 36.970	
Lymphatic metastasis				
Negative	2.294 ± 1.340	0.005	73.967 ± 33.184	0.005
Positive	3.491 ± 1.296		103.537 ± 37.880	

RAGE and TLR4 Are Involved in S100A9-Mediated MDSCs Chemotaxis and Activation

To further investigate the mechanisms by which S100A9 enhances MDSCs migration and activation, we focused on the RAGE and TLR4 that are the most common S100A9 receptors (16, 17, 27). GST-S100A9 (20 μg/ml) up-regulated mRNA levels of *TLR4* and *RAGE* in MDSCs in a time-dependent manner (**Figures 5A,B**). Consistently, the protein levels of TLR4 and RAGE in MDSCs were enhanced by GST-S100A9 (20 μg/ml) (**Figure 5C**). The RAGE inhibitor FPS-ZM1, but not the TLR4 TAK-242, inhibited the migration index of the S100A9-treated MDSCs (**Figure 5D**). In contrast, TAK-242, but not FPS-ZM1, blocked S100A9-induced mRNA expression of *Arg1, iNOS,* and *IL-10* and ROS production in MDSCs (**Figures 5E–H**). Consistent with these results, the suppressive effect of S100A9-treated MDSCs on $CD8^+$ T cell proliferation was also inhibited by TAK-242 but not FPS-ZM1 (**Figures 5I,J**). These results suggest that RAGE and TLR4 are responsible for S100A9-mediated MDSCs chemotaxis and activation, receptively.

Activation of p38 and NF-κB Signaling Are Responsible for S100A9-Induced MDSCs Chemotaxis and Activation

RAGE and TLR4 mediate multiple signaling pathways such as mitogen-activated protein kinase (MAPK), PI3K/Akt, and nuclear factor-kappa B (NF-κB) pathways for inflammation and cancer (28–30). We then focused on these signaling pathways and explored their possible roles in S100A9-mediated MDSCs chemotaxis and activity. P-p38 and p-p65 levels but not p-ERK1/2, p-JNK and p-Akt were increased in MDSCs (**Figure 6A**). FPS-ZM1 and TAK-242 blocked S100A9-induced p-p38 and p-p65, respectively (**Figure 6B**), suggesting that RAGE and TLR4 are involved in S100A9-mediated p38 MAPK and NF-κB pathways, respectively. Exposure of GST-S100A9-treated MDSCs to the p38 inhibitor SB203580 decreased the migration

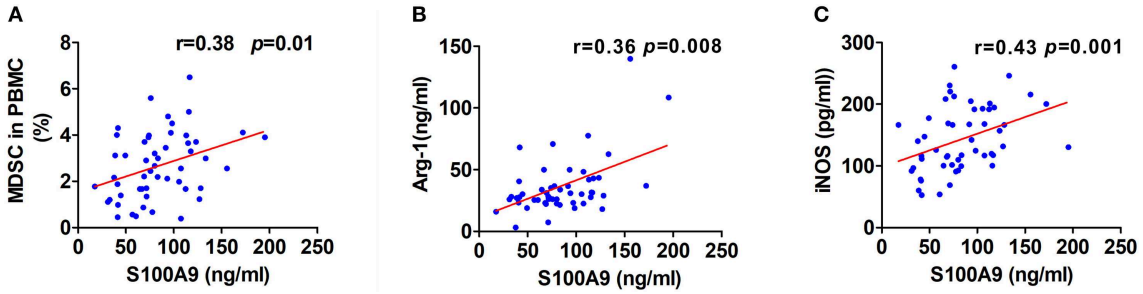

FIGURE 3 | Correlation of serum S100A9 levels with MDSCs frequency or immunosuppressive molecules Arg-1 and iNOS. **(A–C)** Correlation of serum S100A9 levels and MDSCs frequency in PBMC **(A)**, serum Arg-1 **(B),** and iNOS **(C)** in CRC patients ($n = 52$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

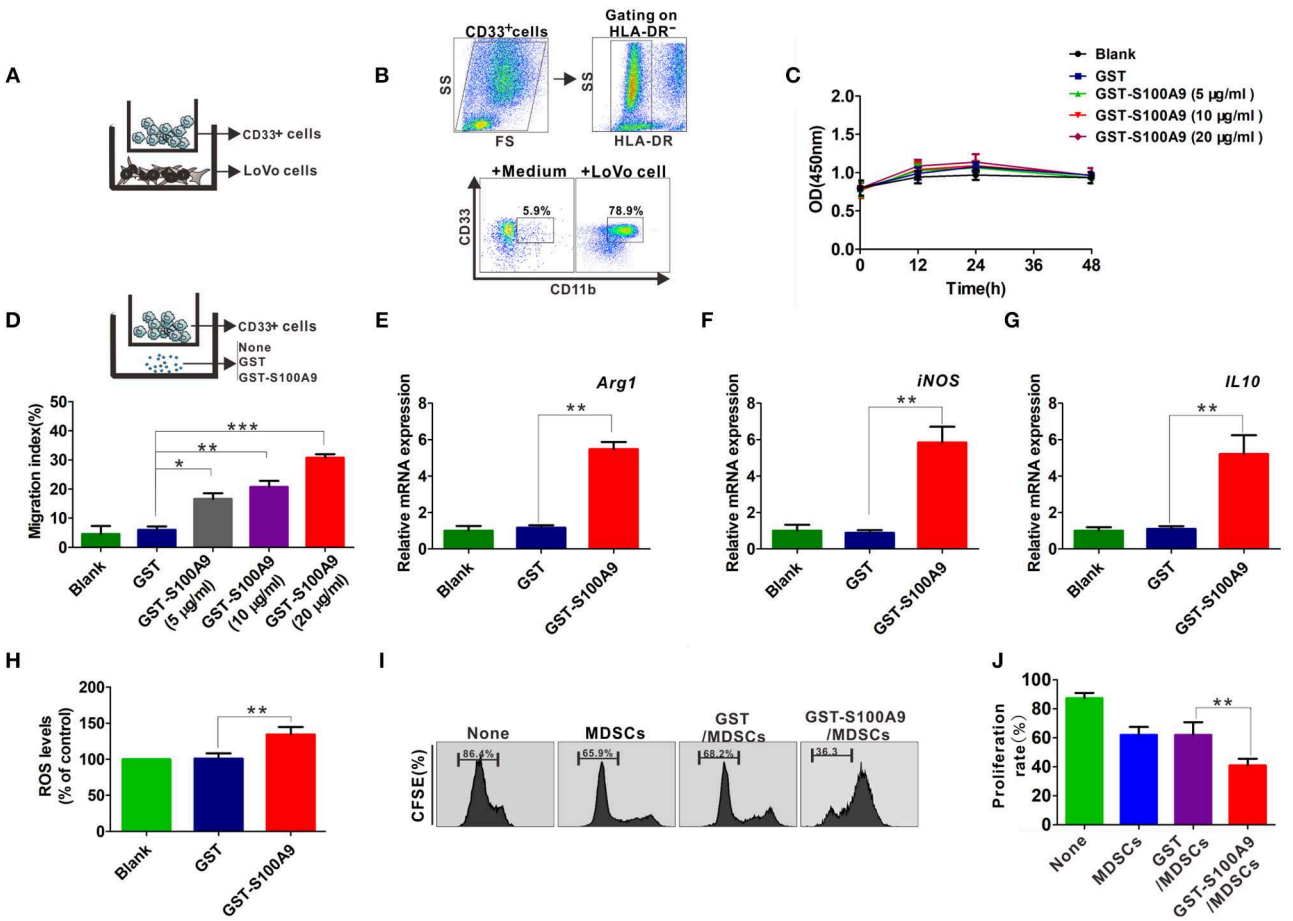

FIGURE 4 | The influence of S100A9 in LoVo-induced MDSCs vitality, chemotaxis and activation *in vitro.* **(A)** The $CD33^{+}$ cells were separated from PBMCs with CD33 positive magnetic beads, and co-cultured with LoVo cells to induce into CRC-associated MDSCs *in vitro*. **(B)** Identification of LoVo induced-MDSCs characterized by the myeloid marker with HLA-$DR^{-}CD33^{+}CD11b^{+}$ by FCM. $CD33^{+}$ cells cultured in medium alone were included as a control. **(C)** CCK8 assay for cell viability of LoVo-induced MDSCs treated with different concentrations of GST-S100A9 (5, 10, and 20 μg/ml) and GST proteins for 12, 24, and 48 h. **(D)** Chemotaxis assay for LoVo-induced MDSCs treated with different concentrations of GST-S100A9 (5, 10, and 20 μg/ml) and GST proteins for 24 h. **(E–G)** Real-time PCR analysis for mRNA expression of immunosuppressive molecules, including *Arg-1* **(E)**, *iNOS* **(F)**, and *IL-10* **(G)** in LoVo-induced MDSCs after GST-S100A9 (20 μg/ml) and GST (20 μg/ml) protein treatment for 24 h. **(H)** Fluorescence intensity analysis for ROS production in LoVo-induced MDSCs treated with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins. **(I)** Suppression of LoVo-induced MDSCs treated with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins on T cells *in vitro.* **(J)** A statistical graph of the suppressive effect of LoVo-induced MDSCs treated with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins on $CD8^{+}$ T cells. Data represent three independent experiments and are represented as Mean±SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

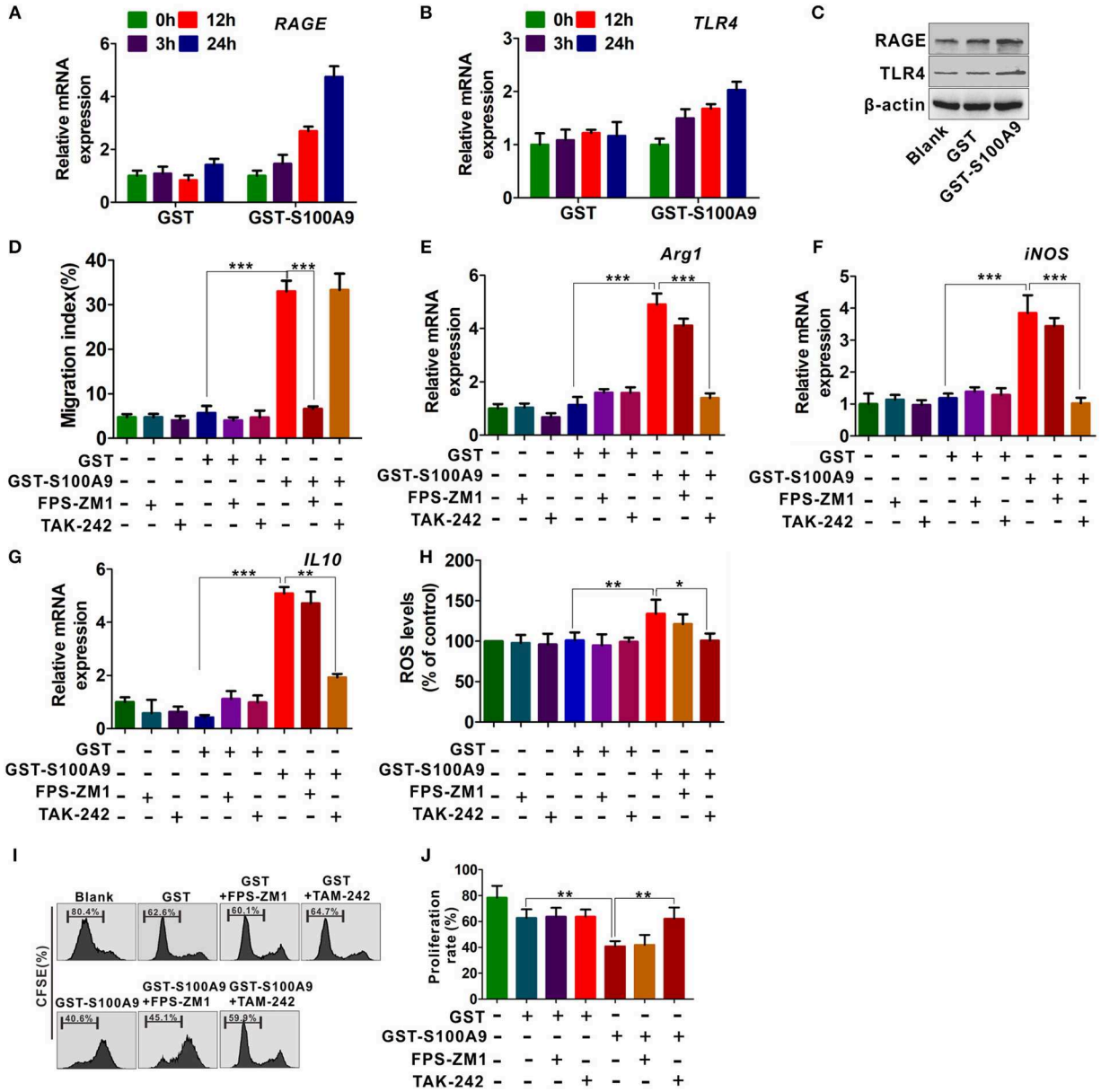

FIGURE 5 | RAGE and TLR4 receptors are responsible for S100A9-mediated MDSCs chemotaxis and activation. **(A,B)** Real-time quantitation PCR analysis of *RAGE* **(A)** and *TLR4* **(B)** mRNA expression in LoVo-induced MDSCs treated with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) for 3, 12, and 24 h. **(C)** Western blot analysis of RAGE and TLR4 expression in MDSCs treated with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) for 24 h. **(D)** Chemotaxis assay for LoVo-induced MDSCs pretreated with and without inhibitors FPS-ZM1 (100 nM) and TAK-242 (100 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins for 24 h. **(E–G)** Real-time quantitative PCR analysis for mRNA expression of immunosuppressive molecules, including *Arg1* **(E)**, *iNOS* **(F)**, and *IL10* **(G)** in LoVo-induced MDSCs pretreated with and without inhibitors FPS-ZM1 (100 nM) and TAK-242 (100 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins for 24 h. **(H)** Fluorescence intensity analysis for ROS production in LoVo-induced MDSCs pretreated with and without inhibitors FPS-ZM1 (100 nM) and TAK-242 (100 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins for 24 h. **(I)** Suppression of LoVo-induced MDSCs pretreated with and without inhibitors FPS-ZM1 (100 nM) and TAK-242 (100 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins on T cells *in vitro*. **(J)** A statistical graph of the suppressive effect of LoVo-induced MDSCs pretreated with and without inhibitors FPS-ZM1 (100 nM) and TAK-242 (100 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins on $CD8^+$ T cells. Data represent three independent experiments and are represented as Mean±SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

index of GST-S100A9-treated MDSCs (**Figure 6C**). On the contrary, exposure of GST-S100A9-treated MDSCs to the NF-κB inhibitor BAY11-7082 decreased the mRNA levels of *Arg1, iNOS,* and *IL-10* and ROS (**Figures 6D–G**). Altogether, these results indicate that RAGE-mediated p38 MAPK and TLR4-mediated NF-κB are responsible for the chemotaxis and activation of MDSCs induced by S100A9, respectively.

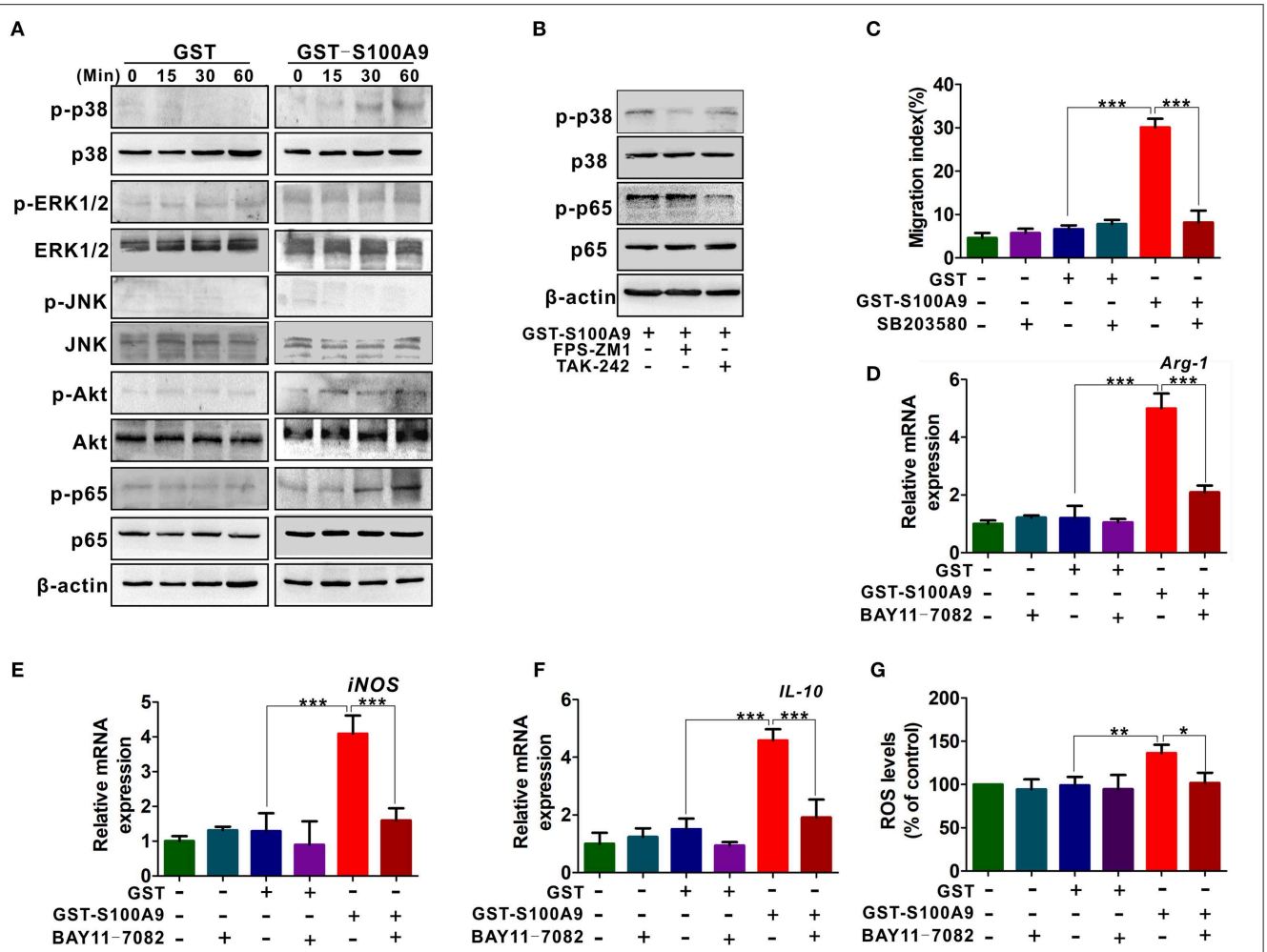

FIGURE 6 | RAGE-mediated p38/MAPK and TLR4-mediated NF-κB are responsible for the chemotaxis and activation of MDSCs resulted by S100A9. **(A)** Western blot analysis of p-p38, p-ERK, p-JNK, p-AKT, and p-p65 expression in LoVo-induced MDSCs treated by GST-S100A9 (20 μg/ml) or GST (20 μg/ml) for 0, 15, 30, and 60 min. **(B)** Western blot analysis of p-p38 and p-p65 expression in GST-S100A9-treated MDSCs with inhibitors FPS-ZM1 (100 nM) and TAK-242 (100 nM) for 60 min. **(C)** Chemotaxis assay for LoVo-induced MDSCs pretreated with and without inhibitor SB203580 (50 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins for 24 h. **(D–F)** Real-time PCR analysis for mRNA expression of immunosuppressive molecules *Arg-1* **(D)**, *iNOS* **(E)**, and *IL-10* **(F)** in LoVo-induced MDSCs pretreated with and without inhibitor BAY11-7082 (50 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins for 24 h. **(G)** Fluorescence intensity analysis for ROS production in LoVo-induced MDSCs pretreated with and without inhibitor BAY11-7082 (50 nM) for 30 min followed by stimulation with GST-S100A9 (20 μg/ml) and GST (20 μg/ml) proteins for 24 h. Data represent three independent experiments and are represented as Mean±SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Diagnostic Power of Serum S100A9 and MDSCs of Peripheral Blood for CRC Neoplastic Progression

We investigated the potential value of serum S100A9 and MDSCs of peripheral blood for CRC progression. ROC analysis showed that the diagnostic value of S100A9, MDSCs and their combination detection yielded an AUC of 0.71, 0.74, and 0.73 (**Figure 7A**, **Table 3**), suggesting that S100A9 and MDSCs, individually or in combination, are weak in discriminating CRC patients from healthy individuals. We next evaluated whether they can distinguish early and advanced stages of CRC. A combination of S100A9 and MDSCs had a better detection efficiency than S100A9 or MDSCs alone, which yielded an AUC of 0.92 with 86.7% sensitivity and 86.4% specificity (**Figure 7B**, **Table 3**). Furthermore, we found that the combination was superior to S100A9 or MDSCs alone in identifying CRC patients with lymphatic metastasis, which yielded an AUC of 0.82 with 75.0% sensitivity and 77.1% specificity (**Figure 7C**, **Table 3**). Those results imply that combination detection of S100A9 and MDSCs could be a serum marker for CRC diagnosis in disease stage and metastasis.

DISCUSSION

MDSCs are immune-modulatory cells that suppress adaptive immunity to promote tumor progression and metastasis. Although the immunosuppressive role of MDSCs in the tumor niche is documented, the detailed regulatory mechanisms

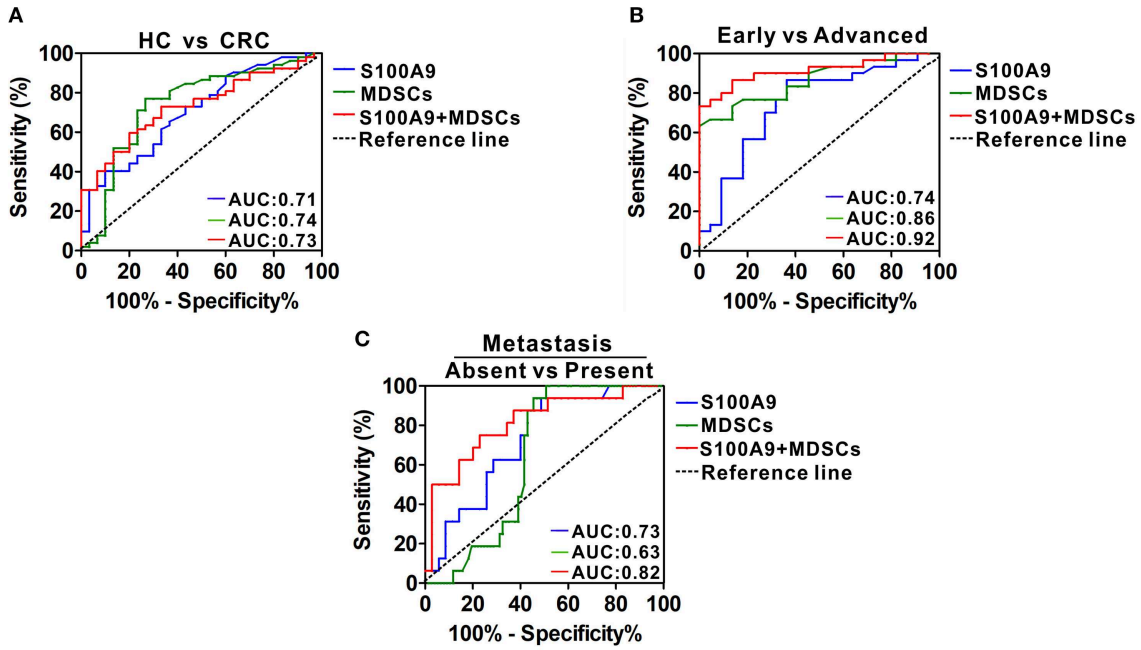

FIGURE 7 | Diagnostic power of serum S100A9 and MDSCs frequency for CRC progression. **(A)** ROC curves of serum S100A9, MDSCs, and their combination for detecting CRC. **(B)** ROC curves of serum S100A9, MDSCs and their combination for identifying advanced stages from early stages in CRC patients. **(C)** ROC curves of serum S100A9, MDSCs, and their combination for detecting metastasis from none in CRC patients.

TABLE 3 | The efficacy analysis of the detection index.

	HC vs. CRC			Early vs. Advanced			Absent vs. Present		
	S100A9	MDSCs	Combination	S100A9	MDSCs	Combination	S100A9	MDSCs	Combination
AUC	0.71	0.74	0.73	0.75	0.86	0.92	0.72	0.74	0.82
Sensitivity	73.1%	73.30%	66.7%	86.70%	73.30%	86.7%	93.8%	68.8%	75%
Specificity	56.1%	76.9%	73.10%	63.60%	81.8%	86.4%	51.40%	80%	77.1%

by which MDSCs are recruited and activated have not been well-elucidated. S100A9 have gained interest because it functions as a chemokine for regulating inflammatory cell or immunocyte, which creates a proinflammatory microenvironment to facilitate tumor growth and metastasis. Here, we provide data suggesting a regulatory role of S100A9 in the CRC microenvironment on MDSCs chemotaxis and activation involving RAGE-dependent p38 MAPK and TLR4-dependent NF-κB signaling pathways (**Figure 8**).

In humans, MDSCs constitute a heterogeneous cell population that is not well-characterized, partially because no unified markers are currently available for these cells. Most studies concur with the observation that MDSCs express CD11b and CD33 but lack the expression of mature myeloid cell markers such as HLA-DR (5). In this study, we identified and characterized the MDSC population in CRC patients and found a strong correlation between MDSCs and CRC neoplastic progression, which is consistent with previous findings in other cancers such as bladder cancer (8), melanoma (31), and hepatocellular carcinoma (32). Our data also demonstrated that high levels of immunosuppression molecules Arg-1 and iNOS mainly expressed in MDSCs in CRC serum samples, demonstrating that MDSCs may not only be significantly increased in CRC but also have more suppressive function compared to those from healthy donor cells with the same phenotype.

We further demonstrated that MDSCs from CRC patients or induced by LoVo cells exerted the MDSCs phenotype, which was characterized by expressing $CD11b^+$ $CD33^+$ $HLA\text{-}DR^-$. The results that show that the CRC cells can induce functional MDSCs *in vitro* agrees with previous studies for other types of cancer cells (14, 33, 34). Various tumor-derived factors have been reported to induce immature myeloid cells ($CD33^+$ cells) to differentiate to MDSCs, these factors include prostaglandin E2, IL-6, IL-10, IL-1β, TGF-β, and proangiogenic factors such as vascular endothelial growth factor (35, 36). The proinflammatory S1008 and S100A9 proteins were reported to regulate MDSC accumulation in all regions of dysplasia and adenoma in a colitis-associated colon cancer model (37). Therefore, we speculate that the induction of MDSCs from $CD33^+$ PBMCs may be

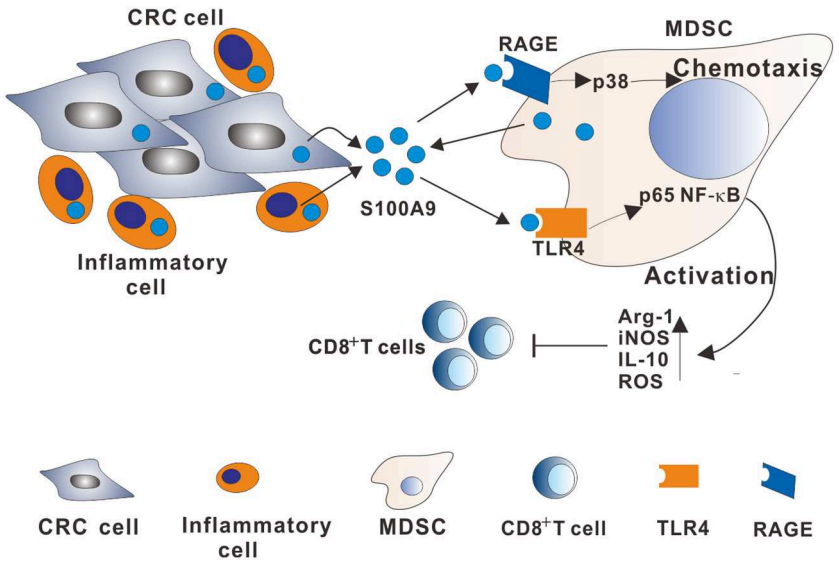

FIGURE 8 | A model describing the mechanisms of the S100A9-induced immunosuppressive microenvironment by regulating MDSCs chemotaxis and activation in CRC. Increased extracellular S100A9 protein derived from CRC cells, inflammatory cells, or MDSCs in TME stimulates RAGE-dependent p38 MAPK signaling cascade, promoting MDSCs chemotaxis. Additionally, increased extracellular S100A9 in TME stimulates the TLR4-dependent NF-κB signaling cascade, promoting MDSCs activation by upregulating immunosuppressive molecules Arg-1, iNOS, and IL10 expression, and ROS production.

associated with these factors derived by LoVo cells, which needs future studies.

Elevated S100A9 expression in CRC tissues and its association with disease progression have been reported in our previous study (21). Here, similar results were obtained. In addition, we explored the relationship of serum S100A9 levels with CRC neoplastic progression. Interestingly, recent research indicated that S100A9 could be one marker for circulatory MDSCs (38), and other studies showed that MDSCs from tumor-bearing mice or peripheral blood in cancer patients express and secrete S100A9 in an autocrine manner (37). Here, a positive correlation between S100A9 and MDSCs as well as immunosuppressive molecules Arg1 or iNOS was also observed. Consistent with the literature, our present findings further suggest that S100A9 participates in immunosuppression during CRC development by regulating MDSCs.

MDSCs have emerged as key effector cells in the immunosuppressive microenvironment of many solid tumor malignancies, and several factors that infuence MDSC recruitment and function have been investigated. For example, tumor-derived granulocyte-macrophage colony-stimulating factor recruits and alters MDSC proliferation and function in pancreatic ductal adenocarcinoma, thwarting $CD8^+$ T cell-mediated anti-tumor immunity (39). Accumulation of CCL2 was found to be correlated to poor prognosis in glioblastoma patients, whereas deficiency of CCL2 reduced the recruitment of MDSCs and Treg cells in a glioblastoma mouse model (40). CXC-motif chemokines such as CXCL12 and IL-8 are also involved in the recruitment of MDSCs (41, 42). Considering the regulatory effect of S100A9 on chemotaxis and activation for inflammatory cells shown in previous studies (43, 44), we assessed whether the protein is responsible for the chemotaxis or activation of MDSCs. Our data showed that S100A9 could intensify the recruitment and function of MDSCs, suggesting that S100A9 plays an important role in the immunosuppressive microenvironment by regulating MDSCs. RAGE-mediated or TLR4-mediated downstream signal cascades have been reported to be involved in the migration or activation function of inflammatory cells induced by S100A9 in inflammation (22, 45–47). Here, we found that the RAGE-mediated p38 and TLR4-mediated NF-κB signaling pathways were involved in MDSC chemotaxis and activation, respectively.

Over the past few decades, increasing experimental evidence has demonstrated that either S100A9 or MDSCs contribute to tumor development (5, 16), suggesting that the detection of S100A9 and/or MDSCs in peripheral blood may be a diagnostic and progression prediction marker for CRC. While the differentiating power of S100A9, MDSCs or their combination is weak for CRC diagnosis, the combination detection could a marker for predicting CRC stages and lymph node metastasis. All this evidence suggests that the combination of S100A9 and MDSCs could be a candidate marker to detect CRC neoplastic progression.

In conclusion, the current observations indicate that accumulative MDSCs and increased S100A9 in CRC patients contribute to immune suppression. A positive correlation of MDSCs and S100A9 was observed in the peripheral blood of CRC patients. Both MDSCs and S100A9 were correlated to CRC neoplastic progression, which could be a candidate marker to detect CRC neoplastic progression. We

further demonstrated that S100A9 plays a role in MDSCs recruitment and activation in CRC by regulating the RAGE-mediated p38 MAPK and TLR4-mediated NF-κB signaling pathways. Thus, inhibiting MDSCs by targeting S100A9-induced signaling pathways may be a beneficial option for CRC patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The serum and tissue samples were obtained from CRC patients with no chemotherapy or radiotherapy prior to surgery. Written informed consent, was obtained from all of these participants. This study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University.

AUTHOR CONTRIBUTIONS

MH and RW performed the experiments, analyzed the data, and wrote the manuscript. LC, QP, YZ, and SL analyzed the data. LD and LZ conceived the study ideas, oversaw the research, and co-wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02243/full#supplementary-material

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Immune Landscape of Invasive Ductal Carcinoma Tumor Microenvironment Identifies a Prognostic and Immunotherapeutically Relevant Gene Signature

Xuanwen Bao[1,2†], Run Shi[3†], Kai Zhang[4], Shan Xin[1], Xin Li[5], Yanbo Zhao[4] and Yanfang Wang[6*]*

[1] Helmholtz Center Munich, German Research Center for Environmental Health, Institute of Radiation Biology, Munich, Germany, [2] Technical University Munich, Munich, Germany, [3] Department of Radiation Oncology, University Hospital, Ludwig Maximilian University of Munich, Munich, Germany, [4] Department of Cardiology, School of Medicine, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou, China, [5] College of Life Sciences, Nanjing University, Nanjing, China, [6] Department of Pharmacy, Pharmaceutical Biotechnology, Center of Nanoscience (CeNS), Ludwig Maximilian University of Munich, Munich, Germany

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***Correspondence:**
Xuanwen Bao
xuanwen.bao@helmholtz-muenchen.de
Yanfang Wang
yangfang.wang@cup.uni-muenchen.de

[†]These authors have contributed equally to this work

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Background: Invasive ductal carcinoma (IDC) is a clinically and molecularly distinct disease. Tumor microenvironment (TME) immune phenotypes play crucial roles in predicting clinical outcomes and therapeutic efficacy.

Method: In this study, we depict the immune landscape of IDC by using transcriptome profiling and clinical characteristics retrieved from The Cancer Genome Atlas (TCGA) data portal. Immune cell infiltration was evaluated via single-sample gene set enrichment (ssGSEA) analysis and systematically correlated with genomic characteristics and clinicopathological features of IDC patients. Furthermore, an immune signature was constructed using the least absolute shrinkage and selection operator (LASSO) Cox regression algorithm. A random forest algorithm was applied to identify the most important somatic gene mutations associated with the constructed immune signature. A nomogram that integrated clinicopathological features with the immune signature to predict survival probability was constructed by multivariate Cox regression.

Results: The IDC were clustered into low immune infiltration, intermediate immune infiltration, and high immune infiltration by the immune landscape. The high infiltration group had a favorable survival probability compared with that of the low infiltration group. The low-risk score subtype identified by the immune signature was characterized by T cell-mediated immune activation. Additionally, activation of the interferon-α response, interferon-γ response, and TNF-α signaling via the NFκB pathway was observed in the low-risk score subtype, which indicated T cell activation and may be responsible for significantly favorable outcomes in IDC patients. A random forest algorithm identified the most important somatic gene mutations associated with the constructed immune signature. Furthermore, a nomogram that integrated clinicopathological features with the immune signature to predict survival probability was constructed, revealing that

the immune signature was an independent prognostic biomarker. Finally, the relationship of VEGFA, PD1, PDL-1, and CTLA-4 expression with the immune infiltration landscape and the immune signature was analyzed to interpret the responses of IDC patients to immunotherapy.

Conclusion: Taken together, we performed a comprehensive evaluation of the immune landscape of IDC and constructed an immune signature related to the immune landscape. This analysis of TME immune infiltration landscape has shed light on how IDC respond to immunotherapy and may guide the development of novel drug combination strategies.

Keywords: immune landscape, immune signature, survival, invasive ductal carcinoma, immune checkpoint inhibitor

INTRODUCTION

Invasive ductal carcinoma (IDC) is a clinically and molecularly distinct disease. IDCs are typically of high histologic grade and high mitotic index. HER2 overexpression or amplification is detected in 20% of these tumors (1). IDC tends to metastasize to bone, liver, and lung, whereas invasive lobular carcinoma (ILC) has a higher tendency to metastasize in gastrointestinal and genital tracts, serosal cavities, and meninges (2). IDCs usually form glandular structures in contrast to the small clusters formed by ILCs. The loss of CDH1 leads to the discohesive morphology in ILCs, whereas IDCs maintain intact cell adhesion (3). Furthermore, the frequency of recurrently mutated genes and recurrent copy-number alterations often differs significantly between IDCs and ILCs (3). These features are generally associated with a poor prognosis. Taken together, these differences suggest that ILCs and IDCs are distinct cancer types and progress along different pathways.

Genetic and epigenetic changes contribute to the progression of tumor progression and recurrence in different cancer types. However, accumulated evidence indicates that the tumor microenvironment (TME) has clinicopathological significance in predicting survival outcomes and assessing therapeutic efficacy factors (4, 5). TME cells constitute a vital element of cellular and non-cellular components in the tumoural niche, including extracellular matrix and cellular components, such as fibroblasts, adipose cells, immune-inflammatory cells, and neuroendocrine cells. Previous studies have revealed that immune cells in the TME modulate cancer progression and are attractive therapeutic targets (6, 7). To date, the comprehensive landscape of immune cells infiltrating the TME of IDCs has not yet been elucidated. We propose that IDCs have a distinct immune landscape and that the immune landscape might lead to different prognoses and treatment responses. In this study, by applying several computational algorithms, we estimated the abundance of immune cells in the TME of IDCs and analyzed the correlation of the immune landscape with genomic characteristics and pathological features of IDCs. Furthermore, we built an immune signature, which is a robust prognostic biomarker and predictive factor for the response to immunotherapy.

Abbreviations: TME, tumor microenvironment; IDC, invasive ductal carcinoma; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; ssGSEA, single-sample gene set enrichment; LASSO, least absolute shrinkage and selection operator; ILC, invasive lobular carcinoma; DEG, differentially expressed gene; WGCNA, weighted correlation network analysis; IRF4, interferon-regulatory factor 4; AICE, AP-1-IRF consensus element.

METHODS

Data Download

TCGA RNA-seq datasets and clinical data for IDCs were downloaded by UCSC Xena browser (https://xenabrowser.net/). GSE20685 and GSE86948 were downloaded from the Gene Expression Omnibus (GEO) database.

Implementation of Single-Sample Gene Set Enrichment Analysis (ssGSEA)

We obtained the marker gene set for immune cell types from Bindea et al. (8). MDSC gene set was imported from MSIGDB gmt file from Broad institute. We used the ssGSEA program to derive the enrichment scores of each immune-related term. In brief, the infiltration levels of immune cell types were quantified by ssGSEA in the R package gsva (9). The ssGSEA applies gene signatures expressed by immune cell populations to individual cancer samples. The computational approach used in our study included immune cells types that are involved in innate immunity and adaptive immunity. Tumors with qualitatively different immune cell infiltration patterns were grouped using hierarchical agglomerative clustering (based on Euclidean distance and Ward's linkage).

The T cell infiltration score (TIS) was defined as the average of the standardized values for $CD8^+$ T, central memory $CD4^+$ T, effector memory $CD4^+$ T, central memory $CD8^+$ T, effector memory $CD8^+$ T, Th1, Th2, Th17, and Treg cells. The obtained cytotoxic activity scores (CYT) score was calculated by the geometrical mean of PRF1 and GZMA (10). The $CD8^+$ T/Treg ratio was the digital ratio of the ssGSEA scores for these two cell types. The correlation between risk score and immune cell ssGSEA score was calculated by Pearson correlation.

LASSO Regularization

LASSO (least absolute shrinkage and selection operator) is an important regularization in many regression analysis methods (e.g., COX regression and logistic regression) (10–12). The idea

behind LASSO is that a L1-norm is used to penalize the weight of the model parameters. Assuming a model has a set of parameters, the LASSO regularization can be defined as:

$$\lambda \cdot \sum_{i=0}^{n} \|w_i\|_1$$

It can also be expressed as a constraint to the targeted objective function:

$$\sum \left\| \boldsymbol{Y} - \boldsymbol{Y}^{*} \right\|_2 , \ s.t. \ \ \|w_i\|_1 < t$$

An important property of the LASSO regularization term is that it can force the parameter values to be 0, thus generating a sparse parameter space, which is a desirable characteristic for feature selection. In our analysis, 19 genes which were highly associated with OS were used as the input. QRSL1, TIMM8A, IGHA1, BATF, KLRB1, SPIB, and FLT3LG were picked after the penalizing process. A risk score (RS) formula was established by including individual normalized gene expression values weighted by their LASSO Cox coefficients:

$$\sum_{i} Coefficient(mRNA_i) \times Expression(mRNA_i)$$

Risk score = (0.210 * expression level of QRSL1) + (0.092 * expression level of TIMM8A) + (−0.046 * expression level of IGHA1) + (−0.066 * expression level of BATF) + (−0.110 * expression level of KLRB1) + (−0.139 * expression level of SPIB) + (−0.262 * expression level of FLT3LG).

Differentially Expressed Gene (DEG) Analysis

DEG analysis was performed by the Limma package (13). The samples were separated into a high-risk score group and a low-risk score group. An empirical Bayesian approach was applied to estimate the gene expression changes using moderated *t*-tests. The *Q*-value (adjusted *p*-value) for multiple testing was calculated using the Benjamini-Hochberg correction. The DEGs were defined as genes with a *Q*-value < 0.05. The clusterProfiler R package was applied for the GO analysis (14). GSEA was applied with the GSEA software.

Co-expression Gene Network Based on RNA-seq Data

The Weighted correlation network analysis (WGCNA) was used to construct the gene co-expression network (15, 16). The co-expression similarity $s_{i,j}$ was defined as the absolute value of the correlation coefficient between the profiles of nodes *i* and *j*:

$$s_{i,j} = |cor\left(x_i, x_j\right)|$$

where, x_i and x_j are expression values of for genes *i* and *j*, and $s_{i,j}$ represent Pearson's correlation coefficients of genes *i* and *j*, respectively.

A weighed network adjacency was defined by raising the co-expression similarity to a power β:

$$a_{i,j} = s_{i,j}^{\beta}$$

with $\beta \geq 1$. We selected the power of $\beta = 5$ and scale-free $R^2 = 0.95$ as the soft-thresholding parameters to ensure a signed scale-free co-expression gene network. Briefly, network construction, module detection, feature selection, calculations of topological properties, data simulation, and visualization were performed. Modules were identified via hierarchical clustering of the weighting coefficient matrix. The module membership of node *i* in module *q* was defined as:

$$K_{cor,i}^{(q)} := cor(x_i, E^{(q)})$$

where, x_i is the profile of node *i*, and $E(q)$ is the module eigengene (the first principal component of a given module) of module *q*. The module membership measure $K_{cor,i}^{(q)}$, lies in [−1, 1] and specifies how close node *i* is to module *q*, $q = 1, \cdots, Q$.

By evaluating the correlations between the immune infiltration status, immune signature of IDCs and the module membership of each module, a brown module was selected for further analysis.

Data Processing and Integration

The mutation datasets were download by R package TCGAbiolinks. The expression profiles of the most powerful prognostic features (QRSL1, TIMM8A, IGHA1, BATF, KLRB1, SPIB, and FLT3LG) were extracted from the whole transcriptome datasets. The immune infiltration status was calculated by the deconvolution algorithm and grouped using hierarchical agglomerative clustering. We summarized the clinic datasets, mutation datasets, expression profiles and immune infiltration status into an integrated dataset (**Supplementary File 1**).

Statistical Analysis

A random forest algorithm was applied to find the most important somatic mutation associated with the immune signature. Survival outcome analysis modeled the results in reference to the patient OS and RFS. *P*-values and Hazard ratios were obtained from univariate Cox proportional-hazards regression models using the R package survival. Multivariate Cox regression was used to calculate the coefficients in the nomogram. The nomogram was plotted by the rms package. The time-dependent AUC value was calculated by the survivalROC package.

RESULTS

Immune Phenotype Landscape in the TME of IDC

Immune cell populations modulate diverse immune responses and lead to anti-tumour effects by infiltrating the IDC TME. The immune cell infiltration status was assessed by applying the ssGSEA approach to the transcriptomes of IDCs. Twenty-four immune-related terms were incorporated to deconvolve

the abundance of diverse immune cell types in IDCs. The IDCs were clustered into 3 clusters (low infiltration: 208; intermediate infiltration: 430; and high infiltration: 130) in terms of immune infiltration by applying an unsupervised hierarchical clustering algorithm (**Figure 1A**). By applying hierarchical cluster analysis and K-means clustering analysis, we constructed a TME cell network, depicting a comprehensive landscape of tumor-immune cell interactions and their effects on the OS of patients with IDC (**Figure 1B** and **Figures S1, S2**). The TME immune cells were clustered into 4 clusters, and the correlation among different immune cell types is shown in **Figure 1B**. The association of OS and RFS with different clusters of IDCs was analyzed by a pairwise log-rank test. The results indicated that the high infiltration group had a favorable survival probability compared with that of the low infiltration group (**Figures 1C,D**).

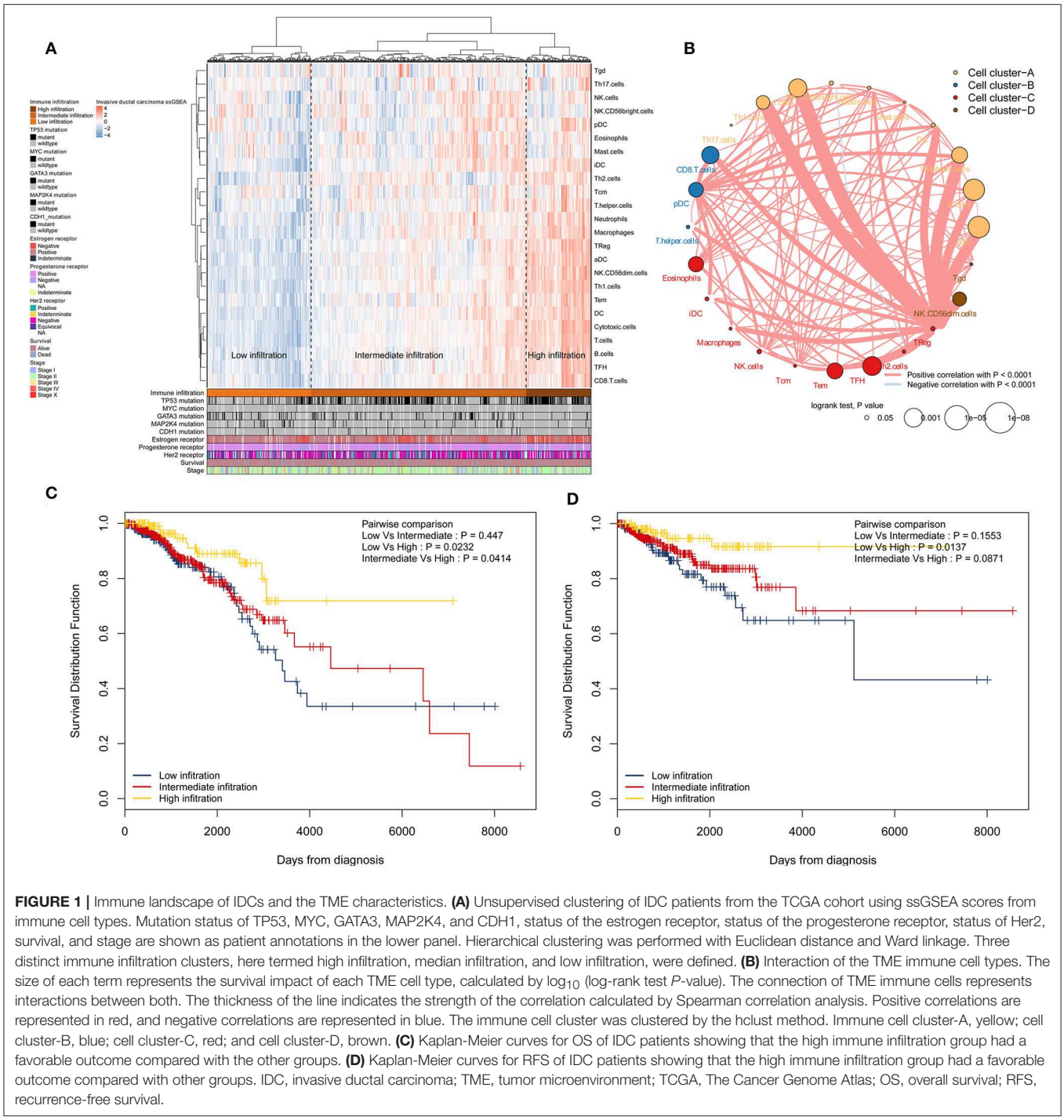

FIGURE 1 | Immune landscape of IDCs and the TME characteristics. **(A)** Unsupervised clustering of IDC patients from the TCGA cohort using ssGSEA scores from immune cell types. Mutation status of TP53, MYC, GATA3, MAP2K4, and CDH1, status of the estrogen receptor, status of the progesterone receptor, status of Her2, survival, and stage are shown as patient annotations in the lower panel. Hierarchical clustering was performed with Euclidean distance and Ward linkage. Three distinct immune infiltration clusters, here termed high infiltration, median infiltration, and low infiltration, were defined. **(B)** Interaction of the TME immune cell types. The size of each term represents the survival impact of each TME cell type, calculated by $\log_{10}$ (log-rank test *P*-value). The connection of TME immune cells represents interactions between both. The thickness of the line indicates the strength of the correlation calculated by Spearman correlation analysis. Positive correlations are represented in red, and negative correlations are represented in blue. The immune cell cluster was clustered by the hclust method. Immune cell cluster-A, yellow; cell cluster-B, blue; cell cluster-C, red; and cell cluster-D, brown. **(C)** Kaplan-Meier curves for OS of IDC patients showing that the high immune infiltration group had a favorable outcome compared with the other groups. **(D)** Kaplan-Meier curves for RFS of IDC patients showing that the high immune infiltration group had a favorable outcome compared with other groups. IDC, invasive ductal carcinoma; TME, tumor microenvironment; TCGA, The Cancer Genome Atlas; OS, overall survival; RFS, recurrence-free survival.

Construction of the Immune Signature

A total of 413 genes were involved in the 24 immune-related terms. We applied the univariate COX regression based on the survival datasets of patients with IDC and the expression profiles of the 413 genes. The 19 most significant genes were selected with the criteria of a p-value < 0.0005 (**Figure 2A**). The expression profiles of the 19 genes are shown in **Figure 2B**. LASSO Cox regression was performed on the 19 genes to identify the most important features in terms of predicting the survival of IDC patients (**Figures 2C–E**). By forcing the sum of the absolute value of the regression coefficients to be less than a fixed value, certain coefficients were reduced to exactly zero, and the most powerful prognostic features (QRSL1, TIMM8A, IGHA1, BATF, KLRB1, SPIB, and FLT3LG) were identified with relative regression coefficients. Cross-validation was applied to prevent over-fitting. A 7-gene immune signature was constructed according to the individual coefficients of the genes. Then, we calculated the risk score for each IDC patient and ranked them (**Figure 2F**). **Figure 2G** shows the survival overview in the IDC patients. A heatmap showed that patients in the high-risk group tended to have increased QRSL1 and TIMM8A expression levels, as well as decreased expression levels of IGHA1, BATF, KLRB1, SPIB, and FLT3LG (**Figure 2H**). The Kaplan-Meier curve and Cox regression suggested that patients with high risk scores had significantly worse OS and RFS than those with low risk scores (HR $= 2.94$, $p < 0.0001$ and HR $= 2.28$, $p = 0.001$, respectively) (**Figures 2I,J**). The effect of the seven genes on the OS and RFS of IDC patients is shown in **Figures S3**, **S4**, respectively. To confirm our findings in the IDC cohort, we validated the prognostic function of the immune signature in two independent GEO cohorts (GSE20685 and GSE86948). The risk score was calculated for each patient by using the same formula as in the IDC cohort. The GSE20685 and GSE86948 cohorts were used to predict the OS of BRCA patients based on our immune signature model. Consistent with our previous findings, the Kaplan-Meier curve suggested a significantly better overall survival in the low-risk group than in the high-risk group (**Figures S5A,B**).

The Low Risk Score Was Associated With Active Infiltration Status and High Cytotoxic Potential

High infiltration status showed a lower risk score than the intermediate infiltration status and low infiltration status showed (**Figure 3A**). Similarly, patients with a low risk score had a higher proportion of high immune infiltration than patients with a high risk score (**Figure 3B**). The presence of high immune infiltration in patients was linked to a low risk score and was associated with a favorable outcome (**Figure 3C**). To compare cytotoxic function with the immune landscape and immune signature that we constructed, the associated signatures were identified for each patient. IDCs with high infiltration status and low risk score were associated with increased levels of immune activation. The TIS ($p < 0.0001$ and $p < 0.0001$, respectively) (**Figures 3D,G**), interferon-γ signature ($p < 0.0001$ and $p < 0.0001$, respectively) (**Figures 3E,H**), and CYT ($p < 0.0001$ and $p < 0.0001$, respectively) (**Figures 3F,I**) were increased in the low-risk score group and high infiltration group. The ssGSEA score of DCs was higher in the low-risk score group than in the high-risk score group. The Kaplan-Meier curve showed that in the low-risk score group, the ssGSEA score of DC cells affected survival but did not affect the high-risk score group (**Figures S6A–C**). Furthermore, the correlation between MDSCs and risk score was analyzed (**Figure S7A**). The ssGSEA score for MDSCs was positively associated with the OS of IDC patients in whole cohorts ($p = 0.017$) (**Figure S7B**). When we stratified the patients into low-risk score and high-risk score groups, the ssGSEA score of MDSCs showed opposite association with the survival of IDC patients (HR $= 2.42$ and 0.63, respectively) (**Figures S7C,D**). These data indicate that compared with high-risk score tumors, low-risk score tumors have a distinct immune phenotype, characterized by increased immune infiltration and increased levels of immune activation.

The Low-Risk Score Was Associated With Increased T Cell Infiltration

The association of risk score and immune-related cells was analyzed by Pearson correlation. Cytotoxic cells, $CD8^+$ T cells, T cells and the 6 other most significant immune-related cell types are shown in **Figure 4**. A high level of correlation was found between the risk score and the T cell-mediated immune response. The ssGSEA scores of 24 immune-related terms in the low, intermediate, and high immune status and low- and high-risk score groups are shown in **Figures S8A,C**. The p-value and difference in the mean ssGSEA score from the high- and low-infiltration status and low- and high-risk score groups are shown in **Figures S8B,D**. The proportions of low, intermediate, and high immune infiltration status in different pathological subtypes and different AJCC stages of IDC are shown in **Figures S8E,F**. The triple-negative subtype of IDCs had a higher proportion of high infiltration status IDCs than other pathological subtypes, indicating an active immune response in triple-negative IDCs. The risk score distribution in different pathological subtypes and different AJCC stages of IDC are shown in **Figures S8G,H**. The luminal A subtype had a lower risk score than the other pathological subtypes.

Functional Annotation and WGNCA of the Transcriptomes of IDC Patients

To identify the underlying biological characteristics of the constructed immune signature, DEG analysis was performed based on the high-risk score group and low-risk score group. The heatmap depicts the significant DEGs between the two groups (**Figure 5A**). The GO analysis indicated that T cell activation, positive regulation of leukocyte cell-cell adhesion, and regulation of lymphocyte activation were the most significantly enriched biological processes between the high-risk score group and the low-risk score group (**Figure 5B**). The GSEA results showed that allograft rejection, IL-6/JAK/STAT3 signaling, the inflammatory response, interferon-α response, interferon-γ response, and TNF-α signaling via the NFκB pathway were the most predominantly upregulated pathways in the low-risk score group. In contrast, the E2F targets, G2M checkpoints, MTORC1 signaling, and protein secretion pathways were significantly

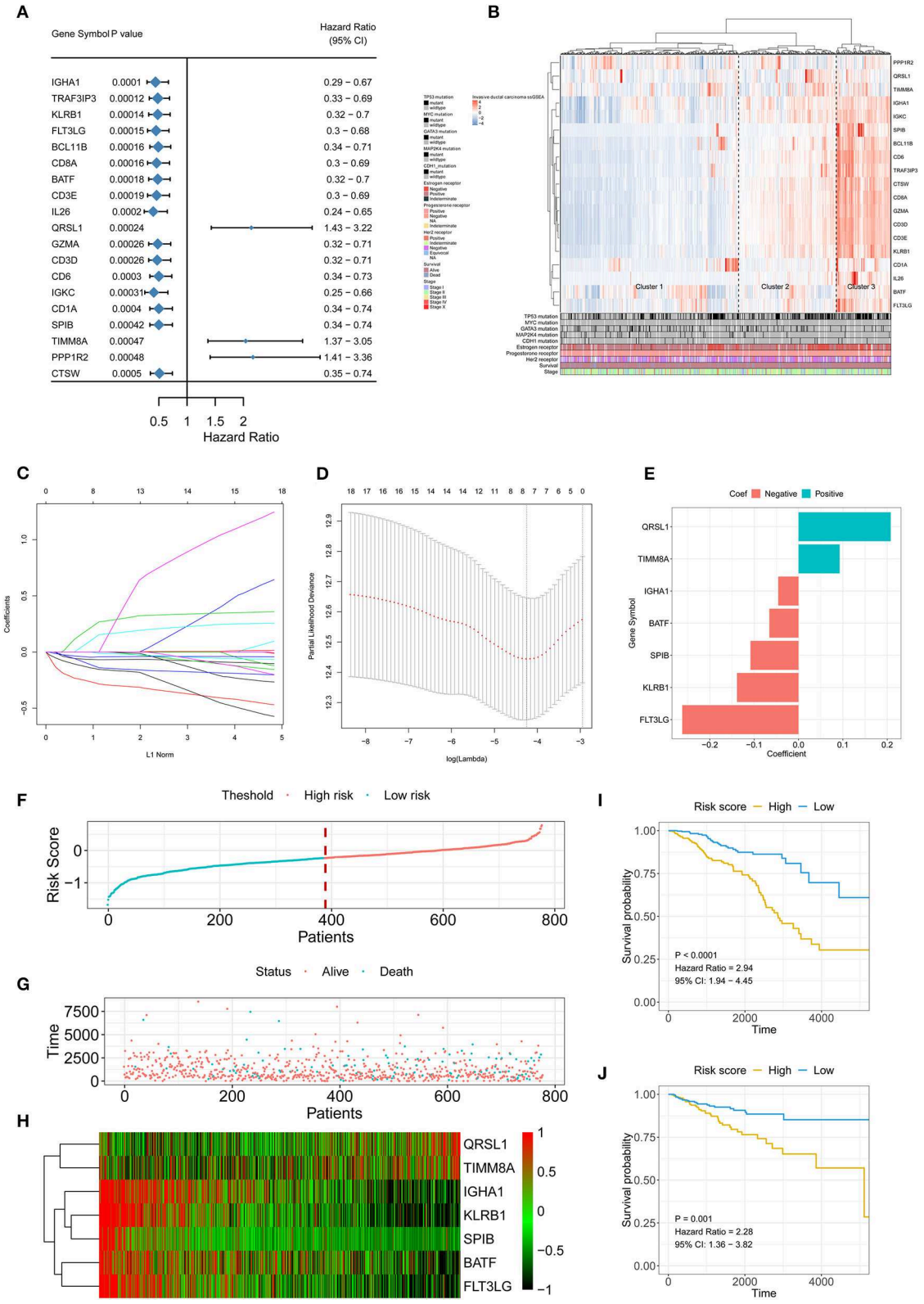

FIGURE 2 | Signature-based risk score is a promising marker of survival in IDC patients. **(A)** The HR and *P*-value from the univariable Cox HR regression of selected genes in the immune terms (Criteria: *P*-value < 0.001). **(B)** The expression of the selected genes shown by heatmap. Mutation status of TP53, MYC, GATA3,

(Continued)

FIGURE 2 | MAP2K4, and CDH1, status of the estrogen receptor, status of the progesterone receptor, status of Her2, survival, and stage are shown as patient annotations in the lower panel. Hierarchical clustering was performed with Euclidean distance and Ward linkage. **(C,D)** LASSO Cox analysis identified seven genes most correlated with overall survival, and 10-round cross validation was performed to prevent overfitting. **(E)** Coefficient distribution of the gene signature. **(F)** Risk score distribution. **(G)** Survival overview. **(H)** Heatmap showing the expression profiles of the signature in the low- and high-risk groups. **(I)** Patients in the high-risk group exhibited worse OS than those in the low-risk group. **(J)** Patients in the high-risk group exhibited worse RFS than those in the low-risk group. IDC, invasive ductal carcinoma; OS, overall survival; RFS, recurrence-free survival.

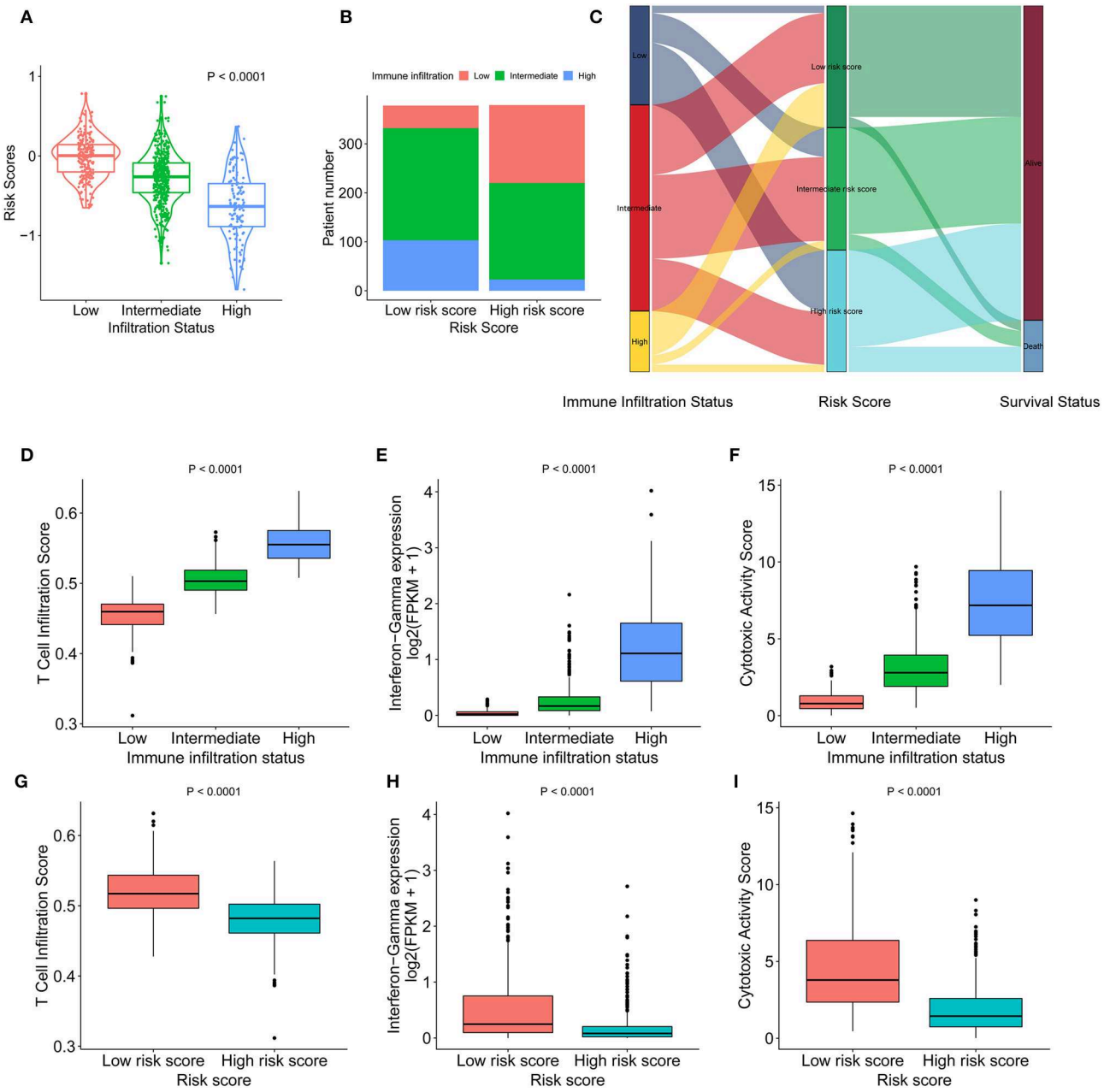

FIGURE 3 | Heterogeneous immune cell infiltration in the low- and high-risk score groups. **(A)** The distribution of risk scores in low, mediate, and high immune infiltration patterns. **(B)** The distribution of immune infiltration patterns in the low- and high-risk score groups. **(C)** Alluvial diagram of immune infiltration patterns in groups with different risk scores and survival outcomes. **(D)** TIS in low, mediate, and high immune infiltration patterns. **(E)** Relative interferon-γ signature in low, mediate, and high immune infiltration patterns. **(F)** Comparison of relative CYT in low, mediate, and high immune infiltration patterns. **(G)** Relative TIS in the low- and high-risk score groups. **(H)** Relative interferon-γ signature in the low- and high-risk score groups. **(I)** Comparison of relative CYT in the low- and high-risk score groups. TIS, T cell infiltration score; CYT, cytotoxic activity scores.

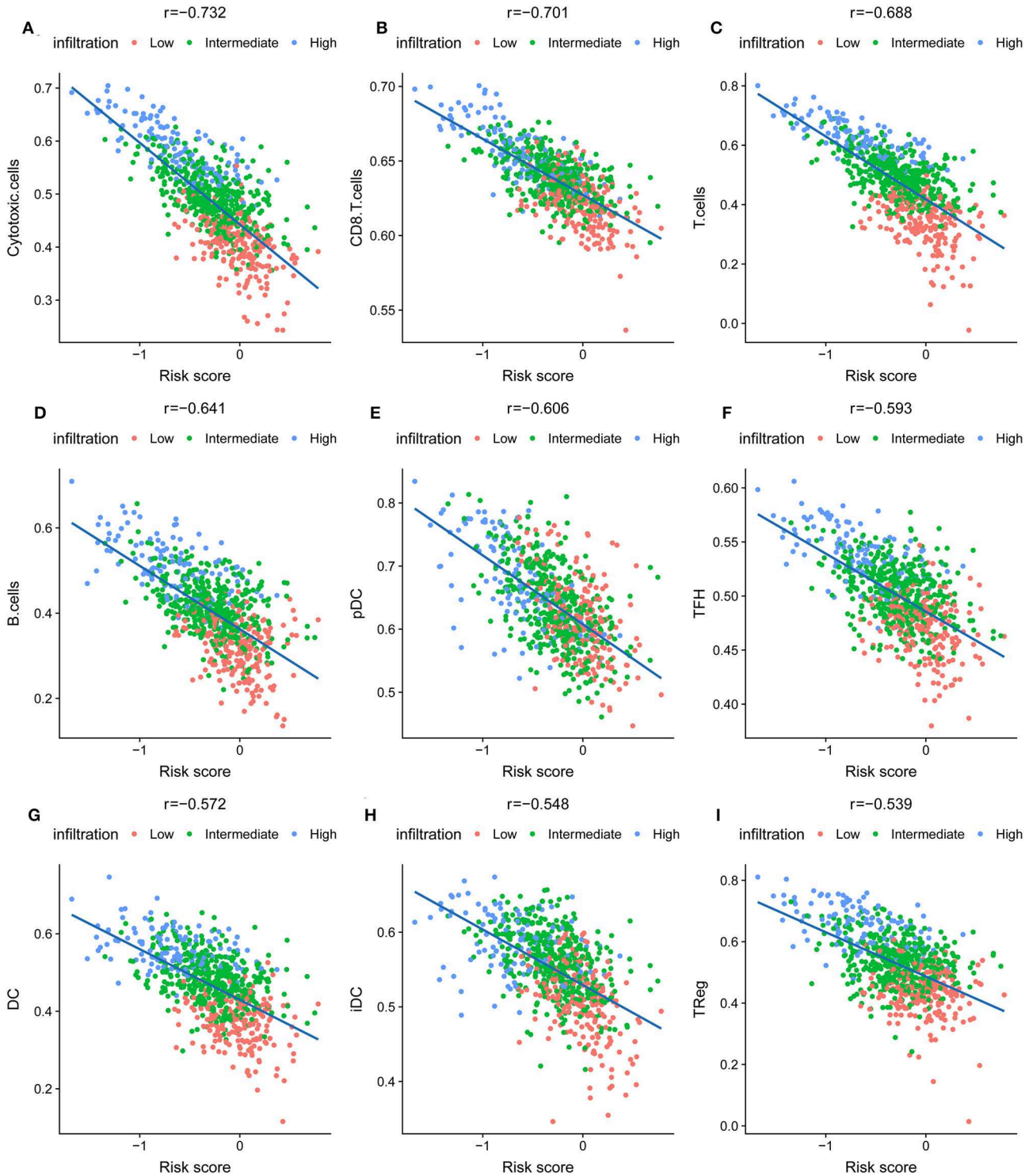

FIGURE 4 | The nine most significant correlations of risk score with immune cell infiltration ssGSEA score. **(A)** Cytotoxic cells, **(B)** $CD8^+$ T cells, **(C)** T cells, **(D)** B cells, **(E)** pDC cells, **(F)** TFH cells, **(G)** DC cells, **(H)** iDC cells, **(I)** Treg cells.

downregulated in the low-risk score group (**Figures 5C,D**). To further identify the underlying biological characteristics in the immune signature, WGCNA was performed, and the correlation of risk score and immune infiltration status with module membership were analyzed. The soft threshold selection is shown in **Figure S9**. The module-trait heatmap illustrates

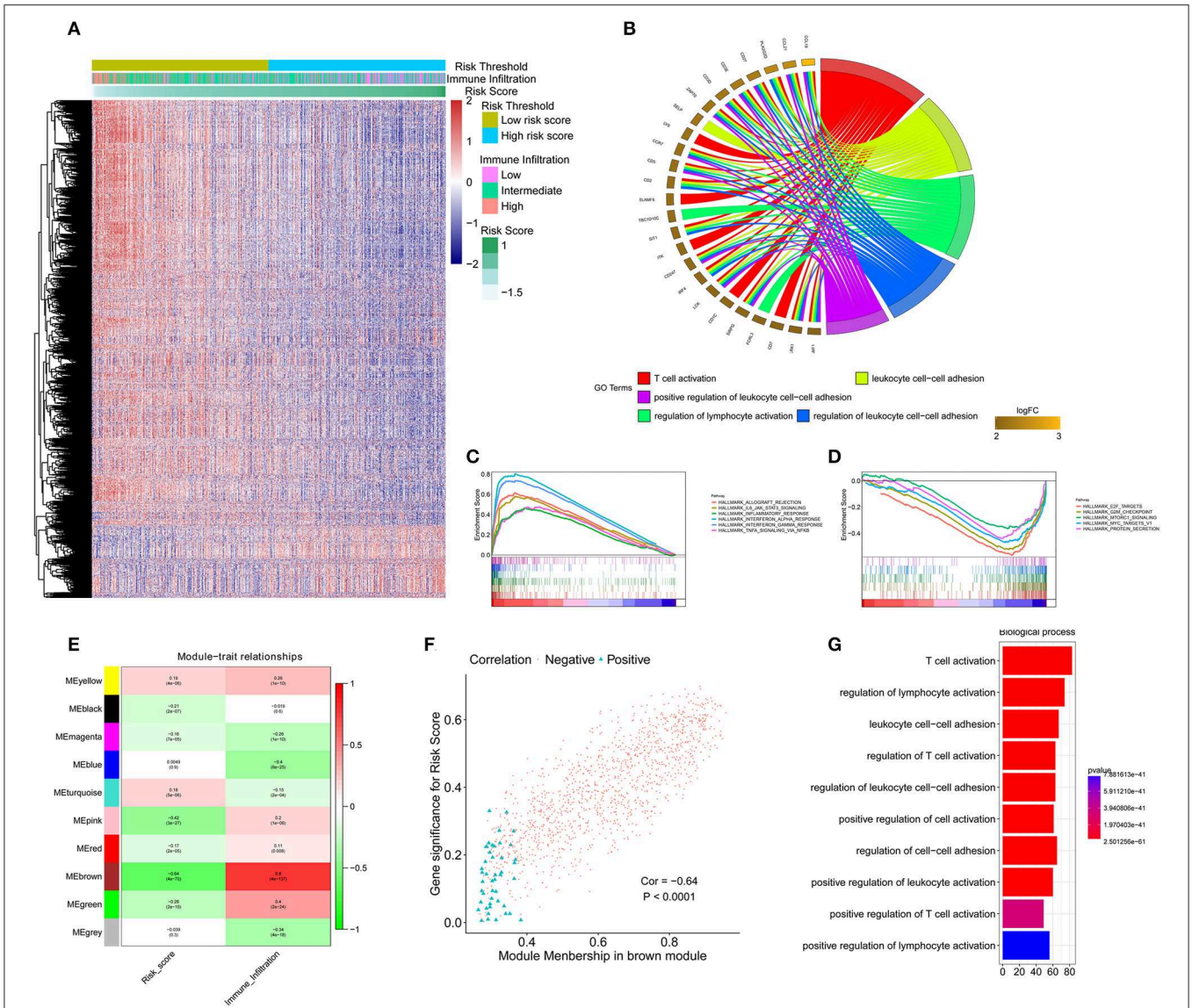

FIGURE 5 | Functional annotation of the immune signature and WGCNA of the IDC transcriptome. **(A)** Heatmap showing the transcriptome expression profiles of the low- and high-risk groups. **(B)** GO analysis based on the significant genes in the comparison between low- and high-risk groups. **(C,D)** GSEA revealed that most significant hallmarks correlated with the immune signature. **(E)** Correlation between modules and traits. **(F)** The correlation between module membership and gene significance in the brown module. **(G)** GO analysis based on the hub genes in the brown module. GO, gene ontology; GSEA, gene set enrichment analysis.

that the brown module had a significant *p*-value with both immune signature and immune infiltration status (**Figure 5E**); the coefficients were −0.64 and 0.8, respectively. The association between module membership and gene significance for each gene in the brown module is shown in **Figure 5F**. The genes from the brown module with a coefficient >0.5 were selected as hub genes, and GO enrichment analysis revealed that T cell activation and lymphocyte activation were the most significantly enriched biological processes, which further confirmed the results from the DEG analysis (**Figure 5G**).

Mutation Load and Immune Signature

The spectrum of somatic mutations in patients with IDCs is known to be varied. We next investigated the distributions of somatic mutations and observed different patterns among IDCs in terms of total mutations. The risk score from the immune signature had a positive correlation with total mutations in IDC patients (**Figure 6A**). By applying a random forest algorithm, we identified 35 highly variable mutated genes that were associated with the immune signature (**Figure 6B**). TP53 was the predominant gene of the 35 identified genes.

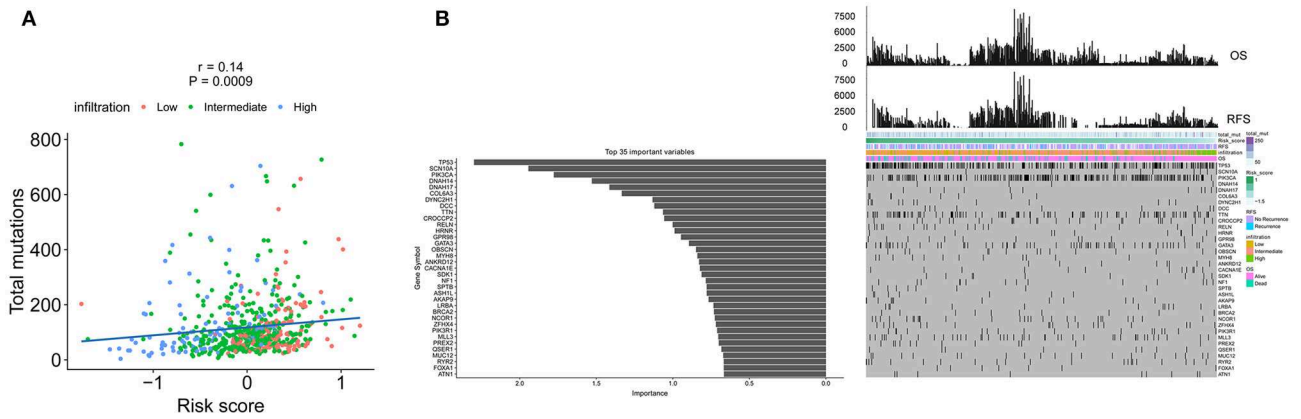

FIGURE 6 | The association of the immune signature with cancer somatic mutations. **(A)** The correlation between the immune signature and IDC somatic mutations. **(B)** Distribution of somatic mutations correlated with the immune signature. The upper bar plot indicates OS and RFS per patient, whereas the left bar plot shows the importance of the somatic mutations correlated with the immune signature. IDC, invasive ductal carcinoma; OS, overall survival; RFS, recurrence-free survival.

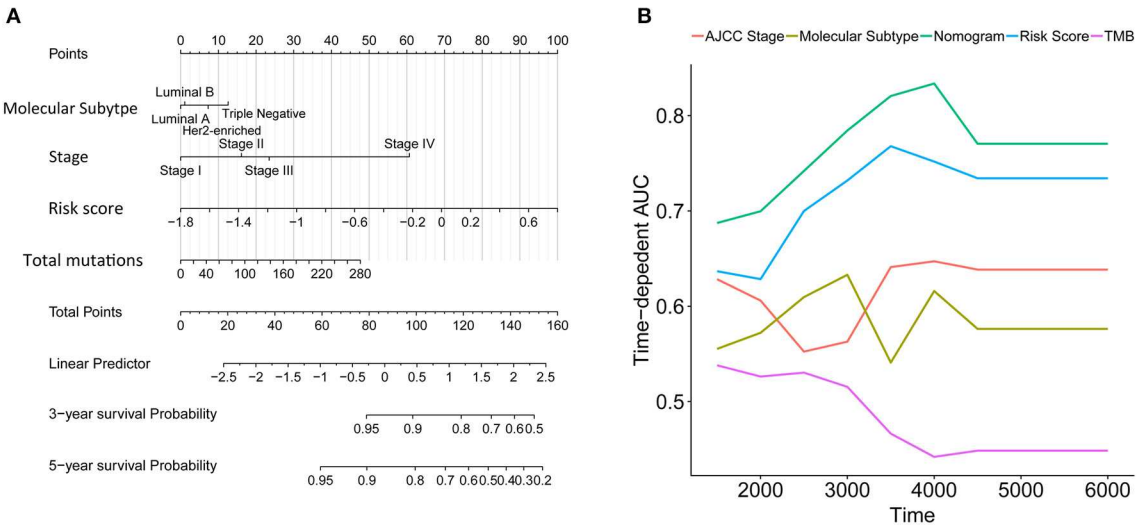

FIGURE 7 | Construction of a nomogram for survival prediction. **(A)** Nomogram combining the immune signature with clinicopathological features. **(B)** The AUC(t) of the multivariable models indicated that the nomogram had the highest predictive power for overall survival.

Construction of a Nomogram to Predict Overall Survival in IDC Patients

We constructed a nomogram that integrated clinicopathological features with the immune signature to predict the survival probability of IDC patients (**Figure 7A**). The AUC(t) functions of the multivariable models were developed to indicate how well these features serve as prognostic markers. Compared to other features, such as signature-based risk score, AJCC-TNM stage, and total mutation burden, the nomogram consistently showed the highest predictive power for overall survival in the follow-up period (**Figure 7B**).

The Immune Signature Predicted the Immunotherapeutic Benefits in IDC Patients

VEGF-A, the main mediator in tumor angiogenesis, hinders T cell infiltration in the tumor microenvironment. Hence, we explored the correlation between VEGF-A expression and the T cell immune response in IDC tumors. Interestingly, the increased VEGFA expression significantly correlated with both decreased levels of activated $CD8^+$ T cells and Th1 cell infiltration in the high immune infiltration tumor microenvironment but not in the low immune infiltration tumor microenvironment (**Figures 8A,B**). Furthermore, perforin, the molecular effector

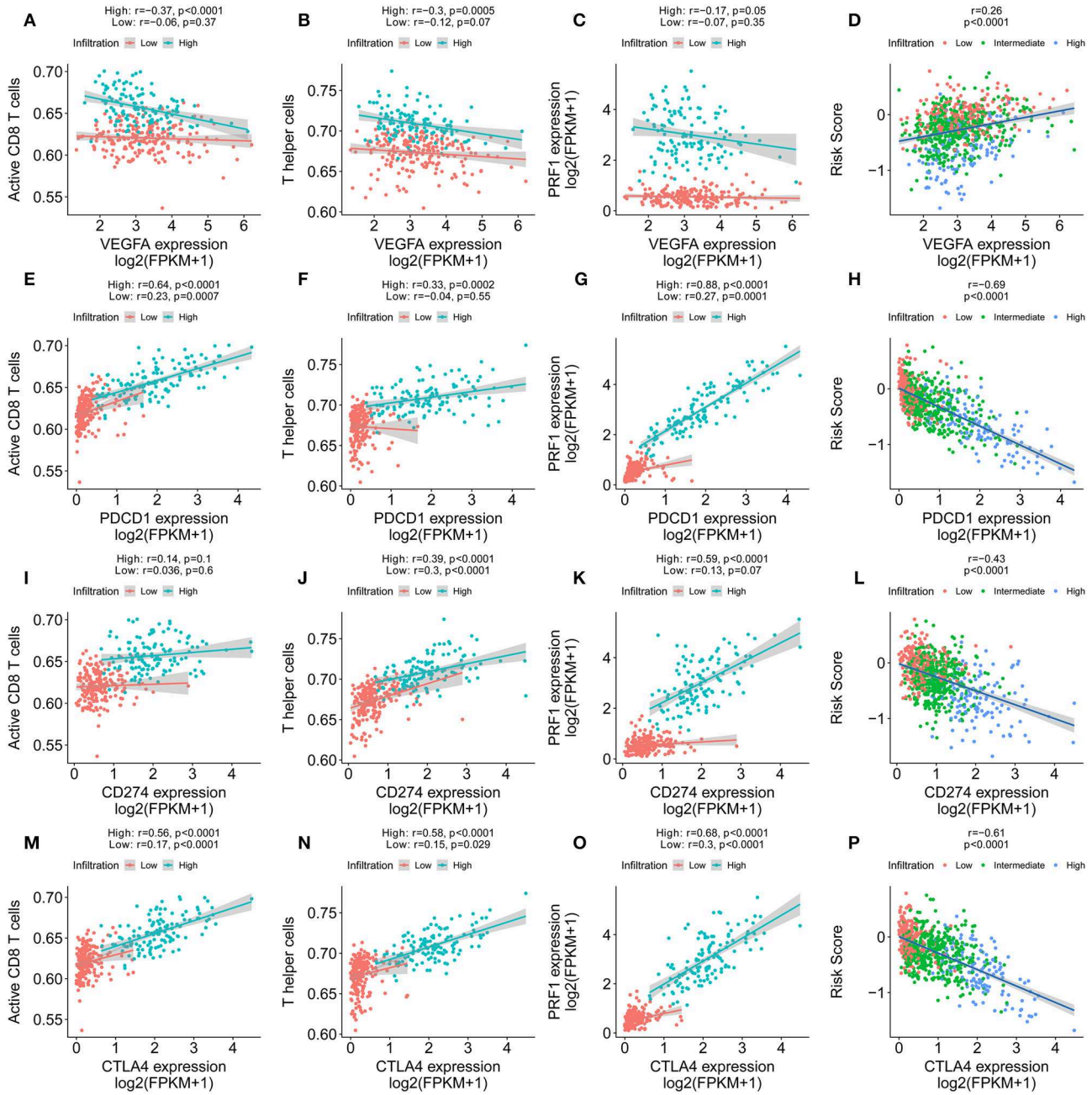

FIGURE 8 | Immune signature predicts immunotherapeutic benefits. **(A–C)** The correlation of VEGFA expression with T cell infiltration, Th1 cells, and PRF1 expression in high and low immune infiltration conditions. **(D)** The correlation of VEGFA expression with the immune signature. **(E–G)** The correlation of PD-1 expression with T cell infiltration, Th1 cells, and PRF1 expression in high and low immune infiltration conditions. **(H)** The correlation of PD-1 expression with the immune signature. **(I–K)** The correlation of PDL-1 expression with T cell infiltration, Th1 cells, and PRF1 expression in high and low immune infiltration conditions. **(L)** The correlation of PDL-1 expression with the immune signature. **(M–O)** The correlation of CTLA-4 expression with T cell infiltration, Th1 cells, and PRF1 expression in high and low immune infiltration conditions. **(P)** The correlation of CTLA-4 expression with the immune signature.

found in the granules of cytotoxic T lymphocytes and natural killer cells, also showed a negative correlation with VEGF-A expression (**Figure 8C**). Finally, the positive correlation of VEGF-A and the risk score was identified (**Figure 8D**). PD-1, PDL-1, and cytotoxic T lymphocyte antigen-4 (CTLA-4) are promising targets for the treatment of patients with breast and non-small cell lung cancer. PD-1, PDL-1, and CTLA-4 antibodies are undergoing studies for the treatment of breast cancer. We analyzed the correlation of PD-1, PDL-1, and CTLA-4 expression in the high- and low-infiltration groups (**Figures 8E–P**). The

expression of PD-1, PDL-1, and CTLA-4 was more significantly correlated with $CD8^+$ T cells, Th1 cell ssGSEA score, and perforin expression in the high-infiltration group than in the low-infiltration group. Furthermore, the mean expression of PD-1, PDL-1, and CTLA-4 was significantly increased in the high-infiltration group, indicating a potentially enhanced response to the corresponding anticancer antibody for IDCs with high immune infiltration status. In our constructed immune signature, the risk score showed a negative correlation with PD-1, PDL-1, and CTLA-4 expression, which implies a potentially enhanced effect of PD-1, PDL-1, and CTLA-4 antibodies in patients with low risk score. Lastly, we checked the correlation of the expression profiles of several immune checkpoint proteins, e.g., CD160, CD274, CD276, CTLA-4, LAG3, and PDCD1, risk score, and VEGF-A in the TCGA and GSE20685 cohorts (**Figure S10**).

DISCUSSION

In this study, we depicted the immune landscape of IDC using a large cohort. The immune landscape might explain the differences in prognoses of patients with IDC and responses to PD1, PDL-1, and CTLA-4 antibodies. Based on the immune landscape, we constructed an immune signature that calculated the risk score per patient. The correlation of signature and immune landscape revealed that the T cell-mediated immune response played a crucial role in the signature. Patients with low risk scores had increased T cell infiltration scores, interferon-γ signatures, and cytotoxic activity scores, indicating active T cell immune responses and favorable survival probability. A random forest algorithm was applied to find the most important somatic mutation correlated with the immune signature. A nomogram was constructed based on the immune signature and other clinicopathological properties of IDCs. A time-dependent ROC analysis showed high accuracy of the immune signature and nomogram in terms of predicting the survival of IDC patients. Lastly, PD-1, PDL-1, and CTLA-4 expression was found to be highly associated with the risk score. The patients with low risk scores had increased expression levels of PD-1, PDL-1, and CTLA-4, indicating a potentially high response rate to PD-1, PDL-1, and CTLA-4 antibodies.

In our analysis, the IDCs were clustered into three main clusters (low immune infiltration, intermediate immune infiltration, and high immune infiltration). The patients in the high-infiltration cluster had the best survival probability compared with patients in the low- and intermediate-infiltration clusters. The T cell immune response is the central event in antitumour immunity (17). T cells are divided into $CD4^+$ (helper T cells, Th) and $CD8^+$ (cytotoxic T cells, Tc) T cells. Their secretomes include IFN-γ, TNF-α, and IL17, which have antitumour effects. Hence, the increased T cell infiltration score, interferon-γ signature, and cytotoxic activity score may lead to an anti-tumor effect in the high-infiltration group. This finding could explain the different OS and RFS in the high- and low-infiltration groups.

From the immune landscape in IDCs, we built an immune signature that included seven features (QRSL1, TIMM8A, IGHA1, BATF, KLRB1, SPIB, and FLT3LG). FLT3LG is a crucial cytokine that controls the development of DCs and is particularly important for CD8-positive classical DCs and their CD103-positive tissue counterparts. A clinical trial is currently underway to treat melanoma patients with a combination of immunostimulatory FLT3LG and a peptide-based vaccine targeting DCs (18). KLRB1, which encodes CD161, a surface marker on several T cell subsets and NK cells, has been found to be most frequently associated with favorable outcomes in many cancer types by enhancing innate immune characteristics (19). SPIB is a member of the ETS family and profoundly affects B cell functions. B cells that lack SPIB fail to proliferate in response to IgM cross-linking, exhibit limited capacity to respond to T-dependent antigens, and produce low levels of IgG1, IgG2a, and IgG2b (20). In addition, SPIB can activate enhancer elements in both Ig-λ and Ig-κ genes, increasing the expression of these two genes. BATF is an inhibitor of AP-1-driven transcription. Recent studies have revealed that BATF can regulate positive transcriptional activity in dendritic cells, B cells and T cells (21). BATF leucine zipper motifs interact with interferon-regulatory factor 4 (IRF4) and IRF8 at AP-1–IRF consensus elements (AICEs), adding additional flexibility to the actions of IRF4 and IRF8, which were previously considered to interact with SPIB and PU.1 (22). The interaction of IRF4 and BATF in T helper 17 cells increases the production of IL-17, IL-21, IL-22, and IL-23 receptor. TIMM8A is involved in the import and insertion of hydrophobic membrane proteins from the cytoplasm to the mitochondrial inner membrane. The Bax/Bak complex mediates the release of DDP/TIMM8a and activates Drp1-mediated fission to promote mitochondrial fragmentation and subsequent elimination during programmed cell death (23). From the expression profiles of the seven genes above, we calculated the risk score for each patient and predicted the survival of IDC patients.

The risk score from the immune signature was most significantly correlated with the ssGSEA score of cytotoxic cells, CD8 T cells and T cells, indicating the important roles of the T cell immune response in the immune signature. Interestingly, DCs in the low-risk group played a more important role than DCs in the high-risk group. The increased proportion of DCs significantly correlated with favorable survival in the low-risk group but did not correlate with favorable survival of patients in the high-risk group. Th innate inflammatory cytokines, such as IL-1, IL-12, and IL-23 expressed by DCs, promote IFN-γ-secreting $CD4^+$ T cell and cytotoxic T lymphocyte responses (24). The high proportion of DCs and T cells cooperate to achieve the antitumour effect in IDC patients with low risk scores. MDSCs were immunosuppressive population. Patients in the high-risk score group had lower infiltration status and poor survival compared with that of patients in the low-risk score group. This might explain why the patients in the high-risk score group with high MDSC score had a poor survival compared with that of patients with low MDSC score. Furthermore, the GSEA results revealed high levels of IFN-γ, TNF-α, and TNF-α secretion in the low-risk group, which contribute to the antitumour activity in IDC patients with low risk scores.

WGCNA revealed opposing directions of the risk score (cor = −0.64) and immune infiltration (cor = 0.8) with the brown module, indicating the high level of correlation of risk score (calculated by immune signature) and immune infiltration. The hub gene in the brown module plays an essential role in regulating immune infiltration. The GO analysis revealed that T cell activation was the most significantly enriched biological process, indicating that the T cell-mediated immune response is the central event in both immune infiltration and the immune signature.

The spectrum of somatic mutations varied in IDC patients. The different mutation burdens in IDCs led us to analyse whether the landscape of immune cells and the immune signature were associated with somatic mutations. The total mutations showed a positive correlation with the risk score in IDC patients. Furthermore, a random forest algorithm was performed to identify the most important variables correlated with the immune signature. TP53, SCN10A, PIK3CA, and 32 other genes were the most significant variables in the analysis. TP53 and PIK3CA mutations are the most common gene mutations in IDCs (44 and 33%, respectively). In the 35 gene variables, GATA3, a key regulator of ER activity, is a newly identified gene that is mutated in IDCs (5% in ILC vs. 13% in IDC, $q = 0.03$) (3). Mutations in GATA3 are more frequent in luminal A IDC and are mutually exclusive with FOXA1 events. The differential expression level and enrichment for mutations of GATA3 in IDCs and of FOXA1 in ILC indicates a preferential requirement for the distinct regulation of ER activity in ILC and IDC (3). Previous studies revealed that the GATA3 mutation correlates with increased expression, which is associated with the immune response (25, 26). Our analysis further confirms the correlation of the GATA3 mutation with immune infiltration. In addition, we constructed a nomogram that integrated clinicopathological features with the immune signature to predict the survival probability of IDC patients. Compared with other clinicopathological features, the immune signature showed the best accuracy in predicting the survival of IDC patients at any time point and would therefore be helpful for the diagnosis and precise treatment of IDC patients.

There have been several studies for the treatment of breast cancer with immunotherapeutic antibodies. PD-1 is expressed by exhausted T cells. PD-1 and PD-L1 exhibit inhibitory receptor–ligand interactions, which are involved in the negative regulation of T cell activation and peripheral tolerance during immune responses by cancer cells. Despite demonstrated successes, only a proportion of patients benefit from PD-1 and PDL-1 antibody treatment. Hence, it is important to determine the mechanism that leads to the varied therapeutic effect of PD-1 and PDL-1 antibody treatment and thus improve individual diagnosis and precision medicine. PD-L1 expression, microsatellite instability and deficient mismatch repair are important biomarkers that predict the response to anti-PD-1/PD-L1 therapies (27–29). Among the three biomarkers, PD-L1 expression has been validated in nearly all tumor types for all approved anti-PD-1/PD-L1 therapies. In our analysis, the expression of PD1, PDL-1, and CTLA-4 was significantly increased in the high-infiltration group. Furthermore, the expression of PD1, PDL-1, and CTLA-4 had a significant correlation with $CD8^+$ T cells, Th1 cell ssGSEA score, and perforin expression in the high-infiltration group, which provides a basis for PD-1/PD-L1 and CTLA-4 treatment. Similarly, the immune signature we constructed also indicated that high expression levels of PD1, PDL-1, and CTLA-4 correlated with low risk score. Therefore, patients with a low risk score could derive more benefit from immunotherapy than patients with a high risk score.

Some limitations should be acknowledged. First, this is a retrospective study, so the robustness of predictive value of the gene signature should be further validated in large prospective clinical trials. Second, experimental studies are required to further elucidate the biological functions underlying the gene signature in IDC.

In the current study, we performed a comprehensive evaluation of the immune landscape of IDC and constructed an immune signature related to the immune landscape. This analysis of TME immune infiltration patterns has shed light on how IDC respond to immunotherapy and may guide the development of novel drug combination strategies.

DATA AVAILABILITY

The datasets supporting the conclusions of this article are available in the Xena browser (https://xenabrowser.net/) repository.

AUTHOR CONTRIBUTIONS

XB and RS conceived and designed the experiments. XB, SX, and KZ analyzed the data. XB and YW wrote the paper. YZ, KZ, and RS reviewed the draft. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00903/full#supplementary-material

Figure S1 | The correlation between different infiltrating immune cells.

Figure S2 | The correlation between the ssGSEA scores of infiltrating immune cells and the OS probability of IDC patients.

Figure S3 | The correlation between the expression level of seven genes in the immune signature and the OS probability of IDC patients. **(A)** BATF, **(B)** KLRB1, **(C)** TIMM8A, **(D)** FLT3LG, **(E)** QRSL1, **(F)** IGHA1, **(G)** SPIB.

Figure S4 | The correlation between the expression of seven genes in the immune signature and the RFS probability of IDC patients. **(A)** BATF, **(B)** KLRB1, **(C)** TIMM8A, **(D)** FLT3LG, **(E)** QRSL1, **(F)** IGHA1, **(G)** SPIB.

Figure S5 | Validation of the immune signature in two external cohorts, GSE20685 **(A)** and GSE86948 **(B)**.

Figure S6 | The correlation between the ssGSEA scores of DCs and the OS probability of IDC patients in the high- and low-risk score groups. **(A)** The ssGSEA scores were higher in the high- and low-risk score groups. **(B)** The correlation between the ssGSEA scores of DCs and the OS probability of IDC patients in the low-risk score group. **(C)** The correlation between the ssGSEA scores of DCs and the OS probability of IDC patients in the high-risk score group.

Figure S7 | The correlation between the ssGSEA scores of MDSCs and the OS probability of IDC patients in the high- and low-risk score groups. **(A)** The correlation between MDSC ssGSEA scores and risk scores. **(B)** The correlation between the ssGSEA scores of DCs and the OS probability of IDC patients in the whole cohort. **(C)** The correlation between the ssGSEA scores of DCs and the OS probability of IDC patients in the low-risk score group. **(D)** The correlation between the ssGSEA scores of DCs and the OS probability of IDC patients in the high-risk score group.

Figure S8 | The ssGSEA score distribution in the low, intermediate, and high immune infiltration patterns and in the low- and high-risk score groups. **(A)** The ssGSEA score distribution in low, intermediate and high immune infiltration patterns. **(B)** The difference and *P*-value from the comparison between the ssGSEA score from low and high immune infiltration patterns. **(C)** The ssGSEA score distribution in the low- and high-risk score groups. **(D)** The difference and *P*-value from the comparison between the ssGSEA score from the low- and high-risk score group. **(E)** The distribution of immune infiltration patterns in different pathological subtypes. **(F)** The distribution of risk scores in different pathological subtypes. **(G)** The distribution of immune infiltration patterns at different pathological stages. **(H)** The distribution of risk scores at different pathological stages.

Figure S9 | The selection of the soft threshold in the WGCNA. **(A)** Scale free topology model fit, **(B)** mean connectivity.

Figure S10 | The correlation of the expression profiles of several immune checkpoint proteins, risk score, and VEGF-A in the TCGA **(A)** cohort and GSE20685 cohort **(B)**.

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REVIEW
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Understanding the Origin and Diversity of Macrophages to Tailor Their Targeting in Solid Cancers

*Karoline Kielbassa†, Serena Vegna†, Christel Ramirez and Leila Akkari**

Division of Tumour Biology and Immunology, Oncode Institute, Netherlands Cancer Institute, Amsterdam, Netherlands

Tumor-associated macrophages (TAMs) are a major component of the tumor immune microenvironment (TIME) and are associated with a poor prognostic factor in several cancers. TAMs promote tumor growth by facilitating immunosuppression, angiogenesis, and inflammation, and can promote tumor recurrence post-therapeutic intervention. Major TAM-targeted therapies include depletion, reprogramming, as well as disrupting the balance of macrophage recruitment and their effector functions. However, intervention-targeting macrophages have been challenging, since TAM populations are highly plastic and adaptation or resistance to these approaches often arise. Defining a roadmap of macrophage dynamics in the TIME related to tissue and tumor type could represent exploitable vulnerabilities related to their altered functions in cancer malignancy. Here, we review multiple macrophage-targeting strategies in brain, liver, and lung cancers, which all emerge in tissues rich in resident macrophages. We discuss the successes and failures of these therapeutic approaches as well as the potential of personalized macrophage-targeting treatments in combination therapies.

Keywords: macrophages, tumor immune microenvironment, solid tumors, immune phenotype, macrophage plasticity

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Kumamoto University, Japan

***Correspondence:**
Leila Akkari
l.akkari@nki.nl

†These authors have contributed equally to this work

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INTRODUCTION

The innate immune system, which consists of macrophages, dendritic cells, neutrophils, and a variety of other effector cells, is indispensable to mount rapid defense mechanisms in the context of homeostatic disruption (1). In the late nineteenth century, Élie Metchnikoff identified macrophages and their phagocytic activity (2), and since then, these cells have been singled out as key players in innate immunity and inflammation while being essential for tissue maintenance (3).

The past decades have emphasized the importance of investigating macrophages, since they not only are responsible for tissue homeostasis but also can contribute to the pathophysiology of several diseases including development and inflammatory disorders as well as cancer, depending on their activation phenotype (4).

For many years, tissue-resident macrophages were thought to originate from bone marrow-derived progenitors and blood monocyte intermediates that differentiate into mature cells once seeded into organs (5). However, the field of development biology has recently expanded our knowledge regarding tissue macrophage ontogeny. Several genetic tracing studies revealed that

multiple macrophage populations develop from embryonic progenitors and are able to self-renew by local proliferation of mature, differentiated cells (6, 7).

Each tissue microenvironment shapes macrophage morphological and functional characteristics according to the homeostatic need of its local surrounding, suggesting that macrophage tissue-specific functions are not strictly dependent on their ontogeny and that the surrounding environment imprints macrophages locally (3). Additionally, several studies reported that macrophage functions are regulated epigenetically (8, 9).

This body of work, mainly performed in the course of development and homeostasis, raises the unanswered questions of macrophage phenotype adaptation in a tissue and ontogeny-specific manner in diseased conditions.

Macrophages present in the microenvironment of tumors are referred to as tumor-associated macrophages (TAMs) and are associated with a poor prognostic factor in several solid cancers (10–12). TAMs promote tumor growth by facilitating immunosuppression, angiogenesis, and inflammation, and can also affect tumor recurrence after conventional therapies (13). These characteristics, together with their genetically stable properties, have rendered macrophages attractive therapeutic targets in the tumor immune microenvironment (TIME) (12, 14). However, the high plasticity and versatility of TAMs, their distinct embryological origins, and their evolution within the TIME during cancer progression and treatment challenge their efficient targeting.

In this review, we build a roadmap of macrophage dynamics within multiple TIME, with a particular focus on tissue and tumor specificity. We will discuss the successes and failures of macrophage targeting with relation to TAM tissue and tumor specialized functions and propose how combinatorial targeting could be implemented to utilize the yet untapped vulnerabilities of these cells in cancer.

Macrophage Ontogeny and Education in Development and Homeostasis

Unlike most immune cells that originate from hematopoietic stem cells (HSCs), certain tissue-resident macrophages derive predominantly from embryonic progenitors, including yolk-sac macrophages and fetal liver monocytes (7, 15). The contribution of these two types of embryonic progenitors varies among different tissue-resident macrophage populations (7, 15).

The subset of resident macrophages in the central nervous system (CNS), referred to as microglia, are widely accepted to be the only tissue-resident macrophages exclusively originating from yolk sac-derived progenitors (16, 17). Embryonic microglia precursors emerge as early as E7.25 (17) and remain the sole source of macrophages in the adult brain parenchyma through their self-renewal potential.

Unlike microglia, Kupffer cells in the liver and alveolar macrophages in the lung are suggested to represent a mixed population of yolk sac macrophages and fetal liver monocytes (18). Kupffer cells and alveolar macrophages develop predominantly from erythro-myeloid progenitors (EMPs) in the yolk sac at E8.5, followed by their migration to the fetal liver by E10.5, which give rise to several cell types including fetal macrophages and monocytes (15). Fetal Kupffer cells are observed in the hepatic sinusoid at E11.0 in mouse embryos and express the macrophage marker F4/80$^+$ (19), while alveolar macrophage differentiation begins with the distribution of fetal macrophages and fetal monocytes throughout the developing lung at around E12.5 and E16.5, respectively (20). Further differentiation of alveolar macrophages from fetal precursors continues until E18.5 and fully colonize the alveolar space during the first days after birth (20).

After birth, Kupffer cells and alveolar macrophages differentially rely on their potential for self-renewal and proliferation for their maintenance. While the pool of adult Kupffer cells is only marginally enriched by HSC-derived cells under steady state conditions (<5%), a substantial proportion of lung alveolar macrophages are progressively replaced during the normal aging process (7, 15).

To differentiate and maintain their tissue-specific functions, tissue-resident macrophages rely on specific growth factors and multiple transcription factors (21). Macrophage colony-stimulating factor-1 receptor pathway (CSF-1R, ligands M-CSF/IL-34) is a crucial signaling node mediating the maintenance of Kupffer cells and microglia (22, 23), while CSF-2R/GM-CSFR appears to be essential in alveolar macrophages differentiation (24). Engagement of the transcription factors (TFs) Pu.1, Irf8, Runx1, and SMAD regulate the development of microglia (**Figure 1**) (17, 23, 25, 26), while myeloid TFs such as Myb, Id2, Batf3, and Klf4 are not required for microglia development (7, 17). Id2 and Id3 TFs are, however, essential to generate and maintain Langerhans cells and Kupffer cells, respectively (27, 28). Differentiation and maintenance of alveolar macrophages are dependent on transforming growth factor-β receptor (TGF-βR) signaling through the upregulation of PPAR-α (29, 30), and TGF-β is a master regulator of microglia development (**Figure 1**) (21, 29).

Further changes in macrophage marker expression profile occur postnatally, which distinguish them from their precursors and other tissue-resident macrophages. *Tim4* is maintained throughout development and postnatally in Kupffer cells, but lost in lung alveolar macrophages and brain microglia (28). Postnatal molecular signatures indicated tissue-specific expression of the TFs *Sall1* (26) and *Sall3* in microglia and *Nr1h3 (Lxra)* in Kupffer cells (**Figure 1**) (28). Molecular signatures of alveolar macrophages revealed important postnatal changes in gene expression, which might be related to their location at epithelial barriers (28). Those signatures showed for example, tissue-specific expression of the TF *Pparg* in alveolar macrophages (28).

Altogether, ample evidence supports the concept that once differentiated, each tissue-resident population of macrophages is distinct in their molecular profiles in a manner that is dependent on their embryological origin and specialized tissue education.

Tissue-Specific Functions of Resident Macrophages

Each tissue microenvironment necessitates macrophages to undertake specific functions to maintain their physiological role in the body homeostasis. Consequently, tissue-resident macrophages adopt morphological and functional characteristics depending on their local surroundings. Unlike Kupffer cells

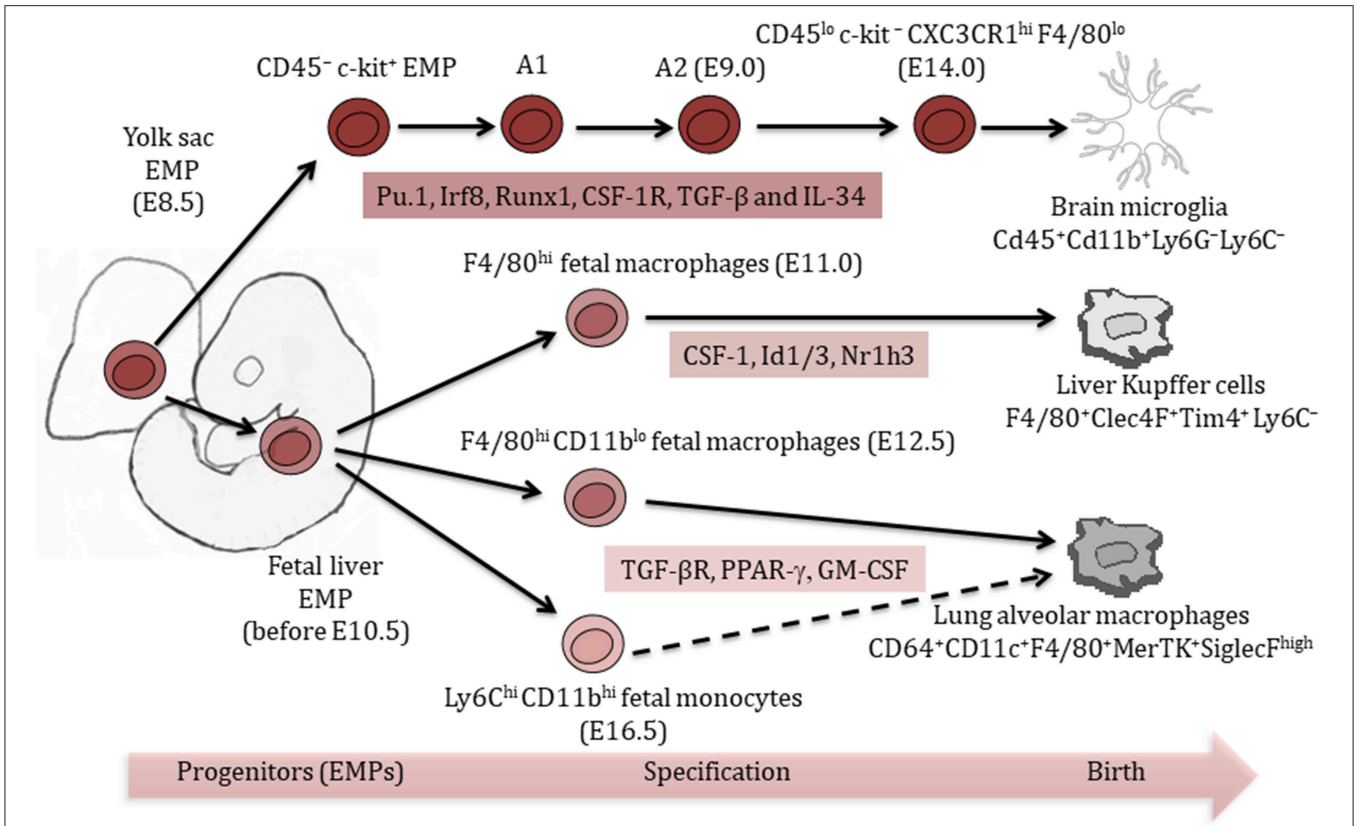

FIGURE 1 | Ontogeny of tissue-resident macrophages. Liver Kupffer cells and lung alveolar macrophages originate from a mixed population of yolk sac and fetal liver erythro-myeloid progenitors (EMPs). Brain microglia exclusively arise from yolk sac EMPs. Multiple tissue-specific transcription factors are important for the differentiation and maintenance of tissue-resident macrophages. Phenotypic markers help identify different tissue-resident macrophage populations.

in the liver or alveolar macrophages in the lung, microglia are the sole myeloid cells present within the healthy brain parenchyma, due to their unique ontogeny and seclusion from the peripheral circulation by the blood–brain barrier (BBB) (31). Microglia regulate the CNS homeostasis through phagocytic clearance of apoptotic neurons (32), regulation of neuronal survival, neurogenesis, and oligodendrogenesis by secreting various neurotrophic factors, including insulin-like growth factor 1 (IGF-1), IL-6, IL-1β,TNF-α, and IFN-α (33–35). Furthermore, microglia are essential in maintaining and remodeling the synaptic network (36). Synaptic pruning includes elimination of undesired synapses, which is mediated by TGF-β signaling and expression of the complement protein C3 (37).

Tissue-resident Kupffer cells mediate the tolerogenic environment of the liver and are important effectors in maintaining tissue and systemic homeostasis (38). Kupffer cells are involved in the clearance of bacteria and microbial products including pathogen-associated molecular patterns (PAMPs), damaged erythrocytes, haptoglobin–hemoglobin complexes and erythrocyte-derived hemoglobin-containing vesicles from the blood. These pleiotropic functions of liver Kupffer cells are mediated by expression of multiple Toll-like receptors (TLRs), Fc receptors, and specific scavenger receptors including SR-A (CD204), MARCO (39), and CD163 (40).

Alveolar macrophage functions are regulated by the surrounding airway epithelium through their interactions with CD200-expressing alveolar epithelial cells in the presence of TGFβ and IL-10. They are involved in surfactant lipid metabolism (41) and multiple cytokine production through the induction of PPAR-α by CSF-2 (GM-CSF) (3, 30).

Kupffer cells and alveolar macrophages have the ability to promote the suppressive activity of regulatory T cells (Tregs) by producing IL-10 (42), TGF-β, or retinoic acid (43, 44), thus leading to effector T cell immunosuppression. Alveolar macrophages additionally induce T cell antigen-specific unresponsiveness, promoting tolerance to innocuous antigens to prevent unnecessary inflammatory responses (45).

Under physiological conditions, the potential of tissue-resident macrophage self-renewal and proliferation is poorly understood. Both metabolic and nutritional signals have been suggested to regulate macrophage self-renewal (46). Similarly, the Th2 cytokine IL-4 promotes macrophage self-renewal during inflammation (47). Because these different signals are generally altered or upregulated in the course of tumorigenesis, they could favor the selective proliferation of tumor-educated,

tissue-resident TAM subsets, thus fueling their pro-tumorigenic participation to the growing tumor. In that case, specific targeting of proliferative tissue-resident TAMs could represent a tantalizing therapeutic avenue to be applied early on in the course of tumorigenesis.

TIME Shaping in Solid Tumors

The TIME consists of a large variety of immune cells with distinctive composition and functions that differ greatly between, but also within, cancer types (14, 48). Immune cells are highly heterogeneous and can exert both anti- as well as pro-tumorigenic activities depending on environmental signals they are exposed to, including inflammation and tumor cell genetic make-up.

Wide-ranging immunogenomic analysis of more than 10,000 tumors comprising 33 distinct cancer types were classified in six different immune content patterns, spanning cancer tissue types, and molecular subtypes (49). This recent study identified that several tumors, including glioma and hepatocellular carcinoma (HCC), displayed a greater range in leukocyte fraction compared to other cancer types. The immune landscape in these tumors displayed a more prominent M2-like macrophage signature, suppression of Th1 cell activation, and a high anti-inflammatory macrophage response. Lower-grade gliomas showed the lowest lymphocyte and highest macrophage responses, dominated by anti-inflammatory, M1-like macrophages (49). On the other hand, *IDH*1 mutations (also found in gliomas) correlated with low immune cell infiltration (50) and decreased leukocyte chemotaxis, resulting in fewer tumor-associated immune cells and were associated with better clinical outcome (49). Lung squamous cell carcinoma exhibited a "wound healing" immune subtype activation with elevated angiogenesis-associated genes, a high proliferation rate, and a Th2 cell bias in the adaptive immune infiltrate (49). However, these immunogenic analyses only partly integrated the tumor's genetics, and it has now been reported that deregulation of several cancer cell-intrinsic pathways influences the inflammatory TIME (48). Indeed, a few examples of how the genetic make-up of cancers affects tumor immunity have been highlighted in recent years. Expression of the tumor suppressor gene p53 together with NF-kB stimulate senescence and a senescence-associated secretory phenotype (SASP) in hepatic stellate cells, which subsequently induced a tumor-inhibiting phenotype in macrophages. Loss of p53, on the other hand, induced the activation of macrophages toward a tumor-promoting phenotype and fueled inflammation-induced HCC (48, 51). NF-kB activity was increased upon loss of p53, which promoted tumor development in the $Kras^{LSL-G12D/+}$; $Trp53^{F/F}$ lung adenocarcinoma model, while NF-kB inactivation resulted in increased immune cell influx and impaired lung cancer formation (52). Moreover, MYC amplification resulted in increased expression of IL-23 and CCL9 by tumor cells in lung adenocarcinoma murine models, which led to a rapid decrease of B, T, and NK cells, while macrophages were increasingly recruited and activated in the TIME (53). Myc activation has also been shown to mediate and maintain the transition from indolent pancreatic lesions (PanIN) to full adenocarcinoma (PDAC), by triggering the inflammatory ensemble of cell types characteristic of aggressive lesions. The reshaping of the PanIN TIME to a PDAC phenotype was dependent on early recruitment of macrophages through CCL9 and CCL2, and fully reversible in PDAC when Myc activity was blocked or deleted, with rapid TAM and neutrophil efflux (54). Deletion or mutations of the tumor suppressor *p53* in murine models of triple-negative breast cancer have recently been reported to enhance neutrophilic inflammation, which is mediated by tumor cell production of WNT ligands promoting the secretion of IL-1β by TAMs (55, 56). Similar results were obtained in prostate cancer models where both Ly6C+ monocyte and Ly6G+ neutrophil recruitment was blocked, giving rise to tumor growth control specifically in *pTEN* and *p53* double KO mice (57). These studies pioneered the concept of personalized immunomodulation of innate cells, in this case targeting neutrophils in *p53*-altered breast and prostate cancers. Such cancer cell genetics-guided approaches to target TAMs have not yet been explored, and could represent potent therapeutic strategies given the abundance of TAMs in multiple solid tumors. However, they also may be complicated by both TAM cell plasticity and ontogeny, highlighting the need for uncovering the dynamics of these cells in a mutational status-dependent manner.

Altogether, these reports emphasize the influence of tumor cell genetics on immune subset recruitment and activation, suggesting that tumors shape their local TIME to their advantage, which could constitute a potential vulnerability to exploit in cancer immune-modulation therapy.

Ambivalent Role of TAM Along Tumorigenic Progression

Several studies explored the dual roles of TAMs in tumor progression of different cancer types (58). Depending on environmental stimuli, TAMs can initiate both pro-inflammatory as well as anti-inflammatory responses through their ability to directly suppress or promote the cytotoxic functions of natural killer (NK) cells and CD8+ T cells, or by triggering Th1 immune response and cytotoxic activity toward cancer cells *via* toxic intermediates production such as NO and ROS (59).

In grade IV gliomas (glioblastoma: GBM), both microglia and monocyte-derived macrophages (ontogenetically different macrophage subsets) contribute to the TAM pool and influence tumor malignancy (60). Among the non-neoplastic cells in the GBM TIME, TAMs account for 30–40% of the total GBM tumor mass, suggesting their importance in tumor maintenance and immunosuppressive features of these aggressive tumors (61). Interestingly, the density of TAMs is lower in grade II and III gliomas, in which they do not display the M2-like phenotype characteristic of grade IV tumors (62). Acquisition of this protumorigenic features in GBM relies on multiple signaling molecules promoting the M2-like polarization of TAMs, as M-CSF (CSF-1) (63).

The role of TAMs in lung cancer progression remains controversial, potentially due to different populations of macrophages analyzed in multiple tumor settings (64–67). Several reports suggest a positive correlation between TAM infiltration and favorable prognosis. Higher tumor islet densities

of pro-inflammatory macrophages were associated with extended survival in non-small cell lung cancer (NSCLC), while the presence of interstitial macrophages correlated with reduced survival (66, 68). Immunostaining using CD68/iNOS (markers for pro-inflammatory macrophages) and CD68/CD163 (markers for anti-inflammatory macrophages) supported these findings with high infiltration of pro-inflammatory macrophages in the tumor islets together with low infiltration of anti-inflammatory TAMs being associated with improved NSCLC patients' survival (69). However, studies also show substantial evidence that TAMs correlate with poor prognosis in human lung cancer (70, 71). For instance, TAMs are associated with tumor IL-8 mRNA expression and increased intratumoral microvasculature, which correlates negatively with survival (70).

In the liver TIME, pro-inflammatory TAMs and endothelial cells produce TNF-α, which activates NF-κB and subsequently protects hepatocytes from apoptosis (72). However, exposure to IL-4, IL-10, and IL-13 triggers a switch toward an anti-inflammatory phenotype and the release of TGF-β, Arg1, and IL-10, as well as factors that promote tissue remodeling and angiogenesis, including VEGF, PDGF, MMP2, MMP9, and MT1-MMP (59, 73–75). Moreover, anti-inflammatory TAMs induce S100A8 and S100A9 expression on HCC cancer cells (76), release CCL22 and epidermal growth factor (EGF) to recruit Treg cells, and promote migration of tumor cells, altogether contributing to HCC malignancy and liver metastasis (**Figure 2**) (77). The role of M2-like TAMs in facilitating the epithelial–mesenchymal transition (EMT) of HCC cells in a TLR4/STAT3 signaling-dependent manner further supports the notion that these cells promote liver cancer malignancies (78).

Overall, it is now generally accepted that anti-inflammatory TAMs are mainly associated with poor survival while pro-inflammatory macrophage infiltration tends to correlate with better outcome (64–67, 70). However, limited studies have distinguished tissue-resident macrophages from monocyte-derived macrophages within solid tumors. This distinction is particularly puzzling in macrophage-rich organs like the brain, lung, and liver. In light of recent results showing that macrophage progenitors are seeded early in these organs, it is of high interest to determine the extent of resident macrophages contributing to the TAM pool and subsequently to pro- or anti-inflammatory cells, or whether they are replaced by recruited monocytes in the course of tumorigenesis.

TAM Recruitment and Activation Within the Lung, Liver, and Brain TIME

During early tumor lesions, TAMs are recruited in the TIME through multiple secreted factors, and can in turn release several cytokines, chemokines, and growth factors that fuel tumor progression (**Figure 2**).

The generation of an immunosuppressive, pro-tumorigenic TIME in which macrophages are one of the most abundant immune cell players is often a consequence of chronic inflammation and organ injury (79). In the lung, inhalation of particulate matter (PM) or cigarette smoke causes activation of alveolar macrophages *via* cell surface receptors, including TLRs, MARCO, or S-RA. Activated alveolar macrophages release a variety of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, as well as IL-8 and GM-CSF (80), which promote tissue injury and recruitment of additional immune cells. Small cell lung cancer cells display high levels of monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), which leads to increased recruitment of blood monocytes to tumors (**Figure 2C**) (81). Increased expression of IL-23 and CCL9 by tumor cells additionally promotes recruitment and activation of macrophages in the $Kras^{G12D}$-driven lung adenocarcinoma model upon MYC amplification, as mentioned in the previous paragraph (53).

When tissue injuries cannot be resolved, chronic liver diseases such as non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), chronic HBV, or HCV infection lead to hepatic fibrosis and cirrhosis, which eventually can favor the development of hepatocellular carcinoma (HCC) (38). Resident Kupffer cells are rapidly activated by various danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) in that context, including lipopolysaccharide (LPS), viral RNA, DNA fragments, free fatty acids, uric acid, and ATP, sensed by multiple TLRs and P2X purinoreceptor 7 (P2X7), respectively (82). Activation of Kupffer cells leads to inflammasome formation and the subsequent release of various pro-inflammatory cytokines such as IL-1β, which contribute to hepatic injury (83). Kupffer cells, hepatocytes, and stellate cells also secrete chemokines, including CC-chemokine ligand 1 (CCL1), CCL2, and CX3CL1, that promote the extensive recruitment of bone-marrow-derived monocytes into the liver (38), where they differentiate into monocyte-derived macrophages (**Figure 2A**). During early tumor lesions, TAMs are recruited in a HCC environment mainly *via* CSF-1, CCL2, VEGF, and TGF-β, and in turn release additional cytokines, chemokines, and growth factors that promote HCC progression (59).

While high immune cell influx to sites of injury is easily achieved in the liver or in the lung, the healthy brain parenchyma is secluded from circulation by the BBB and microglia are the only myeloid cells due to their early seeding during development and specific ontogeny (31). Microglia become activated in response to early tumor stimuli, such as IL-6, TGF-β, prostaglandin E2 (PGE2), ATP, and miRNAs, which leads to the release of various cytokines, growth factors, and MMPs (84). Tumor cells, in turn, release additional mediators that will further recruit and promote another wave of microglial activation, resulting in a cyclic process in GBM, known as reactive microgliosis (**Figure 2B**) (84). Activated microglia switch from a resting state toward an amoeboid phenotype and subsequently release several factors that promote glioma proliferation and migration, including stress-inducible protein 1 (STI1), epidermal growth factor (EGF), TGF-β, as well as IL-1β, which modulate the BBB to further allow invasion of peripheral immune cells into the CNS (85, 86). Complementing the pool of microglia in the brain TIME, monocyte-derived macrophage recruitment is in part mediated by CSF-1, ATP, glial cell-derived neurotrophic factor (GDNF), GM-CSF, CCL2, CX3CL1, and, especially in hypoxic areas, CXCL12 (SDF-1)

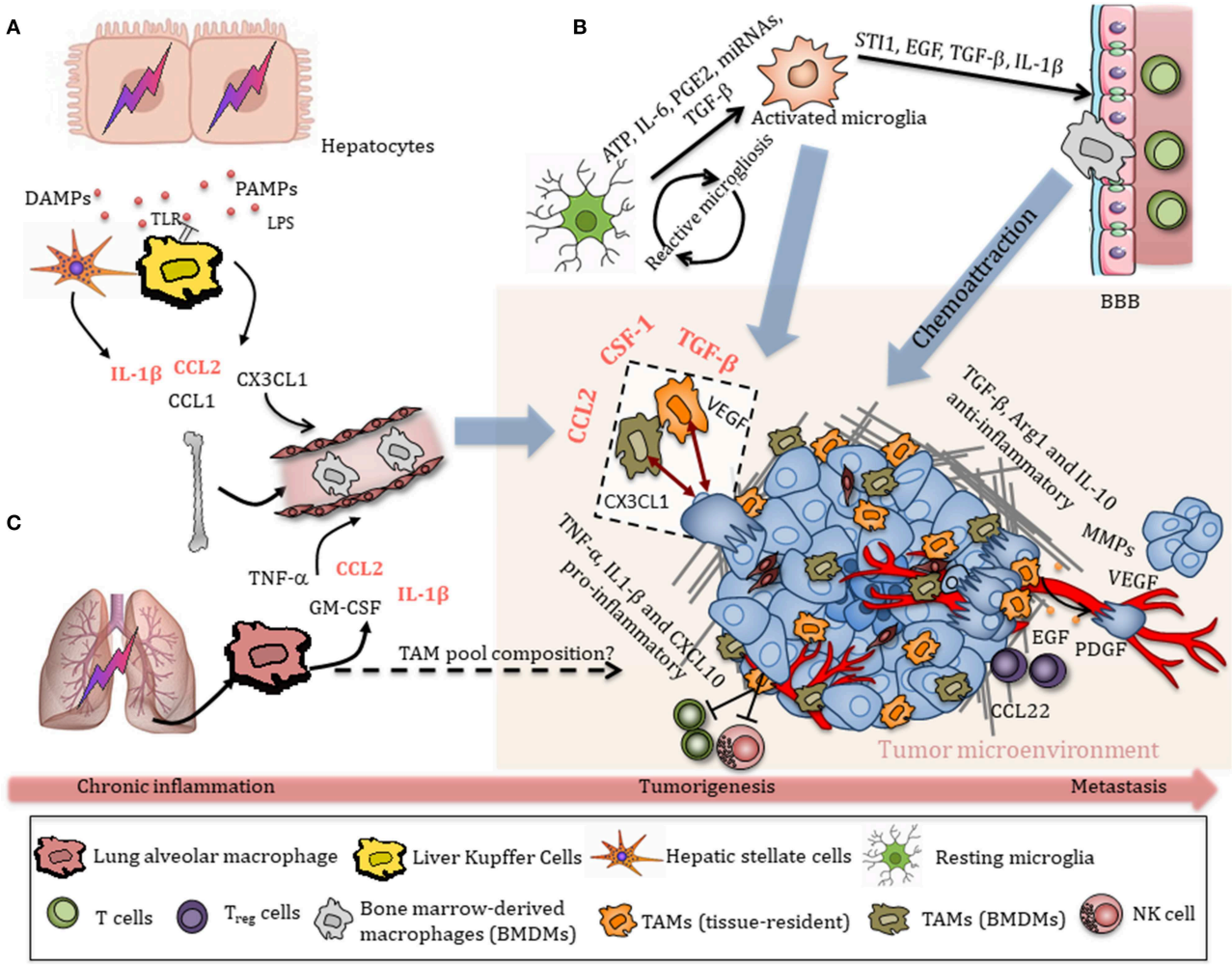

FIGURE 2 | Schematic overview of TAM recruitment in the tumor immune microenvironment (TIME). **(A)** Damaged hepatocytes release a variety of DAMPs and PAMPs, which initiate an inflammatory response through activation of hepatic cells, particularly liver-resident Kupffer cells. Activated Kupffer cells, hepatocytes, and stellate cells secrete chemokines that promote the extensive recruitment of bone-marrow-derived monocytes to sites of injury. Chronic inflammation eventually contributes to tumorigenesis. TAMs are recruited in a HCC environment through CSF-1, CCL2, VEGF, and TGF-β, which in turn release many cytokines, chemokines, and growth factors that promote HCC progression. Anti-inflammatory TAMs release TGF-β, Arg1, and IL-10, as well as factors that promote tissue remodeling and angiogenesis, including VEGF, PDGF, MMP2, and MMP9. TAM-derived EGF and CCL22 recruit Treg cells, promoting metastasis. **(B)** Early tumor stimuli release various chemokines, including ATP, IL-6, PGE2, miRNAs, and TGF-β, that activate resting microglia toward an amoeboid state, which in turn modulate the BBB, allowing circulating monocytes to enter the TIME. Tumor-derived chemokines attract microglia/macrophages to the tumor, where they interact with both bulk glioma cells and glioma stem-like cells (GSCs) and contribute to tumor progression and invasiveness. **(C)** Chronic lung inflammation/injury contributes to NSCLC. Inhalation of particulate matter (PM) or cigarette smoke causes activation of alveolar macrophages *via* cell surface receptors, including TLRs, MARCO, or SR-A. Activated alveolar macrophages release a variety of pro-inflammatory cytokines, which are also released in the peripheral circulation and contribute to systemic inflammation. The relative contribution of alveolar macrophages and interstitial lung macrophages to the TAM pool and subsequently their roles in tumor progression remains unclear.

(61, 63, 85, 87–90). Tumor-secreted CCL2 signals through the CCR2 receptor expressed on TAMs and result in release of IL-6 to promote glioma cell invasiveness (91). In addition to bulk, differentiated glioma cells, glioma stem-like cells (GSCs) have been reported to reside in GBM perivascular niches and to be resistant to radiation and chemotherapy (92). GSCs enhance macrophage recruitment from the periphery by producing chemoattractants, such as periostin (93). Recruited TAMs can in turn influence GSCs by releasing TGF-β followed by production of MMP-9, promoting GSC invasiveness (94).

Distinguishing Macrophage Subsets in Lung, Liver, and Brain Cancers

Macrophage populations are highly plastic and adapt their phenotype in response to microenvironmental influences (95); however, the differential responses to inflammation and tumorigenic progression in the recruited vs. resident macrophage populations is still unclear.

The challenge in distinguishing subpopulations of TAMs resides in the ability of monocyte-derived macrophages to acquire some of the tissue-resident macrophage marker

expression and functionality in the course of tissue injury and tumorigenesis.

Several studies suggest that TAMs in lung tumors are largely monocyte-derived (2). During lung injury/fibrosis, resident alveolar macrophages are identified as $CD64^{+}CD11c^{+}F4/80^{+}MerTK^{+}SiglecF^{high}$, while monocyte-derived alveolar macrophages are characterized as $CD64^{+}CD11c^{+}F4/80^{+}MerTK^{+}SiglecF^{low}$ (96). In the $Kras^{LSL-G12D/+}p53^{fl/fl}$ lung tumor model, fluorescently labeled monocyte precursors differentiate into macrophages in developing tumors (97). Similarly, in the Lewis lung carcinoma (LLC) model of NSCLC, tracing of labeled monocyte progeny demonstrated that $Ly6C^{hi}$ monocytes exclusively differentiate into two main TAM populations: MHC-II^{lo} anti-inflammatory and MHC-II^{hi} pro-inflammatory TAMs (98). Although both TAM subsets are derived from a common $Ly6C^{hi}$ monocyte precursor, MHC-II^{lo} TAMs are found in hypoxic regions and upregulate hypoxia-regulated genes, such as VEGF-A, GLUT-3, GLUT-1, and iNOS, and acquire pro-angiogenic functions (98). However, a limited number of macrophage lineage tracing approaches interrogating their embryological origins have been undertaken in the context of lung tumors, thus restricting our understanding of the TAM pool composition and ontogeny-dependent functions in this disease (99).

Hepatic macrophage populations are highly plastic and adapt their phenotype in response to microenvironmental influences (95). Flow cytometry analysis identified that inflammatory monocyte-derived macrophages express high levels of Ly6C and CCR2 and are able to rapidly infiltrate tissue upon injury. Anti-inflammatory monocyte-derived macrophages, on the other hand, are $Ly6C^{low}$, express high levels of CX3CR1, and exhibit a patrolling behavior along the liver vasculature (74). Resident Kupffer cells are $F4/80^{+}Clec4F^{+}Tim4^{+}$ macrophages and negative for the marker Ly6C (15, 100). This distinction, despite being oversimplified, has been the basis for the handful of studies examining the ontogeny of macrophages in liver injury and tumorigenesis.

In injured livers, bone marrow-derived macrophages (BMDMs) were equally capable of responding to LPS and parasitic insults compared to tissue-resident Kupffer cells (KCs) (101). However, KCs were more effective at accumulating acetylated low-density lipoprotein, while BMDM uptake of a larger range of bacterial pathogens (101). KCs are the first macrophage population to respond to newly formed tumor signals and are therefore involved in HCC onset (102), while BMDMs are essential during later disease progression stages and metastasis formation (103). During HCC progression, monocyte-derived macrophages suppress the functions of effector T and B cells through expression of the immune-checkpoint molecule PD-L1 and the immunosuppressive cytokine IL-10, and are able to directly inhibit NK cells and CD8+ T cells (104). Infiltrating monocytes/macrophages lead to upregulation of S100A8 and S100A9 expression in cancer cells, which was correlated with elevated metastasis formation in HCC (76). While these recent studies shed light into the relative contribution of KC or monocyte-derived macrophages to tumor progression, the extent to which these populations are functionally and transcriptionally distinct in the course of cancer malignancy remains to be fully addressed using lineage tracing tools, as they have now been developed to examine liver macrophage homeostatic functions (100, 105).

The influence of macrophage ontogeny on brain tumor development has been the subject of thorough studies in recent years, significantly enhancing our understanding of the role of macrophage origin in this organ compared to lung or liver cancer. Traditionally, CD45 expression was used to differentiate resident microglia (MG; $CD45^{low}$) from monocyte-derived, infiltrating BMDMs ($CD45^{high}$) (106). Irradiation chimera experiments (where murine heads were protected from irradiation to avoid disruption of BBB) demonstrated that the main source of TAMs in primary brain tumors are resident MG, which showed an upregulation of CD45 expression in gliomas (107, 108). However, these findings were challenged by a recent study employing Cx3cr1 and Ccr2 double transgenic lineage tracing knock-in mice models, showing that recruited $Cx3cr1^{lo}Ccr2^{hi}$ monocytes differentiated into $Cx3cr1^{hi}Ccr2^{lo}$ macrophages and $Cx3cr1^{hi}Ccr2^{-}$ microglia-like cells in glioblastoma. In this study, infiltrating BMDMs represented ∼85% of the total TAM population, while resident MG accounted for only the rest (109). However, using bone marrow chimera experiments and multiple lineage tracing glioma mouse models, Bowman et al. reported that MG and BMDM content was strikingly different, closer to 60 and 40%, respectively. The identification of *Itga4* (Cd49d) as a monocyte-derived macrophage-specific marker in multiple brain malignancies further confirmed these results in glioma patient samples.

While these different results may be explained by the different lineage tracing methods employed, glioma genetics or the choice of surface markers distinguishing MG and BMDMs, they all confirmed that monocyte-derived macrophages are indeed recruited to the brain TIME. Further analyses of these distinct TAM subsets revealed that glioma MG were enriched for the classical complement components C4b, C2, and pro-inflammatory cytokines such as Ccl4 and TNF-α, while BMDMs were enriched immune effectors, such as Cd40, Tlr11, Tlr5, Tlr8, Jak2, and "wound healing" chemokines, including Ccl22, Ccl17, Cxcl2, Cxcl3, and Cxcl16. These distinct signatures remain to be functionally tested and their relevance in human GBM remains to be determined. Nevertheless, the transcriptional programs specific to each macrophage subset in primary brain tumors underline their ability to distinctively respond to tumor progression.

Monotherapies Targeting Macrophages *in vivo*

As mentioned above, numerous studies have shown substantial evidence that TAMs contribute to tumor progression and are associated with poor prognosis in solid cancers (10, 11). Major approaches targeting these cells within the TIME include macrophage depletion, macrophage reprogramming, and macrophage recruitment blockade (13, 110). However, successful macrophage-targeting strategies have been challenging to successfully implement, due to TAM high plasticity, thus

giving rise to therapy resistance (110). Moreover, the vast majority of these therapeutic approaches currently target TAMs as a whole population, without fully considering their ontogeny or phenotype evolution within solid tumors.

Targeting of TAMs that have acquired protumorigenic functions presents the advantage of eliminating cells that are fueling tumor progression while preserving macrophages that may have retained their physiological, anti-tumor functions. Thus, specific depletion of M2-like TAMs is therapeutically interesting, albeit difficult to achieve. The scavenging receptor CD163, is a well-accepted marker of M2-like TAMs, and has been shown to promote their protumorigenic roles in murine and human settings (111). Genetic or nanoparticle-mediated ablation of CD163+ TAMs in melanoma leads to sustained tumor regression, partly through cytotoxic T lymphocyte recruitment and activation (112).

One of the main survival and differentiation cytokines critical to TAM biology is CSF-1, whose downstream signaling pathway can be blocked by targeting its receptor CSF-1R (113). Depending on the cancer type, blockade of CSF-1R signaling showed variable outcomes. CSF-1R blockade using the receptor tyrosine kinase (RTK) small drug inhibitor BLZ945 (Novartis) limits glioma progression and leads to regression of established tumors (114). Mechanistically, CSF-1R inhibition mediated the reprogramming of TAMs toward an anti-tumorigenic phenotype, without depleting cells within the tumor bulk (114, 115). However, long-term exposure to CSF-1R inhibition as a monotherapy was found associated with PI3K hyper-activation driven by IGF-1 production in the TIME (115). Although the CSF-1R inhibitor PLX3397 showed anti-tumor efficacy in a pre-clinical glioma model (63), these findings were not translated in recurrent glioblastoma patients (116). Human glioblastoma frequently bear alterations of PI3K and PTEN (117), which might be associated with inherent resistance to CSF-1R targeting, readily explaining the results of this clinical trial. These results underline the importance of identifying the acquired resistance to long-term macrophage targeting and consequently adapt treatment in a personalized manner, similarly to what has been done for targeted therapies (110).

The effect of CSF-1R targeting was strikingly different in breast and in cervical pre-clinical murine models. CSF-1R inhibition led to macrophage depletion, thereby causing increased infiltration of $CD8^+$ cytotoxic T cells and improving responses to chemotherapy and radiotherapy (10, 118, 119). Thus, CSF1-R targeting leads to different consequences in the TIME, causing either depletion or macrophage reprogramming in a tumor-specific manner. It is yet to be determined whether blockade of CSF-1R affects predominantly tissue-resident or recruited macrophages in solid tumors. In inflammatory models however, CSF-1R blockade mediated depletion of tissue-resident macrophages, which resulted in enhanced recruitment of pro-inflammatory monocytes. These results support the hypothesis that continuous CSF-1R inhibition would then be needed to behold therapeutic effects, as tissue-resident cell depletion could be compensated by recruitment of macrophage progenitors to replenish the pool of tissue/tumor-associated macrophages (120).

Another approach to limiting macrophage pro-tumorigenic roles in the TIME is to prevent their recruitment by inhibiting the chemokine gradients' axes they rely on, including Cxcl12 (SDF-1)/Cxcr4. Blocking Cxcr4 using the inhibitor AMD3100 resulted in reduced metastatic properties of mammary tumors (121). In metastatic melanoma, Cxcl12 favors monocyte differentiation into perivascular macrophages, thus enabling the establishment of an autocrine CXL12/CXCR4 loop promoting further leucocyte infiltration and metastatic progression (122). Tumor cell-secreted CCL2 also acts as a monocyte-attracting chemokine to recruit myeloid cells in several metastatic niches (123). Blockade of the CCL2/CCR2 axis led to reduction of monocyte infiltration in multiple TIME (124) and inhibits breast cancer cell metastatic seeding (123, 125). However, cessation of this CCL2/CCR2 blockade can lead to compensatory phenotype associated with increased breast cancer metastasis, for instance (126).

Several clinical trials are currently testing CSF-1/CSF-1R targeting agents (including the Novartis small-molecule inhibitor BLZ945 and Roche monoclonal antibody RG7155) in, among others, breast cancer, glioma, melanoma, ovarian cancer, and lung cancer (**Table 1**) (132). So far, only RG7155 has yielded therapeutically beneficial outcomes as a single agent in diffuse-type giant cell tumors patients and has been shown to deplete $CSF\text{-}1R^+CD163^+$ macrophages (113). CCR2 inhibitors (MLN1202 and PF-04136309) are utilized for their ability to reduce bone marrow-derived cell recruitment in metastatic cancers and as first-line treatment in pancreatic tumors (**Table 1**).

Combination (Pre-) Clinical Therapies of Macrophage Targeting With Cytotoxic and Immunotherapy Agents

It is only in recent years that strategies using macrophage-targeting agents have been combined with targeted therapies, standard of care treatment or immunotherapies, revealing the potential of these approaches in solid tumors.

Combination of CSF-1R targeting with other RTK inhibitors showed enhanced treatment outcomes in several studies (133, 134). PLX3397 combined with the mTOR inhibitor rapamycin inhibits outgrowth of malignant peripheral nerve sheath tumors when compared to single drug treatment (133). In glioma, when combined with the multi-targeted kinase inhibitors vatalanib and dovitinib, PLX3397 did not induce depletion but depolarization of pro-tumorigenic TAMs and resulted in pronounced glioma regression (135). Moreover, both TAM reprogramming and depletion of TIE2-expressing macrophages (TEMs) together with VEGF ligand inhibition showed anti-tumor effect in orthotopic glioma models (136). In pre-clinical melanoma models, treatment with CSF-1R inhibitors enhanced the efficacy of the BRAF inhibitor vemurafenib, which was associated with depleted macrophages, allowing increased anti-tumorigenic $CD8^+$ cytotoxic T cell infiltration (134).

The efficacy of CSF-1R inhibitors when concurrently administered with chemotherapy has also been reported. TAMs suppress the cytotoxic activity of antimitotic agents, including taxol, in breast cancer and promote early mitotic slippage (137).

TABLE 1 | Summary of recent clinical trials using macrophage-targeting therapies.

Company	Drug	Targets	Phase	References
MONOTHERAPY				
Novartis	BLZ945	CSF-1R *Advanced* *Solid tumors*	I/II	*In progress* NCT02829723
Roche	RG7155	CSF-1R *Locally advanced and/or metastatic ovarian and breast carcinoma*	I	*In progress* NCT01494688
Southwest oncology group	MLN1202	CCR2 *Bone metastasis*	I/II	*In progress* NCT01015560
Pfizer	PF-04136309	CCR2 *First-line metastatic pancreatic cancers*	I/II	*Terminated* NCT02732938
	Sorafenib	VEGFR, PDGFR	III	(127)
Bayer	Regorafenib	VEGFR1-3,PDGFRβ, FGFR *Hepatocellular carcinoma*	III	(128)
Centocor	CNTO888	CCL2 *Castration-resistant prostate cancer*	II	(129)
COMBINATION WITH IMMUNOTHERAPY				
	CP870893 + taxol/carboplatin	CD40 *Metastatic melanoma*	I	(130)
	BLZ945 + PRD001	CSF-1 PD-1 *Advanced solid tumors*	I/II	*In progress* NCT02829723
	LY3022855 + Tremelimumab	CSF-1R CTLA-4 *Advanced solid tumors*	I	*In progress* NCT02718911
COMBINATION WITH CHEMOTHERAPY				
Plexxicon	PLX3397 + Temozolomide	CSF1R, KIT, FLT3 *Advanced solid tumors*	I/II	*In progress* NCT01525602
Pfizer	PF-04136309 FOLFIRINOX	CCR2 *Advanced pancreatic adenocarcinoma*	I/II	*In progress* NCT01413022
Centocor	CNTO888+ Docetaxel Gemcitabine Paclitaxel+Carboplatin Doxorubicin	CCL2 *Advanced solid tumors*	II	(131)
COMBINATION WITH RADIOTHERAPY				
Plexxicon	PLX-3397	CSF1R, KIT, FLT3 *Primary glioblastoma*	I/II	*In progress* NCT01790503

Mechanistically, TAM depletion favors increased levels of cancer cell-DNA damage and cell death in response to taxol, thus enhancing the response to this chemotherapy (137). Moreover, the combination of PLX3397 with the chemotherapeutic drug temozolomide and radiotherapy (NCT01790503) are currently assessed in phase 1b/2 study (18). Immune-cold tumors, such as pancreatic ductal adenocarcinoma (PDA), lack robust T cell infiltrates and are resistant to chemotherapy (138). Targeting TAMs in these types of tumors have thus been tested in order to lessen immunosuppression. Stimulation of the CD40 receptor at the cell surface of macrophages using agonistic CD40 antibody promotes clonal T cell expansion and combination treatment with chemotherapy resulted in optimized myeloid activation and T cell function (138). These results led to the development of a phase I study in which the agonistic CD40 antibody CP870893 (Pfizer) was combined with carboplatin and Taxol, confirming the safety of this treatment regimen (139). Clinical trials in which CSF1R inhibitors are combined with immunotherapy are currently ongoing, such as BLZ945 with PRD001 [monoclonal antibody

targeting programmed cell death-1 (PD1)] and LY3022855 with tremelimumab [monoclonal antibody targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)] in advanced solid tumors (140).

Components of the TIME have pivotal roles in determining treatment outcomes of radiotherapy (RT) (141). RT leads to increased T cell recruitment to the TIME and can prime the immune system against cancer cells *via* immunogenic cell death (ICD). However, enhanced actions of suppressive immune cells such as TAMs constrain the efficacy of RT. TAMs are highly radioresistant and produce increased levels of Arg-1, COX-2, and iNOS post-irradiation, which promote early tumor regrowth (142). Meng et al. administered clodronate liposomes systemically or locally before RT, to deplete circulating and resident TAMs, which increased the anti-tumorigenic effects of ionizing radiation (143). Inhibition of CSF-1R using PLX3397 delays recurrence of GBM after ionizing radiation by altering myeloid cell recruitment and polarization (144). RT also causes increased TNF-α-induced VEGF ligand production by TAMs and VEGF-neutralizing antibodies enhanced the anti-tumor efficacy to RT (143). In PDAC, RT leads to the production of CCL2, which recruits macrophages to tumor sites to support tumor proliferation and neo-angiogenesis after RT. Therefore, disrupting the CCL2–CCR2 axis in combination with RT may improve RT efficacy in PDAC (145).

Personalized Approaches Targeting Macrophages

Personalized macrophage targeting, tailored-based on tissue and tumor types, could represent a significant advance in the development of effective and long-lasting treatments. However, as mentioned in the previous section, more knowledge is needed to take on these approaches, and multiple tumor cell-intrinsic and extrinsic factors should be considered with regard to TAM immunomodulation (146).

High-throughput analysis of GBM samples shows that many types of mutations, including mutations to TP53, PTEN, or NF-1, occur in gliomas, which may profoundly affect tumor–host interactions (117, 147). Different driver mutations can co-exist; therefore, targeting of single activated pathways has led to unsuccessful therapeutic outcomes in GBM. Gain-of-function mutations of TP53 promote inflammation in GBM through upregulation of CCL2 and TNF-α expression via NFκB signaling, which consequently lead to increased infiltration of microglia and monocyte-derived immune cells in the TIME (148). NF1 loss in glioblastoma is also associated with increased macrophage infiltration displaying pro-tumorigenic features (149). Because TP53 and NF1 mutations are characteristic of the mesenchymal and proneural subtypes, respectively, it would be important to select specifically these patients in clinical trials targeting TAMs. Loss of PTEN induces increased expression of PD-L1, which correlates with PI3K expression and immune escape in GBM (150). Therefore, including PD-1/PD-L1 pathway inhibition in PTEN-mutated GBM, together with TAM targeting, could be an efficient treatment strategy, as suggested by the combinatorial effects of PI3K and CSF-1R inhibitors to reprogram macrophages toward an anti-tumorigenic phenotype (110). The recent reports identifying either a dominance of microglia or infiltrating macrophages in treatment-naïve gliomas may be due to the distinct genetic make-up of these tumors, as suggested above, and TAMs adopt distinct programming dependent on their ontogeny. Specific targeting of one or the other subsets of TAMs may thus prove to be advantageous to alleviate the protumorigenic functions of the targeted populations while maintaining the homeostatic functions of the other.

Non-small cell lung cancer (NSCLC) is characterized by driver mutations, including epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and KRAS, permitting the efficacy of targeted therapies [reviewed in (151)]. KRAS mutations, however, are not targetable. The immune landscape of these tumors shows increased intratumoral myeloid and T cells (152), while loss of LKB1 in KRAS-mutated NSCLC results in higher levels of CXCL7, G-CSF, and IL-6, which promote neutrophil recruitment and macrophage activation and thereby suppress T cell activity (153). In these tumors, targeting the LKB1/AMPK pathway by activating AMPK may control tumor growth through limiting myeloid cell infiltration and polarization (154). In lung tumors not harboring targetable oncogenic mutations, T cell immunotherapy has yielded partial success. These could potentially be improved by targeting myeloid cell tissue remodeling and immunosuppressive functions to enhance the efficacy of T cell immunotherapy. The association between major driver mutations in lung cancer and PD-L1 expression on myeloid cells remains debated and EGFR mutated lung tumors have been reported to display both low (155) and high (156) PD-L1 expression. Therefore, a better understanding of PD-L1 expression in specific subsets of TAMs would be useful to target these cells specifically.

The role of tissue-resident macrophages has been well-established in clearing pre-cancerous hepatocytes in the liver (157), and senescence in the liver environment can promote the anti-tumor properties of TAMs in early stages of neoplasia (51). NOTCH signaling amplification limits the anti-tumorigenic response mediated by oncogene-induced senescence via the secretion of the senescence-associated pro-inflammatory cytokines IL-1β, IL-6, and IL-8 (158). Accordingly, targeting amplified NOTCH signaling could increase anti-tumorigenic efficacy through promoting senescence surveillance by myeloid cells. Mutated NRAS in liver cancer results in increased recruitment of anti-tumorigenic TAMs through the senescence-associated secretory phenotype (SASP) and promotes $CD4^+$ T cell-mediated clearance of liver pre-neoplastic cells (159). Similarly, amplification of mTOR in liver cancer leads to increased levels of IL-1β, which activates NF-kB, thereby driving tumor suppressive SASP and immune cell recruitment (160). Furthermore, loss of AKT in liver cancer results in decreased content of pro-tumorigenic Wnt-producing macrophages and thereby limits tumorigenesis (161). Therefore, Wnt/β-catenin targeting may inhibit tumorigenic activities of macrophages (161). Altogether, these studies suggest that generating senescence in established HCC and modulating the SASP may represent a potent approach to reprogram TAMs in liver cancer, which would need to be

tailored to the type of senescence generated in the liver TIME (159).

DISCUSSION

Targeting different subsets of macrophages instead of pan-macrophages could improve disease outcomes by hampering the pro-tumorigenic functions of specific subsets of TAMs and protecting the homeostatic properties of others (114, 136). However, this requires understanding and considering multiple features of these cells such as the following: identifying the adequate surface markers for distinguishing different macrophage subsets in specific organs, deciphering their recruitment and activation dynamics in the course of tumor progression and response to therapy, and defining the shaping they are conditioned to by the genetic make-ups of tumors. In this review, we discussed tissue-specific functions of resident macrophages under homeostatic conditions and in malignancy. We propose that tissue-resident macrophage populations should be targeted during tumor initiation, since they are often involved in early inflammatory processes and are a major contributor to the recruitment of monocyte-derived macrophages. Meanwhile, monocyte-derived macrophage subsets may be best targeted at later time points of tumor progression, since they are often involved in tumor invasiveness and immunosuppression. However, considerable work should be undertaken to better understand the contribution of TAM origin to tumor progression, which requires employing lineage-tracing studies in the context of chronically inflamed tumors in particular. The majority of macrophage-targeting approaches are focusing on CSF-1R inhibition to either deplete or reprogram macrophage populations toward an anti-tumorigenic phenotype and several studies are focusing on combining chemotherapy and immunotherapy with CSF-1R inhibitors (10, 118). However, it is still unclear whether inhibition of CSF-1R similarly affects monocyte-derived and tissue-resident macrophage subsets in different cancer types, which needs to be addressed to fully capture either the efficacy or failures of such treatments.

Importantly, the use of sophisticated mouse models closely reproducing the human genetics of tumors and composition of the TAM pool will be essential to test the efficacy and long-lasting effects of macrophage-centric therapies, as potential resistance could emerge.

Overall, we are only beginning to appreciate the potential of macrophage subset reprogramming. Rather than depleting them, re-educating TAMs into a homeostatic activation state and controlling the recruitment of immunosuppressive subsets could boost anti-tumor immunity. These novel therapeutic avenues could then hold promise for the development of effective anti-cancer treatments, particularly when used synergistically with tumor- or T cell-centric therapies.

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KK, SV, and LA conceptualized the study. KK, LA, SV, and CR wrote the manuscript.

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REVIEW
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Microenvironmental Heterogeneity in Brain Malignancies

Lucas J. M. Perus[1,2] *and Logan A. Walsh*[1,3]*

[1] Rosalind and Morris Goodman Cancer Research Centre, McGill University, Montreal, QC, Canada, [2] Department of Physiology, Faculty of Medicine, McGill University, Montreal, QC, Canada, [3] Department of Human Genetics, Faculty of Medicine, McGill University, Montreal, QC, Canada

Brain tumors are among the deadliest malignancies. The brain tumor microenvironment (TME) hosts a unique collection of cells, soluble factors, and extracellular matrix components that regulate disease evolution of both primary and metastatic brain malignancies. It is established that macrophages and other myeloid cells are abundant in the brain TME and strongly correlate with aggressive phenotypes and distinct genetic signatures, while lymphoid cells are less frequent but are now known to have a pronounced effect on disease progression. Different types of brain tumors vary widely in their microenvironmental contexture, and the proportion of various stromal components impacts tumor biology. Indeed, emerging evidence suggests an intimate link between the molecular signature of tumor cells and the composition of the TME, shedding light on the mechanisms which underlie microenvironmental heterogeneity in brain cancer. In this review, we discuss the association between TME composition and the diverse molecular profiles of primary gliomas and brain metastases. We also discuss the implications of these associations on the efficacy of immunotherapy in brain malignancies. An appreciation for the causes and functional consequences of microenvironmental heterogeneity in brain cancer will be of crucial importance to the rational design of microenvironment-targeted therapies for these deadly diseases.

Keywords: brain cancer, glioblastoma, glioma, brain metastasis, tumor microenvironment, immunotherapy

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The Netherlands Cancer Institute (NKI), Netherlands

Reviewed by:
Catherine Sautes-Fridman,
INSERM U1138 Centre de Recherche des Cordeliers, France
David George Menter,
University of Texas MD Anderson Cancer Center, United States

***Correspondence:**
Logan A. Walsh
logan.walsh@mcgill.ca

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INTRODUCTION

The clinical management of brain tumors remains a significant challenge, as surgery and standard of care (SOC) cytotoxic therapies (including radiation and chemotherapy) often offer minimal survival benefit. The brain tumor microenvironment (TME) is a major component of brain malignancies and is a prominent regulator of disease progression and overall survival. As such, the TME compartment may host new therapeutic opportunities that could improve outcomes for brain tumor patients. Under normal physiologic conditions, the brain hosts a generally immunosuppressive milieu that protects the delicate and non-regenerative neural tissue from inflammatory insult. This is in part regulated by the blood-brain barrier (BBB), a selectively permeable barrier formed by endothelial cells, pericytes, and astrocytes (1, 2), which shields the brain from toxins, pathogens, and inflammatory cells within the peripheral circulation. However, this long-standing dogma of "immune privilege" in the brain is now being reconsidered in light of the recent discoveries of lymphatic vessels in the meninges of humans and mice (3–6), although their function in normal and pathological neurophysiology remains entirely unknown (7).

The brain is largely populated with unique cells that perform tissue-specific functions such as neurons, astrocytes, oligodendrocytes, and other glial cells. Moreover, cellular populations which also reside in other tissues, such as macrophages and endothelial cells, are endowed with distinct phenotypes within this vital organ (8–10). Adding to this complexity, macrophages, the predominant immune cell type in the brain, can arise from multiple ontogenies. Under homeostatic conditions, brain macrophages are known as microglia, which are tissue-resident macrophages that populate the brain during early embryonic development from RUNX1+ yolk sac progenitors, and are sustained through cellular longevity and local proliferation (11–13). In contrast, under inflammatory contexts such as cancer or brain injury, additional macrophages are recruited to the brain from the bone marrow (BMDM); unlike microglia, BMDMs are replenished through peripheral monocytosis (14–17). Interestingly, recent lineage tracing studies have revealed that microglia are phenotypically distinct from BMDMs in both the healthy and diseased brain (16, 17), emphasizing the importance of tissue-specific functionality of the microenvironment.

During malignancy, the brain TME is co-opted to support the growth of cancer cells and shield them from immune destruction. In this review, we discuss variations in the TME of brain cancers as a function of their molecular profile (**Table 1**). The work we present focuses primarily on gliomas and brain metastases, but we include examples drawn from studies on pediatric and rare neurological tumors in order to provide a more complete picture of TME heterogeneity in brain cancer. Finally, we discuss the implications of this heterogeneity in the rational design of brain tumor therapies, including immunotherapies currently under clinical investigation.

TME OF PRIMARY BRAIN MALIGNANCIES

Gliomas are the most common primary tumors of the brain. Glioblastoma (stage IV glioma) is the most frequent type of glioma and represents ∼50% of all adult malignant primary brain tumors and ∼20% of all intracranial tumors including metastases. Glioblastoma patients face dismal survival prospects; even after receiving intensive SOC therapy consisting of debulking surgery, radiotherapy, and temozolomide chemotherapy, the median overall survival is only 14.6 months (35). In addition, temozolomide, a DNA alkylating agent, is only effective against tumors that have epigenetically silenced the DNA repair enzyme O^6-methylguanine-DNA-methyltransferase (MGMT), which occurs in ∼45% of all glioblastomas (36). On the other hand, patients with low grade glioma (LGG; stage I, II, or III), have much more favorable survival prospects and are more responsive to SOC therapies.

Low grade gliomas can be subdivided into astrocytomas and oligodendrogliomas based on cellular morphology assessed by histopathological examination. These histological differences in LGGs are underlined by unique genomic and microenvironmental profiles. Genetically, astrocytomas tend to possess TP53 and ATRX mutations whereas oligodendrogliomas are characterized by mutations in the TERT promoter and co-deletion of the 1p and 19q chromosomal arms (30). Analysis of bulk gene expression data sets has also revealed that astrocytic IDHmut gliomas display a higher signature of macrophage/microglia associated genes whereas oligodendrocytic IDHmut gliomas favor a microenvironmental signature enriched in neuron-associated genes (31). Astrocytomas are also associated with a poorer prognosis compared to oligodendrogliomas across all stages (37, 38).

The salient genomic feature that largely distinguishes LGG from glioblastoma is the mutational status of the two genes encoding the isoforms of isocitrate dehydrogenase (IDH1/2); ∼80% of LGG harbor IDH mutations, compared to only ∼5% of glioblastomas. Interestingly, IDH mutations are an independent prognostic factor in gliomas and are associated with increased survival in all types, including glioblastoma (21, 30, 39). The most common IDH alteration observed in gliomas is a missense mutation in IDH1 that replaces an arginine residue at position 132 with a histidine residue (40). While wild-type IDH converts isocitrate to α-ketoglutarate, the neomorphic enzyme generated by the R132H mutation no longer fulfills this function and instead uses α-ketoglutarate as a substrate to catalyze large amounts of the oncometabolite 2-hydroxyglutarate (2-HG), a hallmark feature of LGG (40).

While it is understood how IDH1 mutations directly shape the phenotypic and epigenetic landscape of glioma cells through 2-HG by significantly altering the methylome of glioma cells and directly causing the glioma CpG island methylator phenotype (G-CIMP), a strong positive prognostic indicator in glioma and glioblastoma (40, 41), it is relatively less clear how these alterations shape the surrounding TME. Naturally, it is highly probable that the unique epigenetic landscape of IDHmut glioma cells alters the expression of key components of the signaling pathways which regulate tumor-microenvironment crosstalk. For example, increased TGF-β signaling has been identified as a G-CIMP driven program in low grade gliomas (41). Another possibility is that 2-HG itself may directly sculpt the TME as a soluble factor. Supporting this notion, in mouse models of glioma, it has been shown that 2-HG is directly taken up by T cells to blunt their abundance and activation in IDHmut tumors in an NFAT-dependent manner (27). This effect strongly impacted adaptive anti-tumor immunity, as combination therapy of a mutant IDH1 inhibitor (BAY1436032) with PD-1 inhibition significantly extended overall survival of glioma-bearing mice (42). Similarly, in RCAS/tva models of glioma, it has been shown that IDH1 mutations are associated with reduced neutrophil chemotaxis and anti-tumor immunity (28). How these associations are regulated mechanistically remains unknown.

In addition to the putative effects of 2-HG on TME composition, there are several defining TME features that distinguish LGG from glioblastoma that may be influenced by IDH status. In both patients and animal models, the TME of LGG has a reduced immune infiltrate, produces less inflammatory cytokines, and is impaired in its ability to recruit peripheral immune cells compared to the TME of glioblastoma

TABLE 1 | Most common brain cancers ordered by type, salient molecular aberrations, and salient microenvironmental or histological features displayed.

Cancer	Molecular classes	Salient molecular aberrations	Salient microenvironmental and/or histological features
PRIMARY BRAIN CANCERS			
Pediatric			
Medulloblastoma (18)			
	WNT	Increased WNT signaling	Fenestrated vasculature enabling access of chemotherapy (19)
	SHH	Increased SHH signaling	Intact BBB that restricts access of chemotherapy (19)
	3	MYC amplification	Higher proportion of PD-1+ CD8+ T cells (20)
	4	CDK4 and MYCN amplification	
Adult			
Glioma			
HIGH GRADE (WHO grade 4)			
Glioblastoma (21–23)			
	IDHwt		
	MES	NF-1 loss	Higher macrophage infiltrate (23–26). More CD4 T cells and neutrophils (23). Higher PD-L1 expression (23).
	CL	EGFR gain and PTEN loss	
	PN	PDGFRA gain	Associated with lower levels of PD-L1 (23)
	IDHmut		Blunted T cell abundance and activation (27). Reduced neutrophils (28) and downregulation of NKG2D (29)
	PN	IDH mutations	
LOW GRADE (WHO grade 1–3)			
Astrocytomas		TP53 and ATRX mutations (30)	Microenvironmental signature enriched in macrophage/microglia-associated genes (31)
Oligodendrogliomas		TERT promoter mutations and 1p/19q co-deletion (30)	Microenvironmental signature enriched in neuron-associated genes (31)
METASTATIC BRAIN CANCERS			
Breast cancer		EGFR gain (only HER2+ tumors)	
Melanoma		BRAF mutations	Stat3+ pro-tumorigenic astrocytes (32). Communication between astrocytes and tumor cells by extracellular vesicles (33) or cx43-dependent gap junctions (34)
Lung cancer		KRAS mutations, ALK translocation, EGFR amplification	

(28). In addition, it has been shown that there are distinct differences in the innate immune infiltrate of LGG compared to glioblastoma. For example, gross macrophage number is positively correlated with glioma grade and inversely correlated with survival; with high grade glioblastoma, particularly the mesenchymal subtype, having the most predominant infiltrate compared to low grade disease (31, 43–46). An increase in tumor-infiltrating neutrophils has also been linked to higher glioma grade (47) and disease progression (48), consistent with the observation that neutrophils are reduced in IDHmut gliomas in mice (28). More specifically, neutrophils may be involved in the pathogenesis of glioblastoma by supporting tumor-initiating cell (TIC) expansion through the secretion of S100 proteins (48). In murine models, neutrophil depletion stunts the development of glioblastoma but not LGG, indicating the specific importance of neutrophils in high grade disease (28). Finally, patient-derived glioma stem cells from IDHmut tumors significantly downregulate the natural killer (NK) cell activating ligand NKG2D compared to those from IDH-wild type patients, leading to blunted NK cell-mediated lysis (29). It remains unknown how these innate immune differences might be regulated by the mutational differences between glioblastoma and LGG; however, given the defined roles of IDH and 2-HG in TME composition (28, 40), it is conceivable that these TME differences could be influenced by similar mechanisms.

In addition to immune cell composition, the structure of the brain extracellular matrix (ECM) appears to help characteristically define both LGG and glioblastoma. The structure and composition of the brain ECM is unique compared to other organs and tissues, and is dominated by glycoproteins, proteoglycans, and glycosaminoglycans (GAGs)

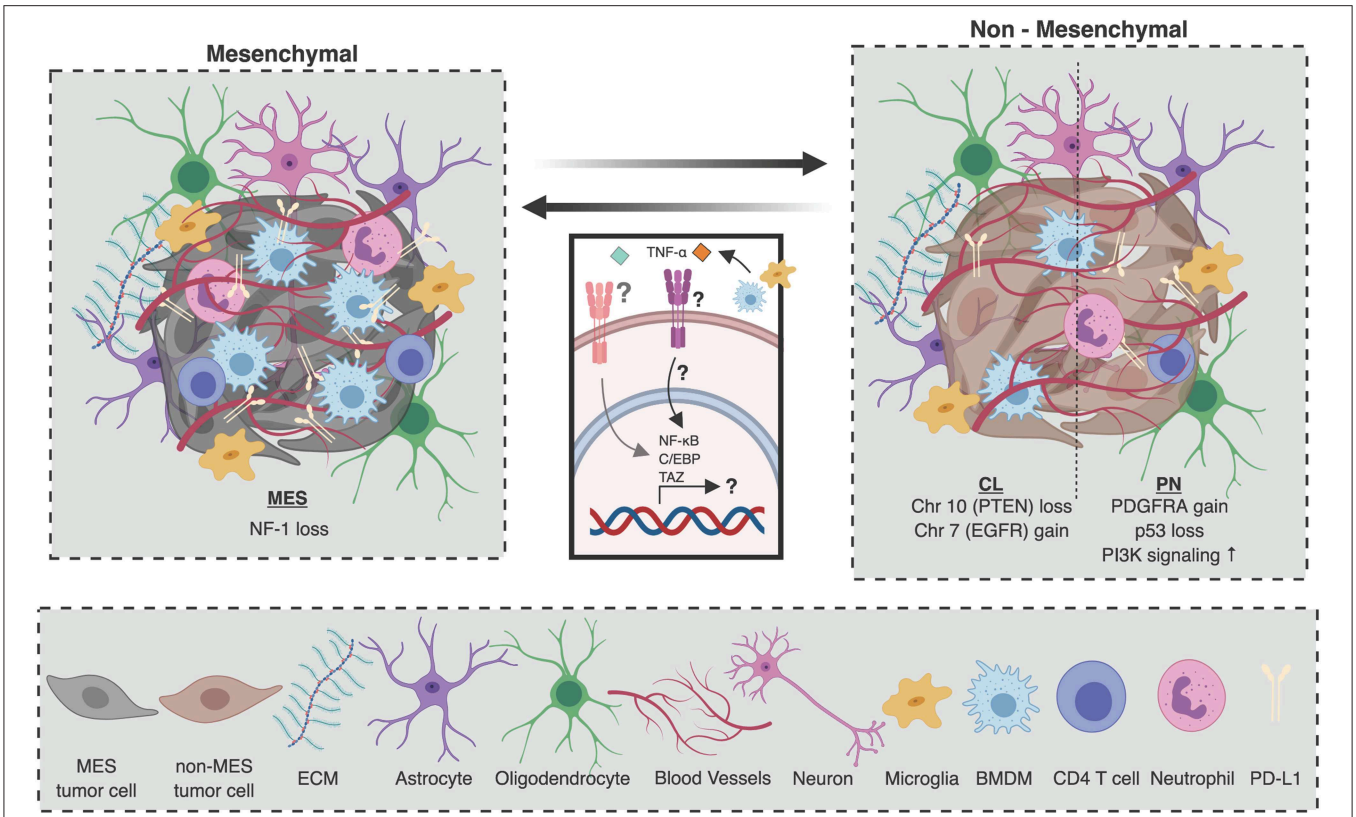

FIGURE 1 | Composition of the glioblastoma tumor microenvironment as a function of molecular subtype. The cellular microenvironment of glioblastoma is composed of many unique cell populations including brain-specific cell types such as astrocytes, oligodendrocytes, and neurons, as well as immune and endothelial cells. Mesenchymal (MES) tumors, which are characterized by *NF1* deletions or mutations with functional consequences, are associated with a higher number of tumor-associated immune cells. Specifically, there are more macrophages and microglia, as well as CD4 T cells and neutrophils in the tumor microenvironment (TME) of MES tumors. On the other hand, the TME of non-mesenchymal glioblastomas [proneural (PN), and classical (CL)] is poorer in immune cells. Similarly, PD-L1 expression is higher in MES tumors than in non-MES tumors. Upon disease recurrence, it is believed that macrophage-derived TNF-α can induce an NF-κB, TAZ, and C/EBP dependent program in tumor-initiating cells (TICs) which promotes transdifferentiation to the MES molecular subtype. Figure created with BioRender.com.

such as heparin sulfate proteoglycans (HSPGs) and hyaluronic acid (HA). In brain tumors, the ECM is dense, leading to hypoxia and tumor aggressiveness. Given the association between ECM stiffness and tumor progression observed in epithelial tumors (49–51), it is possible that ECM stiffness may likewise contribute to glioma progression. Indeed, in mouse models and humans, it has been shown that increased ECM stiffness resulting from HA deposition and tenascin C (TNC) production is associated with higher glioma grade (52). Further, xenograft models revealed that IDHmut tumors displayed reduced aggression in association with reduced ECM stiffness and mechanosignalling, by downregulating HIF-1α-mediated expression of TNC (52). This indicates that the differences in ECM composition are partially regulated by IDH mutational status in gliomas. Since the ECM serves as a scaffold for tissues and regulates cellular architecture and inflammation, ECM differences in IDHmut vs. wild-type tumors may in part underlie the phenotypic differences between LGG and glioblastoma TMEs, and as a consequence influence disease evolution.

TME ACROSS MOLECULAR SUBTYPES OF PRIMARY BRAIN MALIGNANCIES

Glioblastoma is characterized by a high degree of inter- and intra-tumor heterogeneity. Originally, glioblastoma was divided into 4 molecular subtypes based on bulk gene expression data: proneural (PN) characterized by aberrations in platelet-derived growth factor A (PDGFRA), TP53, and increased phosphoinositide 3-kinase (PI3K) signaling; classical (CL) characterized by epidermal growth factor receptor (EGFR) gain and phosphatase and tensin homolog (PTEN) loss underscored by chromosome 7 amplification and chromosome 10 loss, respectively; mesenchymal (MES) characterized by neurofibromin 1 (NF-1) loss and/or mutation; and neural which did not possess any characteristic genomic features (**Figure 1**) (21, 22). Of note, subsequent analyses have shown that the neural subtype is most likely associated with tumor margins where non-malignant tissue typically constitutes the bulk of resected material (21, 23, 53–55). Each of the molecular subtypes of glioblastoma differ in their prognostic outlook, with the PN subtype having the

longest overall survival (22). Interestingly, using unsupervised hierarchical clustering, IDHmut glioblastomas cluster with the PN subtype (23). Consequently, IDH mutations in glioblastoma are considered a hallmark of the PN signature (22, 23). On the other hand, using single-cell RNA sequencing (sc-RNAseq), a recent study has uncovered that glioblastoma cells can be assigned one of 6 distinct molecular "meta-modules" that bear similarities to normal cells of the neuronal lineage; a classification which is recapitulated in pediatric glioblastoma (56). In this framework, glioblastoma cells are classified as either mesenchymal-like (MES-like) 1 (hypoxia independent) or 2 (hypoxia dependent), astrocyte-like (AC-like), oligodendrocyte progenitor cell-like (OPC-like), and neural progenitor cell-like (NPC-like) 1 (inclusion of certain OPC-related genes such as OLIG1 and TNR) or 2 (exclusion of these genes); with 15% of cells deemed "hybrids" as they express two or more of these meta-modules (56). Exactly how these cellular states relate to the three previously-defined glioblastoma molecular subtypes remains to be defined.

In the context of many epithelial tumors, molecular and genetic variation in cancer cells has been shown to translate to phenotypic and functional variation in the TME (57–59). For example, in colorectal cancer, each of the four consensus molecular subtypes has been associated with a distinct TME signature (58). However, very few studies have attempted to comprehensively compare and contrast TME dynamics between glioblastoma molecular subtypes. To date, bioinformatic deconvolution of bulk gene expression data from patient tumors has provided the best insight into the differences in the immune TME between glioblastoma subtypes (60, 61). The most striking differentiating feature is the abundance of cells in the TME, with MES tumors harboring a large fraction of untransformed cells compared to non-MES tumors, a large proportion of which are macrophages and microglia (**Figure 1**) (23). Other analyses of transcriptomic data (24–26), as well as histopathological (23), and flow cytometric (26) quantification of macrophage/microglia markers (e.g., AIF1, CD11b) have corroborated these findings by demonstrating increased macrophages in MES tumors. These differences may partly account for the poor survival associated with MES tumors given that increased macrophage abundance is associated with higher glioma grade (44, 62).

In addition to macrophages, CD4 T cells and neutrophils are also abundant in MES gliomas (**Figure 1**) (23). Within glioblastoma tumors, neutrophils support TIC expansion and contribute to disease progression (47, 48). Moreover, in peripheral blood, high neutrophil to lymphocyte ratio is a prognostic marker associated with poor overall survival (63, 64), highlighting its potential use as a blood biomarker in patients. Diverging roles for neutrophils in the context of other solid malignancies have already been described, where they can exert both pro-tumorigenic or anti-tumorigenic functions (65). Whether such functional heterogeneity exists in glioblastoma, how this may evolve with disease progression, and how the functional contribution of different immune cell types may differ across subtypes remain unclear. Going forward, it will be imperative to characterize the involvement of various cellular immune players in glioblastoma as a function of molecular subtype.

However, delineation by molecular subtype does not uncover the full scope of cellular immune TME heterogeneity in glioblastoma. Unlike myeloid cells, an increased predicted presence of CD8 T cells is not associated with any molecular subtype, but rather with a hypermutated phenotype (23). This finding is consistent with several reports in the context of other solid malignancies (66, 67) as these tumors presumably produce more neo-antigens which can be recognized by T cells. Furthermore, recurrent glioblastomas that display a TMZ-induced hypermutation signature (68, 69) are also associated with a higher predicted CD8 T cell fraction compared to matched primary tumors (23). This suggests that combination treatment of chemotherapy with immunotherapy may help boost anti-tumor immune responses, a concept that is now being explored clinically in glioblastoma patients.

In addition to enlisting the help of specific immune populations, tumors rely on inhibitory checkpoint molecules to shield them from immune destruction by T cells. The most studied of these molecules are programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which canonically inhibit T cell responses in the periphery and secondary lymphoid organs, respectively (70). In glioblastoma, the primary ligand of PD-1, programmed death-ligand 1 (PD-L1), is expressed by both tumor cells and tumor-associated myeloid cells (71). Whole transcriptome profiling of bulk tumors has revealed that PN tumors are more likely to display low levels of PD-L1, whereas MES tumors express higher levels of PD-L1, and CL tumors display more variable expression (**Figure 1**) (72). In addition, PD-1 positive lymphocytes are enriched in MES tumors possessing NF1 and RB1 mutations, and depleted in CL tumors that possess EGFR amplification events and PTEN deletions (73). Corroborating these bioinformatic analyses, the relationship between PD-L1 expression and molecular subtype has been confirmed by histopathological analysis of patient samples (72).

Despite clear differences in TME composition between molecular subtypes, tumor-intrinsic mechanisms that influence these distinctions are poorly defined. The most obvious possibility is that cancer cell expression of subtype-defining molecular features themselves [i.e., EGFR, NF-1, PDGFRA, IDH1 (22)] may regulate the glioma TME landscape. For example, NF-1 deficiency in IDH-wild type glioma cells results in increased recruitment of macrophages (23). In patients, NF-1-deficient tumors exhibit an increased M2-like macrophage signature compared to tumors with normal NF-1 levels, not only amongst IDH-wild type glioblastomas but also specifically in MES tumors (23). Interestingly, the formation of NF-1-associated dermal neurofibromas has previously been reported to be microenvironment-dependent (74), highlighting a potential role for NF-1 in organizing the tumor microenvironment of cancers of the nervous system. Of note, variations in the immune cell composition of the tumor microenvironment as a function of molecular subtype exist in other primary brain malignancies. For example, in pre-clinical models of medulloblastoma, group 3 tumors possessed a higher percentage of PD-1+ CD8+ T cells compared to SHH-driven tumors, which functionally translated to an improved response to PD-1 blockade (20).

Another interesting possibility by which TME composition is regulated in a subtype-specific manner is that the increased immune infiltrate associated with MES tumors is secondary to them being more immunogenic than their non-MES counterparts (25). To support this hypothesis, it has been argued that heightened immunosuppression may be a compensatory response to increased immune activation, as MES tumors are highly enriched in both pro-inflammatory and anti-inflammatory factors (25). However, crucial evidence to test this hypothesis is limited, such as a comprehensive comparison of the tumor mutational burden (TMB) and its impact on immune activation between molecular subtypes. Nevertheless, the increased immune fraction observed in MES tumors would suggest that they may be more responsive to therapies that seek to reinvigorate the anti-tumor immune response. Excitement surrounding this idea is now becoming evident, as a phase I clinical trial using autologous DC vaccination in conjunction with TLR agonists significantly increased survival of patients with MES tumors, but not PN tumors, compared to historical controls (75).

THE VASCULATURE OF PRIMARY BRAIN MALIGNANCIES

The vasculature is an important component of the TME and is often co-opted to support the growth of tumors. One histological hallmark of glioblastoma compared to low-grade glioma is high vascularity. Unlike the healthy brain, the vascular network in glioblastoma is disorganized and displays a high degree of microvascular proliferation (76). As such, the aberrantly structured vasculature fails to adequately perfuse the tumor, leading to the extensive hypoxia, necrosis, high interstitial pressure, and edema. Interestingly, studies have proposed differences in vascular features and/or angiogenic factors according to IDH mutational status. For example, in highly vascularized glioblastoma, it has been shown that IDH1 mutations are associated with lower expression of VEGF and improved overall survival (77). In LGG, IDH status endows a distinct vascular signature, characterized by high TGFβ and hypoxia-associated signaling pathways in IDH-wild type tumors (78). Finally, 2-HG levels have been associated with reduced healthy brain vasculature and increased vascular hyperplasia (79). These studies raise the possibility that IDH status may underlie some of the differences in vascularity observed between low- and high-grade gliomas.

The brain vasculature is also endowed with unique properties owing to the existence of the BBB. The BBB functions as a highly selective barrier between the brain and the periphery, therefore its integrity and functional status significantly impact the trafficking of immune cells, proteins, antibodies, metabolites, and therapeutic agents between the circulation and the tumor. This, in turn, places BBB function as an important regulator of pathology and response to therapy. Although the BBB is often impaired in various brain malignancies (2, 80, 81), not all brain tumors types or subtypes display the same degree of BBB impairment. For example, in medulloblastoma, the composition and integrity of the BBB vary between the four molecular subtypes (19). WNT-driven tumors display an extensively fenestrated vasculature which enables the accumulation of chemotherapeutic agents within the tumor, whereas SHH-driven tumors possess an intact BBB, comparable to that of a healthy brain, and are consequently impermeable to chemotherapy (19). This difference is reflected in the clinical outcome of these subtypes, as SHH-driven tumors have a significantly worse prognosis compared to WNT-driven tumors, a distinction which seems to be partly attributable to variations in BBB permeability (19). Whether variation in BBB function similarly exists across glioblastoma subtypes is unclear. However, these findings raise important considerations for the clinical management of brain tumors as restricted drug access caused by the BBB remains a major challenge. Understanding the mechanisms which control BBB integrity, and how these may be influenced by the molecular and genomic landscape of tumor cells, will enable the development of rational and personalized strategies to improve drug delivery.

Gliomagenesis (cancer development) as well as gliogenesis and neurogenesis (normal brain development) rely on a series of shared mechanisms including notch signaling (82, 83), neurotrophin and trk signaling (84, 85), perivascular VEGF (86), and purinergic signaling (87–90). In fact, VEGF has been demonstrated to be a mitogenic factor for both neuronal stem cells (NSC) and the associated sprouting vasculature (91–94), a phenomenon recapitulated in malignancy where microvascular associated glioblastoma cells appear to acquire a stem-cell-like phenotype (95). This prompted the use of anti-VEGF agents such as bevacizumab in the clinic, although these have failed to confer a significant survival advantage despite modest improvements in progression-free survival (PFS) when combined with SOC chemotherapy (96–99). Purinergic signaling, however, may play a role in establishing the immunosuppressive milieu of glioblastoma. In fact, in many solid tumors, extracellular adenosine metabolism has recently come under intense scrutiny as a key mediator of microenvironmental immunosuppression and cancer progression (100–103). Other perivascular components such as nitric oxide (104), as well as signaling through osteopontin, laminin α2, CD44, and integrin α6 (105–107) have been implicated in supporting tumor-initiating cell (TIC) survival and outgrowth in glioblastoma. Similar pathways have also been described in other primary brain malignancies such as medulloblastoma (104, 108).

INTRATUMOR TME HETEROGENEITY

Glioblastomas are highly heterogeneous tumors. Although they can be classified as PN, CL, or MES based on bulk expression profiles; all glioblastomas, regardless of subtype, possess cells from the other two subtypes in varying proportions (23, 109). Even the IDH mutational status of glioblastoma is not always uniform across a tumor, with some patient samples possessing both IDHwt and IDHmut cells (110). A recent analysis based on bulk gene expression data as well as single cell RNA sequencing has shed light on the extent of intratumoral diversity of molecular states that exists in all glioblastoma tumors (56). Given the unique associations between the molecular profile of glioblastoma cells and their associated microenvironment,

this heterogeneity in molecular state must surely translate into as-of-yet underappreciated microenvironmental heterogeneity within tumors. In addition, microglia tend to dominate the immune compartment outside the tumor core, notably in the leading edges where they support tumor cell invasion of the brain parenchyma (111). This is in contrast to BMDMs which appear to be enriched in the tumor core and in perivascular areas where they support TIC growth through the production of IL-1β (111, 112).

Acknowledging and understanding the variation of tumor-microenvironment interactions across a single tumor is of utmost clinical importance. Following primary debulking surgery, the core of the cellular tumor is removed. Thus, remaining cancer cells inhabit a unique microenvironment, rich in non-neoplastic cells, that is most likely not represented in the resected material. Along with the inflammation triggered by surgical intervention, these elements may shape the molecular profile and behavior of remaining cancer cells in as-of-yet underappreciated ways. As the aim of therapies administered in the adjuvant setting is to eradicate any non-resected tumor cells, consideration of the microenvironmental landscape of residual disease will be crucial to prevent tumor rebound and achieve long term remission.

TME OF RECURRENT BRAIN TUMORS

The molecular subtype of a primary tumor is not a reliable predictor of the molecular subtype of the recurrent tumor (23). In fact, a remarkable degree of plasticity exists between the molecular profiles of matched primary and recurrent tumors (23). While a PN to MES transition upon recurrence has long been speculated to exist (113, 114), it has recently been uncovered that glioblastoma can recur as any molecular subtype (**Figure 1**).

Interestingly, the microenvironmental features associated with each molecular subtype in primary disease appear to be largely recapitulated in the recurrent setting, even in situations of molecular class switching (23). There are a few notable exceptions such as the observations that recurrent MES tumors display a larger predicted fraction of non-polarized M0 macrophages (23, 26) and dendritic cells compared to primary MES tumors. Recurrent glioblastomas display reduced peripherally-derived monocyte numbers without a reduction in total macrophages (23), suggesting that repopulation of the tumor-associated macrophage (TAM) pool in rebound disease is mediated by cells of microglial origin. Nevertheless, the cellular immune TME traits which distinguish MES tumors from non-MES tumors are conserved in recurrent tumors, even in situations of transdifferentiation to and from the MES subtype (23).

The factors which regulate molecular subtype switching in recurrent glioblastoma remain unclear. While primary tumors with a lower simplicity score, indicating a higher degree of intra-tumor transcriptional heterogeneity, tend to give rise to recurrent tumors of a different molecular subtype (23), microenvironmental cues may also dictate molecular plasticity. Glioblastoma rebound is speculated to be largely driven by tumor-initiating cells (TICs) which remain after surgical resection and are resistant to adjuvant chemo/radiotherapy (86, 115). Importantly, the microenvironment is believed to be a key regulator of TIC multipotency (116), and is an important source of factors that promote the survival and outgrowth of TICs. Fittingly, it is believed that the microenvironment may also regulate the molecular profile of TICs. For example, in patient-derived glioma sphere cultures, a subset of PN TICs acquire a MES signature in a TNF-α/NF-κB dependent manner, concomitant with an upregulation of CD44, a downregulation of Olig2, and an increase in radioresistance (**Figure 1**) (117). This transdifferentiated phenotype was also associated with an increase in Stat3, C/EBP, and TAZ signaling; transcription factors that had previously been identified to drive the transition from a PN to MES signature (**Figure 1**) (118, 119). Specifically, the authors proposed macrophages as a potential source of TNF-α, contextualizing the immune TME of glioblastoma as a regulator of molecular subtype plasticity upon tumor rebound (**Figure 1**) (117). Reinforcing the idea that the microenvironment may promote the MES subtype, cultured glioma spheres, which lack any immune cells, are largely of the PN subtype even when they originated from MES tumors. Further, PN glioma spheres derived from MES tumors orthotopically transplanted into immunocompromised mice failed to give rise to MES tumors (117). These results provide intriguing insights into the mechanisms which regulate molecular class switching upon tumor rebound and depict the microenvironment as paramount for determining the molecular fate of TICs and the recurrent tumors to which they give rise.

This is in contrast to a study by Neftel et al. demonstrating that all glioblastoma meta-modules contain cells with the potential of restoring the full diversity observed in human tumors in both immunocompetent and immunodeficient hosts (56). However, their data strongly supports the notion that the microenvironment, at least in part, licenses the molecular state of glioblastoma cells. While certain genetic subclones are skewed towards a certain molecular meta-module, most are capable of giving rise to all modules in similar proportions, suggesting that other factors beyond genetics control the molecular state of glioblastoma cells (56). In fact, it is widely speculated that the microenvironment significantly alters the epigenetic landscape of glioblastoma cells; potentially underlying differences in molecular states, although the mechanisms through which this may occur remain largely unknown (120). To date, it is only known that the histone methyltransferase MLL1 is induced by hypoxia in glioblastoma cells and that loss of MLL1 reduces the expression of HIF transcripts and HIF targets (121). This suggests a feed-forward mechanism between MLL1 and HIF1α targets that sustains the hypoxic response in glioblastoma and consequently may promote TIC self-renewal and tumorigenicity for which hypoxia and HIF1α-mediated transcription are key drivers (121).

TME OF BRAIN METASTASES

Metastases arising from extracranial neoplasms are the most common manifestation of brain cancer (122). Similar to many primary brain tumors, limited treatment options are available

to these patients who succumb, on average, 6–10 months after diagnosis (122, 123). Most brain metastases are derived from tumors of the respiratory system, mammary epithelium, and skin. In fact, ∼15–25% of non-small-cell lung cancer (NSCLC) patients, ∼25% of small-cell lung cancer (SCLC) patients, ∼8% of metastatic breast cancers patients, and ∼20–30% of metastatic melanomas patients will present with at least one brain metastasis at diagnosis (122–124). In the case of metastatic melanoma, nearly half of patients will develop brain metastases throughout the course of disease (125). Naturally, the true proportion of cancer patients with brain metastases is thought to be much higher, and their clinical significance will surely increase over time as patient survival is extended for most malignancies (126).

Metastases follow a distinct evolutionary path from their parent tumor in a site-specific manner. In line with the notion that metastases arise from clones that are best suited to colonizing specific tissues, it is believed that the unique microenvironmental architecture of each organ is the purveyor of the selective drive which guides the evolution of developing metastases. The brain microenvironment appears to exert a particularly harsh evolutionary pressure on circulating tumor cells as only a handful of epithelial malignancies are regularly capable of colonizing the brain, and even then, they do so with very poor efficiency (127).

The absence of brain metastases derived from certain aggressive and highly metastatic neoplasms further highlights the selective nature of the brain microenvironment. Indeed, it seems that brain tropism is dictated by the specific ability of cancer cells to adapt to the brain microenvironment rather than their inherent metastatic potential. Interestingly, neoplasms originating from the same site will metastasize to the brain at different rates as a function of subtype (128, 129). Amongst breast cancers, up to half of HER2+ breast cancers (130) will give rise to brain metastases. Further, HER2− tumors have even been documented to give rise to HER2+ brain metastases while maintaining a global HER2− state at the primary tumor and other secondary sites, emphasizing the strong selective advantage of HER2+ breast cancer cells possess to colonize the brain (131, 132). This highlights the extent to which the brain microenvironment may impose a strict evolutionary program on invading cancer cells (133).

Brain metastases can grow as well-demarcated entities, or as diffusely infiltrating tumors (134). Infiltrating metastases are associated with worse survival outcomes compared to circumscribed tumors which are more amenable to removal by surgical resection (135). Interestingly, there is no association between the infiltration pattern of brain metastases and primary tumor site (134). To date, only higher expression of αVβ6 integrin has been associated with a well-demarcated growth pattern in brain metastases (134). Most studies examining interactions between cancer cells and the microenvironment in brain metastases have focused on extravasation and seeding into the brain parenchyma. Cancer cell seeding into the brain is very inefficient, and most cells die shortly after extravasation (127). Surviving cancer cells populate the perivascular niche (127, 136, 137) which normally supports NSCs or TICs in the context of glioma. This environment is conducive to the survival and outgrowth of neoplastic cells as it is rich in nutrients, oxygen, and endothelial cell-derived angiocrine factors. This process of exploiting this perivascular niche by cancer cells, termed vascular co-option (138), has been extensively demonstrated in brain metastases arising from melanoma, lung cancer, and breast cancer (127, 139–141). However, the specific roles of brain resident cells and peripheral immune cells in the initial stages of metastatic colonization remain incompletely understood.

Studies on heterotypic cell-cell interactions between metastasizing cells and resident central nervous system (CNS) cells have largely focused on astrocytes due to their abundance in the brain, as well as their key role in the physiology of the BBB which invading cells must cross. Immediately after extravasation, invading cancer cells encounter reactive astrocytes that activate neuron-derived plasmin (140, 142). Activated plasmin releases membrane-bound FasL, which then acts as a paracrine death signal on cancer cells, and cleaves L1CAM, an important receptor for vascular co-option, and thus cancer cell survival (142). This is one mechanism that reactive astrocytes can limit metastatic colonization. To counteract this endogenous resistance mechanism, successful cancer cells will express serpins that block plasmin activation (142).

Recently, a multi-cellular communication network between astrocytes and immune cells was discovered during metastatic outgrowth in the brain (32). Metastasizing cancer cells, irrespective of their origin, were shown to induce a Stat3 dependent pro-tumorigenic program in a subset of tumor-associated reactive astrocytes (32). This pro-tumorigenic astrocyte subpopulation also mediated local immunosuppression by inhibiting CD8 T cell activation and educating TAMs toward a pro-tumorigenic phenotype (32). The administration of a Stat3 inhibitor significantly reduced the size of brain metastases. Interestingly, the central role for Stat3 in promoting tumor growth was restricted to brain metastases as Stat3 inhibition had no effect on the growth of extra-cranial metastasis (32). Beyond demonstrating the existence of complex cellular networks in the TME of brain metastases, these results provide evidence for the existence of heterogeneous astrocyte populations that may be differentially involved in the pathology of brain tumors. Of note, the amount of phosphorylated Stat3 (pStat3) is negatively correlated with survival in anaplastic astrocytomas (143), a rare type of grade III/grade IV glioma with an astrocytic morphology. pStat3 has also been implicated in TIC-mediated immunosuppression in both gliomas (144) and glioblastoma (145), underscoring its broad importance in the pathology of brain cancers.

Communication between astrocytes and cancer cells has been reported to support the development and survival of micrometastases (146, 147). Such interactions have also been shown to increase the resistance of metastases to chemotherapeutic agents such as 5-FU, cisplatin, and paclitaxel (148, 149). One proposed mechanism by which astrocytes modulate the molecular landscape of brain metastases is through the delivery of micro-RNAs packaged in extracellular vesicles (33). Delivery of miR-19a has been shown to induce the downregulation of PTEN in breast cancer cells invading the brain parenchyma resulting in accelerated disease progression and reduced overall survival

(33). As discussed, the downregulation of PTEN has also been associated with the CL glioblastoma subtype. This observation suggests that certain oncogenic alterations that are actively selected for in the brain parenchyma confer a similar survival advantage to ontogenetically distinct tumor cells that colonize the brain.

The CL molecular subtype of glioblastoma is strongly associated with an astrocytic signature (22), and amplified or hyperactive EGFR is a hallmark feature of glioblastoma cells with an astrocyte-like signature (56). Astrocytic gliomas, both low grade and high grade, are uniquely capable of forming gap junctions between cancer cells via connexin 43 (cx43) which support the survival, growth, and invasion of the tumor (34). Brain metastases arising from triple negative breast cancer (TNBC), Her2-amplified breast cancer, and non-small cell lung cancer (NSCLC) also form gap junctions with astrocytes through cx43 in order to promote their growth and chemoresistance (147). Indeed, it appears that both primary and metastatic brain tumors that may share a common molecular alteration (in this case, EGFR amplification) employ similar tools in the brain TME to promote disease progression.

Overall, the molecular and genetic profiles of cancers which commonly colonize the brain parenchyma appear to display certain features that mirror the molecular profiles of primary brain malignancies. BRAF-driven melanoma and KRAS-driven NSCLC both exhibit aberrant RAS-MAPK signaling similarly to mesenchymal (MES) glioblastoma (21), which is characterized by the loss of NF-1 (22), a negative regulator of RAS signaling (150–152). Parallels also exist between proneural (PN) glioblastoma and brain metastases which both display increased PI3K signaling (153). ALK-translocation or amplification is a major driver of some lung cancers as well as neuroblastomas (154). Finally, classical (CL) glioblastoma, characterized by high-level EGFR amplification events (22), not only shares a common oncogenic alteration with Her2+ breast cancer brain metastases, but also with EGFR-amplified lung cancer brain metastases. Importantly, numerous cases have been documented whereby brain metastases acquire molecular features associated with various glioblastoma subtypes that are not present in the primary tumor (153). In light of these observations, a comprehensive and comparative analysis of the molecular profiles of primary and metastatic brain cancers is timely. Such studies should also determine to what extent similarities in the molecular landscape of brain cancers translate to similar microenvironmental dynamics.

A recent study of TCGA uncovered associations between oncogenic mutations and various immune signatures irrespective of cancer ontogeny. This connection extends beyond the well-documented association of deficient mismatch repair (dMMR) and an increased cytotoxic T cell infiltrate (67, 155). For example, they found that mutations in STK11 and VHL are associated with a reduced macrophage signature, that loss of p53 is associated with a decrease in cytotoxic lymphocytes, and that mutations in BRAF are associated with an increase in co-stimulatory molecules across all cancer types (155). Further elucidation of these common associations will enrich our understanding of the microenvironmental regulators of brain cancers in a subtype-dependent manner and expand our knowledge on the interplay between the TME and cancer cell molecular networks within primary and metastatic disease.

IMPLICATIONS FOR THERAPY

The TME is a critical regulator of disease progression and response to therapy. In fact, several novel therapeutic strategies against brain cancers leverage the microenvironment to kill tumor cells, including immune checkpoint inhibitors which have been extremely effective in other malignancies such as melanoma, lung, and bladder cancer. Checkmate 143, the first phase 3 randomized clinical trial evaluating the efficacy of adjuvant PD-1 blockade in glioblastoma using nivolumab, concluded that it did not improve overall survival compared to anti-VEGF therapy with bevacizumab (156). However, patients that did respond to nivolumab exhibited more durable responses (156), highlighting the potential of PD-1 blockade in glioblastoma if given to the right patients. PD-1 blockade also seems to hold promise in a small subset of patients with brain metastases originating from NSCLC (157) and melanoma (157–159). In the context of other brain malignancies, immunotherapy has already become part of common clinical practice with Dinutuximab, a monoclonal antibody against GD2 administered in conjunction with GM-CSF, IL-2, and retinoic acid having been approved for post-consolidation therapy in high-risk neuroblastoma patients (160). Overall, many elements of the brain TME are actively being investigated as potential therapeutic targets in the context of various brain malignancies which have been thoroughly reviewed elsewhere (161).

The timing of immunotherapy is also an important consideration. As we have discussed, the microenvironmental landscape of glioblastoma evolves throughout disease progression, most notably after tumor resection and upon disease recurrence. Unsurprisingly, the timing of PD-1 blockade impacts response. In fact, recent trials have revealed that neoadjuvant PD-1 blockade with either pembrolizumab or nivolumab against treatment naïve tumors (162), or even upon disease recurrence (163), favorably impacted outcome. Enhanced survival was associated with distinct changes in local and systemic immunity. Further, responsive tumors were enriched in MAPK pathway alterations whereas unresponsive tumors were enriched in loss-of-function PTEN mutations and concomitantly increased PI3K-Akt signaling (164). Interestingly, loss of PTEN function has also been linked to resistance to checkpoint blockade in melanoma (165), and metastatic uterine leiomyosarcoma (166). There was no correlation between molecular subtype and response to therapy (164). Identifying patients who are likely to respond to various modes of immunotherapy will be a major challenge in future research given the complexity of the brain TME. As such, the search for classical predictive biomarkers such as single genotypic or phenotypic traits is unlikely to be successful. Advances in genomics, the advent of highly multiplexed imaging technologies, and novel machine-learning based algorithms will allow researchers to define multiplex biomarkers which may one day be integrated into clinical protocols in order to facilitate patient stratification and treatment design.

Personalized vaccines have also shown great promise to combat brain malignancies (167, 168). The use of a polio-rhinovirus chimera has proven to be the most promising, inducing long-lasting responses in as much as 21% of treated patients (169). Curiously, the median survival of the experimental cohort was only 12.5 months whereas the median survival of the historical control group was 11.6 months. This small difference can probably be explained by the 21% of patients who did not succumb to disease over the course of the trial, which would suggest that this therapy does not slow down the progression of disease in most patients. Rather, it seems to be uniquely capable of inducing long lasting remissions in a subset of patients while having no effect on the disease course of others. The defining characteristics of this patient subpopulation remain undefined as do the mechanisms by which the introduction of the virus favors tumor eradication. It is feasible, however, that responders shared a functional microenvironmental signature, which may have been imparted through different elements, but that ultimately rendered their tumors susceptible to therapy.

A significant challenge that remains for immunotherapies in brain malignancies is the fact that even if they are successful in priming anti-tumor T cell responses, T cells still face a barrage of local immunosuppression that needs to be overcome. To relieve this inhibition, strategies are actively being developed to reverse the highly immunosuppressive milieu of the brain tumor microenvironment. For example, leveraging the high prevalence of macrophages in glioblastoma, macrophage reprogramming through blockade of CSF-1R has been highly effective in pre-clinical models (170).

Combinatorial strategies will surely yield the most successful clinical results in the future, as they have in the context of many other malignancies. However, the success of future experimental therapies is predicated on an increased appreciation of the complex relationships that exist between the molecular identity and the microenvironmental landscape of brain tumors.

AUTHOR CONTRIBUTIONS

LP and LW conceived the review, wrote, and edited the manuscript.

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Allies or Enemies—The Multifaceted Role of Myeloid Cells in the Tumor Microenvironment

*Lisa Haas and Anna C. Obenauf**

Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Vienna, Austria

For decades, cancer was considered a disease driven by genetic mutations in tumor cells, therefore afflicting a single cell type. This simplified view was slowly replaced by the understanding that interactions between malignant cells and neighboring stromal and immune cells—the tumor microenvironment (TME)—profoundly shape cancer progression. This understanding paved the way for an entirely new form of therapy that targets the immune cell compartment, which has revolutionized the treatment of cancer. In particular, agents activating T lymphocytes have become a key focus of these therapies, as they can induce durable responses in several cancer types. However, T cell targeting agents only benefit a fraction of patients. Thus, it is crucial to identify the roles of other immune cell types in the TME and understand how they influence T cell function and/or whether they present valuable therapeutic targets themselves. In this review, we focus on the myeloid compartment of the TME, a heterogeneous mix of cell types with diverse effector functions. We describe how distinct myeloid cell types can act as enemies of cancer cells by inducing or enhancing an existing immune response, while others act as strong allies, supporting tumor cells in their malignant growth and establishing an immune evasive TME. Specifically, we focus on the role of myeloid cells in the response and resistance to immunotherapy, and how modulating their numbers and/or state could provide alternative therapeutic entry-points.

Keywords: immunotherapy, cancer, myeloid cells, dendritic cells, macrophages, myeloid-derived suppressor cells, immune suppression, tumor microenvironment

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Reviewed by:
Alessandro Poggi,
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McGill University, Canada

***Correspondence:**
Anna C. Obenauf
anna.obenauf@imp.ac.at

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INTRODUCTION

Myeloid cells are a diverse group of cells belonging to the innate immune system that are prone to adapt their phenotype to their tissue of residence (1). Thus, in cancer, they exist in a vast amount of different states and exert a range of distinct functions (**Figure 1**). Among those myeloid cells, macrophages, dendritic cells (DCs), and myeloid-derived suppressor cells (MDSCs) have received much attention in the last decades, due to their ability to both initiate or suppress an anti-tumor immune response (**Figure 2**) (2). In the following, we will specifically focus on those three groups of myeloid cells and provide an overview of their roles as cancer cell allies or enemies.

DENDRITIC CELLS

Since their identification in mice in 1973 by Steinman and Cohn, DCs have become widely accepted as important players in the network of phagocytizing and antigen presenting cells (APCs) that sculpt immune outcomes (3). In tumor immunity, DCs have predominantly an anti-tumorigenic role. DCs arise from a common bone marrow (BM) progenitor—the common dendritic cell progenitor (CDP)—and then differentiate into plasmacytoid (pDCs) and precursors for conventional dendritic cells (cDCs) (**Figure 1**). These immature DCs subsequently migrate out of the bone marrow and colonize peripheral tissues, where they encounter antigens (4–8). The maturation of DCs represents a critical step in their life-cycle, allowing them to gain full APC capacities. Maturation is initiated upon recognition of danger-associated molecular patterns (DAMPs) via pattern recognition receptors (PRRs), where different DC subsets express different PRRs, further contributing to their functional specification. Upon maturation, DCs upregulate their antigen presentation machinery and costimulatory molecules, transforming themselves into potent T cell activators and thus bridging innate and adaptive immunity (9, 10). DCs can license anti-tumor immune responses by processing and cross-presenting exogenous antigens via MHC class I molecules to CD8 T cells, presenting antigens via MHC class II molecules to CD4 T cells, and secreting immune-stimulatory cytokines. In this capacity, they have become an integral part of the cancer immunity cycle and are attractive targets for immunotherapy (11, 12).

cDCs Are Potent Activators of Anti-tumor Immunity

cDCs differentiate into two subsets—cDC1 and cDC2—which are distinguished by their differential marker expression (**Figure 1**), transcription factor (TF) dependency, and functions. The differentiation into cDC1s or cDC2s is instructed by different chemokines and single cell sequencing studies in mice revealed distinct gene signatures that become evident early after the differentiation from CDPs (**Figure 1**): cDC1s are instructed by FLT3L and express the TFs IRF8, BATF3, and ID2, cDC2s are instructed by GM-CSF and are dependent on the TF IRF4, Notch2, and RelB (4, 8, 13, 14).

The role of cDC1 cells in anti-tumor immunity is well-established (15, 16). cDC1s are present as both lymph node resident ($CD8^+$) and migratory ($CD103^+$) populations. Lymph node resident DCs sample antigens in blood and lymph fluid, and migratory cDC1s transport antigens from the peripheral tissue to lymph nodes and spleen. This is indicated by the ability of $CD103^+$ cDC1s to transport tumor-derived fluorescent proteins to the lymph node in a CCR7-dependent manner (17, 18). A substantial fraction of intratumoral $CD103^+$ cDC1s does not migrate to the lymph node, yet they still play a crucial role in anti-tumor immunity. In mouse models those intratumoral, non-migratory $CD103^+$ cDC1s were shown to mediate their effects via direct antigen presentation and establishment of a favorable chemokine environment and were found necessary for tumor control in a lymph node-independent manner (13, 17). They are an important source of CXCL9 and CXCL10 in tumors, which makes them indispensable for the infiltration of both naïve and pre-activated T cells. In patients, the levels of $CD103^+$ transcripts correlate with the levels of CXCL9 and CXCL10, and degree of T cell infiltration (19, 20). The crucial role of $CD103^+$ cDC1s has been further substantiated using $BATF3^{-/-}$ mice devoid of $CD103^+$ cDC1s, which fail to reject immunogenic cancer cell lines and are unresponsive to immune checkpoint inhibition (17, 19, 21, 22).

In contrast to cDC1s, the role of the more heterogeneous population of $CD11b^+$ cDC2s in anti-tumor immunity is less well-explored. They are superior to cDC1s in the induction of CD4 T cell responses via antigen presentation on MHCII, and have been shown to activate TH17 cells, a cell type with controversial roles in cancer that produces high levels of pro-inflammatory cytokines (23, 24). Compared to cDC1s, cDC2s fail to deliver antigen to lymph nodes. Because they have lower levels of endocytic receptors, higher levels of lysosomal enzymes and a lower phagosomal pH, it was hypothesized that antigens are directly degraded during migration instead of being further processed and presented on the surface (8, 13, 25). However, reduced antigen presentation of cDC2s may (also) be due to a lack of appropriate stimuli in the tumor and if stimulated, cDC2s may still play an important role in anti-tumor immunity. This is supported by studies showing that immune responses induced by the TLR7 agonist R848, acting on cDC2s, or anthracyclines, also induce protection in BATF3-deficient mice (26, 27).

pDCs and moDCs Have Antagonistic Roles in Cancer Immunity

DCs display high functional plasticity and despite having largely anti-tumorigenic capacities, they can under certain circumstances for example when present in an immature state, act immune suppressive. This is illustrated by the complex role of pDCs in tumor immunity. pDCs express MHCII, costimulatory molecules, and a narrow set of TLR receptors and have been identified as the main producers of Type I IFN upon activation by DAMPs (1, 28). Despite their capacity to produce Type I IFN, the presence of pDCs is a poor prognostic marker in breast cancer, melanoma, and ovarian cancer in human and animal models (29–32). This could be due to the poor activation of pDCs in the TME and an active instruction of pDCs by the tumor to fulfill immune-suppressive functions, such as production of IDO, IL10, or OX40 expression (33). Monocytic DCs (moDCs or inflammatory DCs) have a different origin and differentiate from $Ly6C^{high}$ monocytes in the context of cancer or inflammation (**Figure 1**). They are efficient in the uptake and processing of antigens and correlate with $CD8^+$ T cell infiltration in several tumor models (34). Yet in direct contrast, they can also display an immune-suppressive phenotype, based on high expression of iNOS, TNF-α, IL-6, IL-10, and their capacity to hamper T cell proliferation *in vitro*, as it was shown using moDCs isolated from murine lung cancer models (23, 34). Thus, further investigation is needed to understand the pro- vs. anti-tumorigenic functions of this complex cell type, the tumor-derived signals that skew them, and particularly how this plays out in patient settings.

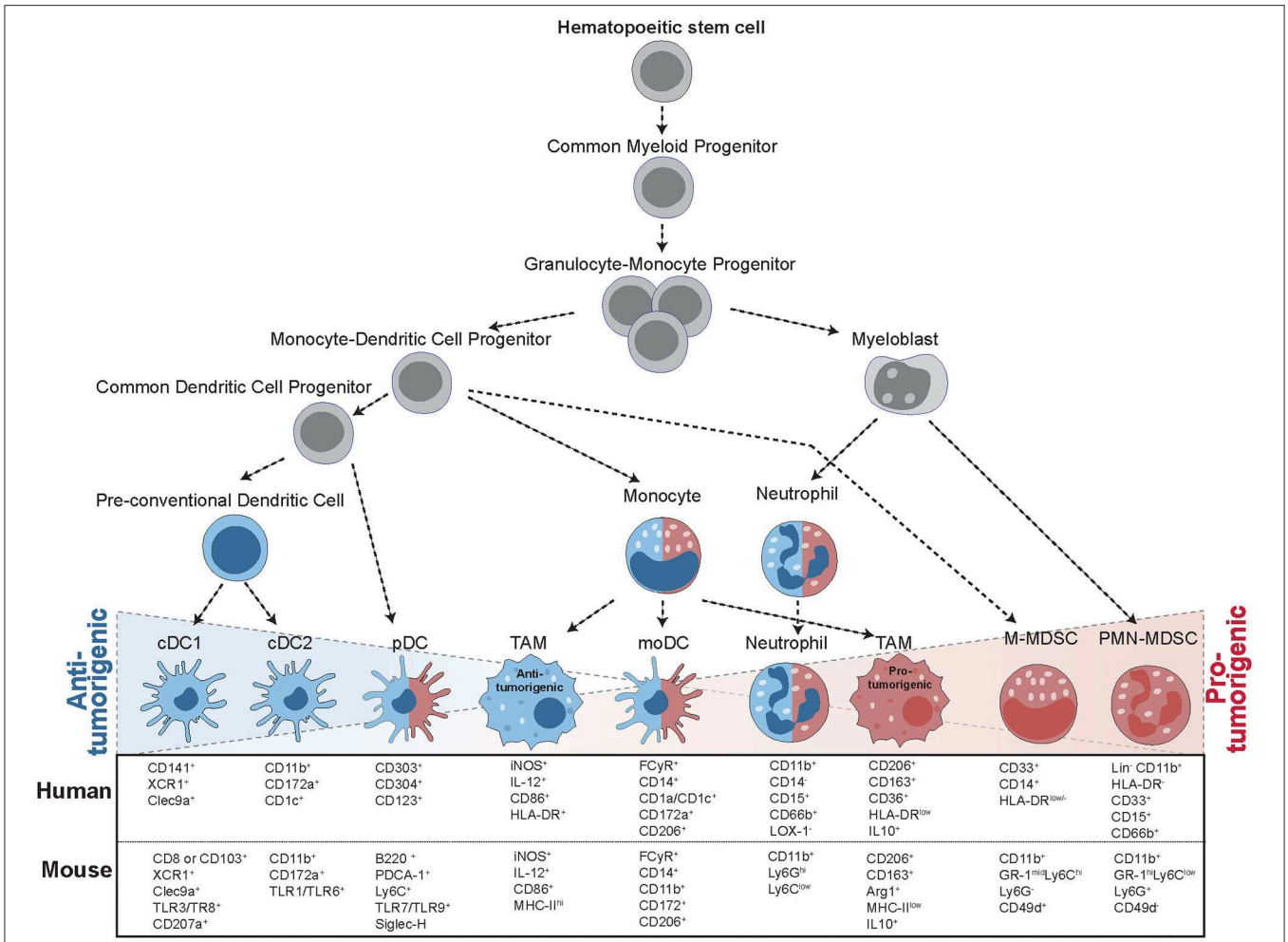

FIGURE 1 | Progression from hematopoietic stem cells (HSC) to tumor-infiltrating myeloid cells. The formation of tumor-infiltrating myeloid cells occurs in a step-wise process: In the bone marrow, HSCs give rise to common myeloid progenitors (CMP), which give rise to granulocyte-monocyte progenitors (GMP). GMPs then further specify into myeloblasts (MB) and monocyte-dendritic cell progenitors (MDP). These precursors then differentiate into a range of different cell types with anti-tumorigenic (blue) and pro-tumorigenic (red) capacities. MDPs can give rise to a common dendritic cell progenitor (CDP), further leading to the formation of conventional DCs (cDCs) or plasmacytoid (pDCs). MDPs also form monocytes, giving rise to monocytic DCs (moDCs) or differentiating into tumor-associated macrophages (TAMS) upon instruction by the tumor. Macrophages can display multiple different activation states, ranging from anti-tumorigenic to pro-tumorigenic subsets. MBs give rise to mature neutrophils or polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), while monocytic MDSCs (M-MDSCs) arise from MDPs upon instruction by inflammatory signals. All of these cell types are characterized by specific surface marker expression, indicated for both human and mouse cells.

Tumors Inhibit DC Functionality on Multiple Levels

In addition to the diverse effects of DCs on tumor cells, in return, the tumors can interfere with DC functionality, either by affecting their differentiation or by suppressing their activation and maturation at the tumor site. Many tumor-secreted factors affect DC differentiation. For example, IL-6 and CSF-1 promote lineage commitment toward suppressive monocytes (35), and vascular endothelial growth factor (VEGF) inhibits DC maturation by suppressing NFκB signaling in hematopoietic progenitors (36). In addition, secreted factors can also directly inhibit the anti-tumor activity of DCs, such as TGF-β, which can inhibit antigen uptake *in vitro* and it was shown that inhibiting TGF-β signaling synergizes with immunotherapy in pre-clinical mouse models (15, 37, 38). In the local TME, metabolic dysfunction can hamper DC activity. For example, high levels of lactic acids were shown to interfere with DC activation and antigen presentation (39). Studies in mouse models showed that lipid peroxidation byproducts can induce continuous activation of the TF XBP1 in DCs, resulting in abnormal lipid accumulation and DC dysfunctionality (40). Recently, it became clear that the TME is strongly influenced by the oncogenic pathways driving cancer progression, which have a profound impact on the immune cell infiltrate (41, 42). In the context of DCs, upregulated beta-catenin signaling reduces infiltration of cDC1s via reducing the production of CCL4, among other chemokines

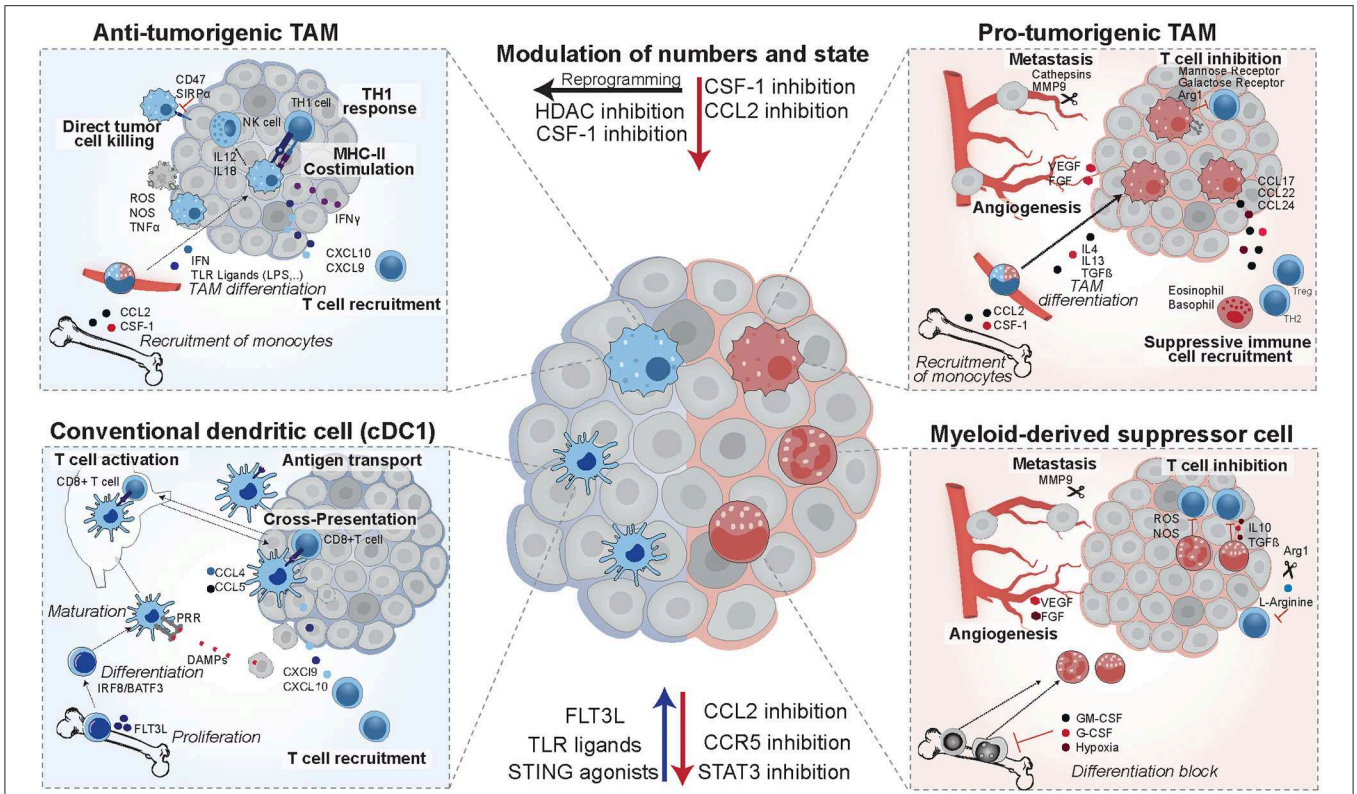

FIGURE 2 | Opposing functions of tumor-infiltrating myeloid cells. *Anti-tumorigenic TAMs* arise from circulating monocytes in response to TLR ligands and interferon. They are characterized by high expression of costimulatory molecules and MHCII. In mouse models they were shown to induce potent TH1 responses and augment NK cells responses. *cDC1* dendritic cells differentiate in response to FLT3L, mature upon recognition of danger associated molecular patterns (DAMPs), and then induce T cell activation via antigen presentation on MHCI. They establish a favorable cytokine environment in the tumor (CXCL9, CXCL10) and murine studies revealed that they are recruited in response to CCL4 and CCL5. In patients, they have positive prognostic value, correlate with T cell infiltration and are enriched in immunotherapy responders. Their numbers and maturation state can be enhanced by FLT3L, TLR ligands, or STING agonists. *Pro-tumorigenic TAMs* arise from circulating monocytes in response to IL4, IL13, and TGFβ, and establish an immune suppressive environment via recruitment of eosinophils, basophils, Tregs, and TH2 cells. They are pro-metastatic and induce angiogenesis, and their recruitment can be reduced by CSF-1 and CCL2 inhibitors in pre-clinical models. In addition, mouse models identified that they can be re-educated to an anti-tumorigenic state using HDAC inhibitors. *MDSCs* form from immature myeloid progenitors upon stimulation by the tumor and suppress T cell activity via IL10, TGFβ, and production of reactive oxygen and nitrogen species (ROS and NOS). They deplete intracellular L-arginine pools and hamper T cell proliferation in murine models and in patients their presence is a negative prognostic factor.

(43). Elevated COX activity in tumor cells results in production of prostaglandin E2, which reduces NK cell infiltration and thus reduced the cDC1 recruitment factors XCL1 and CCL5 in the tumor microenvironment. Consequently, tumors with high prostaglandin E2 displayed reduced cDC1 levels, contributing to reduced effector T cell infiltration (44).

DCs Promote Response to Immunotherapy

CD8 T cell priming against tumor-specific antigens requires cross-presentation of the antigen on an MHC I complex by DCs and marks a crucial step for mounting a functional T cell response (45). Indeed, the presence of cDC1s in human tumors correlates with T cell infiltration levels and increased survival in breast, lung, and head and neck cancer patients (13). Moreover, murine studies using cDC1-deficient $BATF3^{-/-}$ mice highlighted their crucial importance for the response to immunotherapy (13, 17, 19, 46). Recently, a systematic comparison of biopsies from patients responding vs. non-responding to immunotherapy identified intratumoral abundance of cDC1s ($CD141^{+}$ in humans) as predictive for immunotherapy success (47). This is in line with a second study that characterized IL-12 producing $BATF3^{+}$ DCs as crucial for immunotherapy success in mice and showed that IL-12 activates lymphocyte effector functions in patients (48).

Targeting DCs as a Therapeutic Strategy

The central role of DCs in the initiation of immunity and their positive effect on patient survival provide a strong rationale to harness DCs and boost an endogenous anti-tumor immune response. To this end, different approaches are being explored, including: (1) increasing intra-tumoral DC numbers, (2) boosting DC maturation and function, and/or (3) alleviating tumor cell-mediated DC repression (8, 49). Vaccination strategies to increase DC numbers using both non-targeting and targeting vaccines represented a first wave

of therapies that was initiated more than two decades ago (50). Non- targeting vaccines composed of peptides together with adjuvant agents showed limited clinical success and were later improved to contain patients' antigenic peptides in combination with the chemokine GM-CSF, resulting in clinical responses (51, 52). In addition, GVAX—a vaccine containing cancer cells overexpressing GM-CSF—was shown to attract and activate DCs in patients, and later to have some clinical activity (53). There remains however a big discrepancy between the capacity of these vaccinations to induce DC activation and their actual clinical efficacy. This could be due to a suppressive TME and exhaustion of T cells, and thus combination therapies may be the key to their success and are being actively explored in pre-clinical and clinical studies (12, 50, 53, 54). In 2012, a combination trial of GVAX and checkpoint inhibition was shown to be clinically safe (55) and more recently in 2016, an overall response rate of 38% was achieved in patients receiving transfer of modified, autologous DCs with checkpoint inhibition (56). Intra-tumoral injection of FLT3L increases numbers of circulating cDC1s, mobilizes DCs to the TME and has been successful in murine studies in combination with Poly I:C induced maturation (17). In patients, injections of FLT3L resulted in an increase of circulating cDCs (57).

An alternative approach is the maturation of DCs, which results in high expression of chemokines, costimulatory molecules and antigen presentation (9). Different maturation cocktails, comprising proinflammatory cytokines or TLR ligands have been evaluated in clinics and were shown to induce robust T cell activation capacities in DCs (58). To reduce side effects a direct intra-tumoral administration of maturation stimuli may be preferred and direct and abscopal effects of the TLR ligands Poly I:C or CpG are being evaluated (59). The STING pathway, sensing cytoplasmic DNA and inducing prominent Type I interferon release from DCs, has been another focus of intense research and modified cyclic dinucleotides, mimicking the endogenous STING ligands, have progressed into clinical trials (60). In addition, in 2018 a small molecule STING agonist has been published to induce potent, long-lasting responses in mice bearing colon cancer (61). While many of these approaches focus on cDC1s, triggering the release of IFN by pDCs could be an alternative entry point, which is under active investigation in checkpoint inhibitor resistant melanoma patients (62, 63).

TUMOR-ASSOCIATED MACROPHAGES (TAMs)

Macrophages are a heterogeneous population of myeloid cells and are highly abundant in many cancer types. Their heterogeneity is influenced by: (1) their developmental origin, (2) their tissue of residence, and (3) the environmental cues they are exposed to (64). This is reflected by the vast number of different activation states, ranging from anti-tumorigenic to strongly pro-tumorigenic phenotypes.

TAMs Are a Heterogeneous Population of Myeloid Cells With Different Developmental Origins

In tumors, it was predominantly believed that TAMs arise from circulating monocytes that are recruited from the BM or spleen via cytokines such as CCL2 and CSF-1. However, macrophages can also arise from embryonic precursors and develop into tissue-resident macrophages, such as microglia in the brain, alveolar macrophages in the lung, or Kupffer cells in the liver (65, 66). In recent years, it has become clear that both monocyte-derived and tissue-resident macrophages play a role in tumorigenesis. Lineage tracing studies in mouse brain tumors revealed that both tissue-resident and monocyte-derived macrophages populate brain tumors, and macrophages of dual origin were reported in pancreatic ductal adenocarcinoma (67–70). The identification of a unique marker to characterize these heterogeneous TAM populations has proven difficult. In murine macrophages the glycoprotein CD68 is fairly specific and in combination with F4/80 identifies the majority of TAMs. In humans, CD68 is less specific and also expressed on granulocytes, dendritic cells, endothelial cells, fibroblasts and some lymphoid subsets (71). Due to the lack of a specific marker, the scavenger receptor CD163 (in humans M130) is often used in combination with CD68 to identify TAMs in humans (2). Moreover, CD49D can be used as a discriminatory marker between bone-marrow derived macrophages recruited to the brain and tissue-resident microglia in both mouse and humans and CD45 expression levels allow to distinguish these cell types in murine tumors (67).

Due to their substantial heterogeneity, TAMs need further sub-classification. They are commonly divided into "classically activated" M1 and "alternatively activated" M2 macrophages, with M1 referring to anti-tumorigenic and M2 to pro-tumorigenic macrophages. However, this classification is an oversimplification and the M1/M2 activation states present the extremes of a large spectrum of different functional states with various features (72, 73). Pro- and anti-tumorigenic TAMs are instructed by different sets of stimuli: anti-tumorigenic TAMs arise in response to TLR ligands and IFN, whereas pro-tumorigenic TAMs expand in response to IL4, IL13, TGFβ, and glucocorticoids (73–76). TAMs with anti-tumorigenic potential produce IFNγ, have high levels of MHCII and costimulatory molecules and secrete TH1-recruiting chemokines such as CXCL9 and CXCL10. They are strong promoters of TH1 responses, which results in production of IFNγ and IL12, and induces a positive feedback loop. In addition, anti-tumorigenic macrophages augment NK cell responses by producing IL18 and IL22 (**Figure 2**) (2, 77–79). In contrast, TAMs acting in a pro-tumorigenic manner are more phagocytic, express higher levels of mannose and galactose receptors, and have a highly active arginase pathway (79). The depletion of arginine pools by Arg1, an enzyme converting L-arginine into L-ornithine, is detrimental to T cells and has been shown to drive their cycle arrest in murine models (80, 81). Additionally, pro-tumorigenic TAMs express a distinct set of chemokines, including CCL17, CCL22, and CCL24. This, in turn, recruits TH2 cells, regulatory T cells, eosinophils and basophils, and induces a more immune

suppressive microenvironment (76). Bulk sequencing studies of breast and endometrial cancer patient-derived monocytes and TAMs published earlier this year, provided further insight into human TAMs and identified CCL8 as an additional pro-tumorigenic TAM effector molecule, inducing the expression of an invasive gene expression profile in the cancer cells (82).

Moreover, the spatial distribution of macrophages and the respective environmental conditions in different tumor areas has a profound impact on their function. At the leading edge of tumors, macrophages can drive invasive cellular states through a paracrine signaling loop involving CSF-1 and EGF (83). They act as a major source of matrix metalloproteinases, cathepsins, and serine proteases, which promote degradation of basement membranes and promote invasion and metastases (84–86). In growing tumors TAMs frequently accumulate in regions of hypoxia, where the hypoxic conditions could induce a switch to a pro-angiogenic, invasive phenotype, mediated via diverse range of angiogenic factors, such as TGFβ, VEGF, PDGF, and fibrin (83, 87).

TAM Activation Influences Patient Prognosis and Response to Immunotherapy

High levels of TAMs are associated with poor prognosis, such as in patients with breast, lung, head and neck cancer, as well as Hodgkin's lymphoma. However, high levels of $CD68^+$ cells (consisting largely of TAMs but also granulocytes, dendritic cells and fibroblasts, which also express this marker) are reported to correlate with better prognosis in patients with colon, gastric, and endometrial cancer (2, 71, 88, 89). In consideration of the vast heterogeneity of this cell type, the activation state of TAMs may be a better prognostic marker than cell numbers. Especially the strong immune-suppressive effects of pro-tumorigenic TAMs and their expression of PD-L1, PD-L2, CD80, and CD86, which are ligands for the T cell checkpoints PD-1 and CTLA-4, would suggest TAM infiltration to have a negative effect on immunotherapy. Indeed, in several studies using mouse models, depletion or re-education of TAMs using HDAC inhibitors or blockade of CSF-1 signaling, has shown synergism with checkpoint inhibition (90, 91). However, clinical proof of this treatment modality has yet to be obtained (89). What is needed first, are better markers of the different activation states so that they can be characterized in patients.

Targeting TAMs as a Therapeutic Strategy

The recruitment of TAMs into the TME is strongly dependent on the CCL2 and CSF-1 signaling axes mediating their replenishment from circulating monocytes. Thus, multiple treatment strategies including mAbs, small-molecule inhibitors, and RNAi targeting these pathways have been developed (49). In pre-clinical pancreatic cancer models, CSF-1R signaling inhibition reduces both the numbers of tumor-infiltrating TAMs and their expression of immune-suppressive molecules and therefore acts synergistically with checkpoint inhibition (90). In 2017, a promising study reported response to immunotherapy in combination of CSF-1R and PD1 antagonists in pancreatic cancer patients and is now moving on to a phase II clinical trial (64). Conceptually similar, the humanized CSF-1R Ab emactuzumab reduces TAM infiltration and increases T cell infiltration, which was also confirmed in patients with diffuse type giant cell tumors (64). Several CCL2 blockade combination trials are underway and first results showed a 40% increase in chemotherapy response in pancreatic cancer patients (64, 92). Blockade of the CCL2 axis has however limitations, as it is rapidly compensated by granulocytes and cessation of the therapy induces a burst of monocytes from the bone marrow, increasing metastasis and invasion in a breast cancer mouse model, warranting caution (93).

Other than modulating TAM numbers, alternative strategies have focused on directly targeting immune suppressive TAM effector molecules, such as Arg1 inhibitors (94), or on reprogramming TAMs into an anti-tumorigenic population. In murine glioblastoma, inhibition of CSF-1R regressed established tumors and increased survival, which was attributed to a re-education from an M2 to an anti-tumorigenic phenotype (95). Loss of the receptor tyrosine kinase MERTK triggers a proinflammatory TAM phenotype and induces T cell activation (96, 97), while HDAC inhibition reprograms TAMs into highly phagocytic tumor suppressors (91). However, cancer cells can escape phagocytosis by expressing the membrane receptor CD47—the "don't eat me signal," which binds to SIRPα on macrophages, inhibiting phagocytosis. Several clinical compounds targeting this suppressive axis are currently in clinical trials (98). Despite many encouraging results, TAM targeting still needs further investigation, since it was recently shown that classical monocytes and macrophages are required for better response to checkpoint inhibition in mouse models (99, 100) and that binding of antibodies to FC receptors of macrophages contributes to the success of several therapeutic responses (101). Thus, a depletion strategy specific for pro-tumorigenic TAMs or strategy to convert TAMs is needed, further highlighting the need to identify specific markers.

MYELOID-DERIVED SUPPRESSOR CELLS

Soluble factors released into systemic circulation can cause a differentiation block in normal hematopoiesis and promote the expansion of immature myeloid precursors (IMCs), which fail to terminally differentiate. These so-called MDSCs are best characterized in the field of cancer, but also accumulate in infectious diseases, aging or obesity. They are distinct to terminally differentiated mature myeloid cells (e.g., DCs and TAMs), yet their distinction from neutrophils is often a topic of controversy. As evident from their name, these pathologically activated cells exhibit strong immune suppressive capacities and are crucial drivers of an immune-suppressive microenvironment.

MDSCs Are a Heterogeneous Population of Highly Immune Suppressive Cells

Myeloblasts give rise to neutrophils and myeloid-dendritic cell progenitors (MDPs) can specify into monocytes, however, upon tumor mediated instruction these fail to fully mature and

form MDSCs (**Figure 1**). MDSCs arise in response to many tumor-derived factors and are further subdivided into two groups: monocytic (M) MDSCs ($LY6G^-/LY6C^{high}$), which are morphologically similar to monocytes, and polymorphonuclear (PMN) MDSCs ($Ly6G^+/LY6C^{low}$), which are morphologically similar to neutrophils (102, 103). The distinction between PMD-MDSCs and neutrophils has proven difficult, as they share cellular origin and many phenotypic and morphological features. Thus, a few reports suggest the use of the term N1 and N2 neutrophils for describing different neutrophil activation states, where N2 refers to a more PMD-MDSC like phenotype (104, 105). While a few advances to delineate these cell types have been made, further knowledge and additional markers are needed to faithfully distinguish them (106).

MDSCs are mobilized from the bone marrow via G-CSF, GM-CSF, or hypoxia, and recruited to the tumor site, where inflammatory mediators such as IL-6, TNF-α, and prostaglandin E2 then further enhance their immune suppressive functions. PMN-MDSCs mainly inhibit T cell functions via production of reactive oxygen (ROS) and nitrogen species (NOS), inducing T cell apoptosis or anergy, and do so in an antigen-specific manner. Their relatively weak suppressive role has led to speculations about their contribution to immune suppression. However, their high prevalence in cancer patients and several reports showing improved immune responses upon PMN-MDSCs depletion in mouse models, indicate that further investigation is required to delineate their role. In contrast to PMD-MDSCs, M-MDSCs are considered more suppressive and inhibit both antigen-specific and non-specific T cell responses (1, 78, 106). They exert their suppressive functions via high expression of Arg1, driving T cell anergy by depleting arginine pools (80, 81). In addition, MDSCs can express high levels of IL-10 and TGF-β, and produce reactive nitrogen species, negatively affecting T cell recruitment and activation (1, 78, 107). They also harbor tumor-promoting functions that are independent of immune suppression, such as the promotion of metastasis and angiogenesis via the production of VEGF, bFGF, and MMP9 (**Figure 2**) (2, 108).

MDSC Levels Correlate With Poor Patient Prognosis and Resistance to Immunotherapy

In lung, breast, and colorectal cancer the abundance of MDSCs in the tumor has been correlated with advanced stage and decreased overall survival (2), also circulating MDSCs negatively influence patient outcome (109, 110). Circulating neutrophils in clusters with cancer cells were recently reported to promote cell cycle progression and metastatic potential in mouse models and patients (111). While high levels of neutrophils are often associated with poor clinical outcome, they can also have anti-tumorigenic functions, especially in early-stage, small-sized tumors, where they are capable of stimulating T cell responses and secreting proinflammatory mediators. Larger, more advanced tumors preferentially recruit immune suppressive MDSCs (104, 112, 113), which negatively correlates with immunotherapy response in melanoma (110, 114). In conclusion, despite difficulties to faithfully distinguish between PMD-MDSCs and neutrophils, these studies indicate that neutrophils can be both pro- and anti-tumorigenic, whereas MDSCs are exclusively supportive of tumor progression (115).

Targeting MDSCs as a Therapeutic Strategy

MDSCs can be modulated in several ways, by targeting (1) their formation in the bone marrow, (2) their recruitment to the tumor site, or (3) their immune suppressive activities. For targeting MDSC formation and inhibiting their expansion, all-trans retinoic acid was shown to differentiate MDSCs into mature DCs and macrophages and confirmed to reduce numbers of circulating MDSCs in patients (116, 117). MDSC formation is also reduced as an advantageous side-effect of several cancer cell-targeting therapies, such as the tyrosine kinase inhibitor sunitinib, via blockade of VEGF, and c-kit signaling (118, 119), or the cytotoxic drugs gemcitabine and 5-fluorouracil that induce selective apoptosis of MDSC in several tumor models, while leaving T cells, DCs, B cells and NK cells unharmed (120, 121). In order to inhibit the recruitment of MDSCs to the tumor, targeting of the CCL2 axis is being evaluated. Conceptually similar, antagonists for CCR5 are known to reduce MDSC recruitment (122). Targeting of effector functions can be achieved via inhibition of phosphodiesterase, reducing expression of Arg1 and iNOS (123, 124), similar to the HDAC inhibitor entinostat, which reduces expression of COX2, Arg1, and NOS2 in mouse models of melanoma and renal carcinoma (125). HDAC inhibition was shown to act synergistically with PD-1 inhibition in murine models and clinical trials are underway (126, 127). siRNA or decoy nucleotides targeting the TF STAT3, which drives the immune suppressive activities of MDSCs, represent another therapeutic approach to block immune suppressive features (122). Overall, targeting MDSCs is conceptually very attractive due to the wide range of immune suppressive effector molecules. However, due to their heterogeneous nature and the lack of highly specific surface markers, it remains a challenging task that requires further investigation.

PERSPECTIVES AND OUTLOOK

The recent success of T cell targeting agents has validated immune-cell based therapies as an innovative approach to treat cancer. However, immune suppressive mechanisms hampering their success are manifold and myeloid cells are crucial mediators of the suppressive TME. They are a heterogeneous population of cells and rapidly adapting their phenotype to the surrounding tissue. Tumors provide a unique, complex milieu with distinct oncogenic drivers, altered metabolism, hypoxia, and many secreted factors that drive the emergence of myeloid phenotypes unique to the disease. This induces a very complex situation, with many different cell types and activation states that need to be characterized in-depth to allow an understanding of their contribution to immunotherapy success and the development of new therapeutic tools. Technical advances and high dimensional-analytic tools with single cell resolution, ranging from sequencing to mass cytometry, now

give us the opportunity to investigate these cell types at an unprecedented rate of detail during steady-state and disease conditions and in different phases of therapy. These tools need to be implemented in immune-oncology in both, patient samples from different cancer entities with clinical follow-up data available, and pre-clinical models that allow their perturbation and experimental testing of therapeutic targets. Together this will allow a deeper understanding of how activation state, localization, and phenotype of myeloid cells in the tumor shape the microenvironment and provide the basis for modulating the tumor microenvironment in targeted approaches, ultimately improving therapeutic outcomes of cancer patients treated with immunotherapy.

AUTHOR CONTRIBUTIONS

LH and AO researched the data for article, contributed to the discussion of the content, wrote the manuscript, and reviewed and/or edited the manuscript before submission. The authors apologize that due to space limitations, only selective original articles could be cited.

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REVIEW
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Dendritic Cells and Their Role in Immunotherapy

Alycia Gardner[1,2], *Álvaro de Mingo Pulido*[1] and *Brian Ruffell*[1,3]*

[1] Department of Immunology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, United States, [2] Cancer Biology PhD Program, University of South Florida, Tampa, FL, United States, [3] Department of Breast Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, United States

Despite significant advances in the field of cancer immunotherapy, the majority of patients still do not benefit from treatment and must rely on traditional therapies. Dendritic cells have long been a focus of cancer immunotherapy due to their role in inducing protective adaptive immunity, but cancer vaccines have shown limited efficacy in the past. With the advent of immune checkpoint blockade and the ability to identify patient-specific neoantigens, new vaccines, and combinatorial therapies are being evaluated in the clinic. Dendritic cells are also emerging as critical regulators of the immune response within tumors. Understanding how to augment the function of these intratumoral dendritic cells could offer new approaches to enhance immunotherapy, in addition to improving the cytotoxic and targeted therapies that are partially dependent upon a robust immune response for their efficacy. Here we will discuss the role of specific dendritic cell subsets in regulating the anti-tumor immune response, as well as the current status of dendritic cell-based immunotherapies, in order to provide an overview for future lines of research and clinical trials.

Keywords: dendritic cells, immunotherapy, immune checkpoint blockade, vaccines, cancer

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Van Andel Research Institute (VARI), United States

***Correspondence:**
Brian Ruffell
brian.ruffell@moffitt.org

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INTRODUCTION

Immunotherapy has revolutionized the treatment of many solid and hematological malignancies, with immune checkpoint blockade (ICB), adoptive cell therapy (ACT) using tumor infiltrating leukocytes (TIL), and vaccine strategies targeting different aspects of the immune-oncology cycle to improve the functionality of T lymphocytes. Each of these strategies, however, is necessarily predicated on the initiation of the cycle, namely the presentation of tumor antigens by professional antigen-presenting cells (APCs) (1). APCs can be defined by their ability to capture, process, and present exogenous antigen to T cells, and are usually identified by their constitutive expression of major histocompatibility complex (MHC) II and costimulatory molecules. Thus, dendritic cells (DCs), macrophages, and B cells are normally considered to be the three major populations of APCs. It should be noted that other populations also constitutively express MHCII, including thymic epithelial cells, while still others can acquire exogenous antigen, and express MHCII following activation, including eosinophils and basophils (2, 3). However, in the context of solid tumors, antigen uptake, and presentation are primarily the domain of macrophages and DCs (4). While macrophages are the dominant phagocytic population in tumors, they do not migrate to the lymph nodes and are unable to activate T cells *ex vivo* (4). Instead, macrophages are usually found to blunt T cell responses against tumors via multiple mechanisms and act to suppress therapeutic response to ICB as well as chemotherapy and irradiation (5, 6). DCs thus have a unique ability to transport tumor antigen to the draining lymph nodes to initiate T cell activation, a process that

is required for T cell-dependent immunity and response to ICB (4, 7–10). Tumor-resident DCs also have an emerging role in regulating the T cell response within tumors during therapy (4, 11–14). These functions place DCs at the fulcrum of the anti-tumor T cell response and suggest that regulating the biological activity of these cells is a viable therapeutic approach to indirectly promote a T cell response during therapy.

DENDRITIC CELLS IN CANCER

DCs are the quintessential APCs of the immune system, responsible for bridging the gap between innate and adaptive immunity, including the activation of anti-tumor T cells (4, 7–10). DCs arise from bone marrow progenitors known as common myeloid progenitors (CMPs). From here, two cell subtypes diverge. Expression of the transcription factor Nur77 drives the differentiation of CMPs into monocytes, which can further differentiate into monocyte DCs (moDCs) under inflammatory conditions (15–18). In the absence of Nur77, CMPs differentiate into the common dendritic cell progenitor (CDP), which gives rise both to plasmacytoid DCs (pDCs) and conventional DCs (cDCs) (15). Differentiated cDCs are initially immature, requiring maturation signals (for instance, damage or pathogen associated molecular patterns [DAMPs or PAMPs], or inflammatory cytokines) to fully effect their role in the immune response (15, 18). Upon maturation and activation, DCs downregulate phagocytosis, increase MHC and costimulatory molecule expression, increase cytokine production, and display enhanced migration to lymph nodes, likely driven by higher expression of C-C chemokine receptor 7 (CCR7) (15). As a result of the phenotypic changes that occur during activation, mature DCs are able to prime naïve T cells and initiate the adaptive immune response.

cDCs can be further divided into two subsets, known as type one (cDC1) and type two (cDC2) conventional DCs. cDC1 are defined by reliance on the transcription factors BATF3 and IRF8 for development, and express several common surface markers across species, including XCR1, CLEC9A, CADM1, BTLA, and CD26 (19). However, the cells were originally identified by surface expression of CD8α (lymphoid organ resident) or CD103 (peripheral tissue resident) in mice (20–22) and CD141 (BDCA-3) in humans (23–25), making these the most commonly used markers. In both organisms, the cDC1 subset displays enhanced ability to cross-present exogenous antigen and activate $CD8^+$ T cells (15, 18, 26), but this functional demarcation between the cDC1 and cDC2 subset is more pronounced in mice than in humans (19). In both mice and humans cDC1s represent a small percentage of immune cells in circulation. cDC1 accounted for $<0.01\%$ of $CD45^+$ cells in the blood of healthy human donors, as well as $<0.1\%$ of $CD45^+$ cells in surveyed tissue sites (27).

cDC2 are easiest to identify by the absence of cDC1 markers, but higher expression of CD11b, CD1c, and SIRPα (CD172α) is also frequently used to distinguish the population, with IRF4 acting as the key transcription factor (28–31). No specific markers identify migratory from resident cDC2 populations in mice, but differential expression of CD11c and MHCII can be used as a distinguishing feature (15). In mice, cDC2 are primarily responsible for presentation of endogenous antigen to $CD4^+$ T cells and shaping the resulting polarization of the cells, with the ability to polarize $CD4^+$ T cells also observed with human cDC2 (32). As mentioned, however, human cDC2s can cross-present antigen and produce high levels of interleukin (IL)12, properties that are largely restricted to the cDC1 subset in mice (19). Thus, despite the critical role of cDC1s in the development and maintenance of anti-tumor immunity in experimental models (15), it is possible cDC2s have an unidentified role in human cancers. Indeed, a recent study demonstrated a correlation between cDC2 abundance and non-T_{reg} $CD4^+$ T cell infiltration into head and neck squamous carcinomas. High cDC2 and low T_{reg} infiltration was also associated with longer progression-free survival (33).

Type 1 Conventional DCs

In mice, cDC1 are responsible for the induction of the "cancer-immune cycle," as *Batf3*-deficient mice are unable to reject even highly immunogenic tumors or respond to immune-mediated therapies such as checkpoint blockade and adoptive T cell transfer (7–10, 13, 34). This has been traced to the ability of cDC1s to transport antigen from tumors into draining lymph nodes, with migratory cDC1s being the only APC subset capable of causing robust activation and proliferation of $CD8^+$ T cells *ex vivo* (9, 10). Additionally, migratory cDC1 represented the only cDC subset able to transport antigen to the lymph node in two studies using melanoma models (9, 10). cDC trafficking to the lymph node and generation of a systemic anti-tumor immune response is governed by CCR7 expression (9). Mice lacking CCR7-expressing cDC1 failed to recruit $CD8^+$ T cells to the tumor, and the T cells that were present in the tumor microenvironment failed to proliferate, leading to an overall lack of immune control (9). Similarly, the inability of tumors to recruit the cDC1 subset prevents an effective $CD8^+$ T cell response from developing (35, 36), while increasing the number of cDCs in the tumor can restore response to immunotherapy (10, 35). Taken together, these studies strongly support $CD103^+$ migratory cDC1 as critical for the induction of anti-tumor immunity. In non-tumor models of immunity, lymph node-resident cDC also acquire antigen from migratory cDCs and are needed to initiate an optimum $CD8^+$ T cell response (37, 38). Whether there are sequential roles for migratory and resident cDC1s during the development of an anti-tumor response is not yet known. However, cross-presentation by cDC1s is critical for the induction of an adaptive immune response by cytotoxic $CD8^+$ T cells, with mice specifically deficient in cross-presentation-capable cDC1s unable to reject highly immunogenic fibrosarcoma tumors (39). In addition, cross-presentation by cDC1 is enhanced by type I interferon (IFN) signaling (40). The absence of type I IFN in the tumor microenvironment, or the inability of cDC1 to sense type I IFN, are sufficient to impair the development of a $CD8^+$ T cell response (34, 40). Taken together, these studies emphasize the importance of cross-presentation of tumor antigen to naïve $CD8^+$ T cells in the lymph node in the induction of a successful anti-tumor immune response.

It is also becoming increasingly clear that cDC1s have a critical role in maintaining $CD8^+$ T cell function within tumors. In secondary lymphoid organs and in non-tumor models of immunity, the organization of immune cells is critical for effective signaling (41, 42). The localization of T cells near cDCs, especially, has been shown to be critical to the induction of an adaptive immune response (43, 44). Consistent with this, cytokine production by tumor cDC1s has proven essential for immunotherapy. In the of context adoptive cell therapy (ACT), efficacy required cDCs capable of CXCL9/CXCL10 production in order to drive tumor infiltration by the transferred T cells (13). cDC1 production of CXCL9/CXCL10 and expression of the cognate receptor, CXCR3, on $CD8^+$ T cells, has also recently been shown to be critical for response to anti-PD-1 or anti-TIM-3 therapy (11, 14). Surprisingly however, this was not mediated by increased $CD8^+$ T cell tumor infiltration, but rather enhanced effector function in endogenous $CD8^+$ T cells. How chemokine expression by tumor cDC1s promotes a T cell response is unclear, but may relate to cDC1s being largely responsible for production of IL-12 within tumors (4, 45). In support of this, cDC1 production of IL-12 was found to induce IFNγ production by $CD8^+$ T cells following PD-L1 blockade, and the feedback loop between IL-12-producing cDC1s and IFNγ-producing $CD8^+$ T cells was necessary for therapeutic efficacy (12). Similarly, IL-12, CXCL9/10, and IFNγ are all required for response to the combination of paclitaxel chemotherapy and TIM-3 blockade (11). Taken together, the data indicate the importance of cDC1 and $CD8^+$ T cell crosstalk in the tumor microenvironment and suggest that targeting this interaction is therapeutically viable (**Figure 1**). Interestingly, a recently published study used single cell RNA sequencing (scRNA-seq) to identify a subset of regulatory DCs in lung tumors (46). Although these were shown to arise from both the cDC1 and cDC2 lineage following maturation and uptake of tumor antigen, the authors specifically focused on the regulatory DCs of the cDC1 lineage, and showed that blockade of IL-4 could reestablish IL-12 expression, thus improving $CD8^+$ T cell function and tumor control (46).

Another recent advancement in the field is the characterization of natural killer (NK) cell and cDC1 interplay within tumors. Two groups independently showed that NK cell production of either FMS-related tyrosine kinase 3 ligand (Flt3L) or CCL5 and XCL1 induces cDC1 recruitment into the tumor microenvironment (36, 47). Analyses of gene signatures in human tumors indicate that the presence of NK cells correlates with the presence of cDC1 in this context as well, suggesting that manipulation of NK cell presence within the tumor could indirectly improve the adaptive immune response (36, 47). Communication in the opposite direction has also been shown to be required, with IL-12 production by cDC1 leading to IFNγ production by NK cells (48). Neutralization of IL-12 or the absence of cDC1 in *Batf3*-deficient mice increased lung colonization following tail-vein injection of multiple tumor cell lines (48). The requirement for cross-talk between cDC1 and multiple immune subtypes is indicative of the complexity of the immune response within the tumor and suggests that the localization of leukocytes within the tumor is a critical regulator of their function. Improvements in imaging techniques and analysis platforms will help dissect some of this complexity.

At both the genetic and functional level, human cDC1 show similar characteristics to mouse cDC1 (25, 31), suggesting that mouse models to study cDC1 function will be informative in translating the biology to the context of humans. In particular, a recently published study used scRNA-seq to profile myeloid populations in human and mouse lung cancers, and found a high degree of concordance between DC subsets in the two species, including cDC1 (31). The same study assessed the association of the gene signatures most specific to individual cell types and compared them with patient prognosis. cDC1 genes were generally found to be associated with positive prognosis, suggesting that the presence of cDC1 in human lung tumors is associated with better survival (31). Similar findings have been made in hepatocellular carcinoma (49), and the presence of DCs in breast tumors (11), along with the ratio of $CD103^+$ cDC1 to $CD103^-$ DCs in breast cancer, head and neck squamous cell carcinoma (HNSCC), and lung adenocarcinoma (4), have all been shown to correlate with improved patient prognosis. In addition, the presence of cDC1 within human melanoma tumors correlated with improved response to anti-PD-1 therapy (36) as well as with higher $CD8^+$ T cell infiltration into tumors (33), which is associated with a positive prognosis across multiple tumor types (50). Furthermore, genes specific for cDC1 correlate with the presence of *CXCL9* expression by human tumors in the TCGA database (11, 13), and cDC1 in human breast tumors exhibit expression of CXCL9 by immunofluorescence (11), further indicating that human cDC1 are likely to produce similar chemokines and play a similar role in the tumor microenvironment as mouse cDC1. As *CXCL9* expression also correlates with response to anti-PD-1 (14), there is likely a critical role for cDC1s in the context of patient response to ICB as well, although this has not been directly tested.

Type 2 Conventional DCs

While the aforementioned data suggest that cDC1 may be the only DC subset required for the induction of anti-tumor immunity, this neglects the importance of $CD4^+$ T cells, which play a critical role in supporting $CD8^+$ T cell activity (suggesting a role for cDC2 antigen presentation to $CD4^+$ T cells) (51–54). While cDC2 are dispensable for $CD8^+$ T cell activation and proliferation in some tumors (4, 9), this may be due to the specific models and therapies examined. For example, cDC2s were found to be important during response to anthracycline chemotherapy (55), and certain tumor models are responsive to adoptively transferred $CD4^+$ T cells (56). There are also several reports describing recognition of tumor antigens by human $CD4^+$ T cells (56). As with cDC1, scRNAseq has shown that at the genetic level, mouse and human cDC2 subsets in lung tumors show a high degree of overlap (31). This includes the existence of functionally distinct subsets marked by expression of T-bet and RORγt (57). Additionally, it was recently shown that following depletion of regulatory T cells (T_{reg}), a subset of cDC2 can effectively elicit intratumoral $CD4^+$ T cell responses and subsequent tumor control in a mouse model of melanoma (33). Upon T_{reg} depletion, cDC2 were able to migrate to the draining

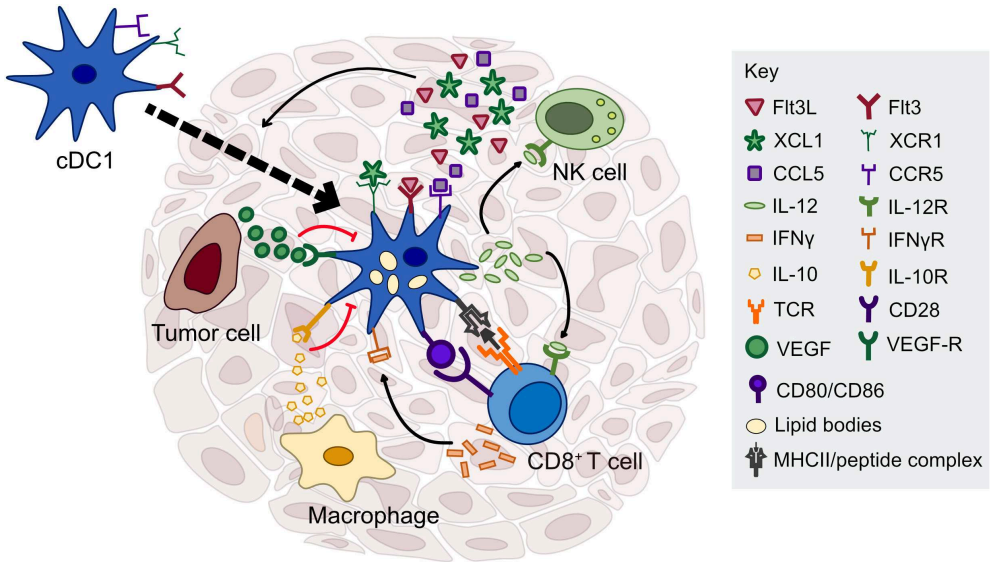

FIGURE 1 | Factors regulating cDC1 function in the tumor microenvironment. cDC1s interact with several immune cell types through cytokine and chemokine signaling, including NK cells, T cells, and macrophages. NK cells are critical for cDC1 recruitment and survival in the tumor through production of Flt3L, CCL5, and XCL1. cDC1 have the capacity to cross-present exogenous antigen to $CD8^+$ T cells and stimulate naïve and previously activated T cells *ex vivo*; however, the importance of antigen presentation by cDC1s in the tumor microenvironment is currently unclear. In contrast, cDC1 production of IL-12, driven by IFN-γ or other inflammatory mediators, is necessary to sustain a T cell response during chemotherapy or immune checkpoint blockade. cDC1 production of IL-12 can be directly inhibited by IL-10 released by macrophages or other immunosuppressive cells, as well as tumor-derived factors that inhibit the maturation of cDC1s such as VEGF.

lymph node and effectively induce differentiation of conventional $CD4^+$ T cells (33). The observed increase in tumor rejection specifically required $CD4^+$ T cell priming in the lymph node, as FTY720 blockade of lymph node egress prevented the anti-tumor immune response (33).

Interestingly, when the cDC2 gene signature was correlated with prognosis for lung adenocarcinoma patients, cDC2 were the DC subset most strongly associated with a positive prognosis (31). Similarly, high levels of cDC2 in HNSCC and melanoma tumors, when combined with low levels of regulatory T cells, correlated with longer progression free survival and higher levels of $CD4^+$ T cell infiltration, further suggesting a role for both cDC2 and $CD4^+$ T cells in human tumors (33). A substantial degree of heterogeneity in the cDC2 subset isolated from draining lymph nodes of human melanoma patients also correlates with the heterogeneity observed in cDC2 isolated from mouse tumors, with similar characteristics observed in both subsets (33). Given these data, it will be interesting to examine whether T_{reg} are also preventing cDC2 function in contexts other than melanoma, and whether depletion of the T_{reg} may augment the anti-tumor immune response in human tumors via increased cDC2 and $CD4^+$ T cell activity.

Plasmacytoid Dendritic Cells

In contrast to cDCs, whose role in anti-tumor immunity is associated with antigen presentation, plasmacytoid DCs (pDCs) are usually associated with response to viral RNA and DNA via production of high levels of type I IFN, along with other inflammatory cytokines such as IL6 and TNFα. However, pDCs do express MHCII and costimulatory molecules and could therefore potentially act as antigen-presenting cells, although the antigen processing capabilities of the cells are unclear (18, 58). Interestingly, pDCs differentiate from myeloid CDP as well as from IL-$7R^+$ lymphoid progenitors (59), resulting in cells that are phenotypically similar but with distinct functional capacities (59). Specifically, only myeloid-derived pDCs were found to process and display antigen (59). The role of pDCs in cancer may therefore depend upon the extent to which they are myeloid derived, in addition to their activation state. At least one study has shown that tumor-associated pDCs are largely inert, but that following intratumoral injection of a TLR7 ligand, pDCs can induce anti-tumor immune responses (60). Whether this response is directly attributable to antigen presentation by myeloid-derived pDCs or is a result of type I IFN activation of cDC function is less clear (61).

In a similar vein, the role of pDCs in human tumors is less established than that of the cDC subsets. As with cDC1 and cDC2, scRNAseq indicates that the human pDCs mirror mouse pDCs (31). The human pDC gene signature also correlates with a positive prognosis in lung adenocarcinoma, although to a lesser degree than either cDC1 or cDC2 (31). In contrast, the presence of pDC in breast tumors, as assessed by immunohistochemical staining, strongly correlated with a poor overall prognosis (62). Additionally, pDCs found in the ascites of patients with ovarian carcinoma induced IL-10-producing $CD8^+$ regulatory T cells and inhibited T cell proliferation (63). High-dimensional analysis has recently been employed by several groups to identify heterogeneity within the classically defined pDC population

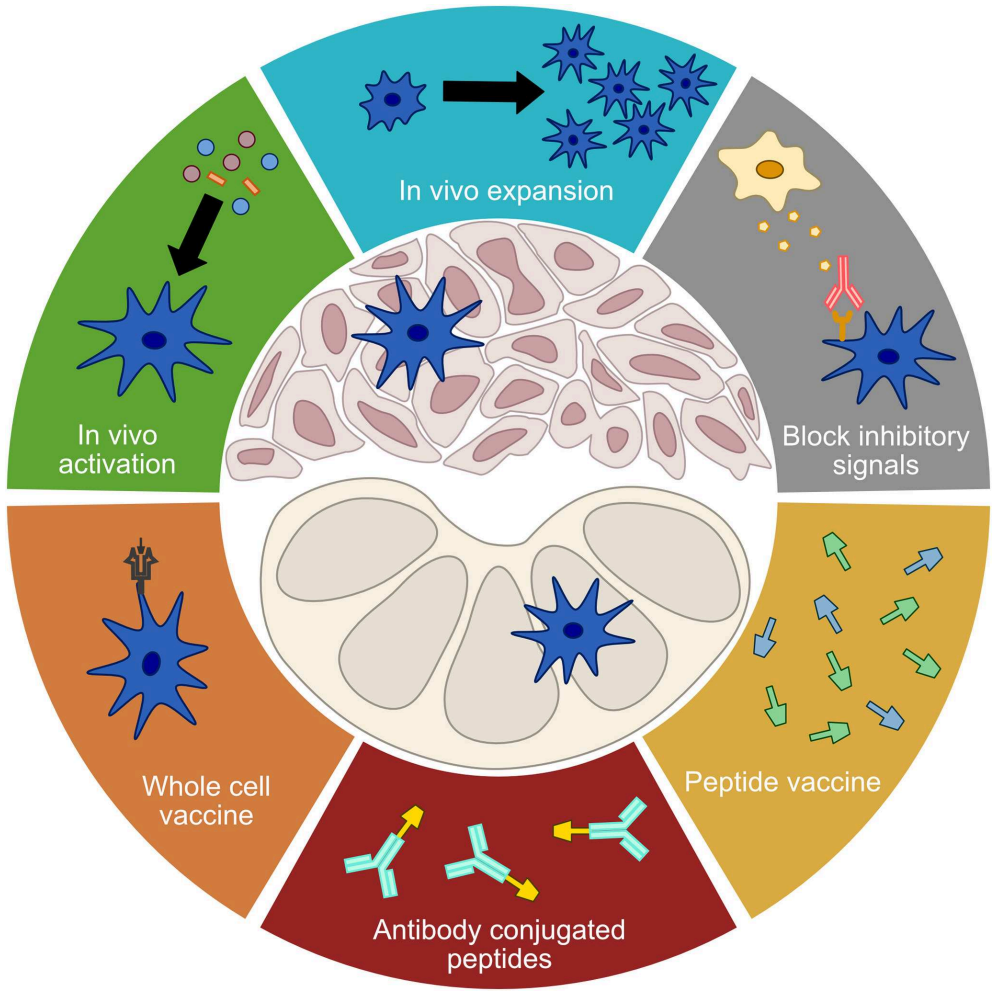

FIGURE 2 | Treatment modalities targeting DCs. A number of current treatment modalities aim to address limited DC functionality in order to elicit or enhance anti-tumor immune responses. Treatments that seek to improve the function of tumor DCs include *in vivo* activation, *in vivo* expansion, and the blocking of inhibitory signals. Vaccination approaches that seek to bypass tumor DCs and directly stimulate a *de novo* T cell response in the lymph nodes include whole cell vaccines, antibody conjugated peptides, and free proteins or peptides.

in human samples (64–66), raising the possibility that the conflicting roles of pDCs in human tumors could be attributed to the conflation of multiple subsets.

Monocyte Dendritic Cells

Monocyte-derived DCs (moDCs) differentiate from Ly6C$^+$ or CD14^{hi} monocytes in mice and humans, respectively, generally under inflammatory conditions (19). Identification of moDCs has historically been difficult, as the markers used for identification overlap substantially with those expressed by macrophages and CD11b$^+$ DCs in mice. Recently, however, expression of the Fc receptors FcγRI and FcεRI were used to distinguish the subset (67). In contrast to the ability of cDCs to present antigen to T cells, moDCs have not been shown to transport antigen to the lymph nodes and activate T cells. As a result, it is unclear what role moDCs can have in inducing a *de novo* T cell response. However, the recruitment of moDCs is enhanced under inflammatory conditions, which can lead to the induction of "TipDCs" (tumor necrosis factor (TNF) and NOS2-producing inflammatory dendritic cells) from moDCs. It was also recently shown that for mice given adjuvant therapy with polyinosinic:polycytidilic acid (Poly [I:C]), moDCs were required for the anti-tumor response, whereas cDC1 were dispensable (68). moDCs have also been shown to enhance the survival of adoptively transferred T cells (69) and may further regulate T cell activity within tumors through production of TNFα and NOS2 (18). Activation of p53 in myeloid precursors can even promote the formation of CD103$^+$ moDCs with the capacity to cross-present antigen and produce high amounts of IL-12 (70). moDCs also appear to play a critical role in the regulation of graft-vs.-leukemia (GVL) responses following therapeutic bone marrow transplants, with inhibition of XBP-1 splicing

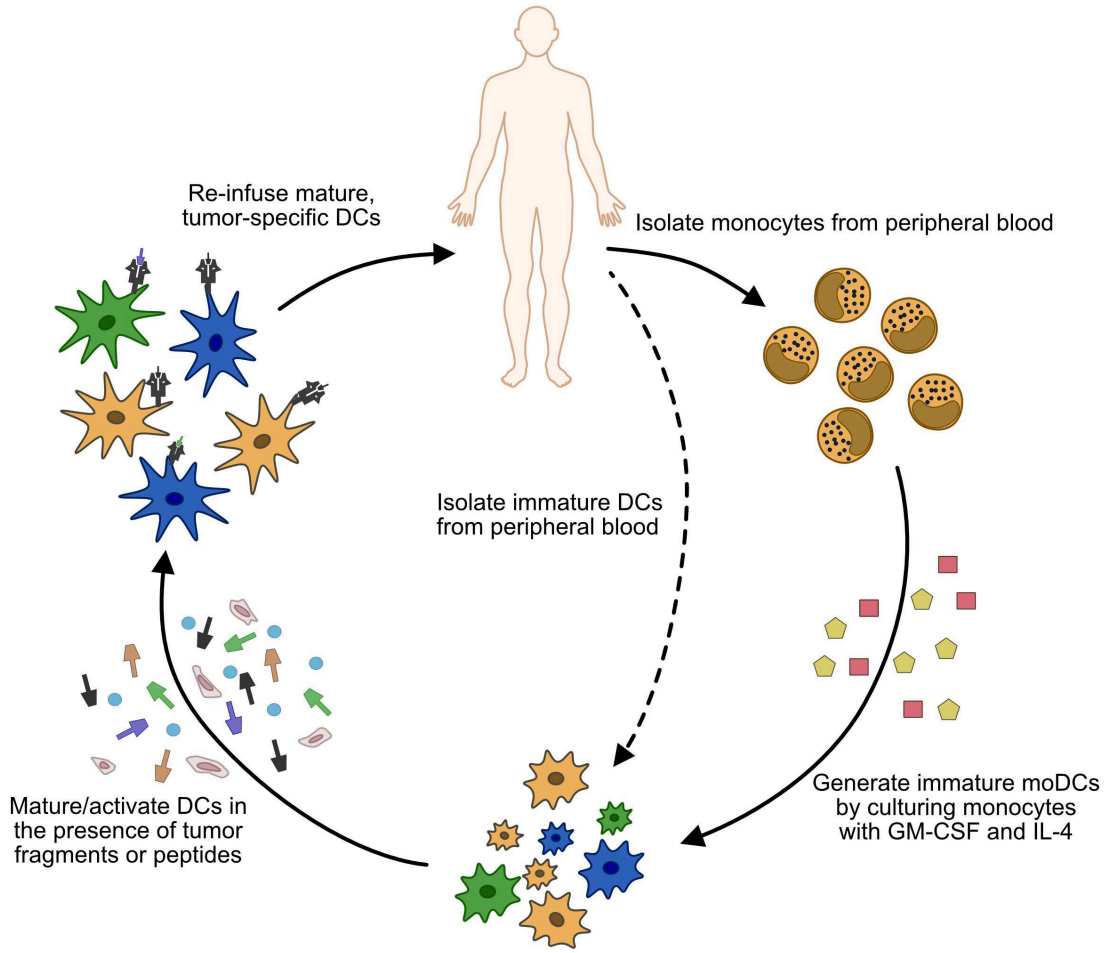

FIGURE 3 | Process of generating whole cell DC vaccines. Monocytes (or less commonly, immature cDCs) are isolated from the patient's peripheral blood. In the case of monocyte isolation, immature moDCs are generated by culturing the isolated cells in GM-CSF and IL-4. Once immature DCs are obtained, they are matured/activated using a variety of cytokine cocktails, and pulsed with tumor antigen or tumor fragments. The matured DCs are then injected back into the patient, usually via subcutaneous (s.c.) or intradermal (i.d.) injections, although intravenous (i.v.) or direct injection into lymph nodes has also been used.

helping to prevent graft-vs.-host disease while maintaining a GVL response in both murine and human xenograft models (71, 72). Thus, while the role of moDCs in the development of spontaneous anti-tumor immunity is unclear, they appear critical in sustaining an immune response during certain inflammatory conditions.

DENDRITIC CELL-BASED THERAPIES

Immunotherapy continues to represent a promising avenue for new cancer therapies, especially since many patients who respond exhibit durable responses. However, response rates for many tumor types are still low, underscoring the need for continued improvement in our understanding of anti-tumor immunity and approaches to enhance it. As expanded upon in the first section, cDCs are central inducers of the immune response, and targeting them may provide a method of improving immune responses in cases where targeting T cells alone is ineffective. As DCs, especially cDC1, tend to correlate with a positive prognosis when they are present in tumors, therapies targeting DCs focus on enhancing DC function, increasing their numbers, or bypassing the tumor microenvironment to promote systemic *de novo* anti-tumor immunity (**Figure 2**).

In vivo Activation

One of the earliest approaches to immunotherapy was the attempt to revert suppression of cDCs in the tumor microenvironment by providing exogenous activation signals. Toll-like receptors (TLRs) are one of the major pathogen- and damage-sensing pathways, with 13 different TLRs present in mice (TLR1-TLR13) and the first 10 also present in humans. DCs subsets display differential TLR expression patterns in both species (73, 74). For example, in humans, pDCs preferentially express TLR7 and 9, cDC1 preferentially express TLR3 and 8, and cDC2 preferentially express TLR1 and 6 (73). This means DCs preferentially recognize different pathogenic/danger-associated

signals and can be targeted with specific agonists, potentially with the goal of optimally shaping the anti-tumor immune response. However, the identification and therapeutic use of TLR agonists predates the classification of the cDC subsets, and limited work has been done in this area.

In humans, TLR7 and TLR9 are among the more widely explored targets given their capacity for inducing a type I IFN response. Topical TLR7 agonists including imiquimod and R848 have been shown to induce an immune response as well as promote some level of tumor control in a variety of cancer types, including melanoma and breast cancer (75–77). Indeed, a number of clinical trials are currently ongoing to test TLR agonists in breast cancer patients, with one having observed immune-mediated rejection of skin metastases following treatment with imiquimod (75). Topical application carries a clear drawback, in that it can only reasonably be used in situations where either the induction of a systemic immune response will be able to induce tumor control, or where tumors are close enough to the body's surface that a local immune response can be induced. As a result, TLR7 agonists with non-topical application methods are also under development. One such agonist is 852A, which has been shown to induce CXCL10 and IL-1RA production, although minimal tumor control was observed in initial clinical trials (78, 79). In addition to TLR7 activation, DCs can be targeted via TLR9 agonists (73), with activation of TLR9 using CpG oligodeoxynucleotides (ODNs) causing pDC maturation and cytokine production. The classes of CpG ODNs have different routes of administration and produce unique downstream effects (73). In addition to CpG ODNs, a novel TLR9 agonist, IMO-2125, has also been shown to engage TLR9 leading to downstream immune signaling and suppression of A20 lymphoma and CT26 colon carcinoma tumor models in mice (80).

TLR3 and TLR8 are preferentially expressed by cDC1, which, owing to their established role in anti-tumor immunity, makes them attractive therapeutic targets (73). Polyinosinic:polycytidylic acid (Poly[I:C]) is one of the most well-known TLR3 agonists and administration of poly(I:C) is effective in inducing cDC1 maturation as well as production of IL-12, type I IFNs, and chemokines. However, as it is not well-tolerated clinically (81), variants have been developed that aim to reduce the toxicity of poly(I:C) administration. One such variant is poly-ICLC, an RNAse resistant form of poly(I:C) that leads to immune activation and some tumor responses, either alone or as an adjuvant to conventional therapies (82, 83). Poly(I:C12U), another poly(I:C) variant, introduces unpaired bases in order to increase the degradation rate of the drug in an effort to reduce adverse effects (84, 85). In addition to TLR3, cDC1 also express TLR8, which can be targeted with the TLR7/8 agonist mentioned previously, R848. Agonists of TLR8 alone are also in development. For example, VTX-2337 was shown to activate cDC1 and monocytes (86) and was well-tolerated in phase I clinical trials, although progression free survival was unchanged in a phase II trial conducted in squamous cell head and neck cancer (73, 87).

STING (stimulator of interferon genes) mediates type I IFN responses following recognition of cytosolic DNA by cGAS (cyclic GMP-AMP synthase) and production of 2′3′-cGAMP (88). Host STING is required for the induction of anti-tumor immunity, as STING-deficient mice fail to develop spontaneous immunity against immunogenic tumor lines and show reduced responses to radiation therapy (89, 90). STING knockout mice also exhibit increased susceptibility to inflammation-associated carcinogenesis following administration of AOM/DSS to induce colitis (91, 92). It is currently unclear whether STING expression by cDCs or other host cells is important for promoting an immune response, and the specifics of the tumor model and therapy being evaluated will likely impact the underlying biology. For example, blockade of CD47 promotes uptake of tumor cells by SIRPα^+ cDC2, leading to activation of the cGAS-STING pathway (93), whereas in other tumor models it is production of 2′3′-cGAMP by tumor cells that is responsible for activation of host STING (94). Regardless, the intratumoral injection of STING agonists such as 2′3′-cGAMP and DMXAA can induce tumor rejection, both alone and in combination with other therapeutic modalities (95, 96).

Despite the pre-clinical efficacy of intratumoral injection of STING or TLR agonists, single agent efficacy in the clinic has remained elusive. This has hampered development of TLR agonists in the past, but in the age of cancer immunotherapy these are now being reevaluated as part of combinatorial therapies. For instance, a recent pre-clinical study showed that treatment with the TLR9 agonist CpG led to increased OX40 expression on CD4$^+$ T cells (97). Accordingly, while intratumoral injection of CpG alone led to rejection of the directly treated tumor, the addition of an OX40 agonist antibody lead to clearance of contralateral tumors (97), and a phase I study testing this combination in non-Hodgkin lymphoma is currently underway (NCT03410901). As STING agonists have been developed more recently, these trials are already incorporating anti-PD-1 into their phase I treatment arms (e.g., NCT03010176). That said, it remains to be seen if this approach will be successful, and the development of systemic therapies targeting these pathways will be important to expand treatments beyond accessible tumors (98).

Blocking Inhibitory Signals

Extracting murine cDCs from tumors allows them to activate and restimulate CD8$^+$ T cells (4), implicating the suppressive microenvironment as a key regulator of cDC function. An alternative approach to enhance the activation state of tumor cDCs is therefore to block inhibitory pathways that reduce cDC functionality. One advantage of this approach is that it allows for systemic administration of inhibitors, as opposed to the local administration required for many immune agonists. One of the first examples of this is targeting vascular endothelial growth factor (VEGF), as VEGF inhibits DC maturation and prevents an effective anti-tumor immune response (99). VEGF inhibitors are already in clinical use to inhibit increased angiogenesis, and evidence indicates that antibodies against VEGF enhance the anti-tumor immune response by counteracting DC inhibition (100, 101). This is supported by several pre-clinical studies showing that inhibitors of VEGF increase immune function and decrease the rate of tumor growth (101–103). VEGF

inhibition has also been shown to enhance DC maturation in human patients (104), suggesting that this may contribute to the efficacy of VEGF inhibitors in the clinical setting. However, it should be noted that the impact of VEGF on the vasculature and other immune populations may be more relevant to the immune impact of VEGF pathway inhibitors (105).

Another potent immunosuppressive signal in the tumor microenvironment is IL-10, which can be produced by tumor cells, macrophages, regulatory T cells, as well as other components of the stroma. Using isolated human DCs in co-culture with human melanoma cell lines, researchers have shown that IL-10 prevents DC maturation and induces a tolerogenic phenotype (106). Blockade of IL-10 in pre-clinical models, either directly or via depletion of macrophages has been shown to improve $CD8^+$ T cell mediated anti-tumor immune responses in both murine and human systems (45, 106–108). At least in a mammary tumor model, this has been directly linked to the ability of IL-10 to suppress IL-12 production by cDC1s, reducing the percentage of $CD8^+$ T cells that display a cytotoxic effector phenotype (45). TIM-3 expression by cDCs has also been shown to prevent response to chemotherapy in several tumor models (11, 109). How this occurs is unclear, but may relate to TIM-3 binding to high mobility box 1 protein (HMGB1) and limiting response to nucleic acids (109). Thus, while anti-TIM-3 antibodies can promote response to PD-1/L1 blocking by reducing T cell exhaustion (110, 111), TIM-3 blockade might prove efficacious even in patients with tumors that do not display substantial T cell infiltration.

Regulation of immunometabolism to increase anti-tumor immunity has been an increasing focus of cancer research. Although our understanding of basic immunometabolism is still evolving, several key insights have been made that are of relevance to tumor-associated DCs. As this has been expertly reviewed previously (99, 112), we will here highlight only two key metabolic aspects of tumor-associated DCs, and the therapeutic approaches being taken to counteract this metabolic inhibition. First, DC expression of indoleamine 2,3-dioxygenase 1 (IDO1) is thought to reduce L-tryptophan availability by converting it to L-kynurenine, leading to an increase in the suppressive capacity of regulatory T cells (113, 114). That said, IDO1 can be highly expressed by tumor cells themselves, and evidence that IDO1 expression by tumor DCs is a major mechanism of immune suppression is lacking. Several IDO1 inhibitors have also failed to demonstrate efficacy over the past few years, raising questions about the validity of this approach. Second, lipid accumulation in DCs has been shown to limit the function of DCs via interference in antigen processing and subsequent antigen presentation (115, 116). Accumulation of lipids in tumor-associated DCs is promoted by DC-specific activation of the endoplasmic reticulum (ER) stress sensor XBP1 (117). DC-specific siRNA silencing of XBP1 led to decreased lipid accumulation by DCs and enhanced immune-mediated tumor control in mouse models of ovarian cancer (117). Although further research will be required before ER stress can be effectively targeted to treat cancer, it is an active area of investigation.

In vivo Expansion

Tumor cDCs are relatively infrequent in human and murine epithelial malignancies (4, 11, 33). Thus, increasing the number of intratumoral cDCs represents an alternative approach to increasing the cumulative function of the population. Rather than the injection of exogenously expanded and activated cDCs (DC vaccination; described below), it has been shown in pre-clinical studies that systemic injection of Flt3L leads to systemic expansion of the cDC1 population, increasing the number of these cells within B16 melanomas and significantly delaying tumor growth (10). This approach also showed promise in increasing both the number of cDCs in pancreatic tumors and overall control of pancreatic tumor lesions in an autochthonous disease model, highlighting the importance of DC infiltration, and expansion even in cancer types with typically low immune infiltration (118). Combined administration of Flt3L with TLR agonists, STING agonists, radiation, and/or checkpoint blockade results in additional tumor control, even in advanced tumors (7, 10, 118, 119). This approach is being tested clinically in several tumor types, including metastatic breast cancer and non-Hodgkin's lymphoma (NCT03789097, NCT01976585). The key advantage of this therapy is the potential for targeting a wider range of antigens, rather than those selected for vaccination, bypassing the need for patient-specific vaccine development. In addition, both systemic T cell activation and local T cell infiltration are enhanced by this combination, increasing the potential for synergy with other immunotherapies.

Dendritic Cell Vaccines

In contrast to *in vivo* expansion, whole-cell DC vaccines rely on exogenous maturation and/or expansion of monocyte-derived DCs or cDC precursors (**Figure 3**), although most trials utilize moDC due to the rarity of cDCs or pre-DCs (27). These cells are isolated from a patient's peripheral blood, loaded with tumor lysate or tumor antigens, and matured using various cytokine cocktails (120, 121). Whole cell DC vaccines are associated with limited toxicities, are therefore considered a relatively safe therapeutic approach, and are being extensively evaluated in the clinic (121, 122). Multiple vaccine formulations can lead to increased antigen-specific T cell responses. There have even been trials in AML involving the fusion of cancer cells with autologous moDCs (123). However, the presence of an immune response has not correlated with clinical efficacy (124), with response rates in general between 8 and 15% in single arm trials (122). The only whole cell DC vaccine approved by the FDA to date is sipuleucel-T, which consists of isolated PBMCs cultured with a GM-CSF/prostatic acid phosphatase fusion protein (125). This approval to treat metastatic prostate cancer was based upon a 4.1 month improvement in overall survival without an accompanying delay in disease progression (125).

Given the ability of most vaccines to induce an immune response against a specific antigen, it is unclear why vaccines have shown limited efficacy to date. One possibility is that the immunosuppressive microenvironment of the tumor blocks T cell infiltration, survival, or effector function. Several

pre-clinical studies have shown that PD-1 and/or CTLA-4 blockade can improve tumor control in combination with tumor cell vaccines (126, 127). Similarly, in a mouse mammary tumor model, the efficacy of a HER2-loaded BMDC vaccine was improved by sequential anti-PD-1 therapy (128). Treatment with DC vaccines have also been shown to augment responses to standard-of-care therapy (129). Clinical trials have begun to evaluate the efficacy of combining DC vaccines with standard-of-care therapies and of vaccination with different DC subsets. In glioblastoma, a phase III clinical trial to assess the efficacy of a whole cell DC vaccine administered in combination with tumor resection, temozolomide, and radiotherapy (NCT00045968) exhibited safety and potential efficacy based on interpretation of early results (130). In contrast, a phase III trial of tumor-RNA loaded whole cell vaccines in combination with sunitinib following surgical debulking for the treatment of renal cell carcinoma (NCT01582672) was terminated early due to a lack of efficacy.

The limited efficacy of DC vaccines could also be a result of protocols that do not produce the optimal T cell response. GM-CSF maturation of PBMCs produces moDCs that are limited in their capacity to migrate to lymph nodes (131, 132), and several studies have shown endogenous DCs are actually required for T cell priming following administration of moDC vaccines (133–135). Murine cDC1s have been used in a vaccine in at least one study (136), but whether this is a viable approach in the clinic remains to be determined, particularly given the paucity of circulating, mature cDC1 in human peripheral blood (27, 137). Instead, studies have largely focused on improving baseline efficacy by assessing activation with different maturation cocktails. For many years, the "gold standard" maturation cocktail consisted of TNFα, IL-1β, IL-6, and PGE_2 (120). However, PGE_2 induces T regulatory cells and lowers IL-12 production, so methods of maturation which omit it are being explored. For example, an interferon cocktail along with TLR3, TLR7, and TLR8 agonists produced superior T cell mediated cytotoxicity against a breast cancer cell line (138), while the combination of TNFα, IL-1β, IFNγ, and a TLR7/8 agonist induced higher levels of the T cell chemoattractants CXCL9/10 (139). At the same time, the "gold standard" cocktail induces the highest level of DC migratory capacity (120). Given that increased DC migration to the lymph node following vaccination has been associated with increased overall survival in a small cohort of patients (140), it is unclear which approach would be better at promoting tumor control. DC migration to the lymph node can also be directly enhanced by pre-treating the injection point with DC activating agents such as tetanus toxoid and CCL3, or TLR agonists such as imiquimod or poly-ICLC (140, 141). The number of DCs injected also plays a role in achieving optimal responses, with 10^6-10^7 DCs per injection representing the optimal rage for efficacy (142, 143). Given the range of approaches, it remains to be seen which, if any, will produce anti-tumor responses that can induce tumor regression, either alone or in combination with other therapeutic modalities.

Peptide/Protein Vaccines

Another possible reason for the failure of many DC vaccines may be the reliance on overexpressed or tissue-specific antigens (e.g., NY-ESO-1, MUC1, MAGEA3, MART1, HER2). In addition to their use in DC vaccines, these antigens have been fused to DC-targeting antibodies against Clec9a, DEC205, or DC-SIGN to enhance their ability to induce an immune response (122). DEC205-fused tumor-associated antigens demonstrate improved ability to induce T cell responses over administration of free antigen (144, 145). Additionally, partial clinical responses were observed following administration of DEC205-fused NY-ESO-1 and TLR agonist adjuvants in a phase I clinical trial (146). While targeting Clec9a generally induces tolerance, different adjuvants can be added in order to drive immune responses (124). For example, when combined with poly(I:C) and other adjuvants, Clec9a-fused antigens induce CD4- and CD8-mediated anti-tumor immunity (147, 148), while fusion of human IFNα to Cle9a led to an anti-tumor response that was improved by treatment with checkpoint blockade in the murine 4T1 mammary tumor model (149). Peptide fusions to antibodies against several other DC surface proteins are also in pre-clinical and clinical development (122). Given that different DC subsets can be targeted using antibodies against specifically expressed surface proteins, this represents another mechanism by which the anti-tumor immune response could be optimally shaped to induce the best outcomes for a given patient. However, one of the most recent advances in the development of cancer vaccines has been the ability to generate vaccines with patient-specific neoantigens. Although expensive and technically challenging, neoantigen vaccines are safe and able to induce strong systemic T cell responses (150, 151). More importantly, complete and durable responses have been observed in patients receiving neoantigen vaccines in combination with anti-PD-1 therapy in early phase clinical trials. Dozens of studies are now underway testing neoantigen vaccines either alone or in combination with ICB (e.g., NCT02950766, NCT03639714, NCT03953235, NCT04161755, NCT03359239).

CONCLUDING REMARKS

Poor responses to current immunotherapies are frequently associated with tumors that have low mutational burdens or low T cell infiltration. For these patients, alternate approaches are likely necessary to elicit favorable responses on par with those observed in disease contexts such as melanoma and lung adenocarcinoma. Increasingly, the role of tumor DCs in the anti-tumor immune response is being recognized as targetable. Although single-agent therapies targeting DCs have been minimally successful, combination with standard-of-care therapies with novel immunotherapies is a promising avenue of investigation. Further research to fully understand the role of the tumor immune microenvironment as a whole is certainly warranted given the complex nature of the interactions between the tumor and immune system.

A more complete understanding will hopefully lead to the development of effective therapeutic strategies that improve patient outcomes.

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

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