THE ROLE OF CHEMOATTRACTANTS IN THE TUMOR MICROENVIRONMENT

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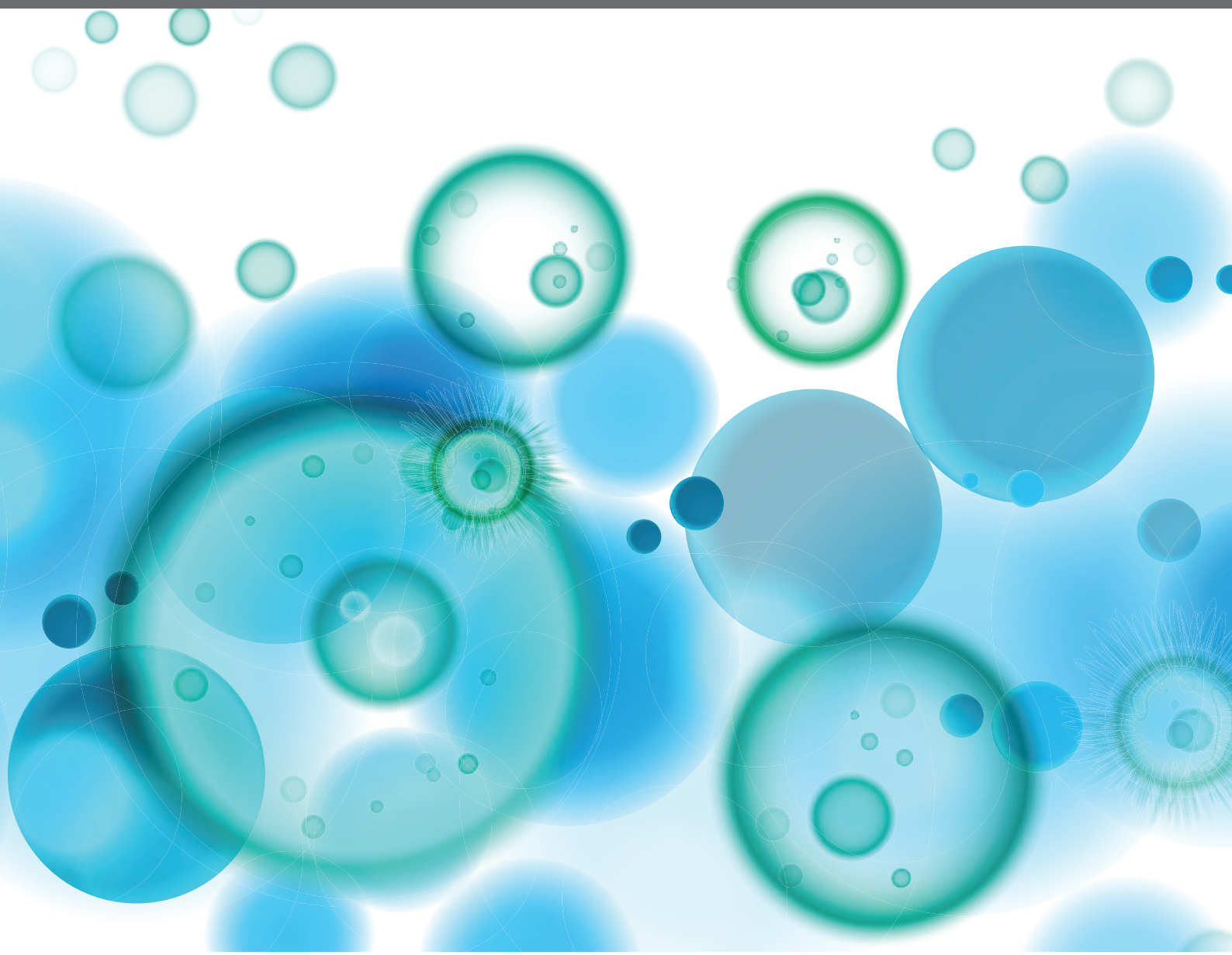

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THE ROLE OF CHEMOATTRACTANTS IN THE TUMOR MICROENVIRONMENT

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EDITORIAL
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Editorial: The Role of Chemoattractants in the Tumor Microenvironment

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Keywords: chemokines, cancer therapy, cancer metastases, anti-tumor immune response, cancer inflammation, immune suppression, chemokine receptor antagonists

Editorial on the Research Topic

The Role of Chemoattractants in the Tumor Microenvironment

Chemokines and other chemoattractants induce directional migration and activation of leukocytes and of non-hematopoietic cells by stimulating specific G protein-coupled Receptors (GPCR). Since the term "chemokine" was officially accepted as the standard nomenclature for "chemotactic cytokines" in 1992, there have been over 100,000 manuscripts published and indexed in Pubmed, ∼20% of which also include the search term cancer. In this *Frontiers in Immunology* Research Topic, leading international investigators in the field have contributed seven reviews, three minireviews, and four original research articles to this Research Topic to provide a comprehensive and timely examination of the role of chemoattractants in the tumor microenvironment (TME). The collection provides an updated overview of the most relevant issues related to the complicated interaction between chemoattractants and other mediators produced by host or tumor cells that contribute to tumor development, growth, metastasis, and immune escape.

Immune cells are fundamental in shaping the balance between a tumor-promoting or tumor-suppressive microenvironment. A fundamental role is played by macrophages, usually referred to as tumor-associated macrophages (TAMs). TAMs can stimulate proliferation of tumor cells, promote angiogenesis and fibrosis and suppress the anti-tumor immune response. In addition, the combined action of TAM at the primary tumors and the so-called metastasis-associated macrophages (MAM) in the metastatic sites promote the metastatic cascade.

The contribution by Argyle and Kitamura in this collection underscore the role of chemoattractants and of their receptors in TAM and MAM accumulation in primary and secondary tumor sites, highlighting the potential therapeutic role of targeting macrophage-recruiting chemokines to prevent malignant tumor development. One key determinant of monocyte recruitment and TAM accumulation provided by the CCL2/CCR2 axis. Indeed, different tumor types can produce CCL2, even though expression of this chemokine is regulated by different means. Consistent with these observations, loss of *Ccr2* or CCL2 blockade inhibits TAM accumulation and is the most promising strategy for inhibition of immune suppression exerted by chemokines.

Ruytinix et al. discuss interesting observations indicating that macrophages recruited into tissues can be polarized toward cells able to produce pro-angiogenic and pro-fibrotic factors as well as to attract other immunosuppressive immune cells according to environmental factors, thus favoring tumor growth in primary site or seeding in distant organs. In addition to several growth factors, an important contribution on monocyte differentiation toward a pro-tumor phenotype is provided by chemokines, including CCL2 and CXCL12.

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An original article from Lepore et al. describes a pro-tumorigenic role for CXCL16/CXCR6 signaling in glioma progression pointing to a critical role in immune-suppression. In a GL261 syngeneic orthotopic implantation model, CXCR6-deficient mice survived significantly longer than WT counterparts, with significantly reduced tumor volumes. Using anti-CXCL16 neutralizing antibodies, the authors discovered that glioma-secreted-CXCL16 induced an immune-suppressive gene expression signature in primary microglial cells.

Strategies able to inhibit macrophage recruiting or polarizing chemokines also create a permissive environment for immunotherapy, favoring activation of effector cells with anti-tumor activities. Nevertheless, activated CD8+ and NK cell populations rely on several receptors for their recruitment and infiltration and immunotherapeutic approaches are less effective in chemokine receptor deficient mice. Indeed, the distribution and phenotype of different NK cell subsets can be affected by specific types of tumor and its location and this often correlate to altered migration and homing. These and other aspects regulating trafficking and tissue localization of NK cells are discussed in this collection by Castriconi et al. Furthermore, by reporting evidence from the literature, Susek et al. revised the effect of CXCR1/2 and CXCR3, highlighting the importance of the formers in suppressive cell recruitment and of the latter in the generation of an effective T and natural killer cell anti-tumor response.

Since the first mechanistic study defining a protective role for leukocyte attractant chemerin in recruiting anti-tumor NK cells to melanoma lesions in 2012, there have been nearly 100 publications exploring the role of chemerin in cancer. The review by Shin et al. provides a comprehensive examination of chemerin in cancer, with a focus on mechanistic preclinical studies and functional consequences of chemerin in tumors. An original research article by Pachynski et al. indicates that chemerin gene expression is significantly downregulated in human breast cancer, which the authors hypothesize to be part of an adaptive tumor evasion strategy. Chemerin overexpression by mouse EMT6 breast cancer cells suppressed tumor growth *in vivo*, which was associated with increased CD4+ and NK cell infiltration into the tumor and mechanistically dependent on NK cells.

Many malignant tumors of non-hematopoietic origin express multiple chemoattractant GPCRs that increase the invasiveness and metastasis of tumor cells. In addition, chemoattractants also enable the interaction of tumor cells with host cells, thus promoting tumor growth and development of distant metastasis.

The review by Jacquelot et al. provides an in-depth look at the chemokines and chemokine receptors involved in melanoma progression. The expression of chemokine receptors by melanoma cells can be a determining factor in metastasis and survival outcomes, with CCR7, CCR10, and CXCR4 being particularly deleterious. The expression of certain chemokine receptors on blood or tumor infiltrating leukocyte subsets from melanoma patients or from preclinical studies can also be a determining factor in prognosis. The authors provide an up-to-date assessment of translational chemokine receptor targeting approaches in melanoma, noting the "double-edged sword" nature of this approach, in that targeting receptors expressed by melanoma may impair effective anti-tumor leukocyte functions.

Triple-negative breast cancer (TNBC) is a subgroup of diagnosed breast cancer patients without targeted therapeutic options. Notch receptor expression and activation strongly correlate with the aggressive clinicopathological and biological phenotypes of breast cancer. Two articles by Liubomirski et al.; Liubomirski et al., together point at the pro-inflammatory microenvironment, and at the Notch pathway, as targets for potential future treatments in TNBC. The authors found that TNBC from patient samples exhibited increased levels of Notch1 and Notch 3 and decreased Notch4 compared to luminal A breast cancers. Moreover, Notch1 expression correlated with TNF-alpha and CXCL8 expression. Notch 1 regulated the contact-dependent induction of CXCL8, and TNF-alpha stimulation led to activation of p65 and subsequently CXCL8 production. The authors conclude that the Notch pathway is a key mechanism for up-regulation of CXCL8 resulting in increased aggressiveness of TNBC.

In an intriguing change of pace from considering the role of chemoattractant receptors on tumor cells or leukocytes, Salazar and Zabel reviews the ways in which chemokine receptor expression by tumor endothelial cells (TEC) can support cancer progression. TEC are highly heterogeneous and express a variety of chemokine receptors such as ACKR1, ACKR3, CXCR4, CCR2, CXCR2, and CXCR3. TME-derived chemokines contribute to the morphological and phenotypic dysregulation of the vascular endothelium, leading to pro-tumorigenic angiogenesis, vasculogenesis, intussusception, vessel co-option, and/or vascular mimicry. The authors speculate that chemokine receptors may be particularly promising targets for future vascular disruption therapies based on their restricted expression (e.g., not by vital organs) and the potential for concomitant effects on leukocytes (e.g., inhibition of immune suppressive regulatory T cells).

Many chemokines are abundantly and concomitantly expressed in the TME and their function is regulated by complex mechanisms. In the latest years it has become clear that complexity is even higher because of the formation of heterocomplexes that exert antagonistic or synergistic effects on selected receptors.

D'Agostino et al. have collected the available scientific literature and their own experience on the phenomenon of heterocomplex formation, concentrating their investigation in cancer. The possible outcomes of heterocomplexes between chemokines, as well as between chemokines and inflammatory molecules (such as HMGB1) on the shaping of the TME is discussed.

Given their role in the pathomechanisms of tumor progression, chemoattractant receptors and their ligands constitute targets for the development of novel anti-tumor therapeutics. Two reviews provide comprehensive insight into the role of chemokines and receptors in tumor pathobiology and targeted treatments. Using publicly available data from The Human Protein Atlas, Vilgelm and Richmond constructed a heat map showing prognostic associations between 25 individual chemokines and 12 different types of cancer. Certain

chemokines contribute to establishing a "T cell-inflamed" TME that is associated with improved prognosis, particularly when checkpoint inhibitor treatments are administered. The authors also describe a variety of chemokine-based countermeasures that can be deployed to populate an "immunologically cold" tumor with anti-tumor leukocytes. Poeta et al. focus on the role of chemokines and chemokine receptors in cancer with considerations on the possibility to be targets for cancer immunotherapy with emphasis on the possibility to optimize the anti-tumoral potential of the immune system. They present an overview on the current use of antagonists or inhibitors of chemokine receptors to treat different type of tumors both in preclinical model and clinical trial.

CONCLUSIONS

It is our hope that this collection will serve to launch new studies that extend our understanding of chemoattractants in the pathomechanisms of tumor progression, and to inspire the discovery and development of new chemokine-focused treatments to make a real impact in the lives of cancer patients and their families.

AUTHOR CONTRIBUTIONS

GB and BZ have both contributed substantially to the work and approved it for publication.

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REVIEW
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Targeting Macrophage-Recruiting Chemokines as a Novel Therapeutic Strategy to Prevent the Progression of Solid Tumors

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Solid tumors are initiated by genetic mutations in non-hematopoietic cells and progress into invasive malignant tumors. This tumor progression often culminates in metastatic disease that is largely refractory to current therapeutic modalities and thus dramatically reduces survival of tumor patients. As solid tumors account for more than 80% of cancer-related deaths, it is necessary to develop novel therapeutic strategies to treat the diseases. An attractive strategy is to target macrophages in both primary tumors [known as tumor-associated macrophages (TAMs)] and metastatic tumors [called metastasis-associated macrophages (MAMs)]. TAMs and MAMs are abundant in most solid tumors and can promote tumor metastasis. Several studies in various models of solid tumors suggest that the accumulation of TAMs, MAMs, and their progenitor cells is regulated by chemokine ligands released by tumor and stromal cells. Consequently, these macrophage-recruiting chemokines could be potential therapeutic targets to prevent malignant tumor development through disruption of the accumulation of pro-metastatic macrophages. This review will discuss the role of chemokine ligands and their receptors in TAM and MAM accumulation in primary and secondary tumor sites, and finally discuss the therapeutic potential of inhibitors against these macrophage-recruiting chemokines.

Keywords: cancer, metastasis, tumor-associated macrophage, chemokine, antagonist, immunotherapy

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INTRODUCTION

Genetic alterations in non-hematopoietic cells can lead to uncontrolled cell proliferation that results in aberrant tissue mass called a solid tumor. Initially the solid tumors grow locally and do not invade adjacent tissues. However, accumulation of genetic alterations in the tumor cells turns them into malignant tumors that spread to different part of the body and establish secondary tumors (metastasis). While early detection techniques have greatly improved patient survival, significant challenges remain in the treatment of tumors following metastasis (1). It has been reported that the establishment of metastatic tumors dramatically increases the mortality rate of tumor patients (1), and thus the presence of solid tumors account for more than 80% of tumor-associated deaths (2). It is therefore necessary to prevent the metastasis formation from solid tumors.

In order to form metastatic tumors, cancer cells in the solid tumors pass through a process called the metastatic cascade (3, 4). In the primary site, cancer cells escape from the anti-tumor

immune responses (immune escape), invade the surrounding tissue (invasion) and enter the blood or lymphatic vessels (intravasation) that disseminate cancer cells into the circulation. The cancer cells also increase the density of blood vessels at the tumor site (angiogenesis), which also enhances tumor cell egress. At the secondary site, the circulating cancer cells migrate from the vessels to the parenchyma (extravasation) and often grow into the lethal metastatic tumors (persistent growth) (5). Through the accumulation of genetic changes, malignant tumor cells acquire several abilities that advance each step of metastasis, e.g., increased proliferation, motility, invasiveness, and survival (6). In addition to these cell autonomous changes, tumor cells require the supports from surrounding stromal cells to progress the metastatic cascade (4–6). It is now widely recognized that both primary and metastatic tumors are composed of numerous stromal cells such as endothelial cells, pericytes, fibroblasts, mesenchymal stem cells, and a variety of immune cells [including regulatory T (T_{reg}) cells, mast cells, neutrophils, myeloid-derived suppressor cells (MDSCs), and tumor associated macrophages (TAMs)]. All of these stromal cells are known to promote tumor angiogenesis, cancer cell invasion, and/or disrupt immune surveillance, which helps progression of the metastatic cascade (5, 7). Among these tumor-promoting stromal cells, TAMs are one of the most abundant cell types in solid tumors (8), and a high number of TAMs in the tumor correlates with poor overall survival in most solid tumors such as breast, gastric, oral, ovarian, bladder, and thyroid cancers (9–13). Furthermore, several mouse models of malignant solid tumors have identified that TAMs recruited to primary tumors and those in the metastatic sites (called metastasis-associated macrophages, MAMs) promote almost all steps of the metastatic cascade (**Figure 1**) (5). Therefore, blockade of TAM and MAM accumulation in the tumor microenvironment could represent a novel approach to prevent the progression of solid tumors and improve the outcome of metastatic disease (14).

Immune cell recruitment into the site of inflammation follows several steps, i.e., tethering to the vessel wall, rolling on it, adhesion to endothelial cells, crawling, and migration through the endothelial monolayer. Since activation of certain set of integrins progress each step of this cascade, blockade of the integrin-induced adhesion cascade has been suggested as a novel therapy for inflammatory diseases (15). Another key factor that regulates the directed migration and positioning of immune cells, including macrophages, are chemokines. Chemokines are a family of small cytokines consisting of more than 50 members in human and mice. They are classified into four subfamilies based on the position of cysteine residues, i.e., XC-, CC-, CXC-, and CX3C-chemokine ligands (XCL, CCL, CXCL, and CX3CL). These chemokine ligands bind to their cognate receptors (XCR, CCR, CXCR, and CX3CR, respectively), and regulate circulation, homing, and retention of immune cells. Although some ligands can bind to multiple receptors and vice versa, the binding affinities of ligands to a cognate receptor are largely different. Furthermore, each immune cell type differentially expresses the receptors, and expression of receptors and ligands is spatially and temporally regulated (16). Therefore, each chemokine ligand-receptor pair selectively regulates the positioning of a certain type of immune cell for host defense and immunity (17). Accumulating evidences suggest that solid tumors utilize chemokines and their receptors to accomplish successful metastasis. In the tumor microenvironment, both cancer and stromal cells produce various chemokine ligands that recruit the tumor promoting immune cells such as T_{reg} cells, neutrophils, MDSCs and TAMs (18). It is therefore likely that blockade of chemokine signals could be an attractive strategy to prevent malignant tumor development by disrupting accumulation of the pro-metastatic cells including TAMs. On the other hand, the target chemokine signal should be carefully considered as it can also affect the recruitment of cytotoxic lymphocytes (CTL) such as $CD8^+$ T and natural killer (NK) cells that have the potential to eliminate malignant tumor cells and thereby are essential for the success of immunotherapies such as checkpoint inhibitors and CTL transfer therapies.

In this review, I will describe the roles of TAMs and MAMs in the metastatic process, and summarize chemokine ligands and receptors that recruit the pro-metastatic macrophages mainly based on results from pre-clinical tumor models in mice. I will also discuss the therapeutic potential of TAM/MAM targeting by chemokine receptor antagonists, and consider the possibility of combining macrophage targeting with emerging immunotherapies for malignant tumors.

ROLES OF MACROPHAGES IN THE METASTATIC CASCADE

The Contribution of TAMs to the Metastatic Steps at the Primary Site

TAMs are macrophages (characterized as $F4/80^+CD11b^+Ly6C^{low}$ in mouse or $CD11b^+CD14^+CD163^+$ in human) that accumulate in the tumor microenvironment and promote tumor progression (4). Although TAMs in solid tumors can be derived from tissue resident macrophages, several animal studies have shown that TAMs originate from classical monocytes in blood that are characterized as $CD11b^+Ly6C^+CCR2^+$ (or $CD14^{++}CD16^-CCR2^+$ in human) (14, 19). For example, a mouse model of glioblastoma has shown that adoptively transferred $CCR2^+$ monocytes are recruited to the tumor and differentiate into TAMs, accounting for 85% of the total macrophage population in the tumor (20). In a mouse model of breast cancer caused by the mammary epithelial restricted expression of the Polyoma Middle T oncogene (PyMT), genetic depletion of $CCR2^+$ monocytes reduces the number of TAMs

Abbreviations: ATF4, activating transcription factor 4; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CITED2, Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain-2; CSF1, colony stimulating factor 1; CTL, cytotoxic lymphocyte; CXCL, CXC-chemokine ligand; CXCR, CXC-chemokine receptor; EGF, epidermal growth factor; EMT, epithelial mesenchymal transition; IGF1, insulin like growth factor 1; LKB1, liver kinase B1; MDSC, myeloid derived suppressor cell; MAM, metastasis-associated macrophage; mTROC1, mammalian target of rapamycin complex 1; NK cell, natural killer cell; PyMT, Polyoma Middle T oncogene; RCC, renal cell carcinoma; RKIP, Raf kinase inhibitory protein; TAM, tumor associated macrophage; TGFβ, transforming growth factor β T_{reg} cell, regulatory T cell; VEGF, vascular endothelial growth factor.

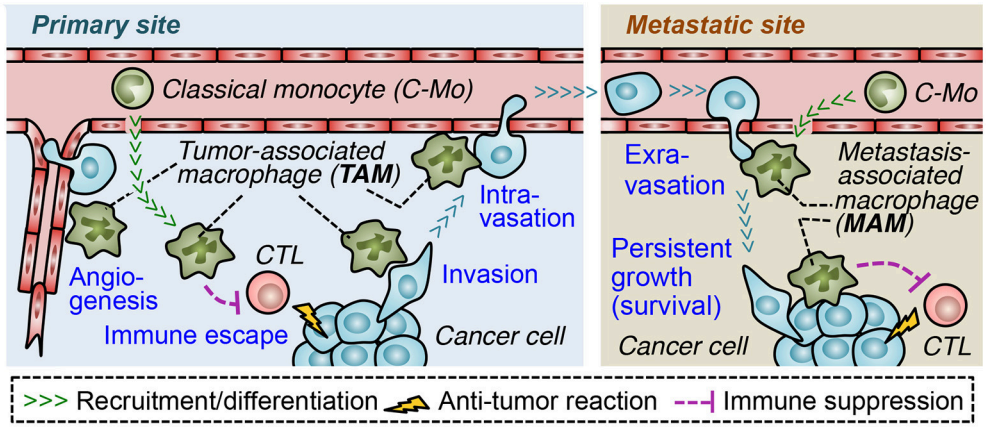

FIGURE 1 | Roles of tumor-infiltrating macrophages in progression of the metastatic cascade. In the primary site, tumor-associated macrophages (TAM) suppress functions of cytotoxic lymphocytes (CTL) and promote angiogenesis, which supports tumor growth as well as dissemination of cancer cells. TAMs also directly help cancer cells to migrate into adjacent parenchyma (invasion) and to enter the blood vessels (intravasation). In the metastatic site, a distinct population of TAMs called metastasis-associated macrophage (MAM) promotes migration of cancer cells from the vessels into the parenchyma (extravasation) and their persistent growth or survival. MAMs may also suppress anti-tumor immune responses in metastatic sites. Both in the primary and secondary site, tumor-promoting macrophages (i.e., TAMs/MAMs) originate from circulating classical monocytes.

in primary tumors. Further, adoptively transferred $CCR2^+$ monocytes are recruited to the tumors and differentiate into $F4/80^+$ macrophages (21). These results suggest that the majority of TAMs in some solid tumors are differentiated from classical monocytes.

Studies using the PyMT breast cancer model have suggested that the accumulation of TAMs promotes progression of the metastatic cascade (**Figure 1**). For example, macrophage ablation in the PyMT mice by genetic deletion of colony stimulating factor 1 (CSF1) suppresses tumor angiogenesis and pulmonary metastasis of cancer cells (22, 23). In this model, macrophage-selective deletion of *Wnt7b* also reduces angiogenesis in primary mammary tumors and suppresses lung metastasis (24). Tumor angiogenesis is known to promote dissemination of cancer cells from the primary tumor into the circulation by increasing the density of leaky vessels and enhancing tumor cell invasiveness (25). It is therefore likely that TAMs enhance the hematogenous dissemination of cancer cells via promoting angiogenesis. TAMs also promote the tumor cell egress by directly helping cancer cell invasion and intravasation. Intravital imaging of the PyMT tumors indicates that mammary tumor cells invade surrounding tissues together with TAMs and enter the blood vessel in association with perivascular TAMs (26, 27). In these processes, TAMs secrete epidermal growth factor (EGF), and activate its receptor in cancer cells, which enhances invasion capability and motility through increasing invadopodium formation and matrix degradation (28). It is also reported that perivascular TAMs transiently increase vascular permeability via secretion of vascular endothelial growth factor (VEGF) and thereby promote intravasation of the PyMT tumor cells (29). Consistent with these results, a high number of TAMs correlates with high density of vasculature in a variety of human solid tumors including breast cancer (30). Furthermore, direct contact between perivascular TAMs, endothelial cells and cancer cells (called tumor microenvironment for metastasis; TMEM) is associated with increased risk of distant metastasis in breast cancer (31). Several studies suggest that TAMs also protect cancer cells from anti-tumor immune reactions. For example, macrophages isolated from the mouse and human solid tumors can directly suppress T cell responses (5, 32) and NK cell cytotoxicity (33, 34) *in vitro*. It is also reported that depletion of TAMs by a CSF1 receptor antagonist enhances $CD8^+$ T cell-mediated anti-tumor immunity under treatment with chemotherapy in the PyMT breast cancer mouse model (35). Mechanistically, TAMs can suppress T cell activities directly via expression of immune regulatory molecules such as arginase-1 (ARG1), IL-10, and transforming growth factor β (TGFβ) (36), as well as via physical contacts with T cells that suppresses full activation of T cells or their access to the tumor cells (37, 38). In addition, TAMs can suppress T cell-mediated immune reactions indirectly by regulating the recruitment of T_{reg} cells (39, 40). These results indicate that TAMs accumulating in primary tumors help cancer cells to disseminate into the circulation via enhancing immune suppression, angiogenesis, cancer cell motility and invasiveness. It is therefore likely that molecules that recruit TAMs can be therapeutic targets to prevent the metastatic seeding of primary tumor cells in certain types of solid tumors.

Metastatic Steps Promoted by MAMs in the Secondary Site

It has been suggested that TAMs contain many different subtypes that play specific roles in tumor development and progression (8). In mouse models of metastatic breast cancer, a population of macrophages characterized as $F4/80^{high}Ly6G^{-}CD11b^{high}CD11c^{low}$ accumulates in the lung with metastatic tumors. This macrophage population is barely found in the normal lung and distinct from lung resident

macrophages that are defined by high expression of F4/80 and CD11c (41, 42). The CD11b-positive macrophages that accumulate in the metastatic sites are thus called metastasis-associated macrophages (MAMs). Recent studies have shown that adoptively transferred classical monocytes are recruited to the metastatic sites where they differentiate into MAMs (43, 44). It is also reported that depletion of MAMs by CSF1 or its receptor knockout reduces metastatic tumor burden in mice that are intravenously injected with mammary tumor cells (41, 42). These results suggest that the recruitment of monocytes and subsequent accumulation of MAMs are required for circulating breast cancer cells to develop metastatic tumors.

In order to establish metastasis foci, circulating cancer cells need to extravasate, survive, and grow at the secondary sites. Several studies using mouse models of metastatic breast cancer have shown that MAMs can enhance the progression of these steps (**Figure 1**) (5, 8). For example, depletion of MAMs by CSF1 knockout reduces the number of cancer cells outside the blood vessels in the lung of mice that are intravenously injected with MET-1 mouse mammary tumor cells (41). It is also reported that macrophage-selective deletion of *Vegfa* reduces pulmonary metastasis formation of breast cancer cells *in vivo,* and suppresses permeability of endothelial monolayers as well as extravasation of cancer cells *in vitro* (43). These results indicate that MAMs promote extravasation of cancer cells via VEGF-A secretion. In the same model, pharmacological or genetic depletion of macrophages following tumor cell extravasation suppresses the metastatic tumor loads in the lung (41). It is also reported that MAMs suppress apoptosis of human breast cancer cells disseminated into the lung of mice by transmitting a survival signal via vascular cell adhesion molecule 1 (VCAM-1) on MDA-MB-231 human breast cancer cells (45). Furthermore, MAMs enhance angiogenesis via a Tie-2-mediated mechanism and thereby promote the outgrowth of micro-metastatic foci in the lung of PyMT mice (46). These results suggest that MAMs promote survival and persistent growth of cancer cells after seeding at the metastatic sites. Moreover, a recent study suggests that MAMs can protect cancer cells from tumoricidal immune reactions in the metastatic sites since MAMs, isolated from the metastatic tumors established by E0771-LG mouse mammary tumor cells, suppress cytotoxicity of $CD8^+$ T cells against cancer cells *in vitro* (44). Given these findings, accumulation of MAMs seems to be a key factor for progression of metastatic steps at the secondary sites during pulmonary metastasis of breast cancer cells, whereas the contribution of MAMs to the development of metastasis in other tumor models or clinical patients has not yet been established.

CHEMOKINES THAT PROMOTE ACCUMULATION OF PRO-METASTATIC MACROPHAGES

Chemokines That Recruit TAMs to the Primary Site

As described above, mouse models of some solid tumors suggest that TAM accumulation in primary tumors is mainly due to the recruitment of classical monocytes that express high levels of CCR2. It is also reported that high expression of a CCR2 ligand (CCL2) in tumors positively associates with the accumulation of TAMs in glioblastoma, squamous cell carcinoma, renal cell carcinoma (RCC), as well as ovarian, endometrial, lung, and breast cancer (47–53). Thus CCL2-CCR2 signals seem to be a key determinant of monocyte recruitment and subsequent TAM accumulation. In line with this notion, several mouse studies have emphasized the importance of CCL2 in the recruitment of TAMs. For example, treatment with anti-CCL2 neutralizing antibodies significantly reduces the number of macrophages in human RCC xenografts transplanted into SCID mice, which reduces micro-vessel density, and growth of xenografted tumors (53). Although the source of CCL2 in this model is not identified, the same group has shown that a RCC cell line, 786-O, expresses high levels of CCL2. They also demonstrated that suppression of the CCL2 expression in 786-O cells reduces the number of TAMs in the xenograft tumor as well as tumor growth and microvascular density (53), suggesting that cancer cell-derived CCL2 promotes the TAM accumulation in this model (**Figure 2A**). In the 786-O RCC cells, the CCL2 production is increased by JunB overexpression via loss of the von Hippel-Lindau (VHL) tumor suppressor gene (54). Since loss of VHL is found in the majority of sporadic RCC and JunB is up-regulated in the VHL-deficient RCC specimens (54, 55), these results suggest that CCL2 production by cancer cells via aberrant JunB expression might be a predominant mechanism to enhance TAM accumulation in RCC. Mouse models of other types of solid tumors have also demonstrated that cancer cell-derived CCL2 plays pivotal roles in the accumulation of TAMs, whereas regulatory mechanisms behind CCL2 production differ between tumor types. For example, in subcutaneous tumors developed by LLC lung cancer cells, deletion of the *Ccl2* gene in LLC cells reduces the number of macrophages in the tumors (56). In this case, CCL2 expression in cancer cells is promoted by activation of the mammalian target of rapamycin complex 1 (mTROC1) pathway (56) that is frequently activated in various types of cancer including lung cancer (57). In endometrial cancers, established in mice by loss of liver kinase B1 (LKB1) tumor suppressor gene ($Lkb1^{-/-}$), the CCL2 level is markedly increased in cancer cells. In this model, genetic deletion of *Ccl2* in the $Lkb1^{-/-}$ tumors significantly reduces the number of TAMs, which results in the delayed tumor progression and prolonged overall survival (52). It is also reported that reduced expression of *LKB1* gene in immortalized human endometrial epithelial cells significantly increases CCL2 secretion (52). Consistent with these data, loss of LKB1 protein is observed in ∼20% of endometrial cancers, and low LKB1 levels in the cancer strongly correlate with high CCL2 expression and high macrophage number (52, 58). Given these results, loss of LKB1 seems to be a trigger for certain populations of endometrial cancer cells to increase CCL2 expression and subsequent TAM accumulation. On the other hand, a recent study showed that AN3CA and KLE endometrial cancer cells produce CCL2 via activating transcription factor 4 (ATF4), and that anti-CCL2 neutralizing antibody treatment suppresses macrophage infiltrations in subcutaneous tumors developed by AN3CA or KLE cells (59). Since high ATF4 expression correlates

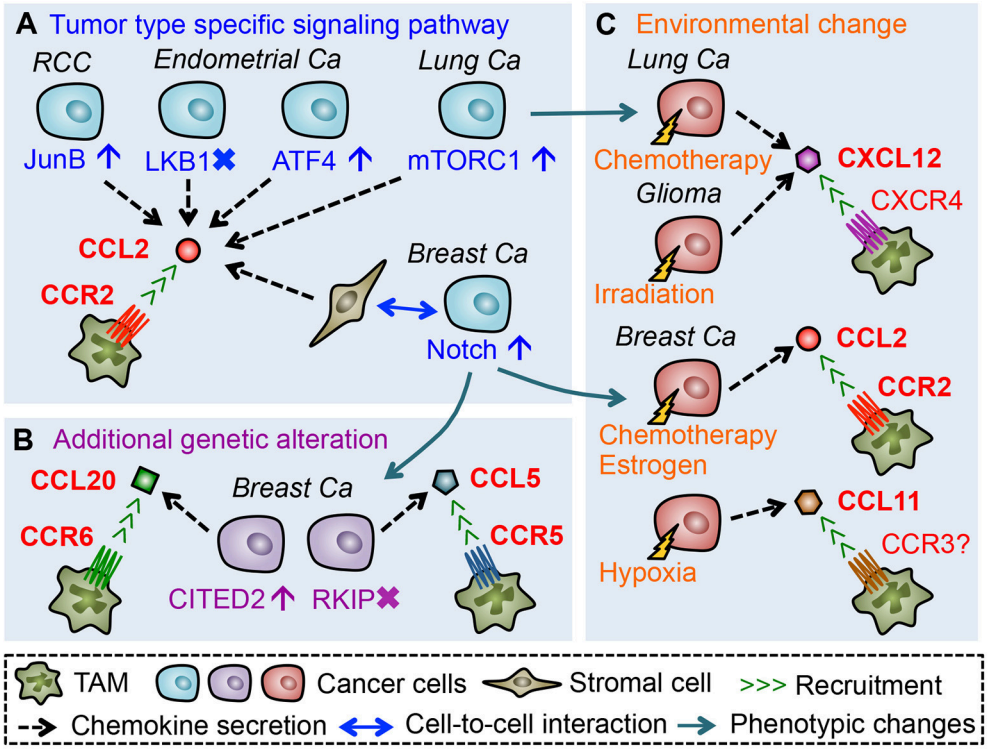

FIGURE 2 | Chemokines that promote accumulation of TAMs in the primary tumors. **(A)** Tumor cells or tumor-activated stromal cells secrete CCL2 through activation or suppression of tumor type specific signaling pathways, which promote TAM accumulation in the tumor microenvironment. **(B)** Additional genetic alteration in cancer cells during tumor progression can induce expression of TAM-attracting chemokines. **(C)** Environmental changes caused by therapeutic treatments or hypoxia also promote *de novo* chemokine secretion from cancer cells.

with high macrophage density in human endometrial cancer (59), up-regulation of this transcription factor in cancer cells might be another mechanism behind CCL2-induced TAM accumulation in endometrial cancer. Collectively, these results suggest that cancer cells promote TAM accumulation by producing CCL2 via tumor type specific signaling pathways (**Figure 2A**).

The CCL2-CCR2 signaling is also required for the accumulation of TAMs and subsequent tumor progression in mouse models of breast cancer. For example, in mice with mammary tumor developed by orthotopic injection of MDA-MB-231 human breast cancer cells, treatment with anti-CCL2 antibody reduces TAM accumulation, which results in the reduced micro-vessel density and tumor growth (60). In this model, however, cancer cells may not be a major source of CCL2 since the number of TAMs does not correlate with mRNA levels of human *CCL2* in MDA-MB-231 cells but does with mouse *Ccl2* in the stroma (60). In line with this notion, immunohistochemical analysis of human breast cancer specimens shows that stromal but not tumoral CCL2 expression significantly correlates with macrophage infiltration, tumor size, and poor prognosis of patients (60). Another study also showed that genetic deletion of *Ccl2* in the host (i.e., stromal) cells but not in cancer cells results in reduced TAM infiltration, deficient angiogenesis, and impaired tumor growth in mice that are orthotopically injected with 4T1 mammary tumor cells (61). It is also reported that CCL2 is expressed in fibroblasts residing in breast cancer biopsies, and that human mesenchymal stem cells increase CCL2 secretion in response to conditioned medium from MDA-MB-231 breast cancer cells (62). Furthermore, conditioned medium from 4T1 mammary tumor cells can increase CCL2 expression in cultured macrophages (63). It is therefore likely that a population of breast cancer cells prompt stromal cells to secrete CCL2 for TAM accumulation in the tumors. Although the precise mechanism behind the stromal CCL2 production is still unclear, a recent study shows that inhibition of Notch1 expression in 4T1 cells reduces CCL2 levels in transplanted tumors and thereby decreases TAM accumulation (64). Since high Notch1 expression associates with transition from ductal carcinoma *in situ* to invasive cancer, as well as worse overall survival of breast cancer patients (65), it is possible that enhanced Notch1 expression in tumor cells during their malignant progression promotes stromal secretion of CCL2 in breast cancer (**Figure 2A**).

Although the above-mentioned studies suggest CCL2 as a dominant TAM attractant in most solid tumors, CCL2 inhibition suppresses TAM accumulation by only around 50% and does not achieve complete TAM depletion in the mouse models (52, 53, 56, 59–61). This suggests the involvement of other

CCR2 ligands such as CCL12 (17) and cytokines such as VEGF and CSF1 that are known to recruit monocytes (66, 67). The incomplete inhibition may also be explained by the contribution of chemokine signals other than the CCL2/CCR2 axis. For example, it is reported that CCL20, a ligand for CCR6, is abundant in PyMT mammary tumors and genetic deletion of *Ccr6* gene in PyMT mice significantly reduces the number of TAMs in mammary tumors (68). Although the cell type that secretes CCL20 is unknown in the PyMT model, a recent study demonstrates that MDA-MB-231 human breast cancer cells express high level of CCL20 and that inhibition of CCL20 expression in cancer cells reduces TAM accumulation in xenografts (69). It is also reported that a highly metastatic derivative of MDA-MB-231 cells (named BM1) expresses high levels of CCL5 (a ligand for CCR5) and treatment of BM1 tumor-bearing mice with a CCR5 antagonist significantly reduces the number of TAMs in tumors (70). These results suggest that breast cancer cells can utilize CCL20-CCR6 and CCL5-CCR5 signaling in order to recruit TAMs. In the MDA-MB-231 breast cancer model (69), expression of CCL20 and TAM accumulation in xenografted tumors are suppressed by knock down of Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain-2 (CITED2), a transcriptional co-regulator whose expression is increased in human invasive ductal carcinoma compared to normal mammary tissues and further enhanced in metastatic breast cancer (71, 72). In the BM1 as well as 4T1 mammary tumor models, forced expression of Raf kinase inhibitory protein (RKIP) suppresses CCL5 secretion from cancer cells and reduces TAM accumulation in the xenograft (70, 73). It is also reported that lower expression of RKIP in breast cancer is associated with higher levels of CCL5, as well as a higher probability of metastasis and poor prognosis (73–75). These results suggest that additional genetic alterations in cancer cells that occur in the course of tumor progression (e.g., overexpression of CITED2 and/or loss of RKIP gene) induce *de novo* chemokines (e.g., CCL20 and/or CCL5) that recruit TAMs to primary tumor sites (**Figure 2B**).

In addition to the genetic alterations in cancer cells, environmental changes may also switch on the *de novo* expression of TAM recruiting chemokines (**Figure 2C**). In mammary tumors in the PyMT mice, the CCL20-CCR6 axis can promote TAM accumulation (68) whereas the CCL2-CCR2 signal plays only a minor role if any (21). However, treatment of PyMT mice with the chemotherapeutic agent, doxorubicin, increases protein levels of CCR2 ligands CCL2 and CCL12 in the tumor stromal area, and promotes the recruitment of $CCR2^{+}$ monocytes into the mammary tumors (76). Furthermore, in mice with the mammary tumors developed by orthotopic injection of PyMT tumor cells, treatment with estrogen enhances TAM accumulation in the tumor via increased expression of CCL2 (77). These results suggest that a chemokine signal used for the TAM accumulation in breast cancer can be switched from CCL20 to CCL2 in response to the environmental changes induced by chemotherapies or hormonal treatments. Such environmental induction of TAM recruiting chemokines can also occur locally in certain areas of the tumor. For example, a hypoxic area in a human breast cancer specimen demonstrates higher levels of CCL11 and a higher number of TAMs compared with a normoxic area (78). Since MDA-MB-231 breast cancer cells under hypoxic conditions increase CCL11 secretion and thereby promote macrophage migration *in vitro* (78), these results suggest that CCL11 is induced by low oxygen and locally recruits TAMs to the hypoxic regions in tumors. Interestingly, therapeutic treatments also promote the regional accumulation of TAMs via localized induction of CXCL12. In a mouse model of glioma, localized radiation therapy induces CXCL12 in the invasion front of xenografts, where TAMs are recruited through activation of the CXCL12 receptor, CXCR4 (79, 80). In subcutaneous tumors established by LLC lung cancer cells, chemotherapy (cyclophosphamide) treatment increases CXCL12 expression around blood vessels and recruits TAMs to the perivascular area through CXCR4 (81), whereas CCL2 from cancer cells promotes TAM accumulation in the LLC tumors without receiving any chemotherapy (82).

Taken together, it is likely that CCL2-CCR2 signaling plays a pivotal role in TAM accumulation in most solid tumors, whereas other signals such as CCL5-CCR5, CCL20-CCR6, CXCL12-CXCR4 can be an alternative or additional chemoattractant pathway (**Figure 2**). However, it is still unclear whether all of these chemokines are required for TAM accumulation in the same tumor microenvironment. Interestingly, a recent study using a mouse model of breast cancer showed that TAMs are recruited via CCR2 signaling to primary tumors where they induce CXCR4 expression in response to tumor-derived TGFβ and then migrate toward the blood vessel via CXCL12 to promote intravasation of cancer cells (83). It is therefore possible that TAMs utilize multiple chemokine signals for their positioning in the primary tumor in order to exert pro-metastatic functions. It is also reported that CCL2 and CXCL12 synergistically enhance the *in vitro* migration of human monocytes and macrophages (84), suggesting that expression of multiple chemokines in the tumor microenvironment is required for the efficient recruitment of monocytes and TAMs. Further investigation is necessary to identify when and how these chemokines are induced in the same tumor microenvironment and to what extent they contribute to TAM accumulation.

Chemokines That Promote MAM Accumulation in the Metastatic Site

A recent study using a mouse model of metastatic breast cancer has shown that transferred classical monocytes ($F4/80^{low}CD11b^{+}Ly6C^{+}$) differentiate into MAMs ($F4/80^{low}CD11b^{high}Ly6C^{low}$) by 42 h after infiltration into the lung with metastatic tumors and that the accumulation of MAMs is continuously increased during metastatic tumor growth (44). This suggests that classical monocytes are constitutively recruited and produce MAMs in metastatic tumors. It is also reported that classical monocytes expressing high levels of CCR2 preferentially migrate to metastatic tumors established by Met-1 mouse mammary tumor cells or those in the PyMT mice. In these models, anti-CCL2 antibody treatment, or genetic deletion of CCR2 inhibits the monocyte migration to the tumor-challenged lung and decreases the number of MAMs, which results in

the reduction of metastatic tumor burden (43). Adoptively transferred human classical monocytes ($CD14^+CD16^-CCR2^+$) also migrate to the metastatic tumors established by 4173 human breast cancer cells (a highly metastatic derivative from MDA-MB-231 cells) in nude mice, and this monocyte recruitment is inhibited by treatment with neutralizing antibodies against either mouse (host stromal cell-derived) or human (cancer cell-derived) CCL2 (43). Collectively, these results indicate that CCL2 secreted from both tumor cells and stromal cells plays a pivotal role in the recruitment of monocytes and subsequent accumulation of MAMs in the site of metastasis (**Figure 3A**).

Consistent with these results, loss of *Ccr2* significantly reduces MAM accumulation and pulmonary metastasis formation in another metastatic breast cancer model using E0771-LG mouse mammary tumor cells (42). In this model, genetic deletion of CCR1 in mice also reduces the number of MAMs in metastatic tumors and results in the decreased tumor burden. Interestingly, loss of CCR1 does not affect the recruitment of monocytes but, instead, prevents MAM-cancer cell interactions and subsequent retention of MAMs in the tumor-challenged lung (42). These results suggest that distinct chemokine signals regulate a specific process of MAM accumulation, i.e., recruitment of monocytes by CCR2 and retention of MAMs by CCR1 in pulmonary metastasis of breast cancer **Figure 3A**. It has been reported that freshly isolated human monocytes reduce expression of CCR2 and concomitantly increase expression of CCR1 when they differentiate to macrophages *in vitro*, and that the differentiated macrophages are more responsive to a CCR1 ligand, CCL3, than monocytes in an intracellular calcium flux assay (85). Therefore, transition of dominant receptor expression might determine differential responses of monocytes and MAMs to distinct sets of chemokine ligands. In a pulmonary metastasis model of renal cancer, MAMs increase CCR5 but not CCR1 expression

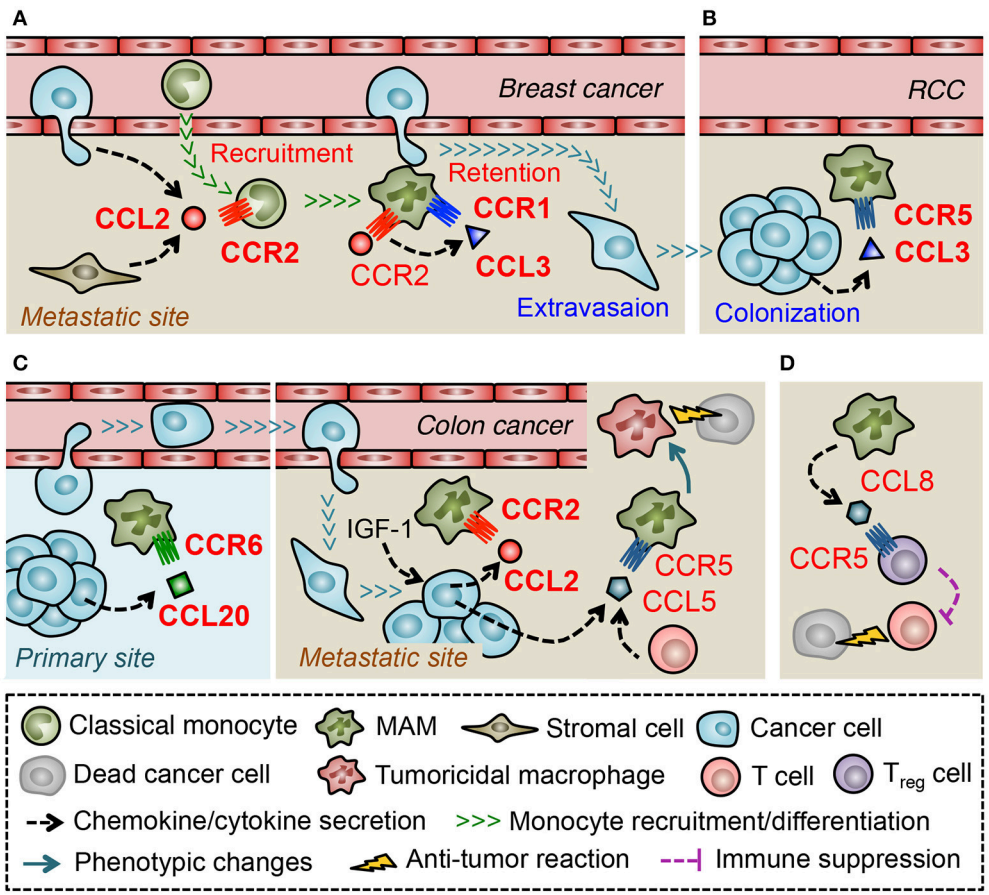

FIGURE 3 | Macrophage-mediated chemokine signals in the metastatic tumors. **(A)** In the lung challenged by metastatic breast cancer cells, CCL2 recruits classical monocytes that differentiate into a distinct myeloid cell population that gives rise to metastasis-associated macrophage (MAM). Activation of CCR2 in MAMs prompts them to secrete CCL3, which in turn enhances MAM-to-cancer cell interaction and subsequent retention of MAMs via a CCR1-mediated mechanism. **(B)** In a later phase of metastasis caused by renal cell carcinoma (RCC), CCL3 in the tumor microenvironment increases MAM accumulation via a CCR5-dependent manner. **(C)** Metastasized colon cancer cells in the liver produce CCL2 via an insulin-like growth factor-1 (IGF-1) signal and recruit MAMs whereas colon cancer cells in the primary site recruit macrophages (i.e., TAMs) via CCL20 secretion. In the liver metastases of colon cancer, tumor cells, or T cells secrete CCL5 that activate CCR5 on MAMs and maintain their pro-tumor features. Thus blockade of CCR5 signal reprograms MAMs to tumoricidal cells. **(D)** In the lung metastases developed by breast cancer cells, MAMs secrete CCL8 and recruit regulatory T (T_{reg}) cells.

at the late stage of metastatic tumor development (by 21 days after tumor injection) and loss of *Ccr5* but not *Ccr1* reduces MAM accumulation in metastatic tumors at this time point (86). On the other hand, CCR5 is not necessary for early MAM accumulation that occurs within 24 h after tumor injection in the E0771-LG breast cancer model (42). It is thus possible that the distinct microenvironments at different phases of metastasis determine predominant chemokine receptors that MAMs utilize for accumulation (**Figure 3B**). In line with this notion, a mouse model of liver metastasis using MC38 colon cancer cells has shown that suppression of the CCL2-CCR2 signal inhibits MAM accumulation until 9 days after intra-splenic tumor injection but fails to do so by day 13 (87).

Regulation of certain ligand expression by environmental factors may also determine the predominant chemokine signals for MAM accumulation. Although MC38 colon cancer cells release high levels of CCL2 and recruit MAMs via a CCR2-dependent manner to the liver (87), these cells produce CCL20 and recruit TAMs via a CCR6-dependent mechanism to primary tumors established by subcutaneous injection (88). Therefore, environmental factors that regulate the expression of MAM recruiting chemokines seem to be specific for the tumor site (**Figure 3C**). A recent study showed that treatment with an antagonist for insulin like growth factor 1 (IGF-1) receptor reduces expression of CCL2 and suppresses MAM accumulation in MC38 metastatic tumors in the liver (89), suggesting IGF-1 as a key regulator of chemokine induction in the microenvironment of tumor-challenged liver. It is notable that treatment with the IGF-1 receptor antagonist also reduces CCL5 levels in the metastatic liver (89). Although the contribution of CCL5-CCR5 signaling to MAM accumulation or the source of CCL5 was not identified in this model, a recent study using a patient-derived organotypic culture model showed that tumor-infiltrating T cells produce CCL5 (90). This study also showed that CCR5 blockade in the organotypic culture induces tumor cell death, which is abrogated by pharmacological macrophage depletion (90). This suggests that CCL5 induced by a specific tumor microenvironment prevents the MAMs to become tumoricidal cells (**Figure 3C**). In the E0771-LG metastatic breast cancer model, a CCR1 ligand, CCL3, is expressed by MAMs at higher level than other types of tumor-infiltrating immune cells or circulating monocytes, and loss of *Ccl3* reduces MAM accumulation in the metastatic lung. Interestingly, the CCL3 expression in MAMs is significantly suppressed by anti-CCL2 antibody treatment and recombinant CCL2 increases CCL3 secretion from cultured macrophages (42). These results collectively indicate that CCL2 in the metastatic tumor microenvironment triggers a chemokine cascade involving CCL3-CCR1 signaling that promotes retention of MAMs in the metastatic lung (**Figure 3A**). Since pulmonary infection with *Cryptococcus neoformans* induces CCL3 expression via a CCL2 dependent mechanism and blockade of CCL3 reduces accumulation of macrophages in the lung (91), CCL2-induced CCL3 expression may be a common mechanism for macrophage accumulation in the lung under pathological conditions. Several *in vitro* studies show such chemokine-induced chemokine production in monocytes or macrophages. For example, human monocytes cultured with CCL5 increase expression of mRNA encoding CCL2, and CCL3 (92). In human monocyte-derived macrophages, CCL18 promotes secretion of CCL2 and CCL3 as well as CCL22 that is known as a chemoattractant of T_{reg} cells (93). Interestingly, a recent report suggests that CCL3 released from E0771 breast cancer cells increases expression of CCL7, CCL8, CCL11, and CCL12 in the lung (94). Although the cell type that releases these chemokines is not clear in this study, another study using 4T1 breast cancer cells indicates that MAMs in the metastatic lung predominantly express CCL8 and recruit T_{reg} cells that express CCL8 receptor CCR5 (95) (**Figure 3D**). It is thus possible that distinct tumor microenvironments increase the level of chemokines such as CCL2, CCL5 and CCL18 that not only recruit monocytes/macrophages but also induce *de novo* chemokines including CCL3, CCL8, and CCL22 and thereby reinforce the accumulation of metastasis-promoting immune cells such as MAMs and T_{reg} cells (96).

Current results have indicated that spatiotemporal expression of chemokine ligands and receptors (e.g., CCL2-CCR2, CCL3-CCR1/CCR5) regulate recruitment, retention, and the phenotype of MAMs. Since these chemokine signals can be attractive targets to prevent the lethal expansion of metastatic tumors, further studies are required to understand which chemokines are expressed in a certain metastatic tumor microenvironment, how their expression is regulated, and what are their precise roles in MAM functions.

THERAPEUTIC POTENTIAL OF CHEMOKINE ANTAGONISTS TO PREVENT MALIGNANT TUMOR DEVELOPMENT

Different studies have identified several chemokines and chemokine receptors that promote the recruitment of TAMs into primary tumors. These chemokine ligands and receptors are potential targets to prevent dissemination of cancer cells from the primary tumors to the circulation. However, since a substantial proportion of patients (4–61% depending on the tumor sites) has already developed metastatic tumors at diagnosis, and their survival rate is <20% in many cases (1), it is possibly more important to consider blocking the metastatic tumor outgrowth in secondary sites rather than dissemination from the primary site if we are going to improve the outcome of cancer patients. As discussed above, the CCL2-CCR2, CCL3-CCR1, and/or CCL3-CCR5 axes enhance MAM accumulation in the metastatic site, especially the lung, in mouse models of metastatic tumors. In these models, blockade of MAM accumulation via genetic deletion of CCR1, CCR2 or CCR5 significantly reduced metastatic tumor burden (42, 43, 86), suggesting that antagonists for these receptors can be novel therapeutic agents to prevent metastatic tumor development through inhibition of MAM accumulation.

CCR1 and CCR2 are well-known key regulators of immune cell accumulation, and thus several pharmaceutical companies have developed monoclonal antibodies and small molecule inhibitors against the chemokine receptors for human autoimmune diseases such as rheumatoid arthritis and multiple

sclerosis (97). CCR5 antagonists have also been extensively explored since this receptor is known as a co-receptor for human immunodeficiency virus (HIV-1) to enter the cell. Consequently, the US Food and Drug Administration (FDA) has approved some CCR5 antagonists as anti-retroviral agents for HIV (97). Although these chemokine receptor antagonists were originally designed for autoimmune and infectious diseases, several pre-clinical studies have indicated their therapeutic potential for metastatic tumors. For example, a CCR1 antagonist (BL5923) can suppress metastatic tumor growth of colon cancer cells in the liver (98), and another CCR1 antagonist (CCX721) reduces tumor burden and osteolysis in a mouse model of multiple myeloma bone disease (99). In mice that have received the subcutaneous injection of LLC cancer cells, treatment with a CCR2 antagonist (RS504393) inhibits the establishment of lung metastatic foci (100). A recent study also showed that another CCR2 antagonist (RS102896) can suppress liver metastasis of MCF-7 human breast cancer cells induced by estrogen (101). Furthermore, in mice that have developed orthotopic tumors by 4T1 mammary tumor cells, treatment with a CCR5 antagonist (maraviroc) reduces metastatic tumor burden in the lung (95). Although clinical trials of chemokine receptor antagonists in cancer are still limited, several positive results have been reported. For example, an anti-CCR2 antibody (MLN1202) has been tested in a phase II clinical trial for metastatic cancer and showed therapeutic effects in 14 out of 43 patients with bone metastases (ClinicalTrials.gov ID: NCT01015560). A phase I trial of a small molecule inhibitor of CCR2 (CCX872) in combination with chemotherapy (FOLFIRINOX regimen) has also been performed in patients with non-resectable pancreatic cancer (ClinicalTrials.gov ID: NCT02345408) in which overall survival (OS) at 18 months was 29% for CCX872/FOLFIRINOX combination therapy, whereas it was 18.6% for FOLFIRINOX alone (102). A small-scale phase I clinical trial of a CCR5 antagonist (Maraviroc) in patients with metastatic colorectal cancer (ClinicalTrials.gov ID: NCT01736813) has demonstrated that maraviroc treatment in combination with chemotherapy showed an objective partial responses in three out of five patients and prolonged overall survival (90).

Despite these encouraging results, a treatment with single chemokine antagonist will not be enough to suppress metastatic tumor growth since even total deletion of CCR1, CCR2, or CCR5 by knockout cannot achieve complete elimination of metastatic tumors in mouse models (42, 86). One possible reason for this is that multiple chemokine receptors support the accumulation of pro-metastatic macrophages (i.e., TAMs and MAMs). It has been reported that solid tumors express several different chemokine ligands. For example, human colorectal cancer specimens concomitantly express CCL2, CCL4, CXCL1, CXCL5, and CXCL8 at a significantly higher level than normal mucosa (103). Further, human breast cancer tissues can express high levels of CCL2 and CCL5 compared to the adjacent normal breast tissues (77, 104). Human ovarian cancer also expresses high levels of mRNA coding CCL2, CCL4, CCL5, CXCL10, CXCL12, and CXCL16 (105). As discussed above, some receptors for these chemokines such as CCR1, CCR2, CCR3, CCR5, and CXCR4 are reported to enhance the recruitment or retention of pro-metastatic macrophages. Interestingly, several *in vitro* studies suggest that CCR1- or CCR2-induced monocyte migration is synergistically enhanced by activation of CXCR4 (84, 106), suggesting that CCR1 and CCR2 cooperate with other receptors such as CXCR4 in order to promote MAM accumulation and subsequent metastatic tumor growth. Collectively, it is possible that MAMs utilize multiple chemokine signals to accumulate in the tumor microenvironment, which makes it difficult to exclude MAMs from metastatic sites by a single chemokine receptor blockade. Therefore, inhibition of multiple chemokine receptors will be required to exercise full therapeutic effects on the MAM-promoting metastatic tumor development. An attractive approach is a treatment with dual-antagonists that inhibit more than one chemokine receptor. So far, several companies have developed dual-antagonists targeting CCR1/CCR3, CCR2/CXCR2, CCR2/CCR5, and CXCR1/CXCR2, and tested their therapeutic effects in inflammatory diseases (96, 107). For example, in genetically engineered mice that develop muscular dystrophy, treatment with a CCR2/CCR5 dual antagonist cenicriviroc reduces macrophage accumulation in the dystrophic diaphragm and slows the progression of the disease (108). In mouse models of non-alcoholic steatohepatitis (NASH), treatment with cenicriviroc reduces macrophage recruitment and ameliorates hepatic inflammation as well as fibrosis in the liver (109, 110). Since cenicriviroc treatment is well tolerated in patients with hepatic impairment without any obvious side effects (111), a phase II trial has been on going in patients with NASH and liver fibrosis (ClinicalTrials.gov ID: NCT03059446). Although clinical trials in cancer patients have not yet been reported, a mouse model where MC38 colon cancer cells were grown intramuscularly has shown that the cenicriviroc treatment can suppress TAM accumulation in the tumor and enhance therapeutic efficacy of local irradiation in suppressing tumor growth (112). These reports suggest that dual chemokine receptor antagonists are attractive drugs for cancer treatment. However, clinical application of dual-antagonists for metastatic diseases requires further identification of chemokine signal combinations that concomitantly promote MAM accumulation in metastatic tumors under different condition (e.g., tumor origin, metastatic site, and progression stage). In addition to proper target receptor selection, it is also important to determine the functional doses of antagonists that are sufficient to provide the continuous receptor coverage *in vivo* (15, 113).

Insufficiency of a single chemokine blockade in metastasis suppression can also be due to a lack of direct cytotoxic effects on cancer cells. As described above, MAMs recruited to the metastatic site can promote tumor cell survival (41). It is also reported that malignant tumor cells express chemokine receptors such as CCR7, CXCR1, and CXCR4 that can increase their invasiveness as well as survival (114). However, it is unlikely that blockade of MAM accumulation by chemokine antagonists can directly induce tumor cell death, and thus macrophage targeting should be combined with another therapeutic modality such as chemotherapy and/or immunotherapy that directly kills the cancer cells. In line with this notion, several animal studies show that blockade of myeloid cell accumulation via chemokine receptor inhibition exerts synergistic therapeutic effects when

combined with cytotoxic drug treatments. For example, reduced monocyte accumulation by genetic deletion of host CCR2 expression enhances the effect of doxorubicin or cisplatin treatment on the relapse of mammary tumors in the PyMT mice (76). Furthermore, a CXCR4 antagonist (AMD3100) prevents macrophage accumulation and delays tumor relapse after cyclophosphamide treatment in subcutaneously transplanted lung cancer and in orthotopic mammary cancers (81). In primary tumors developed by orthotopically injected pancreatic cancer cells, reduced macrophage accumulation by a CCR2 antagonist (PF-04136309) enhances the efficacy of gemcitabine in suppressing the tumor growth (115). Consistent with this pre-clinical study, a recent clinical trial indicates that treatment of pancreatic cancer patients with a CCR2 antagonist (CCX872) in combination with FOLFIRINOX regimen (i.e., a combination of five chemotherapy agents) improve overall survival (102). These results suggest that elimination of macrophages via chemokine receptor antagonists in combination with direct cancer cell killing by chemotherapy is an effective therapeutic strategy to prevent malignant tumor development (**Figure 4**). However, macrophage blockade may not always enhance chemotherapy efficacy. In a mouse model of pancreatic cancer, treatment with a CD40 agonist increases sensitivity of the tumor to gemcitabine via depletion of fibrosis by monocytes/macrophages. Mechanistically, a CD40 agonist induces systemic release of IFNγ that prompts classical monocytes to express matrix metalloproteinase (MMP) and recruit these anti-fibrotic monocytes/macrophages to the tumor via CCL2 (116). Therefore, in such a case, blockade of TAM accumulation by CCR2 antagonists may reduce, instead of enhance, the efficacy of gemcitabine treatment. These results suggest that a certain therapeutic treatment affects features of macrophages in the tumors, and thus application of chemokine receptor antagonists to other therapeutic modalities should be carefully evaluated.

Blockade of TAM/MAM accumulation combined with immunotherapies is another attractive therapeutic strategy to prevent malignant tumor development (117). Since cytotoxic lymphocytes (CTLs) such as $CD8^+$ T and NK cells can eliminate cancer cells if they exert full cytotoxicity, several strategies to utilize their tumor killing ability have been developed. These immunotherapies such as immune checkpoint inhibitors and adoptive CTL transfer have been tested in clinical trials and demonstrated significant therapeutic effects on lymphoma and some solid tumors such as melanoma and lung cancer. However, their efficacy is so far limited in a certain fraction of patients and tumor types due to tumor-cell-intrinsic mechanisms such as the impaired antigen presentation and/or tumor-cell-extrinsic mechanisms including the accumulation of immunosuppressive cells. As previously described, TAMs/MAMs are reported to suppress functions of $CD8^+$ T and NK cells *in vitro* and thus considered as attractive targets to improve efficacy of immunotherapies. In a mouse model of pancreatic cancer, treatment with anti-PD1, and anti-CTLA4 antibodies in combination with TAM depletion by a CSF1 receptor antagonist (PLX3397) blocks tumor expansion more efficiently compared with a single treatment with anti-PD1/anti-CTLA4 or PLX3397 (118). It is also reported that genetic depletion of $CCR2^+$ classical monocytes (i.e., TAM progenitors) enhances accumulation of adoptively transferred $CD8^+$ T cells in the primary tumor, and thereby augments the therapeutic effect of the adoptive T cell transfer therapy on the tumor growth in a melanoma model (119). These results suggest that elimination of macrophages from the tumor microenvironment can improve efficacy of checkpoint inhibitors or adoptive CTL transfer. Therefore TAM/MAM blockade by chemokine receptor antagonists combined with immunotherapies can be a novel therapy for malignant tumors. However, target chemokine receptors should be carefully selected since recruitment of $CD8^+$ T or NK cells in the tumor sites is also regulated by chemokine signals. It has been reported that $CD8^+$ T cells utilize several chemokine receptors such as CCR4, CCR5, CCR7, CCR9, CCR10, and CXCR3 for their trafficking depending on their activation status (120). NK cells also express several chemokine receptors including CCR1, CCR2, CCR5, CCR7, CXCR1, CXCR3, CXCR4, and CXCR6 (121), suggesting that antagonists for these receptors have a potential risk to reduce the efficacy of immunotherapies. However, a recent study using a B16 mouse melanoma model demonstrated that neither *Ccr2* nor *Ccr5* deficiency affect tumor infiltration of adoptively transferred $CD8^+$ T cells, despite the fact that the

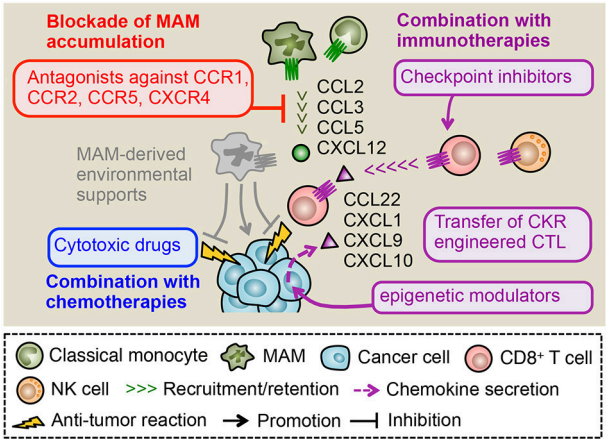

FIGURE 4 | Therapeutic potential of chemokine receptor antagonists. In metastatic tumors, CCL2, CCL3, and CCL5 promote the MAM accumulation and subsequent metastasis formation. Primary tumor models also indicate a pivotal role of CXCL12 in the accumulation of macrophages whereas its role in the metastatic tumor needs to be clarified. Antagonists against receptors for these ligands (i.e., CCR1, CCR2, CCR5, and CXCR4) can reduce the number of MAMs and thus dislodge MAM-derived environmental supports for metastasized cancer cells. Several studies indicate that the MAM targeting by chemokine receptor antagonists synergistically enhance therapeutic effects of chemotherapy and immunotherapy such as immune checkpoint inhibitors and adoptive transfer of cytotoxic lymphocytes (CTL). The tumor microenvironment includes other chemokines such as CXCL9 and CXCL10 that recruit CTLs to the tumor. In some cases, epigenetic modulators can prompt cancer cells to produce these chemokines, which may enhance efficacy of MAM targeting combined with immunotherapies. Genetic manipulation of CTLs to express chemokine receptors for ligands abundantly included in tumors (e.g., CCL22, CXCL1) can also improve the efficacy of MAM targeting combined with CTL transfer therapy.

tumor expresses high levels of CCL2 and CCL5 (ligands for CCR2 and CCR5, respectively). In contrast, *Cxcr3* deficiency significantly reduces the recruitment of $CD8^+$ T cells in the B16 tumors (122). The loss of *Cxcr3* also significantly reduces NK cell accumulation in metastatic tumors established by B16 cells (123). These results suggest that activated $CD8^+$ T and NK cells may predominantly utilize CXCR3 signals for their tumor infiltration. It is thus likely that blockade of MAM-recruiting chemokine receptors such as CCR1, CCR2, CCR5 has minimum effects on the tumor infiltration of $CD8^+$ T and NK cells, which is indispensable for immunotherapy efficacy. In line with this notion, the combined treatment with a CCR1 antagonist and anti-PDL1 antibody significantly reduces tumor burden compared to either of single treatments in a mouse model of breast cancer (124). It is also reported that treatment with a CCR2 antagonist in combination with anti-PD1 antibody suppresses tumor growth in a mouse model of pancreatic cancer, whereas single treatment with anti-PD1 antibody is not effective (125). These pre-clinical data suggest that blockade of macrophage-recruiting chemokine receptors combined with immunotherapy is an attractive approach. However, this combination therapy may not be effective in a certain fraction of solid tumors that do not express sufficient levels of CXCR3 ligands (CXCL9 and CXCL10) and fail to recruit tumoricidal $CD8^+$ T cells (126–128). A recent study using mouse models of ovarian cancer has shown that the reduced production of CXCL9 and CXCL10 from cancer cells is caused by enhancer of zeste homolog 2 (EZH2) mediated histone modification and DNA methyltransferase 1 (DNMT1) mediated DNA methylation of the chemokine genes (129). Interestingly, this study also demonstrates that treatment of tumor-bearing mice with epigenetic modulators, i.e., combination of EZH2 and DNMT1 inhibitors, increases tumor expression of CXCL9/CXCL10 and improves therapeutic efficacy of anti-PDL1 antibody and adoptive transfer of $CD8^+$ T cells by enhancing T cell migration toward tumors. Given the non-redundant requirement of CXCR3 signaling for tumoricidal T cell trafficking to the tumor (122), these epigenetic modulators can enhance efficacy of combination therapy consisting of TAM/MAM blockade and checkpoint inhibitors or CTL transfer. Another attractive approach to enhance efficacy of the combination therapy is engineering of CTLs to express receptors for chemokine ligands that are abundant in the tumor microenvironment. A recent study demonstrated that genetic engineering of $CD8^+$ T cells with CCR4 enhances their migration toward CCL22 secreted from Panc02 pancreatic cancer cells *in vitro*, and that adoptive transfer of the CCR4-engineered T cells into the Panc02 tumor-bearing mice eradicate the established tumor more efficiently than the infusion of non-engineered T cells (130). It is also reported that introduction of CXCR2 in tumor antigen specific $CD8^+$ T cells enhances their infiltration into the tumor that expresses the ligand CXCL1 and thereby reduces tumor growth in a mouse model of colon cancer (131). Collectively, TAM/MAM blockade by chemokine receptor antagonists in combination with immunotherapies seems to be a promising strategy to prevent the progression of solid tumors (**Figure 4**).

FUTURE PERSPECTIVE

Different studies have shown that accumulation of TAMs/MAMs play pivotal roles in the establishment of lethal metastatic tumors. As summarized in this review, several mouse models of metastatic tumors have identified chemokine signals that promote TAM/MAM accumulation and thus can be novel therapeutic targets to block the macrophage-promoting metastasis formation. Pre-clinical studies also suggest that TAM targeting by chemokine receptor antagonists, combined with immunotherapy has the ability to exert synergistic therapeutic effects. Further, this can be enhanced by promoting tumor infiltration of effector CTLs via chemokine signal modification. Further investigation of the synergistic effects of TAM/MAM targeting chemokine antagonists on the CTL recruitment and immunotherapy efficacy will lead to the establishment of effective therapies for metastatic disease. Since predominant chemokine signals utilized for macrophage accumulation can be changed by the tumor microenvironment, a database showing chemokine expression profiles of solid tumors with different subtypes, stages, and treatment history will be helpful to investigate the optimal combination of target chemokine receptors. Identification of environmental factors that induce macrophage-recruiting chemokines is also important since these factors can be alternative therapeutic targets. Another aspect to be considered is that tumor metastasis is supported not only by MAMs but also by other immune cell types such as T_{reg} cells and MDSCs (5). As described above, TAMs can recruit T_{reg} cells to the primary tumors via secretion of CCL20 or CCL22 (39, 40). It is also reported that T_{reg} cell recruitment to primary mammary tumors in mice is promoted by a CCL5-mediated mechanism (132). Several studies have reported that accumulation of MDSCs in the primary tumors is regulated by CXCL5, CXCL8, and CXCL12 depending on the models (133–135). However, the involvement of these chemokine signals in the accumulation T_{reg} cells and MDSCs in the metastatic site has not yet been investigated. A recent study indicates that monocytic MDSCs recruited to the pulmonary metastasis foci originate from circulating classical monocytes (44) that are recruited by the CCL2-CCR2 axis (43), which suggests a significant contribution of CCL2 to MDSC recruitment to the metastatic site. Although their roles at metastatic sites remain to be identified, T_{reg} cells, and MDSCs in the primary tumors are known to suppress CTL functions and are considered as targets to improve immunotherapy. Therefore, deciphering the chemokine signals that recruit T_{reg} cell and MDSC to metastatic tumors, as well as their correlations with MAM-recruiting chemokines will be important to determine effective chemokine receptor antagonists to combine with immunotherapies. Results from these basic studies will lead to novel therapeutic strategies, i.e., TAM/MAM blockade in combination with chemo-/immunotherapies by targeting chemokine signals. Further studies in preclinical models and patient samples are required for the clinical application of combination therapies to metastatic tumors to be realized.

AUTHOR CONTRIBUTIONS

TK writing the manuscript and preparing the figures. TK and DA review and revision of the manuscript and figures.

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REVIEW
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Chemokine-Induced Macrophage Polarization in Inflammatory Conditions

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Macrophages represent a heterogeneous cell population and are known to display a remarkable plasticity. In response to distinct micro-environmental stimuli, e.g., tumor stroma vs. infected tissue, they polarize into different cell subtypes. Originally, two subpopulations were defined: classically activated macrophages or M1, and alternatively activated macrophages or M2. Nowadays, the M1/M2 classification is considered as an oversimplified approach that does not adequately cover the total spectrum of macrophage phenotypes observed *in vivo*. Especially in pathological circumstances, macrophages behave as plastic cells modifying their expression and transcription profile along a continuous spectrum with M1 and M2 phenotypes as extremes. Here, we focus on the effect of chemokines on macrophage differentiation and polarization in physiological and pathological conditions. In particular, we discuss chemokine-induced macrophage polarization in inflammatory diseases, including obesity, cancer, and atherosclerosis.

Keywords: macrophage polarization, chemokines, tumor-associated macrophage, leukocyte migration, inflammation and cancer

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INTRODUCTION

Monocytes arise in the bone marrow from hematopoietic stem cells (HSCs) and develop through a series of sequential differentiation stages. Common myeloid progenitor cells develop into granulocyte/macrophage colony forming units (GM-CFU), which in turn can commit to the macrophage colony-forming unit (M-CFU) or the granulocyte colony-forming unit (G-CFU). The M-CFU differentiates sequentially into monoblasts and promonocytes, which leave the bone marrow and enter the bloodstream, where they differentiate into mature monocytes (1). Mature monocytes represent about 10% of the leukocyte population in human peripheral blood and can circulate in the blood stream for up to 1–2 days before they undergo apoptosis. Alternatively, monocytes can migrate into the tissues and differentiate into specific macrophages (2). The major driver for the homeostatic control of monocyte/macrophage development is macrophage colony-stimulating factor (M-CSF), present in the blood circulation and produced by stromal cells in tissues (3–5). In inflammatory conditions, also other cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and the chemokine CXCL4 influence the differentiation and/or survival of mononuclear phagocytes (6–8).

In contrast to the classical model of macrophage development, where macrophages differentiate from circulating monocytes as described above, recent studies provided evidence that tissue-resident macrophages arise from yolk sac or fetal liver-derived progenitors (9). These

tissue resident macrophages appear to have stem cell-like capacities as they persist independently of monocytes by self-renewal *in situ* (10). One of the major hallmarks of macrophages is their heterogeneity, which is reflected by their specialized function in a particular microenvironment. According to their tissue location, macrophages can take different names including microglia [central nervous system (CNS)], Kupffer cells (liver), alveolar macrophages (lung), osteoclasts (bone), histiocytes (spleen and connective tissue), Langerhans cells (skin), and tissue macrophages in the gut (11). Resident macrophages promote tissue homeostasis, whereas monocyte-derived macrophages primarily assist in host-defense. Moreover, macrophages recruited during and after embryogenesis co-exist in different organs (10, 12).

Besides their heterogeneity, macrophages are known to display remarkable plasticity. In response to different micro-environmental stimuli, a fully differentiated macrophage can adopt a polarized phenotype with specific functional characteristics. Traditionally, macrophages are subdivided into two subpopulations: the classically activated or M1 macrophages and the alternatively activated or M2 macrophages (13). M1 macrophages can be induced by the Th1 cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ and bacterial components such as lipopolysaccharide (LPS). Activated M1 macrophages phagocytose and destroy microbes, eliminate tumor cells and present antigens to T cells to evoke an adaptive immune response. As such, they play an important role in protection against pathogens. The pro-inflammatory phenotype is characterized by the increased production of reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS), which is essential for bacterial killing (14). In response to inflammatory mediators, M1 macrophages express the inducible nitric oxide synthase (iNOS), which uses L-arginine as a substrate to produce nitric oxide (NO) (15). Furthermore, classically activated macrophages release high levels of pro-inflammatory cytokines such as TNF-α, interleukin-6 (IL-6) and IL-1β to deal with infections and thereby promote Th1 responses (16).

M2 activation occurs in response to stimulation with IL-4, IL-10, and IL-13. These macrophages display high surface levels of scavenger, mannose and galactose type receptors involved in debris clearance. Furthermore, they show a more immunosuppressive phenotype characterized by decreased antigen presentation to T cells and production of cytokines that stimulate a Th2 response. In contrast to M1 macrophages, M2 macrophages constitutively express the enzyme Arginase 1 (ARG1), which hydrolyzes L-arginine to L-ornithine (13). L-ornithine is the main precursor for polyamines, essential for cell survival. Furthermore, L-ornithine can also be used as a building block to make proline and hydroxyproline, essential amino acids for the production of collagen, a crucial protein in tissue damage repair (17). As such, these macrophages are involved in long-term tissue repair, promote tumor growth and exert antiparasitic effects (18).

Nowadays the M1/M2 classification is considered as an oversimplified approach that does not fully cover the total spectrum of *in vivo* macrophage phenotypes. Especially, in pathological circumstances macrophages behave as plastic cells modifying in space and time their expression and transcription profile along a continuous spectrum, having M1 and M2 macrophage phenotypes as extremes (19, 20).

The interaction of chemokine receptors on circulating cells with their ligands enables the selective tissue-specific recruitment of subsets of circulating cells such as monocytes. Chemokines are a family of low molecular weight, secreted proteins with a prominent role in leukocyte activation and chemotaxis. Based on the NH_2-terminal motif of two conserved cysteine residues, chemokines can be classified into 4 subfamilies: C, CC, CXC, and CX_3C chemokines. Chemokines signal via G protein-coupled receptors (GPCRs), which are named XCR, CCR, CXCR, CX_3CR according to the chemokine nomenclature (21). Additionally, chemokines can bind with high affinity to atypical chemokine receptors (ACKRs), a subgroup of seven-transmembrane receptors highly related to the classical GPCRs. Since these ACKRs lack or have a modified canonical DRYLAIV motif, activation of ACKRs does not lead to typical GPCR-mediated signaling and chemotactic functions (22).

THE EFFECT OF CHEMOKINES ON MACROPHAGE DIFFERENTIATION AND POLARIZATION IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Neurological Diseases

Microglia, the resident, long-living macrophages in the central nervous system (CNS), act as the major inflammatory cell type in the brain and similar to peripheral macrophages they respond to pathogens and injury (23). Under physiological conditions, microglia are in a "quiescent" state or have a non-activated phenotype (24). Butofsky et al. demonstrated that this "resting" cell' phenotype is different from M1 or M2 microglia and expresses genes associated with neuronal development (25). This particular phenotype was found to be important for synaptic growth, maintenance, and neuronal growth. Furthermore, the "quiescent" state enables the intimate connection between neurons and microglial cells, which is tightly controlled by the CX_3CL1-CX_3CR1 axis (26). CX_3CL1/fractalkine is the only member of the CX_3C chemokine subfamily and differs from most other chemokines, as it can exist as a membrane-associated molecule with the chemokine motif being attached to a long mucin stalk. Alternatively, CX_3CL1 is secreted as a soluble variant (27). CX_3CL1 is expressed on healthy neurons, whereas the transmembrane protein receptor CX_3CR1 is present on microglia (23, 28, 29).

The CX_3CL1-CX_3CR1 axis is an important neuroimmune interaction in the CNS and has been implicated in many neurophysiological and neuropathological conditions (**Figure 1**). For instance, in animal models of Parkinson's disease and amyotrophic lateral sclerosis (ALS), loss of CX_3CR1 increased neuronal cell death (30). Using a murine model of diabetic retinopathy, Cardona et al. showed that in the absence of CX_3CR1 the microglial response is dysregulated and associated with increased IL-1β cytokine release (**Figure 1B**) (31). Additionally, Mattison et al. found that CX_3CL1 suppressed

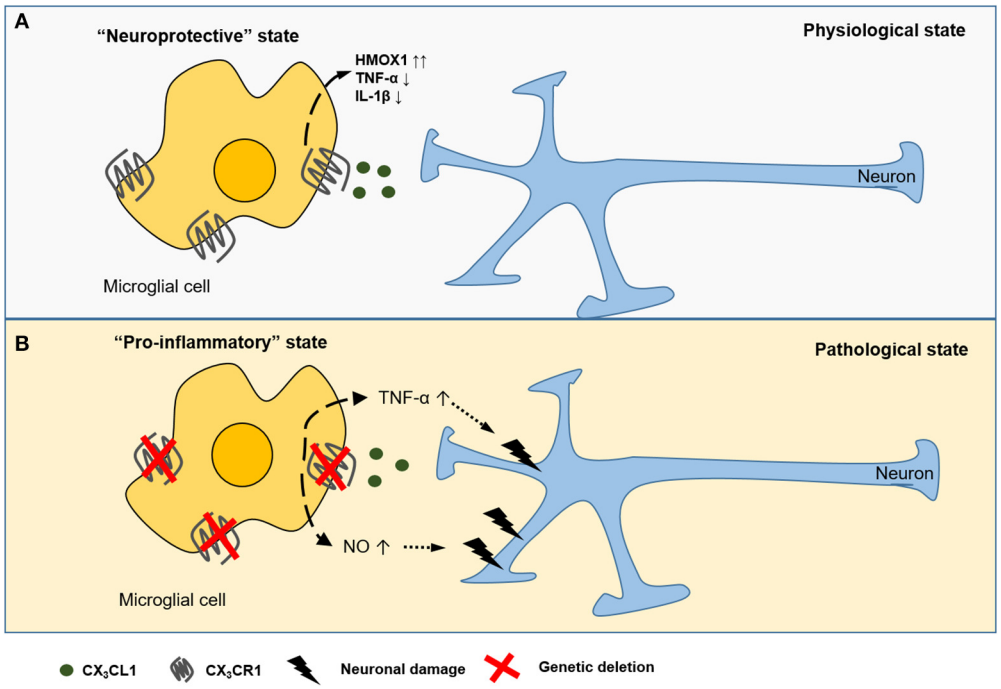

FIGURE 1 | CX_3CL1-CX_3CR1 interaction between neurons and microglial cells in the CNS. CX_3CL1 is released from the neurons and interacts with the CX_3CR1 receptor expressed on CNS microglia. CX_3CL1 signaling induces (dashed arrow) a neuroprotective state **(A)**, characterized by the suppressed release of pro-inflammatory cytokines (TNF-α, IL-1β) and upregulation of heme oxygenese 1 (HMOX1). In several murine models of neurodegenerative diseases, genetic deficiency of CX_3CR1 is associated with potentially detrimental secretion of pro-inflammatory cytokines and reactive nitrogen species (NO) causing (dotted arrow) neurotoxicity **(B)**.

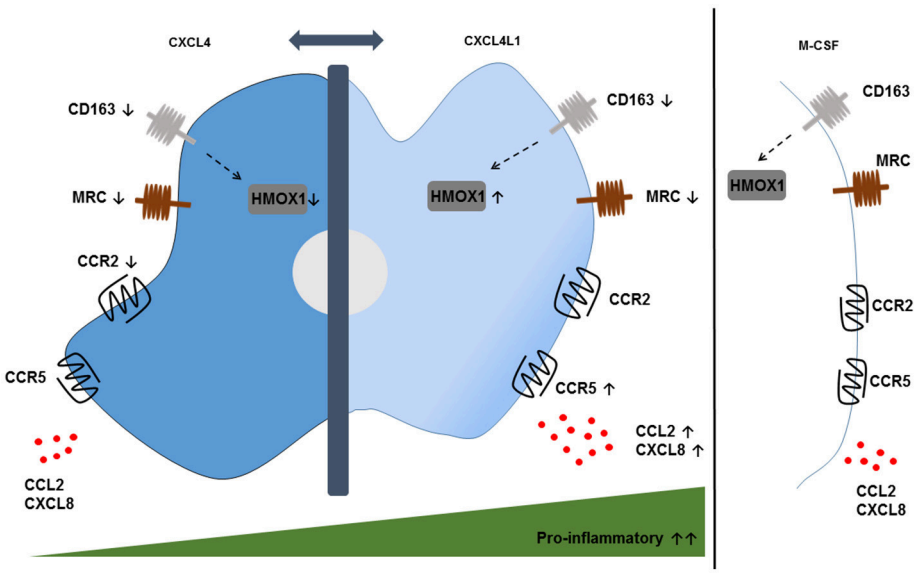

FIGURE 2 | Phenotypic features of CXCL4- and CXCL4L1-induced macrophages. CXCL4-induced macrophages display a pro-atherogenic phenotype, characterized by the downregulation of the hemoglobin-haptoglobin scavenger receptor CD163 and the consequent downregulation of the HMOX1 enzyme compared to M-CSF-treated monocytes. Remarkably, the downregulation of HMOX1 is not observed in CXCL4L1-induced macrophages, which also show reduced expression of CD163. Both phenotypes show a downregulation of the mannose receptor (MRC) CD206. The expression of the chemokine receptors CCR2 and CCR5 and the secretion of pro-inflammatory chemokines CXCL8 and CCL2 are higher on CXCL4L1-treated monocytes compared to CXCL4-stimulated monocytes, thereby indicating more pro-inflammatory characteristics for CXCL4L1- than CXCL4-stimulated monocytes.

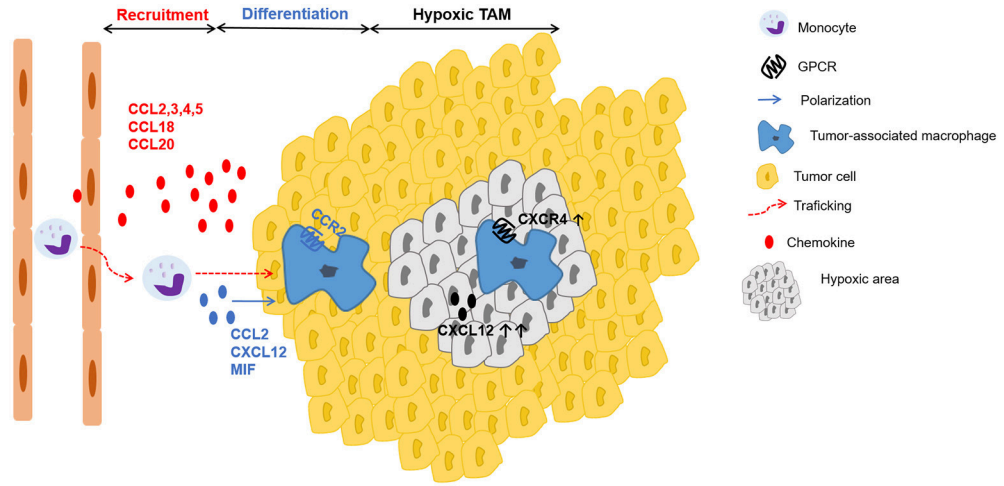

FIGURE 3 | Schematic representation of chemokines involved in recruitment, differentiation and positioning of TAMs. Tumor-derived factors such as the chemokines CCL2, CCL3, CCL4, CCL5, CCL18, CCL20 actively recruit (red arrow) monocytes to the tumor, where they differentiate into tumor-associated macrophages (TAMs). In addition to several growth factors, a particular role in TAM polarization (blue arrow) has been described for the chemokines CCL2, CXCL12 and the chemokine-like protein MIF. In hypoxic areas, higher amounts of CXCL12 and increased expression of CXCR4 on macrophages enhance migration to and retention in these particular sites with low oxygen tension.

the release of pro-inflammatory and neurotoxic factors such as TNF-α and NO in activated microglia during neuroinflammation (**Figure 1B**) (32). Controlling neuroinflammation via CX_3CR1 signaling was particularly beneficial in the pathogenesis of Alzheimer's disease (33). Furthermore, CX_3CL1 promotes microglial phagocytosis of neuronal debris and increases the expression of heme oxygenase 1 (HMOX1), resulting in an anti-oxidant effect, which indirectly promotes neuronal survival (34). Conversely, some studies showed a neurotoxic role for CX_3CL1 in $CX_3CR1^{-/-}$ mice models for Alzheimer's disease (35) and stroke (36). Fuhrmann et al. also reported that neuronal loss neuronal loss in a model of Alzheimer's disease was prevented in CX_3CR1 knock out mice (37).

The atypical chemokine receptor CCRL2 was identified as an important regulator of microglial activation and polarization in experimental autoimmune encephalomyelitis (EAE) (38). Similar to ACKRs, CCRL2 lacks conventional GPCR signaling and chemotactic activity (39). More specifically, it was found that during the chronic disease phase microglia in CCRL2 KO mice develop a profound M1 phenotype compared to wild type (WT) mice after induction of EAE (38). These results highlight a potential role of CCRL2 in EAE-associated inflammatory responses and as such, provide a new potential target to control neuroinflammation.

Finally, using a neuron/microglia co-culture system, Yang et al. found that CCL2/MCP-1 (40) was able to activate microglia and stimulated production of pro-inflammatory cytokines such as TNF-α and IL-1β (41).

Fibrosis

Upon infection, activated macrophages use a set of innate immune defense strategies such as phagocytosis, release of proteases and production of antimicrobial mediators, such as reactive oxygen and nitrogen species. An important side effect of this efficient inflammatory response is partial tissue destruction, which is normally followed by a repair response to regenerate the tissue (42). However, when this repair phase is persistent, it leads to fibrosis or so-called scarring of the tissue, which is defined by the accumulation of excess extracellular matrix components. In the end, this causes progressive loss of function of the affected organ(s) (43, 44). Alternatively activated (M2) macrophages are known to play an important role in wound healing and acquire a pro-fibrotic phenotype (45, 46). Since this phenotype is observed during the peak of the fibrotic immune response, it is suggested that such M2 macrophages are important inducers and regulators of fibrosis (44). For instance, by producing transforming growth factor-β1 (TGF-β1), M2 macrophages directly stimulate collagen production in myofibroblasts (47, 48) and enhance the expression of tissue inhibitors of metalloproteinases (TIMPs) that block the degradation of extracellular matrix (ECM) (48). Additionally, M2-derived chemokines play a role in fibrosis. For instance, CCL18/PARC is pro-fibrotic by promoting collagen production in lung fibroblasts (49–51). Increased collagen deposition, in turn, can enhance CCL18 production in alveolar macrophages, thereby suggesting a positive feedback loop between alveolar macrophages and fibroblasts (50). In idiopathic pulmonary fibrosis (IPF), one of the most common types of interstitial lung disease, CCL18 levels correlated with severity of fibrosis (52). More recently, CCL18 was identified as a marker for early identification of progressive interstitial lung disease in systemic sclerosis (SS) (53). Pechkovsky et al. showed that the Th2 cytokines IL-4 and IL-10 induce M2 polarization of alveolar macrophages (54). Interestingly, IL-10 enhanced the IL-4-induced CCL18 expression (54).

Besides CCL18, also CCL2 directly mediates a pro-fibrotic effect on fibroblasts by affecting TGF-β signaling, which in

turn stimulates collagen production (55). Mice lacking CCR2, the cognate receptor for CCL2, showed reduced infiltration of inflammatory macrophages in two models of hepatic fibrosis (56, 57). These CCR2$^{-/-}$ mice also developed less severe pulmonary fibrosis (58). Macrophages derived from CCR2 KO mice showed reduced production of matrix metalloproteinase (MMP)-2 and MMP-9 (59). Finally, the CCL2-CCR2 axis in macrophages has also been found to be important in renal fibrosis, where mononuclear cell infiltration and expression of chemokine receptors CCR1, CCR2, and CCR5 was enhanced in a spontaneous model of lupus nephritis (60).

Interestingly, in a commonly used model of bleomycin-induced lung fibrosis, CCR4$^{-/-}$ mice showed a decreased inflammatory and fibrotic response compared to WT mice. Further analysis revealed that CCR4 KO alveolar and bone marrow-derived macrophages exhibited a more pronounced M2 activation state, as evidenced by increased expression of the typical M2 markers ARG1 and "found in inflammatory zone 1" (FIZZ1). Further experiments showed that the CCR4 ligand CCL17/TARC (61) plays a role in CCR4-dependent M1 activation leading to iNOS induction and oxidative injury, thereby affecting the development of bleomycin-induced pulmonary fibrosis (62). Additionally, FIZZ1 activates fibroblasts and induces myofibroblast differentiation in bleomycin-induced pulmonary fibrosis (63, 64). Chvatchko et al. reported that CCR4$^{-/-}$ mice were more resistant to the effects of LPS compared to CCR4 WT mice (65). Further analysis revealed that peritoneal macrophages from CCR4 deficient mice possess an altered phenotype, more resembling M2 macrophages with elevated secretion of type 2 cytokines/chemokines and FIZZ1 protein (66). This study underscores the possible role of CCR4 in M1 activation.

In two different murine models of liver fibrosis, Heymann et al. demonstrated a protective role for the CCR8 receptor. Interestingly, hepatic macrophages from CCR8 KO mice showed an altered phenotype with more pronounced dendritic cell-like characteristics and enhanced CCL3 secretion (67).

Macrophage Polarization by Chemokines in Metabolic Disorders

Nowadays it is generally accepted that the immune system and metabolism are tightly connected and recent studies have demonstrated that macrophages, in particular, are critical effector cells in metabolic inflammation (68). Resident macrophages in the adipose tissue of lean mice constitute ∼10–15% of the total cell population. These adipose tissue macrophages (ATMs) express predominantly M2 characteristics and were shown to be critical for maintaining insulin sensitivity in adipocytes (69, 70). Conversely, in obesity, a state of low-grade systemic inflammation (71), adipocytes secrete pro-inflammatory mediators, which recruit monocytes into the adipose tissue mainly via the CCL2-CCR2 and CCL5-CCR5 axis (72–74). During obesity the number of macrophages in white adipose tissue increases fourfold (69) and macrophages acquire an M1 phenotype that contributes to the pro-inflammatory environment (75). Via secretion of pro-inflammatory cytokines, M1 ATMs contribute to insulin resistance by counteracting the insulin sensitizing action of the adipokines adiponectin and leptin (69, 76, 77). More recently, it has been shown that macrophage polarization in obesity can also be modulated by chemokines and their receptors. Kitade et al. demonstrated that inactivation of CCR5 not only resulted in a reduced number of ATMs, but the recruited ATMs switched toward an M2 phenotype (73). Additionally, obesity-induced insulin resistance was attenuated in obese CCR5$^{-/-}$ mice (73). The question how CCR5 regulates M2 polarization is still unanswered. Obese mice with a genetic deficiency in CCR2 showed a reduced number of ATMs combined with a decreased expression of pro-inflammatory genes, compared to matched WT mice (72). Besides the CCR2 and CCR5 ligands, a recent study showed that during obesity CXCL12 recruits macrophages via CXCR4 to the adipose tissue (78). Moreover, CXCL12-CXCR4 signaling induced M1 macrophage accumulation and blocking this signaling diminished secretion of pro-inflammatory cytokines and improved insulin resistance (79).

The recruitment of macrophages, which stimulate the development of insulin resistance in obesity, is also critical in associated metabolic comorbidities such as nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). NAFLD is characterized by excessive fat accumulation in the form of intrahepatic triglycerides in the liver. NAFLD exhibits as a spectrum ranging from steatosis of the liver to a more necro-inflammatory form, NASH, which may develop into hepatic fibrosis, cirrhosis, or hepatic carcinoma (80). In the liver, macrophages consist of distinct populations, namely the resident, self-renewing Kuppfer cells and the inflammatory monocyte-derived macrophages (81–83). Kuppfer cells line the liver sinusoids and are involved in cholesterol metabolism by taking up and clearing modified low-density lipoprotein (LDL) and bacterial endotoxins through their scavenger receptors (84).

In line with the improved insulin resistance in CCR2$^{-/-}$ obese mice, also hepatic steatosis was ameliorated (72). Besides CCR2, Karlmark et al. found that the CX_3CL1-CX_3CR1 axis is involved in the differentiation and survival of intrahepatic monocytes (85). The CX_3CR1-mediated survival depends on the activation of the anti-apoptotic protein BCL2. Furthermore, in the absence of CX_3CR1, hepatic macrophages showed a more pro-inflammatory phenotype characterized by increased TNF-α and iNOS production. These *in vivo* findings confirm earlier published data on elevated *Tnfα* expression and reduced *ARG1* expression in CX_3CR1-deficient macrophages in a carbon tetrachloride (CCl_4)-induced NAFLD mouse model (86). The increased pro-inflammatory response of liver macrophages was associated with enhanced liver fibrosis (85). This latter observation suggests that activation of the CX_3CL1-CX_3CR1 axis can work as an antifibrotic liver therapy.

Macrophage Polarization in Cardio-Vascular Diseases

Cardiovascular disease (CVD) is the most common cause of mortality worldwide and accounts for 45% of all deaths in Europe (87). Atherosclerosis, an arterial narrowing due

to plaque formation, is most often the underlying cause of myocardial infarction (88). The starting point of this pathology is the accumulation of lipoprotein particles in the intimal layer of the blood vessel. These lesions are mostly found at arterial branching points and bends, which are especially prone for local endothelial cell dysfunction. The stored lipoproteins are modified by several mechanisms such as oxidation, enzymatic processing, desialylation and aggregation, become pro-inflammatory and activate surrounding endothelial cells. Activated endothelial cells, in turn, release chemokines which recruit monocytes into the intimal and subintimal space of the artery where they differentiate into macrophages (89). These macrophages actively ingest cholesteryl ester-rich lipoproteins and eventually become "foam cells." Although the uptake of lipoproteins by macrophages seems to be beneficial, these "foam cells" aggravate the disease through their secretion of pro-inflammatory mediators including cytokines and ROS and finally through their eventual death by necrosis or apoptosis. These latter processes result in the release of lipids and the formation of a pro-thrombotic core, which is a key-component of unstable plaques. Rupture of these plaques leads to the initiation of thrombosis, which limits or even blocks the flow of oxygen-rich blood to organs and other parts of the body (90, 91).

The first chemokine implicated in atherosclerosis was CCL2, which is normally not found in the blood vessel wall, but is induced in the early phase of atherosclerosis (92–94). Evidence for a prominent role of the CCL2-CCR2 axis came from a study by Boring et al. who reported that $CCR2^{-/-}$ mice exhibit severely reduced atherosclerotic lesions (95). Later on, CXCR2, CX_3CR1 and CCR1 have been implicated in monocyte/macrophage accumulation in atherosclerotic plaques (96, 97).

Relatively large numbers of pro-inflammatory macrophages were found in plaques and M1 macrophages are associated with unstable plaques (98, 99). M2 macrophages have only been detected later on and are more common in asymptomatic lesions and the stable zones of plaques (100). In addition to M1 and M2 macrophages, atherosclerotic plaques also contain specific macrophage subtypes, which are different from the phenotypes suggested by the classical activation model. For instance, in mice, oxidized lipids induce a distinct proatherogenic phenotype, referred to as Mox macrophages (101). These are characterized by reduced phagocytic and chemotactic capacities compared to M1 and M2 macrophages (101). So far, this phenotype is only observed in mice, whether Mox macrophages are also present in human lesions remains to be investigated. Upon intraplaque hemorrhage, due to rupture of invaded microvessels in the plaque, red blood cells lyse quickly and release hemoglobin and free heme. These heme products can directly polarize macrophages toward the Mhem or M(Hb) phenotype. Functionally, these subtypes are resistant to lipid accumulation and foam cell formation (102). Macrophage polarization to the M(Hb) phenotype occurs via exposure to the hemoglobin-haptoglobin complex (102, 103). This M(Hb) subset expresses high levels of the scavenger receptors CD163 (the hemoglobin-haptoglobin complex receptor) and CD206 (the mannose receptor) and is resistant to cholesterol accumulation because of the increased expression of the cholesterol efflux receptors ABCA1 and ABCG1 (104). Heme induces atheroprotective Mhem macrophages, which have high levels of HMOX1 (105) and are able to engulf extravasated erythrocytes (erythrophagocytosis) (106).

Besides lipids and their derivatives, heme products and also chemokines and growth factors present in atherosclerotic lesions can contribute to macrophage phenotype determination. During the early atherogenic phase, platelets can adhere and act as a rich source of chemokines. The platelet-derived chemokine CXCL4/PF-4 (107), similar to M-CSF, has been shown to prevent monocyte apoptosis and to promote the differentiation into macrophages *in vitro* (8). Later on it was found that CXCL4-induced macrophages acquire a specific phenotype, with a mixture of M1 and M2 characteristics and distinct from their M-CSF-induced counterparts. These so-called M4 macrophages express the pro-inflammatory chemokines TNF-α and IL-6, MMP-7, and MMP-12 and the calcium binding protein S100A8 (108, 109). The complete loss of the hemoglobin-haptoglobin scavenger receptor CD163, which is required for effective hemoglobin clearance after plaque hemorrhage (108, 110) and low expression of the antigen-presenting molecule HLA-DR (8) are typical characteristics of these so-called M4 macrophages. When hemoglobin or the hemoglobin-haptoglobin complexes bind the CD163 receptor, the atheroprotective HMOX1 is induced. Consequently, HMOX1 activity is also completely abolished in CXCL4-stimulated monocytes (111). Interestingly, the marked downregulation of CD163 and the novel phenotype induced by CXCL4 was reported to be irreversible (108). The presence of M4 macrophages within human atherosclerotic lesions is associated with advanced plaque morphology (112). M4 macrophages can be considered pro-atherogenic, since these may promote destabilization of the plaque fibrous cap (113).

More recently, our group studied the effect of CXCL4L1/PF-4var (114), the non-allelic variant of CXCL4, on the differentiation of monocytes into macrophages (**Figure 2**) (115). Both variants are secreted by activated platelets and differ only in 3 amino acids near the carboxy-terminal end. The unique 3D structure of CXCL4L1 results in a decreased affinity for glycosaminoglycans (GAGs) and a more outspoken angiostatic potential compared to CXCL4 (116). Differently to M-CSF and CXCL4, CXCL4L1 is not a survival factor for monocytes. CXCL4L1-exposed monocytes display higher expression levels of the inflammatory chemokine receptors CCR2 and CCR5, suggesting that CXCL4L1 promotes a higher responsiveness to inflammatory chemokines, such as CCL2 and CCL3. Additionally, significantly higher amounts of CCL2 and CXCL8 (M1 marker) were measured in CXCL4L1-stimulated monocytes, whereas CXCL4 did modulate chemokine production in the same way as M-CSF. Finally, we found a lower expression of *IL-1 receptor antagonist (*IL-1RA) in CXCL4L1-treated monocytes, compared to CXCL4-treated monocytes, which is in line with the more inflammatory phenotype of macrophages generated in the presence of CXCL4L1 (115). Similar to CXCL4-treated monocytes, CXCL4L1-stimulated monocytes have a significantly lower expression of the CD163 receptor and the mannose receptor (MRC/CD206) compared to M-CSF treated

monocytes (117). Interestingly, in contrast to M4 macrophages we found that *HMOX1* expression was significantly increased in CXCL4L1-treated monocytes (**Figure 2**) (115). So far, the role of CXCL4L1 in atherosclerosis is not further investigated. However, we showed that patients with stable coronary artery disease have a worse prognosis when CXCL4L1 levels in the serum are low (118).

Role of TAMs in Cancer

It is generally accepted that macrophages are the most abundant component of the leukocyte infiltrate that is influencing tumor development. Macrophages that infiltrate the tumor microenvironment are usually referred to as tumor-associated macrophages (TAMs) (119). TAM infiltration is correlated with a poor prognosis in numerous cancers, suggesting that they promote tumor progression (1, 81, 120, 121). Indeed, TAMs can stimulate proliferation, invasion, metastasis of tumor cells, promote angiogenesis and suppress the anti-tumor response (122). Poor anti-tumoral activities are a consequence of the higher production of IL-10, TGF-β and prostaglandin E2 (PGE2) and reduced synthesis of inflammatory cytokines such as TNF-α and IL-6. Furthermore, TAMs display poor antigen-presenting capacities, leading to suppression rather than stimulation of T cell activation and proliferation (13). The decreased production of inflammatory mediators in TAMs is associated with a defective nuclear factor-kappa B (NF-κB) activation in response to LPS and proinflammatory cytokines (123). In addition to the production of the most potent angiogenic factor VEGF, TAMs were shown to produce platelet-derived growth factor (PDGF) (13) and VEGF-C (124), which was suggested to play a role in peri-tumoral lymphangiogenesis and subsequent lymphatic metastasis. As such, TAMs are generally characterized as M2-like macrophages (125).

However, extensive TAM density is associated with increased survival in some specific tumor types. These findings suggest that TAMs comprise multiple distinct pro- and anti-tumoral subpopulations with overlapping features depending on different micro-environmental stimuli. In an explant model of colorectal cancer liver metastasis, CCR5 blockade with Maraviroc, a highly specific CCR5 inhibitor originally developed to treat HIV patients (126), induced a repolarization from an M2 toward an anti-tumoral M1-like phenotype (127). This phenotypic switch was mediated via increased levels of the signal transducer and activator of transcription 3 (STAT3), which is commonly linked to an M1 activation state, due to abrogation of the suppressor of cytokine signaling 3 (SOCS3) activity (128). This so-called re-education of macrophages induced by CCR5 inhibition in human cancer patients could possibly contribute to the further development of chemokine-based anti-cancer therapy.

TAMs originate from circulating monocytes, which are recruited to the tumor by several growth factors and especially by chemokines, produced by stromal and tumor cells (120). Besides M-CSF, the CC chemokines CCL2, CCL3, CCL4, and CCL5 are well-recognized chemotactic factors for macrophage populations in the tumor (**Figure 3**) (129–133). CCL2 is dominantly expressed by many human carcinomas (134, 135) and detection of CCL2 in TAMs themselves even indicates the existence of an amplification loop for their recruitment (13, 136). Interestingly, once macrophages have entered the tumor microenvironment, the corresponding CCR2 is downregulated. It is suggested that receptor downregulation is a mechanism to trap recruited macrophages in the tumor micro-environment (137). Furthermore, in colon cancer models CCL20/LARC (138) chemoattracts monocytes that differentiate into TAMs. Additionally, in human breast cancer models CCL18 in collaboration with CSF-2 was involved in mobilization and recruitment of monocytes (139). Finally, VEGF-A was identified as a macrophage recruitment factor in an *in vivo* xenograft model, possibly acting indirectly through induction of chemoattractants (140).

Once differentiated, TAMs preferentially accumulate in the hypoxic areas of the tumor (141). Casazza et al. found that the protein Neuropilin-1 (Nrp-1) is essential for TAM mobilization toward Semaphorin 3A (SEMA3A), which is upregulated in hypoxic regions of the tumor. When TAMs enter these hypoxic areas, Nrp-1 expression is downregulated and TAMs are trapped in the hypoxic environment (142). Further, these hypoxic TAMs upregulate hypoxia-regulated genes and alter the gene expression profile, acquiring an even more pronounced pro-angiogenic, immunosuppressive, and pro-metastatic phenotype (143). This hypoxia-induced response is partly mediated via the key transcription factor hypoxia-inducible factor (HIF)-1α (144). Interestingly, in endothelial cells HIF-1α induces CXCL12 expression, which is in direct proportion to the oxygen tension in hypoxic areas (145). Additionally, hypoxia induces the expression of CXCR4 on monocytes and macrophages, thereby highlighting a possible role of the CXCL12-CXCR4 axis for TAM trafficking to the hypoxic tumor areas (**Figure 3**) (146).

Besides functioning as chemoattractants, some chemokines can also affect TAM polarization. Sierra-Filardi et al. disclosed an important role for the CCL2-CCR2 axis in regulating macrophage polarization, since blocking CCL2 led to an upregulation of M1 polarization-associated genes and decreased expression of M2-associated markers in human macrophages (147). Additionally, in several animal models of non–small-cell lung cancer (NSCLC) CCL2 blockade significantly reduced tumor growth. Although the total number of recruited macrophages did not change, there was a clear change in the polarization state of TAMs toward a more anti-tumor phenotype after CCL2 blockade (148). These results are in line with the findings from Roca et al. who showed that CCL2 stimulation shifts human peripheral blood $CD11b^+$ cells toward a $CD206^+$ M2-polarized phenotype (149).

Furthermore, in multiple myeloma (MM) CCL2, CCL3, and CCL14/HCC-1 (150) stimulate macrophage polarization into MM-associated macrophages (139), which induce MM drug resistance *in vitro* and in MM mouse models *in vivo* (151, 152). Tripathi et al. showed that hypoxic cancer cell-derived oncostatin M and the chemokine CCL11/eotaxin skewed macrophages toward an M2 phenotype (153, 154).

Besides factors produced by tumor cells, some chemokines produced by the macrophages themselves can affect their polarization. As such, autocrine CXCL12 production modulated

the differentiation of monocytes toward a proangiogenic and immunosuppressive phenotype (155).

Interestingly, migration inhibitory factor (MIF), a cytokine that is not a chemokine but considered to be a "chemokine-like" molecule, was found to be a regulator of TAM polarization in melanoma bearing mice. A small molecule MIF antagonist attenuated tumor-induced macrophage M2 polarization coinciding with a reduced angiogenic potential (156).

The final step of cancer progression is metastasis, i.e., the dissemination of cancer cells from the primary tumor to distant organs. This highly complex process involves cell detachment from the primary tumor site, local invasion, intravasation into adjacent circulatory blood and lymphatic vessels, extravasation at distant capillary beds and proliferation in/colonization of distant organs (157). Before metastatic tumor cells are able to colonize, primary tumor-derived products prepare a primed microenvironment at secondary sites, also known as the pre-metastatic niche (158). Soluble factors including VEGF and placental growth factor (PIGF) induce the recruitment of VEGF-receptor 1 (VEGFR1) positive myeloid cells, which form clusters in the lungs and liver, preparing a permissive niche for disseminating tumor cells. Depletion of these $VEGFR1^+$ cells inhibited metastasis (158). Disseminated cancer cells, in turn, produce CCL2 that recruits inflammatory $CCR2^+$ monocytes from the blood to the metastatic niche, where they differentiate into so-called metastasis-associated macrophages (MAMs) (159). By secreting VEGF-A, these MAMs cause vessel wall permeabilization, allowing subsequent tumor cell extravasation (159). Interestingly, activation of CCR2 on MAMs induces the expression of CCL3 (160). CCL3 signaling via CCR1, in turn, promotes the retention of MAMs in the lung through vascular cell adhesion molecule (VCAM1)-α4 integrin mediated signaling and promotes cancer cell extravasation and retention at the metastatic site (160). Furthermore, VCAM 1 – α4 signaling protects cancer cells from pro-apoptotic signals (161).

Thus, TAMs and MAMs are not only a target for chemokines but also considered as a source of chemotactic mediators. Among these CCL2, CCL3, CCL17, CCL18, and CCL22 have been found to be produced by TAMs/MAMs (61, 162). In ascitic fluid from ovarian cancer patients CCL18, an attractant for Th2 cells was identified, but this chemokine was not produced by ovarian carcinoma cell lines *in vitro* (163). Therefore, it was suggested that the inflammatory mononuclear cells infiltrating the tumor were the CCL18-producing cells (164). Furthermore, CCL17 and CCL22 induce migration of regulatory T (T_{reg}) cells via interaction with the CCR4 receptor (165). Thus, attraction of immunosuppressive immune cells through chemokine production is one of the pro-tumoral characteristics of TAMs.

CONCLUDING REMARKS

Monocyte-derived macrophages respond to a variety of stimuli to modulate their phenotype, which underlines their phenotypic plasticity, one of the major features of macrophages. M1 and M2 macrophages represent the extremities of a continuum of macrophage polarization states with M1 and M2 representing a rather pro-inflammatory and anti-inflammatory phenotype, respectively. Besides their well-known role in monocyte migration, chemokines have also been found to play a role in long-term regulatory processes by inducing macrophage differentiation and polarization in physiological and pathological processes.

AUTHOR CONTRIBUTIONS

PR wrote the review and designed the figures; JVD and PP corrected the manuscript; SS provided critical feedback, helped shaping, and corrected the manuscript.

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CXCL16/CXCR6 Axis Drives Microglia/Macrophages Phenotype in Physiological Conditions and Plays a Crucial Role in Glioma

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Microglia are patrolling cells that sense changes in the brain microenvironment and respond acquiring distinct phenotypes that can be either beneficial or detrimental for brain homeostasis. Anti-inflammatory microglia release soluble factors that might promote brain repair; however, in glioma, anti-inflammatory microglia dampen immune response and promote a brain microenvironment that foster tumor growth and invasion. The chemokine CXCL16 is expressed in the brain, where it is neuroprotective against brain ischemia, and it has been found to be over-expressed in glioblastoma (GBM). Considering that CXCL16 specific receptor CXCR6 is diffusely expressed in the brain including in microglia cells, we wanted to investigate the role of CXCL16 in the modulation of microglia cell activity and phenotype, and in the progression of glioma. Here we report that CXCL16 drives microglia polarization toward an anti-inflammatory phenotype, also restraining microglia polarization toward an inflammatory phenotype upon LPS and IFNγ stimulation. In the context of glioma, we demonstrate that CXCL16 released by tumor cells is determinant in promoting glioma associated microglia/macrophages (GAMs) modulation toward an anti-inflammatory/pro-tumor phenotype, and that *cxcr6ko* mice, orthotopically implanted into the brain with GL261 glioma cells,survive longer compared to wild-type mice. We also describe that CXCL16/CXCR6 signaling acts directly on mouse glioma cells, as well as human primary GBM cells, promoting tumor cell growth, migration and invasion. All together these data suggest that CXCL16 signaling could represent a good target to modulate microglia phenotype in order to restrain inflammation or to limit glioma progression.

Keywords: CXCL16, CXCR6, tumor microenvironment, glioma, microglia, neuroinflammation

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INTRODUCTION

Modification of local brain microenvironment can be sensed by microglia cells, which respond to preserve brain homeostasis, or to exacerbate brain damage. Understanding the mechanisms of microglia communication in the brain is important to identify molecular players that can be used as targets to counteract brain damage and preserve brain homeostasis. Within the brain, microglia are plastic cells that constantly monitor brain parenchyma to sense local perturbation and, depending on specific environmental cues, can change their phenotype and functional activity promoting inflammatory or anti-inflammatory conditions (1, 2).

While chemokines were originally discovered for their ability to regulate leukocyte trafficking, it is now accepted that, beyond chemotaxis, these molecules exert pleiotropic activities in the context of brain physiology, as well as brain cancer (3–8). A crucial role for chemokines and their receptors as mediators of homeostatic crosstalk between neurons and glia has emerged (9, 10) and we have recently shown that the trans-membrane chemokine CXCL16, through its unique receptor CXCR6, orchestrates cell cross-talk to promote neuroprotection against glutamate-induced excitotoxic insults (11); to mediate endogenous protective mechanisms to counteract neuronal damage during brain ischemia (12); and to modulate neurotransmitter release in the hippocampus (13).

Glioblastoma (GBM) is a high grade tumor with a poor prognosis. Despite aggressive surgical resection and chemotherapy, GBM patients undergo tumor recurrence due to the highly infiltrative nature of the tumor cells, and to the persistence of chemotherapy-resistant cells (14). Glioma cells release molecular regulators, such as cytokines and growth factors, which may act in autocrine ways promoting tumor cell proliferation and invasion or in paracrine ways contributing to the establishment of a pro-tumor-microenvironment (15–17). Non-tumor cells of the brain parenchyma, such as astrocytes, endothelial cells, but also microglia, as well as infiltrating peripheral immune cells, sense glioma, and contribute to the formation of a tumor niche that provides a crucial environment for glioma progression. In this context, the cross-talk between tumor cells and glioma associated microglia/macrophages (GAMs) leads to GAMs polarization toward an anti-inflammatory, immunosuppressive, pro-invasive phenotype that support tumor growth and invasion (18).

GBM cells express chemokines that regulate tumor cell proliferation, invasion, angiogenesis, as well as the maintenance of an immunosuppressed microenvironment (19, 20). CXCL16 is expressed in human glioma (21), while the presence of CXCR6 is controversial, likely associated with glioma-stem cells (21, 22).

In the present paper we highlight for the first time a major role of CXCL16/CXCR6 axis in driving microglia polarization toward an anti-inflammatory phenotype that: in inflammatory context provides a neuroprotective mechanism to limit brain damage; in the context of glioma triggers a pro-tumoral microenvironment. Moreover, we show that CXCL16 produced by glioma cells directly stimulates the CXCR6 expressed by tumor cells, promoting their proliferation, migration and invasion.

MATERIALS AND METHODS

Materials

Recombinant murine CXCL16 (cat#250-28) and CXCL12 (cat#250-20A) were from Peprotech; IL-4 (cat#12340045) and IFNγ (cat#12343536) were from Immunotools; anti-CXCL16 (cat# MAB503-100), mouse CXCR6 PE-conjugated antibody (cat# FAB2145P−025, RRID:AB_2089531), human CXCR6 PE-conjugated antibody (cat#FAB699P−025, RRID:AB_2261441) were from R&D System, APC anti mouse H-2Kb/H-2Db (cat#114613) and APC anti mouse CD1d antibody (cat#123521, RRID:AB_2715919) were from Biolegend; APC rat anti-mouse CD44 (cat# 559250), PE rat anti-mouse CD274 (PD-L1)(cat#558091) were from BD Pharmingen; IgG from rat serum antibody (cat#l4131, RRID: AB_1163627), LPS (cat#L4391), 2′,7′-Dichlorofluorescin diacetate (cat#D6883) were from Sigma-Aldrich; anti-Iba1antibody was from Wako (cat#019-19741, RRID:AB_839504); anti-GFAP (cat#NB300-141, RRID:AB_10001722), anti-5-bromo-2-deoxyuridine (cat#NB500169, RRID:AB_341913) antibodies were from Novus Biological and anti-Arg1antibody was from Santa Cruz (cat#sc-271430 RRID:AB_10648473); anti-CD68 antibody (cat#MCA1957T, RRID:AB_322219) was from AbD Serotec;. Secondary Abs were from DAKO; Microbeads CD11b^{+} were from Miltenyi Biotec; Trans-well inserts were from BD Labware (cat#353097); IPTG (Dioxane-free) was from Thermo Fisher (cat#AM9464). Hematoxylin, eosin, and BSA were from Sigma-Aldrich. All cell culture media, fetal bovine serum (FBS), goat serum, penicillin G, streptomycin, glutamine, the Thermo Script RT-PCR System, and Hoechst (cat#33342, RRID:AB_10626776) were from Invitrogen. 5-Bromo-2′-Deoxyuridine (BrdU) (cat#B5002) and lentiviral shRNA clones targeting murine CXCR6 and CXCL16 were from Sigma-Aldrich. Elisa kit for Interleukin 1 Beta (IL-1β) was from Claude-Clone Corp. (cat#SEA563Mu); Elisa kit for CXCL16was from RayBiotech (cat#ELMCXCL16); Griess reagent kit for Nitrite determination was from Molecular Probe (cat#G-7921), Red fluorescent FluoSpheres (0.03%) were from Invitrogen.

Ethics Statement

This study was approved: by the Institutional Review Board of the Policlinico Umberto I Medical Center according to the Bioethics and Safety Act and the Declaration of Helsinki. Each participant provided oral informed consent (according to the principle 22 of Ethical Principles for Medical Research Involving Human Subjects); by the Institutional Review Board of Neuromed Medical Center according to the Bioethics and Safety Act and the Declaration of Helsinki. Each participant provided written informed consent.

Human Tissue Samples

Tumor specimens (GBM 1, 2, 3, 11, 13, 14, 19, 28, 40, 45, 46, 51, 58) were obtained at Policlinico Umberto I (Rome) and Neuromed (Pozzilli, Isernia) from adult glioblastoma (GBM). Within half an hour from surgical resection GBM tissues were processed to obtain primary GBM cells or frozen for molecular study. Histopathological typing and tumor grading were done

according to the WHO criteria resulting as grade IV. Normal cerebral tissues derived from the prefrontal cortex of patients who died from heart failure were kindly provided by Dr. Eleonora Aronica, with ethics approval of Amsterdam University.

Animals and Cell Cultures

The experiments described in the present work, were approved by the Italian Ministry of Health in accordance with the guidelines on the ethical use of animals from the European Community Council Directive of September 22, 2010 (2010/63/EU). Wild type mice C57BL/6J (cat# JAX: 000664, RRID: IMSR_JAX:000664) and Homozygous cxcr6gfp/gfp knock-in mice (cat# JAX: 005693, RRID: IMSR_JAX:00569) (23), in which the coding region of CXCR6 receptor has been substituted with the coding region of the green fluorescent protein, were from Jackson Laboratory. In the present manuscript, we refer to cxcr6gfp/gfp knock-in mice as *cxcr6ko* mice, and to C57BL/6J as *wt* mice.

The mouse GL261 glioma cell line (RRID:CVCL_Y003; kindly provided by Dr. Serena Pellegatta, Istituto Di Ricovero e Cura a Carattere Scientifico, Besta, Milan) was cultured in growth medium (DMEM with 20% heat-inactivated FBS, 100 IU/ml penicillin G, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM glutamine, and 1 mM sodium pyruvate). GL261/CD133$^+$ cells were obtained as previously described in Garofalo et al. (24). The cell lines were tested for mycoplasma contamination (negative). Primary GBM cells were obtained as previously described (25). Briefly tumor tissues were mechanically dissociated to cell suspensions and red blood cells were lysed with hypotonic buffer. Tumor cells were re-suspended in serum-free growth medium and cultured at 37°C in humidified atmosphere with 5% CO2. Twenty-four hours later, non-adherent cells were removed and the growth medium was supplemented with 10% heat-inactivated FBS. Cells were sub-cultured when confluent. In the current study, primary GBM cells, were used within 1–3 passages, and were named GBM13, GBM19, GBM40, and GBM45.

Microglia Culture and Polarization

Microglia cells were obtained from mixed glia cultures derived from the cerebral cortices of post-natal day 0–2 (p0–p2) *wt* mice. Cortices were chopped and digested in 15 U/ml papain for 20 min at 37°C. Cell suspensions were plated (5×10^5 cells/cm^2) on poly-L-lysine (0.1 mg/ml) coated flasks in growth medium supplemented with 10% FBS. After 9–11 days, cultures were shaken for 2 h at 37°C to detach and collect microglia cells. These procedures gave almost pure microglial cell populations as previously described (26). For microglia polarization, cells were seeded on poly-L-lysine (cat#P2636 from Sigma-Aldrich) coated six-well plate and the day after they were treated with LPS 100 ng/ml + IFNγ 20 ng/ml or glioma conditioned medium (GCM) with rat AbCXCL16 or IgG (1 μg/ml) for 24 h.

CXCR6 and CXCL16 Silencing by shRNA Interference

GL261 cells were transduced by lentiviral particles directing IPTG-inducible expression of CXCR6 shRNA or constitutive expression of CXCL16 shRNA constructs. Cells (1.6×10^4) were plated in 96-well plates and infected for 24 h according to the manufacturer's instructions. Transduced cells were selected with 2 μg/ml puromycin for 3–12 days. IPTG (5 mM) was added for 10 days to culture medium to induce CXCR6 shRNA expression. Knockdown efficiency of CXCR6 receptor and CXCL16 was evaluated by PCR or chemotaxis assay. Silenced cell lines were named GL261shCXCR6 and GL261shCXCL16 in this study.

Chemotaxis and Invasion *in vitro* Assays

GL261, GL261shCXCR6 and human primary GBM cells were pre-incubated in chemotaxis medium (DMEM without glutamine, 100 IU/ml penicillin G, 100 μg/ml streptomycin, 0.1% BSA, and 25 mM HEPES, pH 7.4) with AraC (10 μM, 15 min) to block cell duplication. Cells (4×10^4) were plated in the upper wells of 48-well boyden chamber (NeuroProbe) on 8 μm-pored Poly-L-Lysine coated membrane. The lower wells contained CXCL16 (0.1, 1, 10, 50, or 100 nM), CXCL12 (50 ng/ml), or vehicle (C). Cells were left migrate for 4 h (GBM cells) or 24 h (GL261). For invasion assay, GL261 and GBM19 were plated at a density of 2×10^4 cells/cm^2 on matrigel-coated transwells (8 μm pored membrane) and left invade toward CXCL16 (1, 10 nM) or vehicle, respectively, for 48 or 24 h at 37°C. Migrated/invaded cells were fixed and stained with a solution containing 50% isopropanol, 1% formic acid, and 0.5% (w/v) brilliant blue R 250. For each membrane, stained cells were counted in at least 20 fields with a 32 × objective of a phase-contrast microscope (Zeiss).

MTT Assay

GL261, GL261shCXCR6, and GBM19 cells were seeded into 96 well plates (5×10^3) and treated with vehicle (C) or with CXCL16 (10 nM) for different time points (0, 24, 48, 72, or 96 h). MTT solution (500 μg/ml) was added into each well for 1.5 h. DMSO was then added to stop the reaction and the formazan produced was measured at 570 nm. Viability of cells was expressed relative to absorbance values.

Western-Blot

For protein analysis, microglial cells (6×10^5) were seeded on six-well plates and treated with vehicle, CXCL16 (200 nM), glioma conditioned medium (GCM) with or without rat AbCXCL16 for 24 h; cells were washed with PBS and lysed in hot 2 × Laemmli buffer, boiled 5 min, and sonicated. The same amount of proteins was separated on 12% SDS-polyacrylamide gel and analyzed by Western immunoblot using the following primary antibodies: ARG-1 1:200, ACTIN 1:2,000. HRP-tagged goat anti-mouse and anti-rabbit-IgG were used as secondary antibodies (1:2,000; Dako), and detection was performed by the chemiluminescent assay Immun-Star WesternC Kit (Bio-Rad, CA). Densitometric analysis has been carried out with Quantity One software (Biorad, CA).

Phagocytosis Assay

Microglial cells were seeded on poly-L-lysine-treated 10 mm glass coverslips (7×10^4 cells) and stimulated with CXCL16 (200 nM) or vehicle for 24 h and GCM with or without rat AbCXCL16 for 24 h. Medium was then removed, 0.05% (corresponding to

1.8×10^7 spheres/ml) red fluorescent FluoSpheres were added for 1 h in serum-free medium (0.1% BSA), and nuclei were stained by Hoechst. Cells were washed three times with PBS to remove non-phagocytized spheres and fixed in 4% PFA for 1 min. Phagocytosis was quantified by counting the number of phagocytizing cells (scoring as positive only cells with at least five FluoSpheres to avoid possible false positives due to sphere adhesion to cell surface) in at least 20 random fields per coverslip.

Form Factor Calculation

Microglia were seeded on glass coverslips, treated as necessary, fixed, permeabilized, blocked and stained with Alexa-Fluor 488 Phalloidin (Invitrogen) for 20 min together with Hoechst. Fluorescent images were processed using the MetaMorph 7.6.5.0 software (Molecular Device, Sunnyvale, CA, USA), and form factor was calculated according the formula: 4π area/perimeter^2 (27). Form factor is a parameter taken as 1 for round cells, and correspondingly <1 when the morphology deviates from the spherical shape.

Nitric Oxide (NO) Measurement

NO production by microglia cultures was assessed by measuring nitrite accumulation in the culture medium by Griess Reagent Kit according to manufacturer's instructions (Molecular Probes, MA, USA). The absorbance was measured at 570 nm in a spectrophotometer microplate reader (BioTek Instruments Inc., VT, USA).

ELISA Assay

Microglial cells (6×10^5 cells) were seeded onto a 6-well culture plate, after 24 h cells were stimulated with LPS 100 ng/ml + IFNγ 20 ng/ml or LPS 100 ng/ml + IFNγ 20 ng/ml + CXCL16 (200 nM) for 24 h. Medium was than collected, centrifuged at 1,000 × g for 20 min, and supernatant was stored at −80°C. Control cells were stimulated only with vehicle. IL-1β present in the supernatant was measured using a specific ELISA for mouse IL-1β (Cloud-Clone Corp.) as described by the manufacturer. For each sample, cells were detached and proteins were quantified (BCA assay). For quantification of mouse CXCL16 in glioma conditioned medium (GCM) we used the mouse CXCL16 ELISA Kit (RayBiotech, Norcross, GA, USA) as described by the manufacturer. All supernatants were centrifuged (1,000 × g for 5 min) to eliminate floating cells and then samples were 10-fold concentrated with 10 KDa Microcon Centrifugal Filter devices (Merck Millipore, Darmstadt, Germany). Samples were measured in duplicate and confirmed in two independent experiments.

Reactive Oxygen Species (ROS) Measurement

Primary microglia cultures (3×10^5 cells) were treated for 18 h with 200 nM CXCL16 and then cells were incubated with 20 μM of 2′,7′-Dichlorodihydrofluorescein diacetate (DCF-DA, Sigma-Aldrich, #D6883) for 30 min at 37°C. Cell fluorescence was detected in FL1 channel and analyzed with a FACSCanto II (BD Biosciences). Data were elaborated using FlowJo v9.3.2 software (TreeStar, Ashland, OR, USA).

Cytofluorimetric Analysis

Cells were harvested in PBS with 5 mM EDTA and washed in staining buffer (PBS without Ca^{2+} Mg^{2+}, 0.5% BSA, 2 mM EDTA, 0.025% NaN_3). mAbs directly conjugated to PE and APC fluorochromes and specific for the following antigens were used: MHC class I (APC anti-mouse H-2Kb/H-2Db, BioLegend), CD1 (APC anti-mouse CD1d, clone 1B1, BioLegend), CD44(APC rat anti-mouse CD44, clone IM7, BD Pharmingen), PD-L1 (BD Pharmingen), CXCR6 (R & D systems). Corresponding isotypes were used for negative control. Immunostaining was performed with saturating amounts of Abs for 30 min at 4°C. Samples were acquired with a flow cytometer FACSCanto II (BD Biosciences) and data were elaborated using FlowJo 9.3.2 software (TreeStar).

Reverse Transcript PCR (RT-PCR) and Quantitative Real Time PCR (RT-qPCR)

Samples were lysed in TRYzol reagent for isolation of total RNA. The quality and yield of RNAs were verified using NANODROP One (Thermo Scientific). For RT-PCR one microgram of total RNA was reverse transcribed using ThermoScript RT-PCR System and 150 ng of the reverse transcription products were used as a template for PCR amplification. The PCR protocol was as follows: 95°C for 5′, 30 cycles 94°C for 30″, 55°C for 30″, and 72°C for 30″. MJ Mini Thermal Cycler (Bio-Rad) was used for all reactions and amplification products were analyzed on 1.8% agarose gel stained with ethidium bromide. For RT-qPCR Reverse transcription reaction was performed in a thermocycler using IScript TM RT Supermix (Biorad) under the following conditions: incubation, 25°C, 5′; reverse transcription, 42°C, 45′; inactivation, 85°C, 5′. Real Time-PCR was carried out in a I-Cycler IQ Multicolor RT-PCR Detection System using Sso Fast Eva Green Supermix (Biorad). The PCR protocol consisted of 40 cycles at 95°C, 30″ and 60°C, 30″. For quantification analysis, the comparative Threshold Cycle (Ct) method was used. The Ct values from each gene were normalized to the Ct value of GAPDH in the same cDNA samples. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ Ct method (28) and expressed as fold increase in arbitrary values. Primers sequences are reported in **Supplementary Table 1**. Primers used for CXCR6 and CXCL16 were not intron spanning, and "no-RT" reactions were used as controls to rule out priming off of genomics DNA. As control for *cxcl16* and *cxcr6* mRNA expression, we used RNA from Human fibroblast cell line HFF-1 (ATCC® SCRC-1041™, RRID:CVCL_3285); RNAs from Mouse fibroblast NIH/3T3 cells (ATCC® CRL-1658™, RRID:CVCL_0594); RNA from primary human T lymphocytes kindly provided by Dr. Samantha Cialfi, Department of Molecular Medicine, Sapienza, Rome); RNA from mouse primary $CD4^+$ T cells derived from spleen.

Brain Injection of Glioma Cells and Survival Analysis

Eight week old male mice (*wt* or *cxcr6ko*) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic head frame. Animals were injected with 1×10^5 GL261, GL261shCXCR6, or GL261shCXCL16 cells at 2 mm lateral and 1 mm anterior to the bregma in the right striatum. Cell

suspensions, in sterile phosphate-buffered saline (PBS) (5 μl) were injected with a Hamilton syringe at a rate of 1 μl/min at 3 mm depth. For GL261 shCXCR6, after 10 days of IPTG treatment, cells were injected in mice and shRNA expression was maintained by adding IPTG (10 mM) in drinking water. For survival analysis glioma injected mice were daily monitored. The end points were determined by lack of physical activity or 20% weight loss in glioma-bearing mice. The mean survival time was calculated using the Kaplan–Meier method and statistical analysis was performed using a log-rank test.

Tumor Volume Evaluation and Brain Sections Immunostaining

Seventeen days after tumor cell injection glioma-bearing mice were killed and brains were isolated and fixed in 4% buffered paraformaldehyde. Brains were snap frozen and cut in 20 μm coronal brain cryosections. Tumor volume was evaluated with hematoxylin–eosin staining as previously described. Briefly, after staining, brain slices (20 μm of thickness) were analyzed by the Image Tool 3.0 software (University of Texas, Health Science Center, San Antonio, TX, USA). Tumor volume was calculated according to the formula (volume = t × ΣA), where A = tumor area/slice and t = thickness (29). For tumor cell proliferation *in vivo*, 17 days after tumor cells injection glioma-bearing mice were injected intraperitoneally with bromo-2-deoxyuridine (50 mg/kg). Two hours later, mice were killed and brains processed for BrdU immunostaining. For immunostaing analysis cryosections were washed in PBS and blocked with blocking solution (3% goat serum, 0.3% Triton X-100 in PBS) for 1 h at room temperature. Sections were then incubated with specific antibodies (anti-Iba1 1:500, anti-CD68 1:200, anti-GFAP 1:750, anti-BrdU 1:200) in 1% goat serum and 0.1% Triton X-100/PBS solution overnight at 4°C. After several washes, sections were stained with the respective secondary fluorophore-conjugated antibody and Hoechst for nuclei visualization. For Iba1 staining, citrate buffer antigen retrieval protocol was used. For BrdU immunostaining, sections were pretreated with HCl 1N for 15 min, HCl 2N for 25 min at 37°C, and neutralized with 0.1 M borate buffer. Digitized fluorescent cell images were collected using an inverted fluorescence microscope (Nikon Ti Eclipse) and analyzed with MetaMorph analysis software (Molecular Devices, USA).

BrdU Cell Immunostaining

GL261 cells were grown on glass coverslips at a density of 5×10^4 cells/cm^2 and treated for 4 h with CXCL16 10 nM or vehicle. Cells were then incubated with 10 μg/ml BrdU for 30 min, washed with PBS and fixed in 4% paraformaldehyde for 20 min. Fixed cells underwent immunostaining protocols as described for brain sections. Hoechst was used for nuclear staining. BrdU positive cells were counted out of 800 cells for condition.

Invasion *in vivo* Assay

Seventeen days after GL261 injection, mice brains were isolated and fixed in 4% buffered formaldehyde for morphological evaluation. Coronal brain sections (20 μm), prepared using the standard procedures, were stained with hematoxylin and eosin. For analysis of tumor invasiveness, glioma cells protruding more than 150 μm from the main tumor mass were counted in at least 20 fields, obtained from six slices per mice.

Isolation of CD11b$^+$ Cells

Glioma-bearing *wt* or *cxcr6ko* mice after 17 days from inoculation were deeply anesthetized and intracardially perfused with ice cold PBS. Brains were removed, cut into small pieces and single-cell suspensions were achieved by enzymatic digestion in trypsin (0.25 mg/ml) solution in Hank's balanced salt solution (HBSS). Cell suspensions were labeled with CD11b$^+$ Microbeads, loaded onto a MACS Column (MiltenyiBiotec) and placed in the magnetic field of a MACS Separator. After removing the magnetic field, CD11b$^+$ cells were eluted and used for RNA extraction. CD11b$^+$ cells were also isolated from human GBM tissues surgically removed from patients as described above.

Statistical Data Analysis

Data are expressed as the mean ± SEM. Statistical significance was assessed by Student's *t*-test, Student's paired *t*-test or one-way ANOVA, as indicated; Holm–Sidak, Turkey *post-hoc* test or Student-Newman-Keuls Method were used as a *post-hoc* test. For Kaplan–Meier analysis of survival, the log-rank test was used. All statistical analyses were done using Sigma Plot 11.0 software.

RESULTS

CXCL16 Drives Microglia Polarization *in vitro*

Since we have shown that CXCL16 is neuroprotective in ischemia (11, 12), and neuroinflammation plays a role in brain damage following ischemic insult (30, 31), we considered the possibility that CXCL16, acting on CXCR6 expressed by microglia cells (11), might provide protective effects also modulating microglia phenotype.

We performed *in vitro* experiments treating primary mouse microglia for 24 h with CXCL16 (200 nM) and analyzing the expression of pro- (*nos2, il1b, cd86, tnfa*) and anti- (*arg1, chil3, retnla, cd163*) inflammatory genes (32) by RT-qPCR: as reported in **Figure 1A**, CXCL16 increases the expression of anti-inflammatory genes (right panel; $n = 5$ $p < 0.05$; Student's *t*-test), while no significant modulation of pro-inflammatory genes is observed, with the exception of *nos2* (left panel; $n = 5$ $p < 0.05$; Student's *t*-test).The ability of CXCL16 to induce anti-inflammatory polarization was further supported by an increase in ARG-1 protein expression in microglia treated with CXCL16 (200 nM, 24 h) vs. not treated cells ($n = 4$, $p < 0.05$; Student's *t*-test), **Figure 1B**. Moreover, CXCL16 increases: the number of phagocytizing microglia (measured as number of cells that phagocytized five or more fluorescence beads) ($n = 3$, $p < 0.001$; Student's *t*-test) vs. control, **Figure 1C**; the production of reactive oxygen species (measured as generated DCF fluorescence) vs. vehicle ($n = 3$ experiments in duplicates, $p < 0.05$; Student's *t*-test), **Figure 1D**.

We then wanted to verify the hypothesis that CXCL16 could also modulate microglia polarization in the context of pro-inflammatory conditions (LPS, 100 ng/ml + IFNγ, 20 ng/ml,

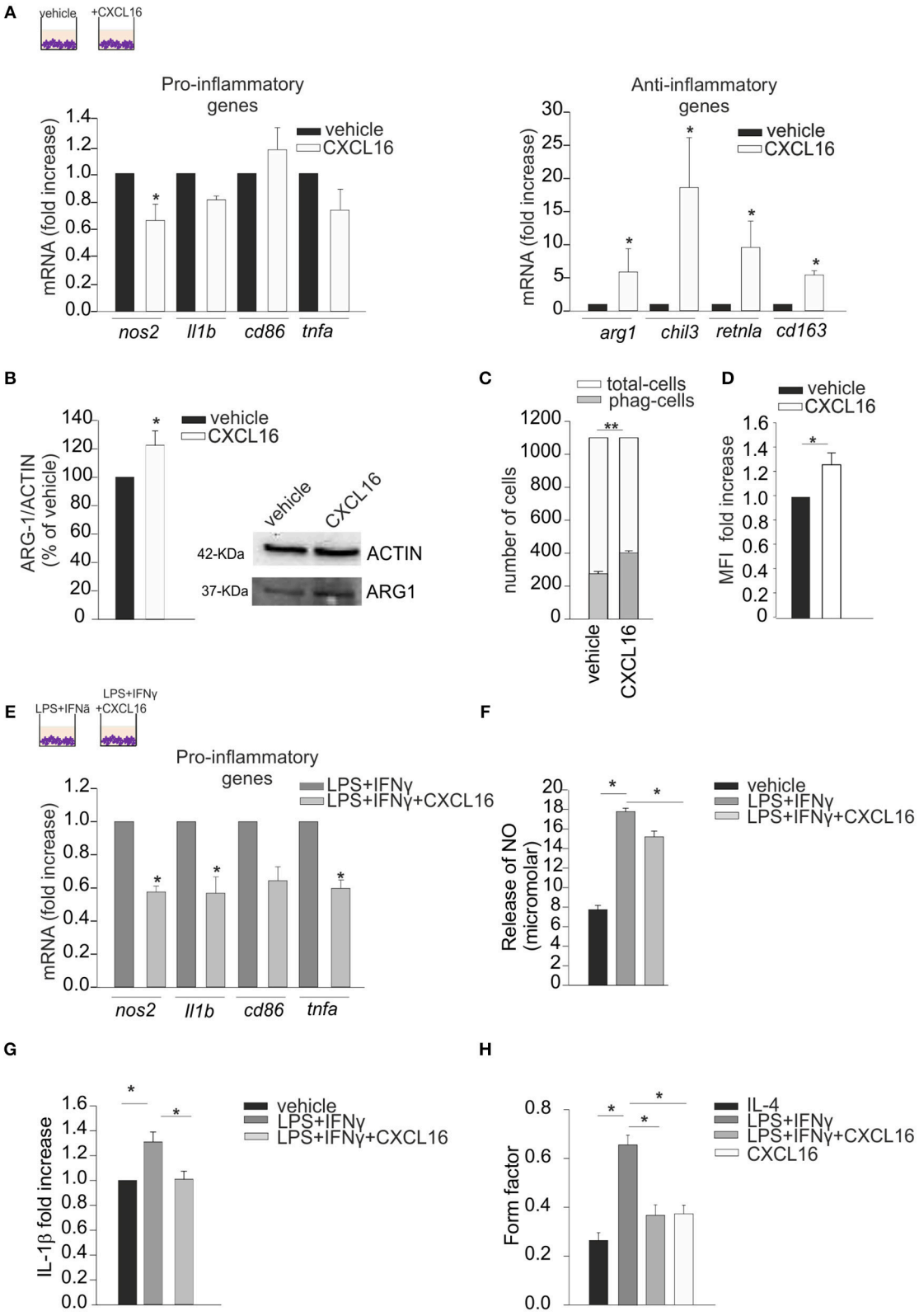

FIGURE 1 | Effects of CXCL16 in modulating microglia phenotype. Expression analysis by RT-qPCR for mRNAs of pro-inflammatory (*nos2, il1b, cd86, tnfa*) or anti-inflammatory (*arg1, chil3, retnla, cd163*) related genes in primary *wt* microglia treated with: **(A)** vehicle or CXCL16 (200 nM); **(E)** LPS+IFNγ (pro-inflammatory

(Continued)

FIGURE 1 | stimulus) in the absence or presence of CXCL16. For each gene data are expressed as specific mRNA fold increase in CXCL16 treated cells normalized to specific mRNA expression in vehicle **(A)**, or in LPS + IFNγ treated cells **(D)**; **(B)** Western-blot analysis of ARG-1 protein expression in microglia cells incubated vehicle or CXCL16. Right, representative image; left histogram bar of the quantification of ARG-1 expression (data are expressed as ARG-1 signal normalized to ACTIN signal). **(C)** Phagocytosis of fluorescent beads in microglia cells stimulated with CXCL16 (200 nM, 24 h), or not (vehicle). Data are expressed as number of cells containing 5 or more beads (gray bars) within total counted cells (white bars); **(D)** ROS production of microglia cells after CXCL16 treatment as evaluated by using the DCF probe. DCF was analyzed as median fluorescence intensity (MFI) by flow cytometry; **(F,G)** Release of NO and IL-1β by microglia cells not stimulated (vehicle) or stimulated with LPS + IFNγ in the absence or presence of CXCL16; for IL-1β data are expresses as fold increase vs. vehicle; **(H)** Form factor analysis of microglia cells treated with IL-4 (anti-inflammatory stimulus), LPS + IFNγ, LPS + IFNγ + CXCL16, or CXCL16. Statistical analysis: Data are expressed as the mean (± s.e.m.) **(A)** $n = 4$, $^{*}p < 0.05$, Student's *t*-test; **(E)** $n = 5$, $^{*}p < 0.05$, Student's *t*-test. For each gene, variability in its expression among control conditions in different experiments never exceeded 10%. **(B)** $n = 4$, $^{*}p < 0.05$, Student's *t*-test; **(C)** $n = 3$, $^{**}p < 0.001$, Student's *t*-test; **(D)** $n = 3$ experiments in duplicates, $^{*}p < 0.05$, Student's *t*-test; **(F)** $n = 7$, $^{*}p < 0.05$, one-way ANOVA followed by Holm–Sidak *post-hoc* test; **(G)** n = 4, $^{*}p < 0.05$, one-way ANOVA followed by Holm–Sidak *post-hoc* test; **(H)** $n = 30$ cells, $^{*}p < 0.05$, one-way ANOVA followed by Tukey *post-hoc* test.

24 h): as reported in **Figure 1E**, the presence of CXCL16 (200 nM) significantly reduced the expression of *nos2, il1b,* and *tnfa* genes ($n = 4$–5, $p < 0.05$; Student's *t*-test). Moreover, we measured the release of nitric oxide (NO) and IL-1β by microglia cells treated with vehicle or LPS + IFNγ, in the presence or not of CXCL16: as shown in **Figures 1F,G**, the release of NO ($n = 7$, $p < 0.05$; One-way ANOVA followed by Holm–Sidak *post-hoc* test) and IL-1β ($n = 4$, $p < 0.05$; One-way ANOVA followed by Holm–Sidak *post-hoc* test) induced by LPS + IFNγ was significantly reduced by treatment with CXCL16.

The activation state of microglia cells has been often correlated with their shape, although it is not possible to strictly associate a morphology to a specific phenotype (33). We measured the ramification grade of microglia calculating the "form factor," a parameter taken as 1 for round cells, and correspondingly <1 when the morphology deviates from the spherical shape. As shown in **Figure 1H**, in analogy with what previously reported (29), the form factor of cells polarized toward an anti-inflammatory ramified phenotype (IL-4 20 ng/ml, 24 h) was 0.26 ± 0.03, while in cells with an inflammatory phenotype (LPS+IFNγ) was 0.66 ± 0.04. The form factor of cells stimulated with CXCL16 (0.37 ± 0.03) or treated with LPS + IFNγ + CXCL16 (0.37 ± 0.04) were similar to cells treated with IL-4, and statistically different from those treated with LPS + IFNγ ($n = 30$ cells in three different experiments, $p < 0.05$; One-way ANOVA followed by Tukey *post-hoc* test), further confirming that CXCL16 polarizes cells toward an anti-inflammatory phenotype.

CXCL16 Released by Glioma Promotes Microglia Polarization Toward an Anti-Inflammatory Phenotype *in vitro*

We analyzed the expression of CXCL16 and CXCR6 in human GBM tissues acutely (<2 h) removed from patients, and in normal cerebral tissues (controls) derived from the temporal and frontal cortex of patients who died for heart failure: RT-qPCR analysis revealed a significant higher expression for *cxcl16* and *cxcr6* mRNAs in GBM, compared to controls (**Figure 2A** left panel) ($p < 0.001$ and $p < 0.05$, respectively; Student's *t*-test). We also analyzed the expression of *cxcr6* in human $CD11b^{+}$ cells (microglia/macrophages) isolated from GBM tissues, and found a considerable expression of *cxcr6* also in these cells (**Figure 2A**, right panel). To study the role of CXCL16/CXCR6 in glioma development in a mouse model, we analyzed *cxcl16* and *cxcr6* mRNAs expression in GL261, and in the more aggressive derived glioma stem cells (GL261/$cd133^{+}$ cells): as shown in **Figure 2B**, both chemokine and its receptor are expressed by GL261 cells, with higher expression of *cxcr6* in GL261/$cd133^{+}$ cells (both by RT-PCR and RT-qPCR analysis). Mouse T-cells and fibroblasts were analyzed as positive controls for *cxcr6* and *cxcl16* mRNAs expression, respectively. Furthermore, we analyzed the expression of CXCR6 membrane protein by flow cytometry, using a mouse CXCR6 PE-conjugated antibody and confirmed the expression of CXCR6 on GL261 cells (**Figure 2B**, right panel).

It is known that glioma cells secrete soluble factors that contribute to the establishment of a pro-tumor microenvironment switching GAMs toward an anti-inflammatory phenotype (2, 32, 34); thus, considering that microglia cells do express CXCR6, we speculated that CXCL16 released by tumor cells might act as an effector in driving such microglia polarization.

Primary microglia cells were incubated for 24 h with glioma conditioned medium (GCM) in the presence of neutralizing anti-CXCL16 antibody (AbCXCL16) (GCM + AbCXCL16), or control IgG (GCM + IgG), and analyzed for the expression of pro- or anti-inflammatory genes. In the presence of AbCXCL16, microglia increases the expression of *nos2, il1b, cd86, tnfa* (pro-inflammatory genes, **Figure 2C**, left panel), and decreases the expression of a*rg1, chil3, retnla, cd163* (anti-inflammatory genes, **Figure 2C**, right panel) compared to cells treated with GCM+IgG ($n = 14$, $p < 0.05$; Student's *t*-test). As control, in each experiment we checked the expression of pro- and anti-inflammatory genes of GCM-incubated microglia (data not shown). These results suggest that CXCL16 in GCM is determinant to promote microglia polarization to establish a pro-tumor/anti-inflammatory microenvironment. To further support these data we analyzed the phagocytizing activity of microglia cells incubated with GCM or GCM + AbCXCL16 (**Figure 2D**), and the expression of ARG-1 in these cells (**Figure 2E**): we found a significant reduction in the number of phagocytizing cells ($n = 4$, $p < 0.001$; Student's paired *t*-test) and in the expression of ARG-1 protein ($n = 3$, $p < 0.05$; Student's *t*-test) in cells treated with GCM + AbCXCL16 vs. cells treated with GCM. The presence of soluble CXCL16 in GL261 conditioned medium was also confirmed by ELISA measurement (0.47 ± 0.03 pg/ml).

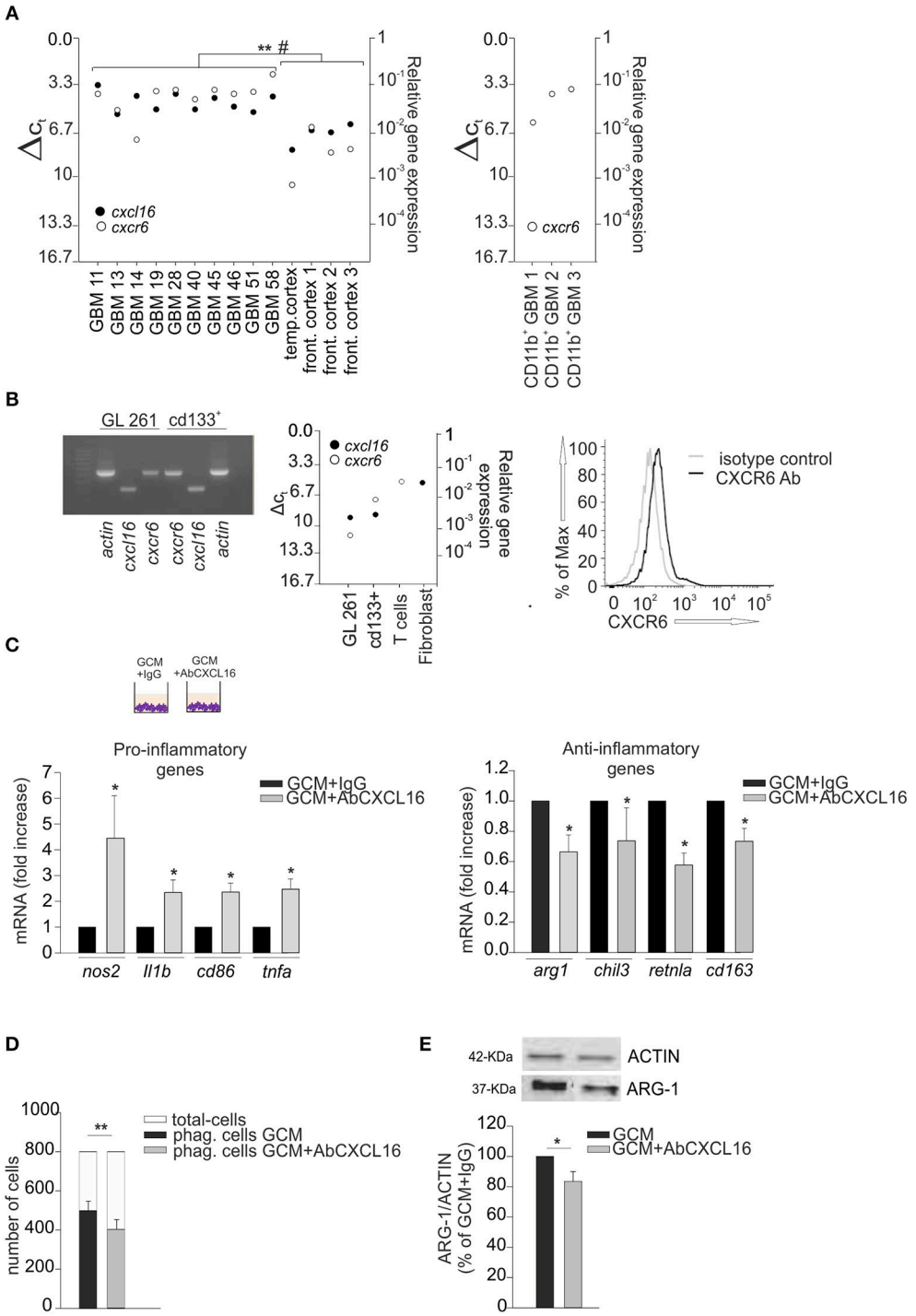

FIGURE 2 | CXCL16 released by glioma cells modulates microglia phenotype. **(A)** Left panel, RT-qPCR for *cxcl16* and *cxcr6* (black and white circles) mRNA expression in human GBM tissues and in human control tissues (temporal or frontal cortex); right panel, qRT-PCR for *cxcr6* mRNA expression in human CD11b$^+$ cells (microglia/machrophages) isolated by GBM tissues. Mean values of double measurements from individual patients; ΔCT values = CT gene of interest−CT *gapdh* (housekeeping gene). A ΔCT of 3.33 corresponds to one magnitude lower gene expression compared to *gapd*h; **(B)** Left panel, representative PCR for *cxcl16*, *cxcr6*, and *actin* mRNAs expression in mouse GL261 cells and mouse GL261 derived stem cells (cd133$^+$); right panel RT-qPCR for *cxcl16* and *cxcr6* (black and white circles) mRNAs expression in GL261, cd133$^+$ cells, and in mouse T-cells and fibroblasts; CXCR6 surface expression on mouse GL261 cells as evaluated by flow cytometry. Black and gray lines represent CXCR6 staining and isotype control, respectively. **(C)** Expression analysis by RT-qPCR for mRNAs of pro-inflammatory related genes (*nos2, il1b, cd86, tnfa*) (left panel), or anti-inflammatory related genes (*arg1, chil3, retnla, cd163*) (right panel) in primary *wt* microglia treated with GCM in the presence of AbCXCL16 neutralizing antibody (GCM + AbCXCL16) or control IgG (GCM + IgG). For each gene data are expressed as specific mRNA fold change

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FIGURE 2 | in GCM + AbCXCL16 treated cells normalized to specific mRNA expression in GCM + IgG treated cells and are the mean (± s.e.m.); **(D)** Phagocytosis of fluorescent beads in microglia cells stimulated with GCM or GCM + AbCXCL16. Data are expressed as number of cells containing 5 or more beads (black/gray bars) within total counted cells (white bars); **(E)** Western-blot analysis of ARG-1 protein expression in microglia cells incubated with GCM or GCM + AbCXCL16. Top representative image; bottom histogram bar of the quantification of ARG-1 expression (data are expressed as ARG-1 signal normalized to ACTIN signal). Statistical analysis: data are expressed as the mean (± s.e.m.) **(A)** $^{**}p < 0.001$ *cxcl16* expression, $^{\#}p < 0.05$ cxcr6 expression, Student's *t*-test; **(C)** $n = 14$, $^{*}p < 0.05$, Student's *t*-test; for each gene, variability in its expression between control conditions in different experiments never exceeded 10%; **(D)** $n = 4$, $^{**}p < 0.001$, Student's paired *t*-test; **(E)** $n = 3$, $^{*}p < 0.05$, Student's *t*-test.

CXCR6 Expression in Glioma Recipient Mice Is Determinant for Tumor Microenvironment

Since tumor micro-environment plays an important role in glioma progression, and considering the ability of CXCL16 to promote microglia anti-inflammatory phenotype *in vitro*, we decided to investigate the effect of CXCL16/CXCR6 signaling on tumor micro-environment *in vivo*: we therefore orthotopically implanted GL261 cells into the brain of *wt* and *cxcr6ko* mice. Some animals were used for a survival-analysis, others were sacrificed 17 days after implantation for tumor volume analysis (**Figure 3A**). As reported in **Figure 3B**, tumor volume was strongly reduced (62%) in *cxcr6ko* mice compared to *wt* mice (*cxcr6ko*: 3.83 ± 0.67 mm^3; *wt*: 10.07 ± 0.55 mm^3; $n = 7$–12, $p < 0.001$; Student's *t*-test). Moreover, survival studies (Kaplan–Meier analysis) revealed that *cxcr6ko* mice survive longer than *wt* mice (*cxcr6ko*: 45 ± 2.9 days; *wt*: 22.8 ± 2.3 days, $n = 6$–10; $p < 0.001$ Log rank test, **Figure 3C**). These data suggest that the CXCL16/CXCR6 axis plays a key role in establishing a pro-tumoral microenvironment in the brain of glioma-bearing mice. Due to the importance of GAMs in glioma progression, we also investigated Iba1 and CD68 cell immuno-reactivity. As shown in **Figure 3D** there was no difference in $Iba1^+$cells (measured as % of Iba^+ staining per tumor area) in *wt* and *cxcr6ko* mice (*cxcr6ko*: 0.69 ± 0.02%; *wt*: 0.72 ± 0.04%; $n = 4$, p = 0.56; Student's *t*-test), but there was a strong reduction in $CD68^+$cells (measured as % of $CD68^+$ staining per tumor area) in *cxcr6ko* mice compared to *wt* mice (53% reduction, *cxcr6ko*: 0.26 ± 0.02%; *wt*: 0.55 ± 0.02%; $n = 4$, $p < 0.05$; Student's *t*-test), indicating that although there was no difference in the recruitment of total GAMs in tumor mass, they were differently activated in *cxcr6ko* mice.

To confirm a role of CXCL16 in driving GAMs toward a pro-tumor phenotype *in vivo*, we implanted GL261 cells into the brain of *wt* and *cxcr6ko* mice and, after 17 days, $CD11b^+$ cells were isolated from the ipsi- and contra-lateral brain hemispheres of each mice and analyzed by RT-qPCR. Data reported in **Figure 3E** show no significant differences in the expression levels of pro-inflammatory genes (n= 4–5, $p > 0.05$; Student's *t*-test); instead we found a significant reduction in the expression of anti-inflammatory genes such as a*rg1, chil3, retnla, cd163* in $CD11b^+$ cells from *cxcr6ko* mice (**Figure 3F**; $n = 4$–5, $p < 0.05$; Student's *t*-test). In order to look at differences in the brain tumor microenvironment in the two genotypes, we also analyzed astrocytic activation and tumor cells invasion in the surrounding brain tissue.

As reported in **Figure 3G** (left), the brain of glioma-bearing *cxcr6ko* mice showed reduced astrogliosis (measured as % of $GFAP^+$ area in brain slice) compared to *wt* mice (46% reduction, 0.53 ± 0.03% in *wt*, 0.29 ± 0.03% in *cxcr6ko*; $n = 4$, $p < 0.001$; Student's *t*-test). In addition, as revealed by the analysis of the number of glioma cells protruding more than 150 μm from the main tumor mass, *cxcr6ko* mice presented a reduction in the number of glioma cells invading the brain parenchyma (10.1 ± 1.0 cells for brain slice) compared to *wt* mice (24.8 ± 1.5 cells for brain slice) ($n = 3$, $p < 0.05$; Student's *t*-test), **Figure 3G** (right).

Direct Effects of CXCL16/CXCR6 Axis on Glioma Cells

We investigated the direct effects of CXCR6 stimulation on GL261 cells: at this aim cells were stimulated with CXCL16 and analyzed for migration in the Boyden chamber assay. Data reported in **Figure 4A** (left panel) demonstrate that the chemotactic index of GL261 increased with CXCL16 dose, starting at 0.1 nM CXCL16, with maximal effect at 10 nM ($n = 3$ experiments in triplicate, $p < 0.05$; One-way ANOVA followed by Holm–Sidak *post-hoc* test). *In vitro* matrigel invasion assay with GL261 shows significant increase of cell invasion upon CXCL16 stimulation (**Figure 4A**, central panel; $n = 3$, $p < 0.05$; One-way ANOVA followed by Holm–Sidak *post-hoc* test). In accordance with this effect, we also found that CXCL16 stimulation increased the expression level of the matrix metalloproteinases *mmp9* and *mmp2* (**Figure 4A** right panel; $n = 6$, $p < 0.05$; Student's *t*-test), whose activity is reported to be involved with the invasion ability of glioma cells (35). To investigate whether CXCL16 might directly promote glioma cell proliferation, in analogy with CXCL12 (20), we analyzed GL261 proliferation upon stimulation with CXCL16. **Figure 4B** shows that CXCL16 (10 nM) significantly increased GL261 cell number after 24 and 48 h, as revealed by MTT analysis (left panel; $n = 3$ experiments in six-replicates, $p < 0.05$; One way ANOVA followed by Student-Newman-Keuls Method). Similar results were obtained measuring BrdU incorporation in GL261 cells: CXCL16 administration (10 nM, 4 h) increased the number of proliferating $BrdU^+$ cells compared to vehicle (C) stimulated cells (middle and right panels; $n = 3$, $p < 0.05$; Student's *t*-test).

CXCR6 Silencing in GL261 Reduces Tumor Migration and Proliferation *in vitro* and *in vivo*

To confirm the role of CXCR6 activation on glioma cells, GL261 cells were engineered for CXCR6 silencing, using an IPTG-inducible shCXCR6 construct. As shown in **Figure 4C**, we selected a GL261shCXCR6-inducible cell clone that, after 10 days of treatment with IPTG, presented a strong reduction in CXCR6 mRNA expression compared to control cells (not treated with IPTG). To further confirm CXCR6 silencing in the

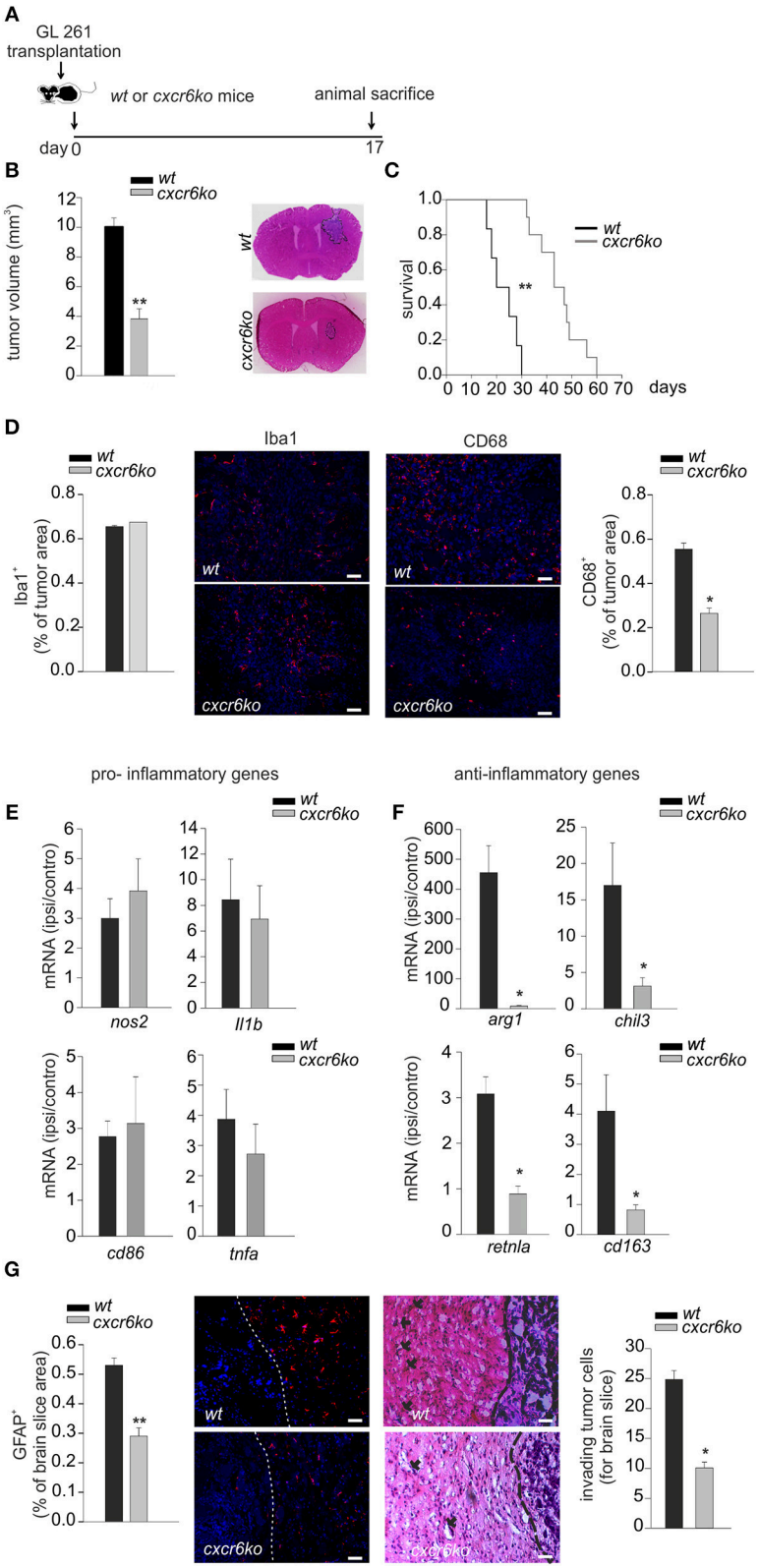

FIGURE 3 | CXCL16/CXCR6 axis is involved in establishing a pro-tumor microenvironment. **(A)** Representative scheme of GL261 transplantation in *wt* and *cxcr6ko* mice; **(B)** bar histogram of the mean (± s.e.m.) of tumor volume in *wt* and *cxcr6ko* mice, and representative hematoxylin-eosin stained coronal brain sections of GL261

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FIGURE 3 | bearing mice; **(C)** Kaplan–Meier survival curves of *wt* and *cxcr6ko* GL261 bearing mice; **(D)** Immunofluorescence analysis of Iba1 and CD68 expression in tumor bearing brain slice 17 days after GL261 transplantation in *wt* and *cxcr6ko* mice: (central panels) representative immune-fluorescence images for Iba1$^+$, CD68$^+$ (red signals), nuclei are evidenced with Hoechst (blue signal), scale bar = 20 μm; (left and right panels): bar histograms representative of the immunofluorescence analysis of Iba1$^+$, CD68$^+$. Data are expressed as % of Iba$^+$ or CD68$^+$ staining per tumor area; **(E,F)** RT-qPCR for pro-inflammatory and anti-inflammatory genes in CD11b$^+$ cells isolated from the ipsilateral and controlateral brain hemispheres of *wt* and *cxcr6ko* mice transplanted with GL261 cells. Data are expressed as mRNA fold increase in the ipsilateral hemisphere vs. the controlateral hemisphere, normalized for *gapdh* mRNA, and are represented as the mean (± s.e.m.); **(G)** Left, bar histogram representative of immunofluorescence analysis of GFAP$^+$ positive cells in tumor bearing brain slice (data are expressed as % of GFAP$^+$ area in brain slice), and representative images of GFAP$^+$ staining (red signal); right**,** analysis of glioma cell invasion of surrounding brain tissue in *cxcr6ko* or *wt* mice injected with GL261.Representative coronal brain sections stained with hematoxylin/eosin. Black arrows indicate glioma cells invading the brain parenchyma beyond the main tumor border (dashed line) for more than 150 μm (scale bars, 20 μm) and bar histogram of the number of invading tumor cells 17 days after glioma cell transplantation. Statistical analysis: Data are expressed as the mean number (± s.e.m.) **(B)** $n = 7$–12, $^{**}p < 0.001$, Student's *t*-test; **(C)** $n = 6$–10, $^{**}p < 0.001$, long-rank test; **(D)** $n = 4$, $^{*}p < 0.05$, Student's *t*-test; **(E,F)** $n = 4$–5, $^{*}p < 0.05$, Student's *t*-test. **(G)** $n = 3$–4, $^{**}p < 0.001$, $^{*}p < 0.05$, Student's *t*-test.

selected clone, we performed chemotaxis experiments toward CXCL16: **Figure 4D** shows that shCXCR6 cells (+IPTG) did not respond to CXCL16 ($n = 4$ experiments in duplicate, $p < 0.05$; Two- way ANOVA followed by Holm–Sidak *post-hoc* test). Since CXCL16 is present in GCM and CXCL16 stimulation increases GL261 proliferation (**Figure 4B**), we speculated that basal cell proliferation might be altered in shCXCR6 cells: as reported in **Figure 4E**, cell proliferation measured at 24, 48, 72, 96 h was reduced in IPTG induced GL261shCXCR6 cells (+IPTG) compared to GL261shCXCR6 not treated cells (-IPTG) ($n = 4$ experiments in quadruplicate, $p < 0.05$;One way ANOVA followed by Student-Newman-Keuls Method).

To confirm a role for CXCR6 in glioma development also *in vivo*, we orthotopically implanted GL261shCXCR6 cells, silenced or not with IPTG (+/-IPTG), into the brain of *wt* mice. Tumor-bearing mice were supplied with drinking water with or without IPTG, respectively, and, 17 days after implantation, were sacrificed for tumor volume analysis (**Figure 4F**). Mice injected with tumor cells silenced for CXCR6 revealed a significant reduction (67%) in tumor volume compared to mice injected with GL261 expressing CXCR6 (2.19 ± 0.47 mm^3 shCXCR6 + IPTG mice vs. 6.54 ± 1.26 mm^3 shCXCR6–IPTG) ($n = 5$, $p < 0.05$; Student's *t*-test). These mice were also i.p. injected with BrdU 4 h before sacrifice, in order to analyze tumor cell proliferation *in vivo*: as shown in **Figure 4G**, shCXCR6+ IPTG cells revealed a significant reduction in BrdU incorporation (0.52 ± 0.08% of tumor area) compared to control cells (0.81 ± 0.05% of tumor area) ($n = 3$, $p < 0.05$; Student's *t*-test). Moreover, as reported in **Figure 4H**, in mice injected with tumor cells silenced for CXCR6 there was a reduced number of glioma cells that migrate and invade the surrounding brain tissue (15.00 ± 1.8 cells for brain slice) compared to mice injected with cells not silenced (28.6 ± 3.9 cells for brain slice) ($n = 3$–4, $p < 0.05$; Student's *t*-test).

CXCL16 Released From Glioma Plays a Role in Tumor Development *in vivo*

To prove a role of CXCL16 released from glioma cells in tumor progression, GL261 cells were engineered for constitutive CXCL16 silencing, using shCXCL16 construct. As shown in **Figure 5A**, we selected a shCXCL16 cell clone with 80% reduction in CXCL16 mRNA expression compared to GL261 cells. GL261 or shCXCL16 cells were implanted into the brain of adult *wt* mice and 17 days after implantation mice were sacrificed for tumor volume analysis (**Figure 5B**). As reported in **Figure 5C**, mice injected with shCXCL16 cells revealed a significant reduction (67%) in tumor volume compared to mice injected with GL261 (3.3 ± 0.40 mm^3 shCXCL16 vs. 9.6 ± 0.67 mm^3 GL261) ($n = 4$; $p < 0.001$; Student's *t*-test). Glioma-bearing mice were also injected with BrdU 4h before animal sacrifice: analysis of BrdU incorporation in tumor cells (**Figure 5D**) revealed a significant reduction in BrdU$^+$ cells in mice implanted with shCXCL16 cells (0.45 ± 0.02% of tumor area), compared to mice implanted with GL261 cells (0.8 ± 0.15% of tumor area) ($n = 3$; $p < 0.05$; Student's *t*-test). In addition (**Figure 5E**) we found that mice injected with tumor cells silenced for CXCL16 presented a reduced number of glioma cells that migrate and invade the surrounding brain tissue (12.16 ± 0.82 cells for brain slice) compared to mice injected with GL261 (24.85 ± 1.49 cells for brain slice) ($n = 3$, $p < 0.05$; Student's *t*-test). To exclude that differences observed in *vivo* between tumor volume in GL261 and shCXCL16-GL261 bearing mice might be due to clonal differences between cells rather than to the CXCL16 silencing, we checked for the expression of markers involved in tumor immune recognition (specifically MHC class I, CD1 and PD-L1) as well as tumor cell migration and invasion (CD44). Flow cytometry analysis revealed no differences in the expression of all these markers (**Supplementary Figure 1**). All these data confirm that CXCL16 released by glioma cells concurs to tumor progression, and promotes tumor cell proliferation.

Effects of CXCL16/CXCR6 Axis in Patient's Derived GBM Cells

To investigate whether CXCL16/CXCR6 also modulates cell migration, invasion and proliferation in human GBM, we isolated tumor cells from patient's derived biopsies (GBM 13, 19, 40, 45) and analyzed their expression of CXCL16 and CXCR6 by RT-qPCR. We first compared the expression level of *cxcl16* and *cxcr6* mRNAs in whole patient's tissue, and in the corresponding isolated tumor cells. RNAs from primary human T-cells and a fibroblasts were used as positive controls for *cxcr6* and *cxcl16* expression, respectively. All the examined tissues express high levels of *cxcl16* and *cxcr6*, compared to normal brain tissues (see **Figure 2A**); however, the corresponding primary cells, even if cultured for only few passages (from 1 to 3), showed a strong reduction in their expression level (**Figure 6A**). This reduction could be due to a culture-dependent variation in the expression level in tumor cells, but we cannot exclude that the differences

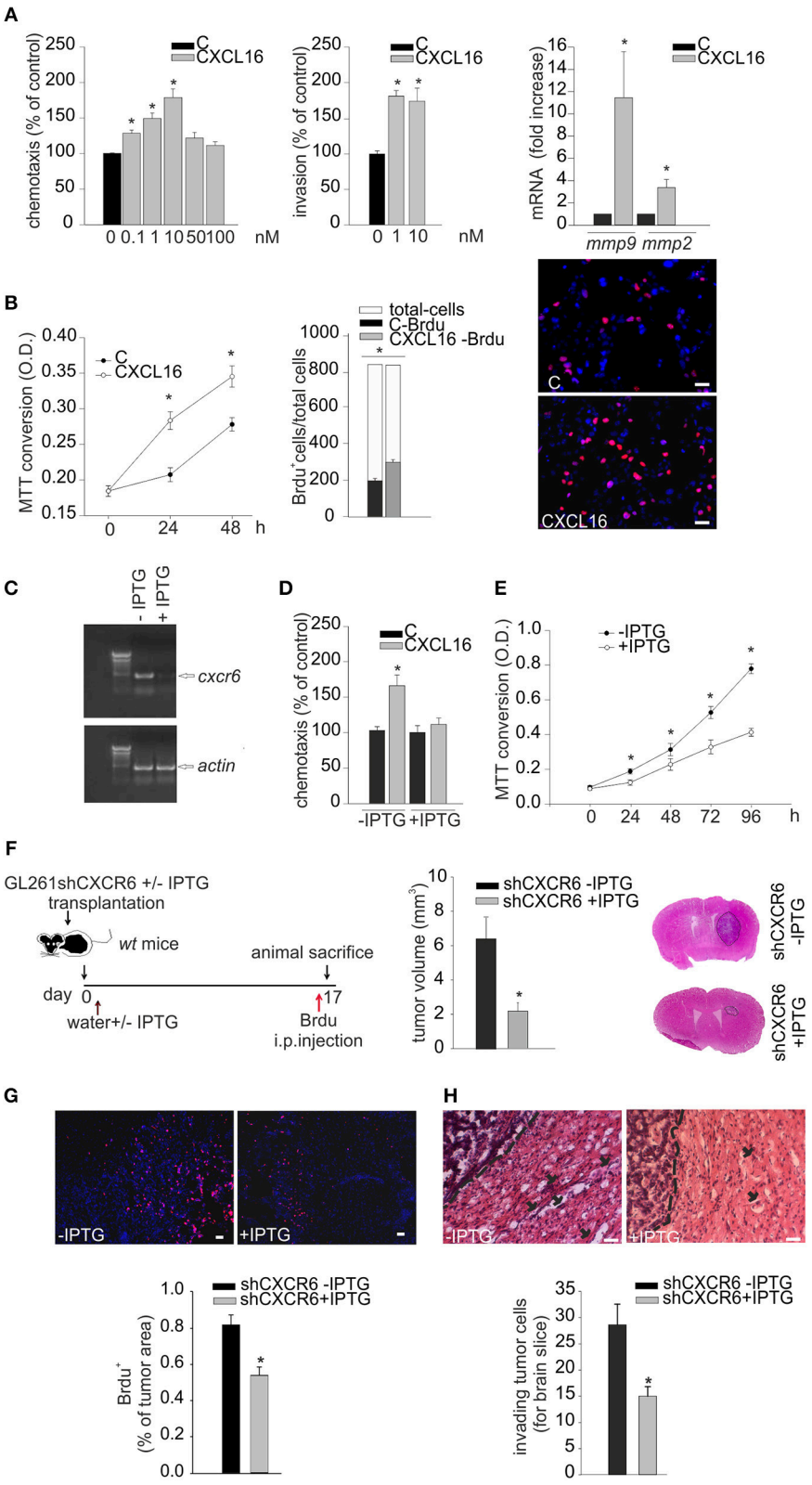

FIGURE 4 | Biological effects of CXCL16/CXCR6 axis on mouse glioma cells. **(A)** Left panel, chemotactic assay (24 h) of GL261 cells in the absence (C) or presence of different doses of CXCL16. Data are expressed as percentage of cell migration toward CXCL16 *vs.* control. Central panel, invasion assay of GL261 cells in

(Continued)

FIGURE 4 | the absence (C) or presence of different CXCL16 concentrations (1, 10 nM, 48 h). Data are expressed as percentage of cell invasion in CXCL16 stimulated cells vs. control. Right panel, expression analysis of *mmp2*, *mmp9* metalloproteinases mRNAs by RT-qPCR in GL261 cells not stimulated (vehicle), or stimulated with CXCL16 (10 nM, 24 h). For each gene data are expressed as specific mRNA fold increase in CXCL16 treated cells normalized to specific mRNA expression in vehicle; **(B)** Left panel, growth curve (0, 24, 48 h) of GL261 cells unstimulaed (C) or stimulated with CXCL16 (10 nM); data are expressed as MTT conversion optical density; middle panel, BrdU incorporation assay on GL261 stimulated with CXCL16 (10 nM, 4 h), or not (C). Data are expressed as number of $BrdU^+$ cells (black or gray bars) within total counted cells (white bar); Right panel, representative images (scale bar = 20 μm) of BrdU incorporation in GL261 cells unstimulated or stimulated with CXCL16 (BrdU, red signal; Hoechst, blue signal); **(C)** RT-PCR for *cxcr6* and *actin* mRNAs expression in GL261 shCXCR6 cells induced or not with IPTG (+/–IPTG); **(D)** Chemotactic assay of GL261shCXCR6 induced or not with IPTG, not stimulated (C) and stimulated with CXCL16 (10 nM; 24 h); data are expressed as percentage of cell migration toward CXCL16 vs. control; **(E)** Growth curve (0, 24, 48, 72, 96 h) of GL261shCXCR6 cells induced or not with IPTG (+/–IPTG); data are expressed as MTT conversion optical density; **(F)** (left) representative scheme of GL261shCXCR6 cells transplantation in *wt* mice; (right) bar histogram of the mean (± s.e.m.) of tumor volume in shCXCR6-IPTG or shCXCR6+IPTG treated mice, and representative hematoxylin-eosin stained coronal brain sections of glioma bearing mice; **(G)** BrdU proliferation analysis in mice transplanted with GL261shCXCR6 cells induced or not with IPTG. (Top) representative images (scale bar = 20 μm) of proliferating BrdU+ cells (red) within tumor area; (bottom) bar histograms of immunofluorescence analysis of $BrdU^+$ cells; data are expressed as % of $BrdU^+$ staining per tumor area. **(H)** Analysis of glioma cells invasion of surrounding brain tissue in mice injected with GL261shCXCR6 treated or not with IPTG. (Top), Representative coronal brain sections stained with hematoxylin/eosin. Black arrows indicate glioma cells invading the brain parenchyma beyond the main tumor border (dashed line) for more than 150 μm. Bottom, bar histogram of the number of glioma invading cells. Statistical analysis: Data are expressed as the mean number (± s.e.m.) **(A)** left panel $n = 3$ in triplicate, $^*p < 0.05$, one-way ANOVA followed by Holm–Sidak *post-hoc* test; right panel $n = 3$, $^*p < 0.05$, one-way ANOVA followed by Holm–Sidak *post-hoc* test; **(B)** left panel $n = 3$ six-replicates, $^*p < 0.05$, one-Way ANOVA followed by Student-Newman-Keuls Method; central panel $n = 3$, $^*p < 0.05$, Student's *t*-test; **(D)** $n = 4$ in duplicate, $^*p < 0.05$, two-way ANOVA followed by Holm–Sidak *post-hoc* test; **(E)** $n = 4$ in quadruplicates, $^*p < 0.05$, one-Way ANOVA followed by Student-Newman-Keuls Method; **(F)** $n = 5$, $^*p < 0.05$, Student's *t*-test); **(G)** $n = 3$, $^*p < 0.05$, Student's *t*-test; **(H)** $n = 3$–4, $^*p < 0.05$, Student's *t*-test.

observed are due to the selection of specific cell subpopulation (or the elimination of infiltrating cells in the tumor tissue) during the cell culture procedures. By flow cytometry analysis, using specific human CXCR6 PE-conjugated antibody, we confirmed the expression of membrane CXCR6 protein in primary GBM19 and GBM45 cells (**Figure 6B**). As reported in **Figure 6C**, primary GBM19 and GBM45, cells responded to CXCL16 stimulation (10 nM, 4 h) increasing their chemotactic index compared to unstimulated cells (control), thus suggesting the expression of functional CXCR6 ($n = 4$, $p < 0.05$; One way ANOVA followed by Holm–Sidak *post-hoc* test). Since it is known that CXCL12 is able to induce migration of GBM cells (36) and GBM19 and 45 cells do express CXCR4 (data not shown), CXCL12 stimulation was used as positive control of migratory activity.

GBM19 cells were also used to investigate CXCL16-induced cell invasion trough matrigel substrate (CXCL16 10 nM, 24 h) and cell proliferation (CXCL16 10 nM, 24, 48, 72 h). As shown in **Figure 6D**, upon stimulation with CXCL16, there was a significant increase in cell invasion (left panel; $n = 3$, $p < 0.05$; Student's *t*-test), and in cell proliferation (right panel; $n = 3$ experiments in five replicates, $p < 0.05$; Student's *t*-test) compared to un-stimulated cells (control). All these data indicate that activation of CXCL16/CXCR6 axis in human primary GBM cells is able to promote tumor cell proliferation, migration and invasion. To further support our data, we used cBioportal Database to look at a possible correlation between patient survival and CXCR6 expression in glioma tumor. We first explored the alteration in *cxcr6* gene in a merged cohort of low grade glioma (LGG) and GBM (TCGA, Cell 2016) and found a significant increase in patient months survival associated with *cxcr6* deletion (median months survival 130.7 vs. 20.6 in normal cases, Log rank Test *P*-value 0.0339); we than looked at *cxcr6* mRNA expression data in a cohort of Glioblastoma (TCGA, Cell 2013) and found a significant decrease in months survival associated with *cxcr6* mRNA overexpression (median months survival 5.2 vs. 14 in normal cases, Log rank Test *P*-value 0.00417), **Supplementary Figure 2**.

DISCUSSION

Communication among cells in the brain parenchyma, including neurons, astrocytes and microglia, is determinant to maintain brain homeostasis. The identification of key players in the cellular cross-talk within the brain, and their alterations in pathological conditions, can be useful to develop specific tools to limit brain damage.

In this paper we report for the first time that: (i) CXCL16 drives microglia toward an anti-inflammatory phenotype, able to counteract inflammatory conditions "*in vitro*"; (ii) CXCL16 released by glioma cells drives GAMs polarization toward an anti-inflammatory phenotype which is determinant to promote glioma progression; (iii) CXCL16 released by tumor cells contributes to glioma cell proliferation, migration, and invasion of brain parenchyma.

Recently we reported that CXCL16, acting on astrocytes, drives neuroprotective effects in brain ischemia, counteracting glutamate excitotoxic damage (11, 12). Besides glutamate-excitotoxicity, also neuroinflammation is a common feature to many chronic or acute neurodegenerative disorders, including brain ischemia. Following acute brain damages, microglia cells at the site of injury produce anti-inflammatory cytokines, scavenger receptors, and trophic factors thus promoting restorative processes. However, later on, microglia acquire a pro-inflammatory phenotype releasing pro-inflammatory cytokines, chemokines, and inducible nitric oxide synthase, all involved in the exacerbation of brain damage (37, 38).

In this paper we report that CXCL16 modulates the inflammatory phenotype of microglia *in vitro*: in particular, we found that CXCL16 *per se* is able to drive microglia toward an anti-inflammatory phenotype and that, in the context of an inflammatory microenvironment (LPS and IFNγ), CXCL16 can contrast the acquisition of a pro-inflammatory phenotype. Considering these data, we speculate that, in addition to limit neuronal damage, counteracting excitotoxicity, the release of

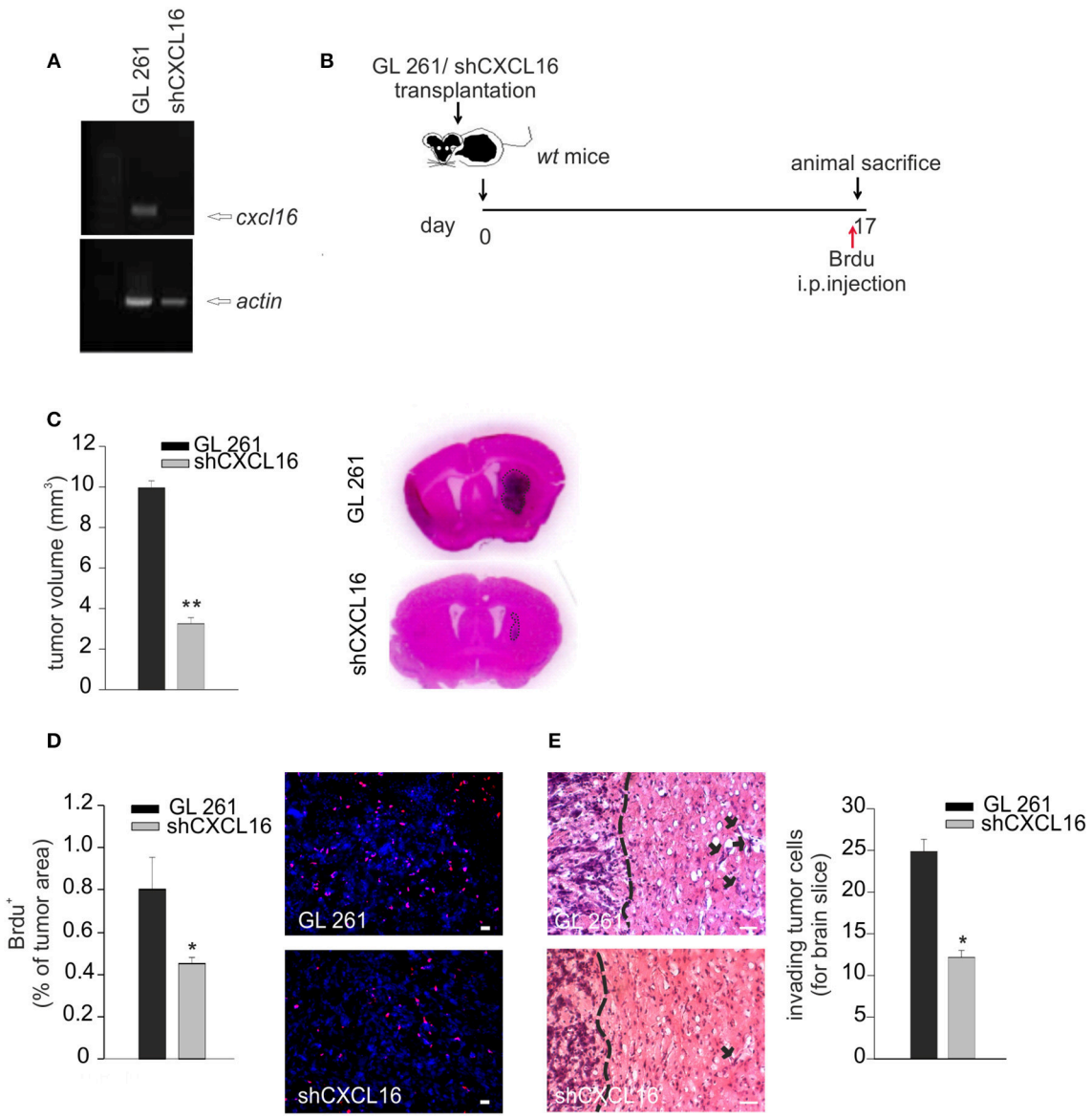

FIGURE 5 | CXCL16 released by glioma cells acts promoting tumor proliferation *in vivo*. **(A)** RT-PCR for *cxcl16* and *actin* mRNAs expression in GL261 and shCXCL16 cells; **(B)** representative scheme of GL261 and shCXCL16 cells transplantation in *wt* mice; **(C)** bar histogram of tumor volume in GL261 or shCXCL16 cells bearing mice, and representative hematoxylin-eosin stained coronal brain sections; **(D)** BrdU proliferation analysis in mice bearing GL261 or shCXCL16 cells. Left panel, bar histograms of immunofluorescence analysis of $BrdU^+$ cells; data are expressed as % of $BrdU^+$ staining per tumor area; right panel, representative images (scale bar = 20 μm) of proliferating BrdU+ cells (red) within tumor area; **(E)** Analysis of glioma cells invasion of surrounding brain tissue in mice injected with GL261shCXCL16 or GL261. Representative coronal brain sections stained with hematoxylin/eosin. Black arrows indicate glioma cells invading the brain parenchyma beyond the main tumor border (dashed line) for more than 150 μm. Right, bar histogram of the number of glioma invading cells. Statistical analysis: Data are expressed as the mean (± s.e.m.) **(C,D)** $n = 4$, $^*p < 0.05$, $^{**}p < 0.001$, Student's *t*-test; **(E)** $n = 3$, $^*p < 0.05$, Student's *t*-test.

CXCL16 in response to ischemic insult (12) might also trigger neuroprotection by limiting neuroinflammation.

The same microglia phenotype triggers different effects on brain homeostasis, in a context-dependent way. During the first phase of glioma development, microglia reacts to counteract tumor growth, phagocytizing tumor cells and activating pro-inflammatory T-cell immune response; at later stages, glioma-released factors produce chronic stimuli, contributing to the establishment of a pro-tumoral microenvironment, also switching GAMs toward an anti-inflammatory/pro-tumor phenotype (2, 32, 34, 39). In line with what already reported (40), we found that CXCL16 is over-expressed in human GBM tissues obtained from patients and demonstrated, *in vitro*, that CXCL16 released by glioma cells acts as a mediator for microglia polarization. We report that neutralization of soluble CXCL16 in GCM results in a strong reduction in the expression of

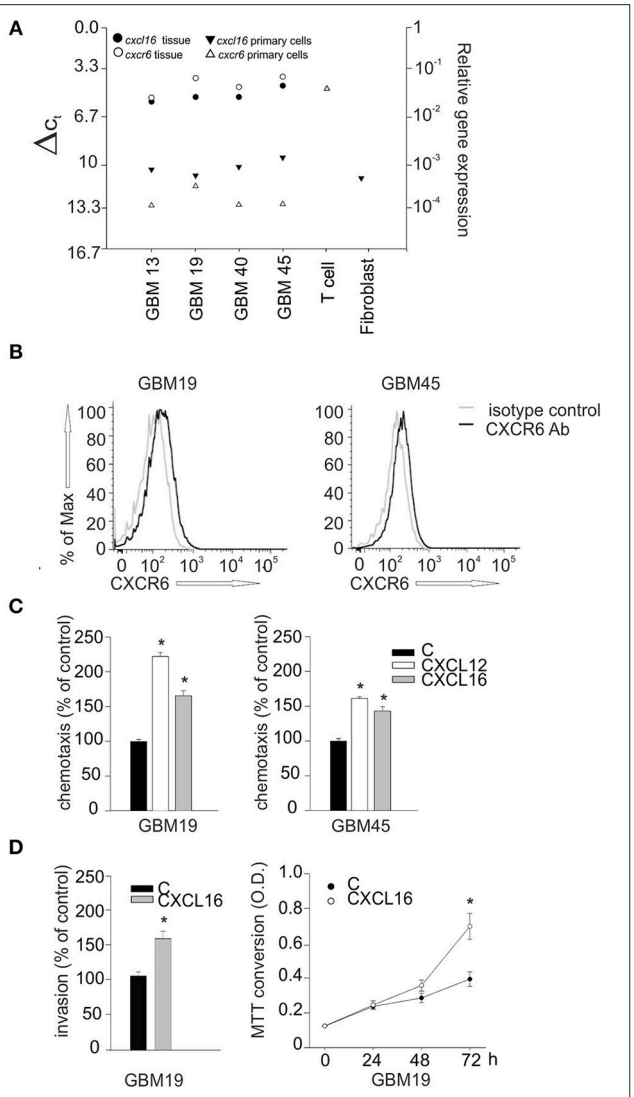

FIGURE 6 | Biological effects of CXCL16/CXCR6 axis on primary human glioblastoma cells.**(A)** *cxcl16* and *cxcr6* mRNAs expression in human GBM tissues (black and white circles), in human primary GBM cells derived from the same tissues and in human primary T cells and fibroblasts (black and white triangles) determined by RT-qPCR; **(B)** CXCR6 surface expression on primary GBM19 and GBM45 as evaluated by flow cytometry. Black and gray lines represent CXCR6 staining and isotype control, respectively; **(C)** Chemotaxis assay of human primary glioblastoma cells: GBM19 and GBM45, toward CXCL16 (10 nM, 4 h), CXCL12 (50 nM, 4 h) and vehicle (C). Data are expressed as percentage of cell migration vs. control; **(D)** Left panel, matrigel invasion assay on human GBM19, toward CXCL16 (10 nm, 24 h) and vehicle (C). Data are expressed as percentage of cell invasion vs. control ($n = 3$, $p < 0.05$, Student's *t*-test); right panel, proliferation assay of GBM19 upon stimulation with CXCL16 (10 nM) at different time points (0, 24, 48, and 72 h), data are expressed as MTT conversion optical density. Statistical analysis: Data are expressed as the mean (± s.e.m.) **(C)** $n = 4$, $^*p < 0.05$, one-way ANOVA followed by Holm–Sidak *post-hoc* test; **(D)** left panel $n = 3$, $^*p < 0.05$, Student's *t*-test; right panel $n = 3$ in five replicates, $^*p < 0.05$, Student's *t*-test.

anti-inflammatory genes in microglia *(arg-1, chil3, retnla, cd163*), and in a significant increase of pro-inflammatory genes (*nos2, il-1b, cd86, tnfa*), compared to microglia cells exposed to control GCM, suggesting that soluble CXCL16 released by tumor cells promotes microglia pro-tumor phenotype.

Using *cxcr6ko* mice, we confirmed the crucial role of the CXCL16/CXCR6 axis in the establishment of a pro-tumor microenvironment. These mice, transplanted with GL261 cells, have a strong reduction in tumor volume and a significant increase in mice survival when compared to *wt* animals. Moreover, analysis of Iba1 and CD68 immune-reactivity within the tumor mass reveals a different activation state of GAMs in *cxcr6ko* mice, indicating an effect of CXCR6 signaling on GAMs activation. Accordingly, the analysis of $CD11b^+$ cells derived from the brain hemispheres of tumor injected mice, confirms the role of CXCL16 signaling in determining GAMs polarization: indeed, the strong up-regulation of anti-inflammatory genes observed in the brain of *wt* animals did not occurred in *cxcr6ko* mice. Other chemokines released by glioma cells, such as CCL2, have been reported to play a role in the recruitment of GAMs within the tumor mass, but do not contribute to their phenotypic changes (18). Thus, CXCL16 is the first chemokine released by glioma cells that has been proven to drive the interplay with GAMs to acquire a phenotype that supports tumor growth.

GBM are characterized by extensive proliferation and dissemination of the tumor cells within the brain that hinders complete surgical resection (41, 42). The high invasion ability of GBM is due to multiple autocrine motility-enhancing signaling systems, and to distinct signals derived from non-tumor infiltrating and stromal cells.

For the first time we demonstrated that CXCL16/CXCR6 axis plays a role in promoting glioma growth, directly acting on tumor cells. Specifically, we demonstrated that: (i) GL261 cells express both CXCL16 and CXCR6; (ii) stimulation with CXCL16 promotes GL261cell migration, invasion, and proliferation; (iii) the silencing of CXCR6 on glioma cells reduces their proliferation rate and migration ability; (iv) *in vivo*, transplantation of CXCR6-silenced GL261 cells in *wt* mice leads to a reduced tumor cell proliferation and infiltration and tumor volume compared to mice injected with not silenced glioma cells.

The absence of CXCR6 on glioma cells, but not on other cells of tumor microenvironment, reduces but does not block tumor development, suggesting that other signals are important for tumor progression, and again confirming that CXCL16/CXCR6 signaling acts also on cells of the tumor microenvironment. The hypothesis that CXCL16 released from tumor cells acts in an autocrine/paracrine way to promote tumor progression is further confirmed by the significant reduction in tumor volume, proliferation, and infiltration in mice bearing glioma cells silenced for CXCL16. We have previously shown that $GL261/cd133^+$ cells grafted in mice resulted in a higher tumor volume compared to mice grafted with GL261 (43), we now report that within glioma, $GL261/cd133^+$ cells do present a higher *cxcr6* mRNA expression compared to GL261, thus suggesting that autocrine CXCL16 signaling plays also a role in cancer stem cells.

The role of CXCR6 in human glioma cells is controversial: high expression of CXCL16 has been reported in several human GBM cell lines, as well as in human glioma tissues (TCGA database), in contrast to a very low, sometimes almost

undetectable, expression of CXCR6 (21). Moreover, by *in situ* hybridization, it has been shown that CXCR6 is expressed in glioma only on a small population of cells that are positive for markers of embryonic or neural stem cells (21). Considering the very low expression level of *cxcr6* in GBM cells, as measured by real-time PCR, authors speculated that CXCR6 could not play a role in glioma cell biology (22). While we confirm that primary human GBM cells from patients express low levels of *cxcr6* and *cxcl16*, we report that the original GBM tissues, acutely dissected from patients, over-express both *cxcl16* and *cxcr6*, compared to human control brain tissues. In spite of the low expression level of *cxcr6*, we demonstrate that the human primary GBM cells do express CXCR6 protein (as revealed by flow cytometry analysis) and respond to CXCL16 stimulation, modulating migration, invasion, and proliferation, thus suggesting an important activity of CXCL16 in glioma cell biology also in humans. According to Hattermann et al. (22), soluble CXCL16 might act with an "inverse signaling" mechanism that is independent by its receptor, and dependent by the transmembrane form of the chemokine expressed by cells; however, we demonstrated that the direct effects of the soluble CXCL16 on GL261 cells, in terms of proliferation, migration and invasion, are prevented when these cells are silenced for the CXCR6 receptor, but still expressing transmembrane CXCL16, both *in vitro* and *in vivo* (**Figures 4D–H**), highlighting an important activity of CXCR6 at least in these cells. In analogy to what has been recently reported for another GBM-derived molecule, osteopontin, which regulates glioma cell invasiveness and tumor growth (44) and the pro-tumorigenic reprogramming of microglia (45), we demonstrate that soluble CXCL16 released by glioma cells drives GBM growth directly promoting tumor cell proliferation, invasion, and acting on GAMs establishing a pro-tumor microenvironment. We also prove that human infiltrating GAMs do express *cxcr6*, further supporting the idea that also in human, CXCL16 released by tumor cells, might act on these cells promoting a pro-tumor microenvironment.

For the first time we show that CXCL16/CXCR6 axis plays an important role in driving the cross-talk among cells within the brain and microglia, as well as infiltrating macrophages, triggering a phenotype that, depending on environmental cues, can be either neuroprotective or detrimental. These data highlight the potential use of CXCL16 as pharmacological tool to augment the anti-inflammatory cellular response and to restrain inflammatory stimuli. Moreover, since disruption of CXCL16 signaling counteracts glioma progression limiting cell proliferation and migration but also microglia pro-tumor polarization, a multi-target therapy including the use of a CXCR6 antagonist, together with drugs approved by Food and Drug Administration (FDA) and currently used to treat GBM patients (such as Temozolomide or checkpoint inhibitors that target programmed cell death protein 1,PD-1) could be potentially considered in the future.

ETHICS STATEMENT

The study was approved by the Policlinico Umberto Primo Ethics Committee and Neuromed Ethics Committee, and the animal experiments were approved by the Italian Ministry of Health in accordance with the guidelines on the ethical use of animals from the European Community Council Directive of September 22, 2010 (2010/63/EU).

AUTHOR CONTRIBUTIONS

FL and GD designed, performed and analyzed the experiments. FA performed cytofluorimetric analysis. AS and VE surgically resected glioma tissue form GBM patients. CL and FT designed the experiments, analyzed data 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.2018.02750/full#supplementary-material

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

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Molecular Mechanisms Directing Migration and Retention of Natural Killer Cells in Human Tissues

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A large body of data shows that Natural Killer (NK) cells are immune effectors exerting a potent cytolytic activity against tumors and virus infected cells. The discovery and characterization of several inhibitory and activating receptors unveiled most of the mechanisms allowing NK cells to spare healthy cells while selectively attacking abnormal tissues. Nevertheless, the mechanisms ruling NK cell subset recirculation among the different compartments of human body have only lately started to be investigated. This is particularly true for pathological settings such as tumors or infected tissues but also for para-physiological condition like pregnant human uterine mucosa. It is becoming evident that the microenvironment associated to a particular clinical condition can deeply influence the migratory capabilities of NK cells. In this review we describe the main mechanisms and stimuli known to regulate the expression of chemokine receptors and other molecules involved in NK cell homing to either normal or pathological/inflamed tissues, including tumors or organs such as lung and liver. We will also discuss the role played by the chemokine/chemokine receptor axes in the orchestration of physiological events such as NK cell differentiation, lymphoid organ retention/egress and recruitment to decidua during pregnancy.

Keywords: natural killer cells, chemokines and chemokine receptors, migration and residency, tumor and inflammation, pregnancy

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INTRODUCTION

The initial view describing Natural Killer (NK) cells as a quite homogeneous $CD3^{neg}$ $CD56^{+}$ circulating lymphocyte population has been largely overcome. NK cells have been recently included in a wider innate lymphoid cell (ILC) family, and circulating cells are just the tip of an iceberg formed by a conspicuous and heterogeneous lymphoid population colonizing both, lymphoid and non lymphoid tissues (1–3). Moreover, cytometry by time-of-flight (CyTOF) highlighted the existence in peripheral blood (PB) of a single individual of at least 30,000 different NK cell phenotypes (4). These findings consolidate the concept that observed phenotypic and functional NK cell status actually represents a single crystalized picture of a very dynamic process. Nevertheless, in healthy individuals, two main circulating PB NK cell populations have been extensively studied, $CD56^{bright}$ and $CD56^{dim}$ NK cells, which represent sequential stages of maturation and show a dichotomy in phenotypic and functional properties (5). These include the

expression of MHC class I-specific inhibitory Killer Ig-like Receptors (KIRs), restricted to $CD56^{dim}$ NK cells that represent the majority of cells circulating in blood. KIRs are involved in NK cell "education," a phenomenon that provides the basis of self-tolerance and generates "armed" cells, i.e., NK cells fully responsive to the engagement of activating receptors (i.e., NCR, NKG2D, and DNAM-1) (6, 7). $CD56^{dim}$ NK cells also express high levels of CD16, thus exerting strong antibody-dependent cellular cytotoxicity (ADCC). Moreover, they efficiently respond to cytokines stimulation and are characterized by a chemokine receptor repertoire giving them the potential to colonize lymphoid and non-lymphoid tissues in response to a proper chemokine milieu.

The composition of the milieu can greatly vary in perturbed tissues. This justifies the prevalence in some tumors of immature, poor cytolytic $CD56^{bright}$ NK cells that are undetectable in matched healthy tissues (8). Tumor parenchyma, as well as the immune cells participating to the inflammatory processes, may change the microenvironment providing NK cells with a plethora of stimuli. These include membrane-bound or soluble molecules such as chemokines or cytokines (TGF-β, IL-12, IL-18), which either promote or dampen innate and adaptive immune responses. Cytokines, in addition to shape the functional activity of NK cells, modify their chemokine receptor repertoire altering their native migratory potential (9–13) and at the same time provide signals essential to generate, expand and recall memory NK cell populations (14). Interestingly, recent data showed that non-hematopoietic organs such as liver can be colonized by peculiar tissue resident NK cell populations that belong to the memory NK cell reservoir able to mediate "recall" responses (15).

Here, we will recapitulate studies that analyzed the main mechanisms regulating NK cell trafficking in lymphoid and non-lymphoid tissue under either steady state or "perturbed" conditions, including tumors, inflammation and pregnancy.

DEFINING DYNAMICS OF NK CELLS IN HEALTHY TISSUES

NK cells are not exclusively found in PB but populate different tissues and organs. The traditional view of NK cells as "armed" effector cells, which patrol human body through blood ready to extravasate to the site of injury, has been partially revisited and a growing number of studies show that NK cells might also stably reside in most peripheral tissues, under steady-state conditions.

Until recently, the task of depicting NK cell distribution in human compartments has suffered from several methodological shortcomings. Earlier analyses often relied on the use for NK cells detection of markers poorly specific and/or unable to distinguish the two main NK cell subsets, i.e., $CD56^{bright}$ $CD16^{low/neg}$ $Perf^{low}$ and $CD56^{dim}$ $CD16^{pos}$ $Perf^{high}$. The advent of new OMICS technologies, and the possibility to perform single-cell analyses have expanded our understanding on the distribution of NK cells across human body. Indeed, in the recent years, our knowledge about NK cell diversity has further increased with the identification of NK cell subsets specifically populating various peripheral solid organs, such as lung, liver, lymphoid tissues, and uterus. These findings have challenged the classical view of NK cells as a lineage comprising a relatively homogeneous population of cells with similar functions and longevity. Nonetheless, at variance with B and T cells, we know little about recirculation and trafficking of NK cells across peripheral tissues. Although NK cells express an ample array of chemotactic receptors, the role of the different chemokines in guiding *in vivo* the distribution of NK cells through the body compartments still remains unclear. The distribution of NK cells seems to be subset-specific in mouse, as different NK cell subsets showed organ-specific localizations (16). Conversely, this issue has been poorly investigated in the human system. As the two major PB-NK cell subsets display a chemokine receptors pattern that only partially overlaps, they may have a peculiar tissue-specific compartmentalization (**Figure 1**). PB-$CD56^{bright}$ NK cells are uniquely characterized by the expression of CCR7, CXCR3, and L-selectin (CD62L), which justify their abundance in secondary lymphoid tissues (SLTs). Conversely, PB-$CD56^{dim}$ NK cells, despite sharing the CXCR4 receptor with $CD56^{bright}$ NK cells, are equipped with receptors specific for inflammatory chemokines, such as CXCR1, CXCR2, CX_3CR1 (8, 16, 17). Additionally, $CD56^{dim}$ NK cells can migrate in response to factors that do not belong to the chemokine superfamily. These include the proinflammatory protein chemerin and the sphingosine 1-phosphate (S1P) molecule that affect trafficking of NK cells during inflammation or steady-state conditions, respectively (18, 19). Based on the different expression of chemotactic receptors, the tissue distribution of human NK cell subsets observed under steady-state conditions is dependent on the expression of local tissue-specific environmental signals. In order to shed light on the mechanisms lying behind the migratory properties of PB NK cells, a wide array of samples derived from different body compartments was analyzed to investigate the presence and distribution of functionally different NK cell subsets (8). The study showed that the relative distribution of $CD56^{bright}$ and $CD56^{dim}$ NK subsets in the various human districts does not parallel that in PB. $CD56^{dim}$ NK cells represent the major NK cell subset in bone marrow (BM), lung, spleen, subcutaneous adipose tissue and breast tissue, whereas $CD56^{bright}$ NK cells abundantly outnumber cytotoxic NK cells in gastric and intestinal mucosa associated lymphoid tissues (MALTs), liver, uterus, visceral adipose tissue, adrenal gland, and kidney (8, 20, 21). Importantly, the relative distribution of the two main NK cell subsets matched with the specific patterns of chemotactic factors expressed in the tissues (8).

A main question arising from the detection of NK cells in many organs is whether NK cells stably reside in those tissues or could eventually exit and recirculate. Studying the dynamics of NK cells under steady-state conditions is limited by the difficulty of having access to samples from human body districts. On this regard, useful hints may be derived from studies in which human subjects have been treated with monoclonal antibodies directed against molecules pivotal in lymphocytes migration, such as integrins. This is the case of natalizumab, a humanized monoclonal antibody directed against the α4-chain of VLA-4 (α4β1) and α4β7 integrins, widely expressed on many different lymphocyte populations including T cells,

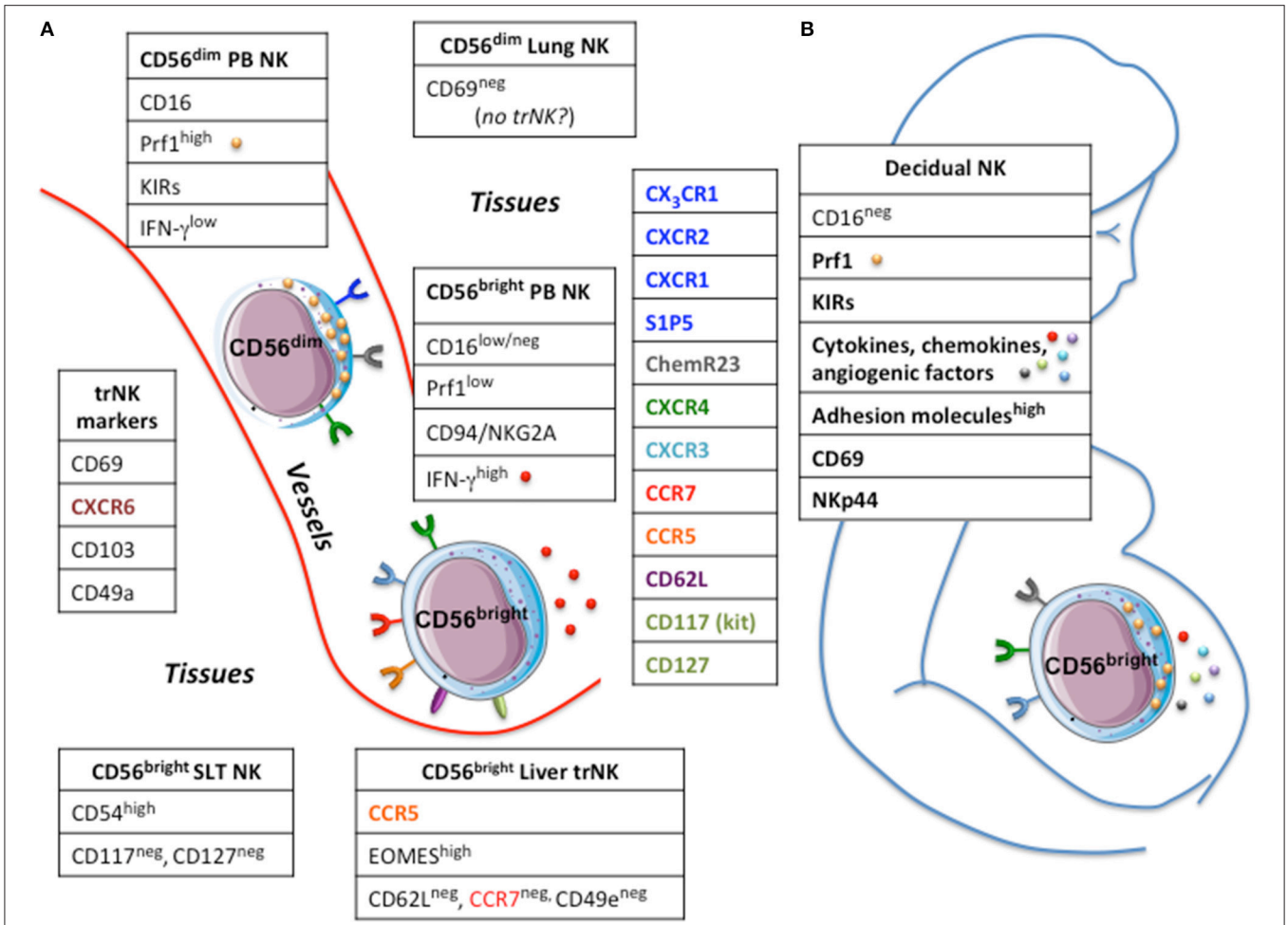

FIGURE 1 | Circulating and tissues resident NK cells. **(A)** In human two main NK cell subsets, CD56bright and CD56dim, can be detected in peripheral blood (PB NK) having a different repertoire of chemokine receptors. Tissue resident (tr) NK cells share the expression of certain markers but express molecules typical of the hosting tissue.**(B)** large numbers of NK cells populate the decidua, particularly in the first trimester of pregnancy. Decidual NK cells have unique phenotypic and functional characteristics, which contribute to support nutrition of the fetus, ensure maternal-fetal tolerance and control viral infections. Prf1, perforin; KIRs, killer cell immunoglobulin-like receptors; SLT, secondary lymphoid tissues.

B cells, and NK cells as well as on a majority of monocytes and macrophages. Interestingly, it has been reported that 1-year treatment with natalizumab in multiple sclerosis patients resulted in a pronounced accumulation (almost 2-fold increase compared to baseline levels) of NK cells in PB (22), which then gradually decreased upon treatment interruption (23). These data are in evident agreement with a dynamic passage of circulating NK cells across the endothelial barriers for patrolling peripheral tissues, although it remains to be determined whether it might occur also in steady-state or just under inflammatory conditions.

In addition to extravasation from PB to solid tissues, NK cells may eventually egress from peripheral tissues and trafficking to SLT. This re-circulation has been suggested by the direct investigation of afferent lymph draining from normal skin (24) and analysis of cellular content in seroma fluid upon axillary lymph nodes (LN) dissection, which represents an accumulation of *bona fide* afferent lymph (25, 26). Interestingly, most seroma NK cells expressed high level of CCR7 and CD62L, as well as CXCR4, CXCR3, a chemokine receptor repertoire identifying lymphocyte populations migrating toward SLTs. These data indicate that high endothelial venules (HEVs) might not represent the only route for NK cell entrance in SLTs. Conversely, very little information is available regarding the egress of NK cells from SLTs. It has been described in the murine model that changes in responsiveness of sphingosine-1 phosphate receptor 5 (S1P5) to its ligand (S1P) play a key role in allowing NK cell egress via lymphatics (27). However, whether this mechanism might also be effective in human has not yet been confirmed. Notably, NK cells have been detected in efferent lymph fluid and NK cells exiting from LN have a phenotype slightly different from that of NK cells found within SLTs. In particular, a portion of NK cells express significant amounts of KIR and CD16, implying that $CD56^{bright}$ NK cells might acquire

these molecules in the LN during inflammation and then egress through the efferent lymph for recirculating in PB (28).

All these previous studies have so far depicted the distribution of the two main "conventional" human NK cell subsets across the human body (8, 29). Recently, this issue reached a higher level of complexity because of data showing that various body districts harbor "unconventional" subsets of NK cells that apparently do not recirculate in the blood or lymphatics and adopt a unique phenotype that is distinct from that of circulating NK cells. Tissue residency has been described for NK cells as well as for other "helper" innate lymphoid cells (ILCs), T cell subsets (memory CD8, CD4 and Treg cells) and "innate-like" T cell types, including subpopulations of γ/δ T cells and natural killer T (NKT) cells (30). Tissutal NK cells, similarly to other lymphocytes residing in tissues, may display markers such as CD69, CD103 (also known as αE integrin) and CD49a (also known as α1 integrin), which are functionally involved in retaining them in tissues and, hence, can be useful for the identification and isolation of tissue-resident (tr) NK cells (**Figure 1**). As discussed earlier, at least three-quarters of NK cells in non-reactive lymph nodes display a CD56^{bright} Perf^{low} phenotype (20, 31). This accumulation is compatible with the pattern of adhesion molecules (CD62L) and chemokine receptors (CCR7) expressed on circulating PB-CD56^{bright} NK cells but not PB-CD56^{dim} NK cells. From recent data, it is possible to speculate that a fraction of NK cells reaching the LN could be retained within the structure as trNK cells. Supporting this hypothesis is the presence of a distinct subset of NK cells in human SLTs characterized by co-expression of CD69 and CXCR6, high expression of CD54 (ICAM-1) but lacking CD117 (c-kit) and CD127, the latter specifically expressed by CD56^{bright} NK cells (32). Because of the high level of CD54, these SLT-NK cells are also reminiscent of CD56^{bright} NKG2A^{pos} CD94^{pos} CD54^{pos} CD62L^{neg} NK cells that accumulate in tonsils of EBV carriers, which produce high amount of IFNγ, show very low plasticity even after prolonged cytokine stimulation, and are able to potently restrict EBV-induced transformation of B cells (33).

Among solid tissues, liver is abundantly populated by NK cells, where they represent up to 30–40% of all the lymphocytes populating this organ (34). At steady-state, NK cells are preferentially located in the hepatic sinusoids, often adhering to the endothelial cells (35). Similar proportion of CD56^{dim} and CD56^{bright} NK cell populations have been reported to populate this organ (36), but only CD56^{bright} has been described to own features of trNK cells. Indeed, liver CD56^{bright} NK cells are characterized by higher level of EOMES transcription factor, expression of CXCR6 and CD69 as well as CCR5 but absence of CD62L and CCR7 (37). Interestingly, the expression of CD49e (also known as α5 integrin or VLA-5 α chain) has been recently identified has a reliable marker able to distinguish conventional "circulating" NK cells from *bona fide* liver-NK cells, which are otherwise negative for this marker (38). Many reports have suggested the importance of CCR5 and CXCR6 in their localization and retention within liver parenchyma, since their cognate ligands (CCL3, CCL5, and CXCL16) are constitutively expressed by various parenchymal and non-parenchymal cells in the liver, including cholangiocytes, sinusoidal endothelial cells, hepatocytes and Kupffer cells (34). Investigation of human liver transplants has indicated that EOMES^{high} trNK cells can persist *in situ* for very long periods (up to 13 years in one human study), further supporting the idea that subsets of NK cells may stably reside within liver tissues. At the same time, circulating CD56^{bright} EOMES^{low} cells may be recruited to the liver and have the potential to become CD56^{bright} EOMES^{high} NK cells (39).

An exception to the aforementioned tissues is represented by lungs since: (i) the majority (∼80%) of NK cells populating these organs belongs to the CD56^{dim} Perf^{high} subset (40); (ii) only a limited fraction of Lung-NK cells is characterized by expression of markers consistent with tissue-residency (i.e., CD69). Interestingly, this fraction is mainly composed of CD56^{bright} CD16^{neg} and only a small proportion of CD56^{dim} CD16^{bright} NK cells (41), thus suggesting that "genuine" lung-resident NK cells may share some commonalities with CD56^{bright} trNK cells found in the uterus, liver, and lymphoid tissues (37). Lung-NK cells were detected in the parenchyma only, and were not found outside of the parenchyma, (i.e., blood vessels or bronchi) (8, 41). Therefore, overall, these data support a model in which human lungs mainly contain highly differentiated NK cells recirculating between lung and blood, rather than a stable pool of tissue-resident NK cells (41). Consistent with this hypothesis, using a parabiotic mouse model, it has been recently shown that parabiont-derived donor NK cells are able to rapidly replenish the majority of NK cells in the lungs of recipient mouse (42).

Development of tissue-resident lymphocytes seems to involve a transcription program inducing the expression of genes involved in tissue-retention while inhibiting that of genes important for tissue egress and trafficking. In mice, it was recently described that the transcription factor Hobit (homolog of Blimp-1 in T cells or ZNF683), a zinc finger protein, acts in concert with Blimp-1 (B lymphocytes-induced maturation protein) to serve as a master regulator of tissue-residency for lymphocytes. Thus, Hobit and Blimp-1 mediate a common transcriptional program that is shared among tr memory (Trm) T cells, NKT, trNK cells, and helper-like ILCs. Together with Blimp-1, Hobit sustain unresponsiveness to signals for SLT re-circulation from peripheral tissues by suppressing expression of *S1pr1* (which encodes S1P1), *Sell* (which encodes CD62L) and *Ccr7* (which encodes CCR7) (30). The role of Hobit in human Trm cells is less clear. Recent reports have shown peculiar results with regard to the expression of Hobit/ZNF683 in the two major human PB-NK cell subsets. Indeed, Hobit has been detected at high levels in circulating CD56^{dim}NK cells (despite this transcription factor is almost absent in circulating NK cells in mice) while only poorly expressed by PB-CD56^{bright} NK cells (43, 44).

However, it has been found that a strong Hobit/ZNF683 expression identifies a subset of intrahepatic CD56^{bright} NK cells in human liver, which additionally express a distinct set of adhesion molecules (CD69, CD49a) and chemokine receptors (CXCR6) consistent with tissue residency (44). These data may suggest that Hobit expression in humans may instruct unique migratory properties in the two distinct circulating NK cell subsets. Whilst low expression of Hobit in circulating CD56^{bright} NK cells could maintain high levels of CCR7 and CD62L

necessary for SLT entry, high level of Hobit in $CD56^{dim}$ and $CD56^{bright}$ trNK cells might down-regulate these markers on their surface, thus limiting their recirculation to SLT and tissue egress, respectively.

NK CELLS IN PREGNANCY

Pregnancy is a quite peculiar situation, in which an immunocompetent individual (the mother) is in contact for a long period of time with a genetically different immunodeficient individual (the fetus), and is characterized by a deep modification of mother's tissues. During the first trimester of pregnancy, extravillous trophoblast cells (EVT) from the fetus invade the maternal decidua penetrating through the basement membrane of the uterus epithelium with remodeling of the maternal spiral arteries. These changes ensure adequate nutrition of the fetus and are supported by immune cells present at the maternal-fetal interface (45). In normal pregnancy many different mechanisms exist to ensure tolerance of the semi-allogeneic fetus by the maternal immune defense, thus preventing fetus rejection and allowing the reproductive success.

The decidua is populated by a large variety of leukocytes, which represent approximately 30–40% of decidual cells. The most represented leukocyte populations are NK cells, $CD14^{pos}$ myelomonocytic cells and T lymphocytes (46). Decidual NK cells (dNK) represent 50–90% of total decidual lymphoid cells in the first trimester of pregnancy (47) (**Figure 1**). The number dwindles by the end of second trimester, and returns to basal levels at the end of pregnancy. NK cells have also been identified in non-pregnant endometrium (eNK) and their number changes throughout the menstrual cycle, reaching the maximal level in the post-ovulatory phase of the cycle (48). Most uterine NK cells do not express CD16 and show high levels of CD56. The dNK cells have been shown to exhibit unique phenotypic and functional properties. Indeed, relevant differences exist in the gene expression of the NK cell subsets present in peripheral blood and early pregnancy decidual tissues. CD9 tetraspanin, galectin, α-1 integrin and other adhesion molecules are overexpressed in dNK (49). Unlike resting PB NK cells, dNK cells express the CD69 marker and a large percentage express the NKp44 activating receptor. The expression levels of activating receptors/co-receptors (NKp46, NKp30, DNAM-1, NKG2D, and 2B4) are similar in dNK and PB NK cells and, regarding to inhibitory MHC class I-specific receptors, the dNK cells have been shown to express Killer Immunoglobulin receptor (KIRs), CD94/NKG2A and LILRB1 (also known as ILT2, LIR1, and CD85j). Interestingly, the KIR repertoire of dNK cells is skewed toward recognition of HLA-C, the only classical MHC Class I molecule expressed by trophoblast cells (46, 50). Although expressing both perforin and granzymes dNK cells are poorly cytotoxic, a characteristic that has been linked to the block in the polarization of cytolytic granules to the immunological synapse (51). Importantly, cytokines, such as IL-15 can restore the dNK cell cytotoxic function, a phenomenon that is crucial in normal pregnancy to control viral infection (52).

Various studies have shown peculiar functional capabilities of dNK cells. Indeed, they release a wide panel of cytokines, chemokines, and angiogenic factors that are involved in the development of placenta, tissue remodeling, trophoblast invasion and neoangiogenesis (48). Several studies analyzed the chemokine repertoire in endometrium and decidual tissues of women undergoing elective pregnancy termination, studying its involvement in NK recruitment. CXCL9 (Mig), CXCL10 (IP10), CXCL12 (SDF-1), CCL3 (MIP-1α) e CCL4 (MIP-1β) are constitutively expressed in the endometrium. First-trimester human trophoblast expressed and released chemokines able to exert their activity on NK cells, including CXCL12 and CCL3 (53). In line with these results studies have shown that chemokines produced by endometrial or trophoblast cells induce the peripheral blood NK cell chemotactic response. Decidual endothelial and stromal cells express CCL2 (MCP-1), CXCL8 (IL-8), CXCL10, CX_3CL1 (fractalkine), and CXCL12 while only stromal cells express detectable levels of CCL5 (Rantes) and CCL4. Noteworthy, CXCL10, CXCL12 and CX_3CL1 induce the migration of PB NK cell across primary cultures of decidual endothelial and stromal cells (54). Furthermore it has been shown that also chemerin is expressed in the uterus by EVT and stromal cells but not by decidual endothelial cells (DEC) (55–58). The treatment of DECs and stromal cells with progesterone enhanced CXCL10, CX_3CL1, and CCL2 but not CXCL12 levels, while estrogen treatment of stromal cells resulted in up-regulation of CXCL10 and CX_3CL1 (54–57). Moreover, the treatment of stromal cell primary cultures from pregnant, fertile non-pregnant, or menopausal women with progesterone and estrogen resulted in a significant up-regulation of chemerin secretion.

Although it is unclear how and when the various chemokines participate in the recruitment of dNK cells, it has been shown that dNK cells express high levels of CXCR3, low level of CXCR4 and very low levels of CXCR1, CXCR2, CX_3CR1 or CCR1, 2, 3, 5, 6, and 7. In this regard, CXCR3 and CXCR4 are involved in migration of decidual NK cells to CXCL9, CXCL10 and CXCL12 respectively (59). Moreover dNK cells migrate through stromal cells in response to CXCL10 and CXCL12 but not to CX_3CR1 (54). Interestingly, dNK cells from pregnant women express chemerin receptor (ChemR23 or CMKLR1) that induces their migration through stromal cells in response to chemerin. The different chemokine receptor profile between dNK and PB NK cells suggests that the phenotypic features of leukocytes recruited from peripheral blood during pregnancy can be influenced by the decidual microenvironment. In this regard, evidence indicates that the pregnant uterus is a good source of cytokines acting on NK cells including IL-15 (60). Interestingly *in vitro* culturing of PB NK cell with IL-2 or IL-15 induced a down-regulation of ChemR23 (18). In line with these observations studies have shown that co-culture of PB NK cells with stromal cells results in a chemokine receptor profile similar to that of decidual NK cells (54).

Nevertheless, it is noteworthy that the precise origin of dNK cells is not yet clear. It is possible to speculate that a pool of dNK cells may originate from PB NK cells recruited in decidua at early stages of pregnancy. On the other hand, studies suggest that they could also originate from *in situ* progenitor cells that,

in response to uterine stromal environment, differentiate into CD56^{bright} CD16^{neg} NK cells (61).

NK CELLS IN TUMOR TISSUES

A consolidated view considers NK cells as the more effective lymphocyte subset involved in immune surveillance of hematological malignancies, initial stages of solid tumors and blood spreading metastatic cells (62–64). Conversely, NK cells appear to be poorly efficient in controlling advanced, consolidated tumors due to different reasons, which comprise the plethora of immune suppressive factors characterizing the tumor microenvironment (63, 65). These include the expression by cancer cells of MHC class I molecules and immune checkpoint-ligands such as PD-Ls and B7-H3 (63, 66), the lack of expression or the release of soluble forms of ligands of activating receptors, and the presence of soluble immunomodulators, the prototypic one being represented by TGF-β1 (63, 65). Additional aspects impacting on the NK-mediated tumor immune surveillance are the low frequency and/or the quality of NK cells attracted in tumor tissues (**Figure 2**). Indeed, highly cytolytic CD56^{dim} CD16^{pos} NK cells are rare and immature CD56^{bright} CD16$^{low/neg}$ NK cells with low perforin content represent the majority of tumor-associated NK cells. Although some authors suggested the possibility of an *in situ* expansion of CD56^{bright} NK cells (67), a shared hypothesis considers as primum movens the type of chemokines/receptors interactions occurring in the tumor microenvironment.

The tumor orchestrates escape strategies and creates a chemokine milieu consisting of reduced expression of CXCL2, CX_3CL1, CXCL1, and CXCL8, attracting CD56^{dim} NK cells, and increased expression of CXCL9, CXCL10, CXCL19, and CCL5 that drives migration of CD56^{bright} NK cells. The dichotomy between high (CD56^{dim}) and low (CD56^{bright}) cytolytic NK cells has been widely studied and data show that pro-inflammatory cytokines can increase the killing properties of CD56^{bright} NK cells (68). However, this cytokine-mediated rescue mechanism might be deeply affected by TGF-β (69), which is highly represented in tumor tissues. This is because the tumor environment is rich in both TGFβ-1 producing cells and in factors that induce TGFβ activation, such as acidic pH, reactive oxygen species, proteases and specific members of integrin family (70). Active TGF-β1 decreases the expression of activating NK receptors and, by up-regulating mir27a-5p, of perforin 1 (Prf1) and granzyme B (GzmB), thus hampering NK cell cytotoxicity. Moreover, TGF-β1 might dampen CD56^{dim} recruitment and favor that of CD56^{bright} by modifying their respective chemokine receptor repertoires (13). In particular, TGF-β1 increases the expression of CXCR3 and CXCR4 in CD56^{brigh} and CD56dim NK cells, whereas, via mir27a-5p, down-regulates CX_3CR1 expression in CD56^{dim} cells (13, 71). CX_3CR1, whose cognate ligand is represented by CX_3CL1 (also known as fractalkine), is selectively expressed by CD56^{dim} NK cells and together with CXCR4 has been demonstrated to regulate NK cell-egress from bone marrow and NK cell extravasation (72). Interestingly, in agreement with this ability of tumors in inducing a regulatory milieu, an unusual low expression of CX_3CR1 has been reported in CD56^{dim} NK cell population of tumor-infiltrated bone marrow and peripheral blood of Neuroblastoma (NB) patients (13). Although a more detailed analysis should be performed to deepen whether this unusual chemokine receptor repertoire actually defines a peculiar CD56^{dim} population (73) mirroring the "broad spectrum of human Natural Killer Cell Diversity" (2), it is conceivable that CX_3CR1^{low} CD56^{dim} cells show defective migration toward tumor (or inflamed tissues). Conversely, the recruitment of CD56^{bright} NK cells in a CXCL9 and CXCL10 rich milieu might be favored by their constitutive expression of high levels of CXCR3 and CXCR4, which further increase under the influence of TGFβ-1 (8, 16, 17). Along this line, CD56^{bright} CD16^{low} represented the predominant NK cell population in the ascitic fluids of ovarian cancer patients (74). The concomitant up-regulation of CXCR3 and CXCR4 by TGF-β1 represents an interesting event if considering that these receptors are subject to cross regulation. Indeed, chemokine receptors' function can be modulated by desensitization, which is a physiological process that prevents overstimulation due to prolonged agonist exposure by signal attenuation or termination (27). Desensitization of a receptor can be dependent on its ligand (homologous desensitization) or by other ligands present in a complex chemokine gradient, a cross-desensitization called heterologous desensitization. In this context it has been shown that pre-stimulation of NK cells with CXCL9 inhibited NK cell migration not only to CXCR3 ligands but also to CXCL12, thus indicating that triggering of CXCR3 can promote both homologous and heterologous (CXCR4) desensitization (75).

In solid tumors a "fast track entrance" for CD56^{bright} CX_3CR1^{neg} CXCR3^{high} CXCR4^{high} NK cells might be the ectopic, neo-generated High Endothelia Venules (HEV) that contribute to the architecture of Tertiary Lymphoid Structures (TLS) (76, 77). These transient, un-capsulated lymphoid aggregates resembling Secondary Lymphoid Organs (SLO) have been detected in peri- or intra-tumor sites as well as in other chronic inflamed tissues. TLS share with SLO the presence of distinct T and B cell compartments, reactive Germinal Center (GC), Follicular Dendritic Cells (FDC), fibroblastic reticular cells (FRC) and lymphatic vessels, as well as HEV whose lining endothelial cells express highly specific addressin molecules, collectively termed peripheral node addressins (PNAd) (76). These are known to dictate adhesion and consequent extravasation of immune cells, including NK cells, within paracortical region of lymph nodes, an event that might occur also at TLS levels. In different tumors including lung, breast or gastrointestinal stromal tumors (GIST) (78), tumor-associated TLS might contribute to the preferential recruitment of CD56^{bright} NK cells that constitutively express the homing receptor CD62L and high levels of CCR7 specific for the lymph node chemoattractants CCL19 and CCL21. For example, in TLS associated to human lung cancer intra-tumoral PNAd^{+} HEV exclusively co-localized with CD62L^{+} lymphocytes (76). Notably, while TGF-β negatively impacts on CD56^{dim} NK cells recruitment in perturbed tumor tissues, upregulation of CCR7 may promote their migration to SLO and TLS. Accordingly, enrichment in CD56^{dim} CCR7^{+}

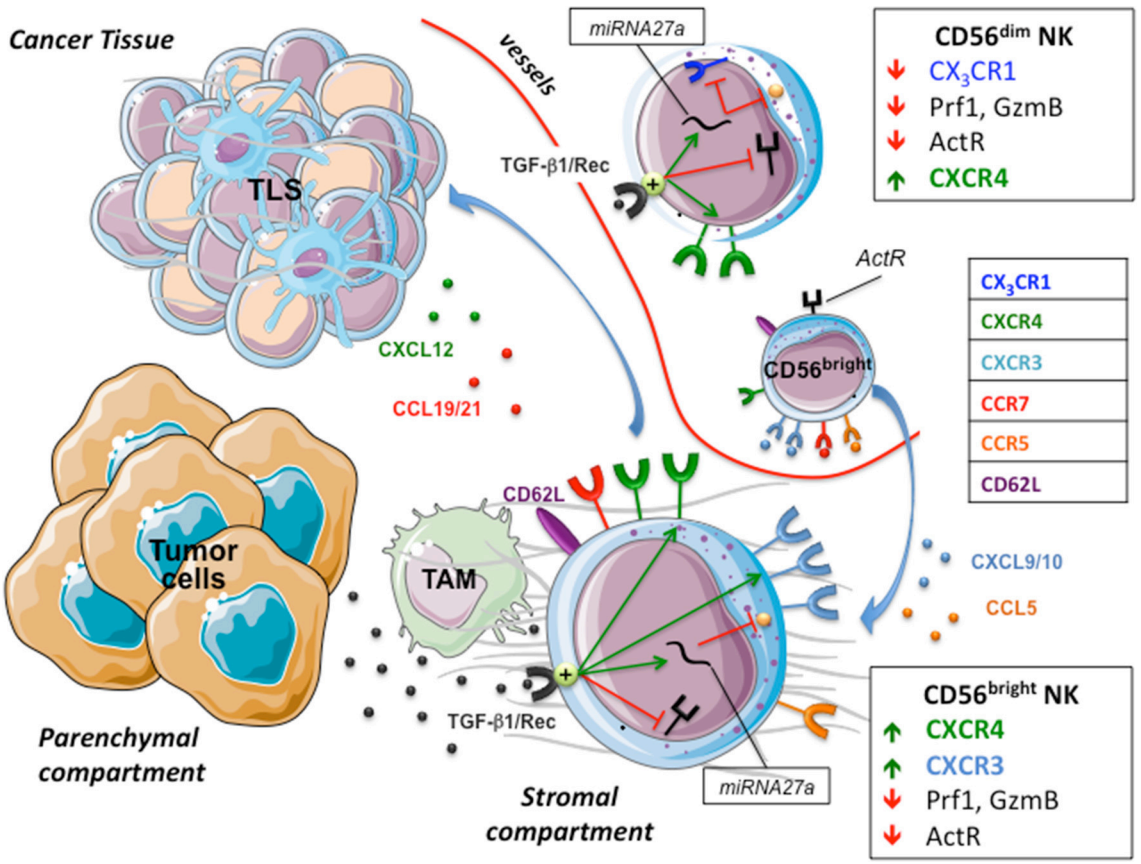

FIGURE 2 | NK cells in tumor microenvironment. The tumor chemokine milieu presents a reduced expression of chemokines attracting CD56dim NK cells, and an increased expression of CXCL9/10, CCL5, and CXCL19/21 that drives the migration of CD56bright NK cells toward the stromal compartment and tertiary lymphoid tissue (TLS). Tumor cells and tumor-associated macrophages (TAM) release/activate TGF-β1 that decreases the capability of NK cells to recognize and kill targets and modify their chemokine receptor repertoire, hampering the recruitment of CD56dim NK cells and favoring that of poor cytolytic CD56bright. ActR, Activating receptors; GzmB, Granzyme B.

KIR^+ $CD57^+$ highly cytotoxic NK cells has been documented in tumor-infiltrated lymph nodes of melanoma patients (79). Several mechanisms involved in the acquisition of CCR7 by $CD56^{dim}$ NK cells have been identified that include the crucial role of IL-18, highlighted by Mailliard et al. (80), and the possible uptake of CCR7 from surrounding cells by trogocytosis (81). Soluble IL-18 is produced by stimulated antigen presenting cells, in particular by macrophages that, as M2-polarized cells, might represent the most abundant immune population in the tumor microenvironment (82). Interestingly, a variable subset (30–40%) of unpolarized (M0) and M2 macrophages and most tumors associated macrophages (TAM) express a membrane form of IL-18 (mIL-18) (74, 83, 84). Upon TLR stimulation, macrophages polarize toward M1 and loose mIL-18, an event paralleled by the release of small amounts of soluble IL-18 (sIL-18) that, acting in close proximity, induces the expression of CCR7 in $CD56^{dim}$ NK cells (83). It is of note that, since M1 polarizing macrophages also acquire CCR7 expression (83), a contribution of trogocytosis-mediated uptake cannot be ruled out. Although mechanisms responsible for IL-18 membrane retention and release have to be clarified, this cytokine shows many predictable cleavage sites for extracellular proteases such as Matrix metallopeptidase (MMP) −2 and −9, which characterize the secretory profile of parenchymal tumor cells and TAM. Thus, also in the absence of pathogen-derived stimuli, the action of MMPs (or other still unknown mechanisms), may allow IL-18 shedding from TAM and the induction of CCR7 expression in $CD56^{dim}$ tumor-associated NK cells (TA-NK), thus promoting their migration to SLO and TLS.

In solid tumors CCR7 acquisition by NK cells may depend on close cell-to-cell contacts with macrophages or dendritic cells, whereas it is less plausible that tumor cells could play a relevant role. Indeed, TA-NK cells were found to be predominantly located in the stromal compartment, whereas they were rare/absent in the parenchyma in direct contact with tumor cells (40, 85). Regarding the compartmentalization of TA-NK cells, in an adenocarcinoma colon model, stromal-infiltrating NK cells had morphology compatible with actively migrating cells, and in some instances migrating NK cells co-localized with degraded matrix (85). In the same model,

most of the NK-poor tumor nodules were surrounded by a capsule-like structure with collagen IV and laminin, two major components of the basement membrane. On the contrary, tumor nodules lacking these containment structures were more infiltrated by NK cells. These observations, together with data showing that poor NK cells infiltration have been equally detected in both chemokines-rich and -poor tumors, strongly indicate stromal barriers as a hindrance impacting on possible NK-to-tumor cell contacts. Along this line, during imatinib mesylate therapy in GIST patients, the frequency of NK cells did not change in fibrous trabeculae, whereas significantly increased in the core of both localized or metastatic tumors, an observation that correlated with a better prognosis (78). Interestingly, a recent study analyzing the off-target effect of imatinib mesylate on immune cells showed that this drug causes a significant up-regulation of CXCR4 in both T and NK cells (86). Accordingly, NK cells *ex-vivo* isolated from peripheral blood of chronic myeloid leukemia patients receiving imatinib mesylate showed levels of CXCR4 significantly higher than those detected in healthy individuals (86). A study by Goda S. and colleagues (87) may in part explain how increased CXCR4 surface levels can facilitate NK cells to cross the bridge connecting the stroma and the tumor parenchyma compartments. In particular, they showed that human $CD56^{dim}$ $CD16^{pos}$ NK cell invasion into type I collagen is enhanced by CXCL12, the CXCR4 ligand, in a matrix metalloproteinases (MMP)-dependent manner. Notably, CXCL12 has been shown also to promote the production in monocytes (88) and megakariocytes (89) of MMP-9, which has protease activity on collagen IV. With this assumption, it is conceivable that therapies strengthening the CXCR4/CXCL12 axis could potentiate extracellular matrix degradation favoring NK (and T) cells migration toward tumor cells.

In light of these considerations, data on the NK cell phenotype and density in tumor sites cannot be considered "*per se*" a favorable prognostic factor and should be more and more integrated with data on NK cell localization with respect to stroma, parenchyma tumor cells and with the analysis of the whole immune landscape. For instance, high NK cell infiltration has been associated with improved survival in metastastic renal cell carcinoma but not in colorectal carcinoma (90). Contradictory results may depend on the method used to unequivocally identify NK cells, which still represents a major challenge as NKp46, the more reliable marker, is also expressed by other subsets of ILCs (91). Opposite clinical impact of NK cell infiltration in solid tumors might also depend on the targeted tissue, the tumor phase and the ratio between NK and tumor cell numbers. It has been demonstrated that NK cells can edit tumor cells modifying their immunogenicity. In particular, in NK and melanoma cell co-cultures performed at low effector/target ratios, which reflect the level of NK cell infiltrates observed at the tumor site, an initial tumor cell lysis is followed by an equilibrium phase characterized by decreased susceptibility to killing due to up-regulation of both classical and non-classical MHC class I molecules on melanoma cells. This effect is mediated by IFN-γ released by NK cells activated upon melanoma cell recognition. Importantly IFN-γ and TNF-α are also potent inducers of the expression of the immune checkpoint ligands PD-L1 and PD-L2 in macrophages/dendritic cells, tumor cells and tumor-associated endothelial cells (92, 93). Moreover, TNF-α is known to promote Epithelial-Mesenchymal Transition (EMT), a process leading epithelial tumors to acquire a less differentiated, pro-metastatic phenotype. Along this line, in lung cancer, a recent report showed an important correlation between PD-L1 expression and EMT score (93, 94). Thus, low number of NK cells contacting tumor cells might have more undesirable than beneficial effects, being unable to efficiently eliminate tumor cells while causing a gradual accumulation of cytokines that exert a paradoxical tumor promoting effect by modifying the immunogenicity of tumor cells.

Whatever the case, when designing NK cell-based immunotherapeutic approaches for cancer patients, we should take into account the relevance of the molecular mechanisms regulating NK cell migration into tumors. For instance, a recent and promising approach is represented by the infusion of NK cells engineered to express chimeric antigen receptor (CAR) specific for tumor-associated antigens (65, 95, 96). The efficacy of adoptively transferred CAR-NK might be deeply limited by their inability to cross stromal barriers and to adhere to parenchymal tumor cells, as recently suggested for T cells by Caruana and colleagues (97). It was pointed out that *in vitro* manipulation aimed to the CAR engineering of T cells leads to silencing of heparanase (HPSE), an endoglycosidase that cleave heparan sulfate proteoglycans of ECM, thus reducing the invasive potential of CAR-T cells in solid tumors. Thus, cell-based therapy may also include strategies to favor migration of effector cells through stromal compartment and tumor parenchyma, a phenomenon unlikely to occur, particularly in advanced solid tumors.

NK CELLS IN INFLAMED TISSUES

The perturbation mediated by pathogens in peripheral tissues results in the early activation of resident or recruited cells of the innate immunity with a consequent boost of chemotactic factors, which attract different immune cells including peripheral blood mature conventional $CD56^{dim}$ NK cells. These cells mainly differentiate in the bone marrow and express CXCR1, CXCR2, chemR23, S1P5, CXCR4, and CX_3CR1 (16, 17, 26). This chemokine receptor repertoire drives NK cells to inflamed tissues. Importantly, the relative expression of CXCR4 and S1P5 in developing mouse NK cells has been described to regulate bone marrow egress into circulation (98). Moreover, in bone marrow, prevalent CX_3CR1 expression by $KLRG1^+$ NK cells located in sinusoids suggested its crucial role for NK cell entry into the vascular compartment (99).

The presence of NK cells in healthy and inflamed peripheral tissues (18, 26, 29) has been well documented and different studies demonstrated the existence of a crucial crosstalk between $CD56^{dim}$ NK cells and DC or macrophages (**Figure 3**). NK/DC interactions resulted in a bidirectional activation leading to killing of immature DC (iDC) by autologous $NKG2A^+$ KIR^{neg}

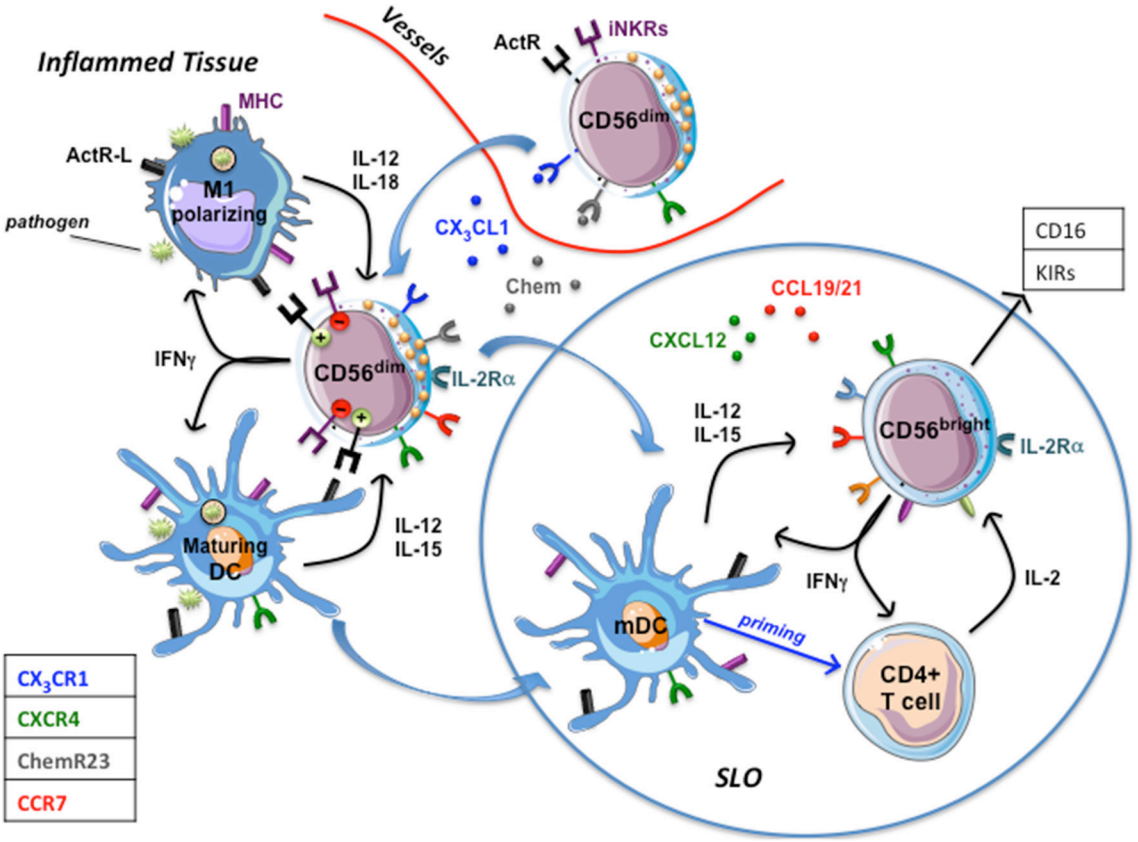

FIGURE 3 | NK cells in inflamed tissues. Inflamed tissue produce chemokines (Chemerin, CX3CL1) that drive the migration of circulating CD56dim prf1high NK cells. Once in tissues they interact with DC and macrophages that, upon pathogen recognition, have begun to mature (mDC) or polarize toward a proinflammatory functional phenotype (M1). Maturing DC and M1-polarizing macrophages release immunostimulatory cytokines that induce NK cells to produce large amounts of IFN-γ (which potentiate phagocytes' functions) and to express IL-2Rα and CCR7, which drive their migration into secondary lymphoid organs (SLO). mDC migrated to SLO and DC-primed T cells producing IL-2 stimulate CD56dim NK cells and CD56bright NK cells that acquire a $CD16^{pos}$ KIR^{pos} phenotype. ActR-L, Activating receptors ligands; iNKRs, MHC class I specific inhibitory receptors (KIRs, CD94/NKG2A); Chem, chemerin.

NK cells. NK-mediated DC lysis, due to a pivotal role of NKp30 and DNAM-1 activating receptors (20, 100), is restricted to iDC undergoing an unfruitful maturation process, characterized by an inadequate MHC class I up-regulation. This mainly impacts on the expression of the non-classical HLA-E molecule, the cognate ligand of the CD94/NKG2A inhibitory receptor. This effect has been interpreted as a negative selection strategy aimed to avoid inappropriate antigen presentation by MHC class I low DCs, which could result in tolerogenic responses. DC that underwent an appropriate maturation program (mature dendritic cells, mDC) are spared from NK-cell mediated attack since they express very high levels of classical MHC class I and significantly up-regulated HLA-E (101, 102). The principal NK cell-derived mediators shaping DC immune-phenotype are represented by TNFα and IFNγ, whose release depends on the synergistic activity of IL-12 and IL-15 produced by pathogen stimulated DC. mDC *de novo* express CCR7, (a phenomenon also occurring during pathogen-driven macrophage polarization toward M1) which confers to these cells the competence for migrating to SLO. In SLO T cell zone, DCs co-localize with NK cells belonging to the $CD56^{bright}$ $CD16^{low/neg}$ subset, which constitutively express CCR7 (101).

The complex interactions among NK cells expressing IL-2Rα (CD25), DC-primed T cells producing IL-2 and mDC result in a conspicuous IFN-γ production by NK cells shaping T cell priming, polarization and adaptive immune responses (101). Whether, in SLO, NK cells may also shape macrophages' functions remains to be elucidated. $CCR7^{pos}$. NK cells can migrate to SLO via HEV since they also express high levels of CD62L. However, a predominant population of $CCR7^{pos}$ $CD56^{bright}$ $CD16^{neg}$ NK cells has been described in seroma fluid, thus depicting afferent lymph as an alternative way for $CD56^{bright}$ NK cells to colonize SLO (8). Interestingly, it has been observed, in seroma, the presence of low numbers of $CD56^{pos}CD3^{neg}$ cells expressing CX_3CR1, KIRs and CD16 molecules, a phenotype usually characterizing classical $CD56^{dim}$ NK cells (8). Although a multiparametric analysis providing information about a possible co-expression of CCR7 was lacking, these data support the hypothesis that, *in vivo*, cytolytic $CD56^{dim}$ NK cells might also migrate to "perturbed" SLO. Along this line,

the interaction of $CD56^{dim}$ NK cells with M0 or M2 macrophages polarizing toward M1 upon TLR engagement results in the acquisition of CCR7 and of a fully activated NK cell status characterized by high CD69 and IL2Rα expression, release of large amount of IFN-γ and increased cytolytic activity (83). Thus, in inflammatory conditions, M1-activated $CD56^{dim}$ NK cells, becoming competent for SLO migration thanks to the acquisition of CCR7, might deeply contribute to both immunosurveillance of tumor metastases and control of infected cells. Migration of fully functional $CD56^{dim}$ NK cells to SLO, could be particularly relevant in the context of KIR/KIRL-mismatched haploidentical stem cell transplantations (haplo-HSCT). Indeed, in SLO, NK-mediated killing of recipient mDC and residual T cells might contribute to the low rate of graft vs. host disease (GVHD) and graft rejection documented in this clinical setting (103).

Nevertheless, is there any *in vivo* evidence that $CD56^{dim}$ NK cells might traffic through and leave SLO, thus recirculating via efferent lymph? A few preliminary reports indicate this possibility. Non-reactive LNs or LNs characterized by sinus hyperplasia lack or show low expression of $KIR^{pos}CD16^{pos}$ cells. Interestingly, reactive LNs characterized by paracortical/follicular hyperplasia harbor a significant percentage of cells expressing KIR and CD16 and a similar $KIR^{pos}CD16^{pos}$ cells enrichment was observed in the efferent lymph (i.e., toracic duct). Several observations, including a difference in the telomerase length, strongly suggest that $CD56^{bright}$ $CD16^{neg}$ KIR^{neg} cells can acquire a KIR^{pos} $CD16^{pos}$ phenotype thanks to the influence of the different pro-inflammatory cytokines present in LNs (28). However, the hypothesis that $CD16^{pos}$ KIR^{pos} NK cells might migrate to and expand in LNs before egressing via efferent lymph cannot be ruled out. In this context, in pathogen-perturbed tissues, $CD56^{dim}$ NK cells interacting with macrophages acquire the competence to SLO migration and, expressing high levels of IL2Rα (83), become highly responsive to IL-2 produced by T cells in the paracortex area of LN.

Pro-inflammatory cytokines are capable of shaping innate and adaptive immune responses also acting on the establishment of the NK cell memory reservoir. Both in mouse and human, it has been described that the CMV-driven onset of memory NK cell populations requires the presence of pro-inflammatory cytokines such as IL-12 and IL-18. Cytokines represent the third signal essential to generate, expand and recall NK cell memory. Signal 1 is represented by receptor-mediated antigen recognition, LY49D in mouse and NKG2C or KIR2DS1 in humans, and signal 2 by co-stimulatory signals, DNAM-1 and CD2 in mouse and human, respectively (15, 104). In addition, cytokines by themselves are capable of generating memory-like NK cells in an antigen–independent setting (14), as NK cells, shortly cultured in the presence of IL-12, IL-15 and IL-18, showed superior IFN-γ and TNF-α production and cytotoxicity in response to tumor targets and conferred more protection to leukemia or melanoma in xenograft mouse models. Thus, full NK cell activation and antigen-dependent or -independent generation of NK cell memory requires cytokines-mediated signals. It should be considered that cytokines also strongly impact on the chemokine receptor repertoire of NK cells. Beside sIL-18 whose capability of inducing CCR7 expression has been discussed above, IL-15 has been shown to down-regulate CX_3CR1 expression in mouse bone marrow-derived NK cells (10) and in human PB NK cells (12), thus reducing the chemotactic response to CX_3CL1 ligand (12). IL-12 in association with IL-2 significantly decreased the CXCR3 mRNA and their surface expression in NK cells (9). Additionally, IL-2 alone has been shown to down-regulate the surface expression of CXCR1 as well as of CXCR4 inhibiting NK cell migration in response to CXCL12. On the other hand, IL-2 up-regulated the surface expression of CXCR3 increasing NK cell migration in response to its ligands CXCL9 and CXCL10 (11).

Regarding the migratory properties of memory NK cells, different questions remain unanswered. Do cytokines that drive their onset, impact on their chemokine receptor repertoire contributing to the generation of tissue-resident memory NK cells in various anatomical areas? Does the maintenance of the NK cell memory pool involve tissue-restricted reactivation of resident memory NK cells or do these cells maintain the potential to recirculate? Studies focused on mouse recall response to haptens provided some relevant indications. These studies showed that memory NK cells responsible for the immune response were the $CD49a^{+}$ $DX5^{neg}$ liver resident NK cells, and that the activity of CD18 and P-selectin, molecules involved in trafficking of NK cells, was needed (15). In this scenario, the characterization of human memory NK cells in terms of chemokine receptor expression, before and after cytokine-stimulation, could be particularly relevant.

CONCLUDING REMARKS

It has become evident that NK cells are not constituted by a homogeneous population of innate lymphocytes but rather by different subtypes with specific abilities as well as distinct homing properties. Investigating how NK cell subsets distribute in human body has relevance not only for a better understanding of our immune defenses but also for exploiting these cytotoxic cells in therapeutic settings.

It is worth noting that the migratory properties of NK cell subsets are relevant not only for identifying the region in which they should exert their activity. Recent reports indicate that NK cells could acquire specific properties, such as cytotoxicity, only upon their migration to secondary lymphoid organs where the cytokine milieu would induce their further differentiation. At the same time, although the picture of tissue-resident NK cells is still fuzzy, it is conceivable that these subsets of NK cells might locally acquire peculiar properties, such as release of specific soluble factors able to affect their properties but also influence other cells present in the microenvironment. As a matter of fact, NK cells (as well as all other innate lymphoid cells of which they represent the prototype) are more and more emerging as accessory cells able to modulate the functions of neighboring cells, including antigen presenting cells, in the environment in which they are attracted/hosted.

Despite the relevance of these issues, several open questions still remain to be addressed regarding the ability of NK cells

to infiltrate and reside in either healthy or pathological tissues. Decades after the discovery of NK cells as lymphocytes able to recognize and kill cancer cells without prior sensitization to them, we still miss a clear and complete depiction of the phenotype and properties of tumor-infiltrating NK cells. Similarly, although a number of studies have now highlighted the relevance of NK cells in the control of viral infections, how these cytotoxic lymphocytes recirculate and/or are retained in infected tissues still remain to be clearly determined, at least in humans.

On the other hand, novel technologies allowing extensive multiparametric analyses, either by mass cytometry or classical flow cytometry, not even conceivable until only a few years ago, might now open new avenues for a comprehensive mapping of tissutal NK cells. The path appears already tracked since we have now, as reported in the present review, a better appreciation of at least some of the molecules and the signaling ruling the homing properties of these innate lymphocytes.

AUTHOR CONTRIBUTIONS

RC, PC, AD, GF and CB wrote the manuscript. FB, BC and SR read the manuscript and provided critical input.

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The Role of CXC Chemokine Receptors 1–4 on Immune Cells in the Tumor Microenvironment

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Chemokines govern leukocyte migration by attracting cells that express their cognate ligands. Many cancer types show altered chemokine secretion profiles, favoring the recruitment of pro-tumorigenic immune cells and preventing the accumulation of anti-tumorigenic effector cells. This can ultimately result in cancer immune evasion. The manipulation of chemokine and chemokine-receptor signaling can reshape the immunological phenotypes within the tumor microenvironment in order to increase the therapeutic efficacy of cancer immunotherapy. Here we discuss the three chemokine-chemokine receptor axes, CXCR1/2–CXCL1-3/5-8, CXCR3–CXCL9/10/11, and CXCR4-CXCL12 and their role on pro-tumorigenic immune cells and anti-tumorigenic effector cells in solid tumors. In particular, we summarize current strategies to target these axes and discuss their potential use in treatment approaches.

Keywords: chemokines, cancer immunotherapy, metastasis, NK cells, T cells, myeloid cells

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INTRODUCTION

Immune evasion is a hallmark of carcinogenesis (1). Tumor cells interact closely with stromal cells, immune cells and the extracellular matrix (ECM). Via complex mechanisms these communications support tumor growth, metastatic spread, and immune escape (2). A family of small chemotactic proteins, called chemokines, has key roles in these interactions. Depending on their protein sequence, and more specifically, the location of the cysteine (C) residues at their N-terminus, chemokines are subdivided into four main classes: the C-, the CC-, the CXC-, and the CX3C-chemokines (3). Irrespective of their class, chemokines signal through binding to cognate seven-transmembrane spanning G protein-coupled receptors (GPCRs), found on the migratory cells. To date, 48 chemokines and 18 signal-transducing receptors have been identified in humans. Each chemokine can activate several different receptors. Immune cell subsets differentially express chemokine receptors, which results in their selective recruitment, according to the special needs of each environment. Within the tumor microenvironment (TME), chemokine ligand secretion is often altered compared to healthy tissue. This facilitates recruitment of pro-tumorigenic immune cells such as myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TAN), tumor-associated macrophages (TAM), and regulatory T cells (Treg). These cells expand during tumor progression, suppress effector lymphocytes, and are associated with worse prognosis in patients with various solid malignancies (4–7). Several studies demonstrate that tumor cells secrete chemokines in an autocrine and paracrine fashion to directly promote cancer cell growth, survival and metastasis (8). Here we focus on the impact of the CXCR1/2, CXCR3, and CXCR4 chemokine axes on recruitment of pro-tumorigenic and anti-tumorigenic immune cells in solid malignancies.

We highlight the role of the CXCR1/2 axis on promoting immunosuppressive cells and the impact of CXCR3 and CXCR4 axes on increasing effector cell recruitment. Furthermore, we summarize preclinical and clinical studies that shape the therapeutic potential of chemokine-targeting and their implication in combinatorial immunotherapeutic treatment approaches.

THE ROLE OF CXCR1 AND CXCR2 IN SOLID MALIGNANCIES

CXCR1 and CXCR2 are expressed by several cell types, especially neutrophils, fibroblasts and vascular endothelial cells. CXCR1 and CXCR2 bind the ligands CXCL6 and CXCL8 (IL-8) with similar affinity, while binding of CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7 is mediated by CXCR2 (9). Mice do not have a CXCL8 (IL-8) gene. Moreover, the gene product of murine CXCL5, called LIX, is homologous to human CXCL6 and binds both CXCR1 and CXCR2 (10). High levels of these chemokine receptors and ligands in tumor tissues and serum are correlated with worse prognosis in several tumor types, including ovarian cancer, lung adenocarcinoma, colorectal carcinoma and pancreatic ductal adenocarcinoma (PDA) (11–15). One explanation for the poor prognosis could be the preferential recruitment of pro-tumorigenic immune cells via the CXCR1/ 2 axis (summarized in **Table 1**). Altered signaling pathways in tumor cells can increase chemokine secretion. For instance, overexpression of the transcription factor Snail in ovarian cancer cells upregulated CXCL1, CXCL2, and CXCL5 through the NF-kB pathway and promoted MDSC recruitment (11). Snail depletion or antibody-mediated CXCR2 targeting diminished MDSC cell numbers within tumors and increased T cell and NK cell numbers (11). Similarly, CXCL1 and CXCL2 secretion by breast cancer cells resulted in increased infiltration of pro-tumorigenic myeloid cells and was further augmented by chemotherapeutic treatment, leading to chemoresistence (16). The role of CXCL5 in recruiting $CXCR2^+$ MDSC and TAN has also been shown in models of renal cell carcinoma (RCC) (17), PDA (18), melanoma (19, 20), and hepatocellular carcinoma (HCC) (21). In patients with RCC, intratumoral CXCL5 and CXCL8 levels correlated with increased MDSC infiltration (17). Targeting CXCR2 reduced MDSC numbers and increased effector T cells (17). While targeting CXCR2 alone only modestly decreased tumor burden in a murine RCC model, combination with immune checkpoint inhibition significantly reduced tumor weight (17). Similarly, high CXCL5 expression was found in PDA and mediated recruitment of $CXCR2^+$ neutrophils (18). Abrogation of CXCR2 diminished neutrophil infiltration and increased the ratio of effector T cells (18). In genetically modified mice that expressed human CXCL8, MDSC were efficiently recruited to the tumor site and suppressed T cell activity (22). Collectively, these data indicate that CXCR1/2 blockade reduces pro-tumorigenic immune cell infiltration and increases T and NK cell recruitment. This supports attempts to combine CXCR1/2 blockade with other immunotherapies, such as checkpoint inhibition or adoptive cell therapy. CXCR1/2 blockade also helps to overcome chemoresistance mediated by pro-tumorigenic immune cells (16, 23). It was recently shown that chemokine signaling within the TME displays high plasticity: $CXCR2^+$ TAN numbers within tumor biopsies increased in PDA patients that were previously treated with an inhibitor of CCR2 (23). Inversely, depletion of TANs resulted in increased TAM numbers and only dual inhibition of both the CXCR1, CXCR2, and CCR2 axis disrupted myeloid infiltration and improved responses to chemotherapeutic treatment (23).

CXCR1 and CXCR2 are highly expressed by cytotoxic $CD56^{dim}$ NK cells (37, 38). We recently showed that CXCR2 expression is downregulated on tumor-infiltrating NK cells in RCC and genetic modification to re-express CXCR2 enhanced recruitment of NK cells to the tumor site (39). Similarly, Ali et al. showed that CXCL8 was released within the TME of melanoma-infiltrated lymph nodes and could efficiently recruit highly cytotoxic NK cells (24). The percentage of this NK cell population among all NK cells within the affected lymph node was associated with improved prognosis among patients with stage III melanoma. Likewise, genetically modified CXCR2+ T cells displayed increased *in vivo* migration in murine melanoma models (40, 41). A clinical phase I/II trial in patients with metastatic melanoma infused with genetically modified CXCR2+ T cells has been initiated (**Table 2**).

Findings from pre-clinical studies have already been translated into clinical phase studies (summarized in **Table 2**). The combination of paclitaxel with reparixin—a CXCR1 and CXCR2 inhibitor—was well tolerated in patients with metastatic breast cancer and resulted in 30% response rate (42). Based on these findings, a phase II study was initiated (NCT02370238). Combination therapies with CXCR1/2 inhibitors are also in clinical phase trials for prostate cancer and metastatic melanoma.

THE ROLE OF CXCR3 AND ITS LIGANDS IN SOLID TUMORS

CXCR3 is expressed on different subtypes of T and NK cells (37, 44) and binds to CXCL9, CXCL10, and CXCL11. During homeostasis, CXCL9, CXCL10 and CXCL11 are expressed at low levels by monocytes, endothelial cells and fibroblasts, but are upregulated upon cytokine stimulation, especially by IFNγ and TNFα (45, 46). CXCR3 and its ligands are expressed by various solid tumors, although their prognostic role greatly differs among the entities. This underlines a role in tumor suppression as well as tumor growth promotion and metastasis. While high CXCR3 expression in glioblastoma, colorectal, and breast cancer is associated with poor prognosis, it correlated with better outcomes in patients with gastric cancer (28, 47, 48). In contrast, high CXCL9, CXCL10, and CXCL11 expression in the TME of patients with colorectal, oesophageal, non-small cell lung (NSCL) and ovarian cancer is an indicator of improved overall survival (27, 49–51), while it is a poor prognostic marker in patients with localized clear-cell RCC (52).

CXCR3 is a key receptor in recruitment of activated T cells as it is absent in naïve T cells, but highly expressed on activated

TABLE 1 | The effect of chemokine ligands and their receptors on immune cells within the tumor microenvironment.

Chemokine receptor	Chemokine (systematic name/common name)	Cell type	Role within the tumor microenvironment	References
CXCR1/CXCR2	CXCL1 (GROα) CXCL2 (GROß) CXCL5 (ENA-78)	MDSC	- Targeting CXCR2 in Snail^{+} ovarian cancer xenograft models inhibits MDSC recruitment and prolongs overall survival of tumor-bearing mice	(11)
	CXCL1 (GROα) CXCL2 (GROß)	CD11b(+)Gr1(+) myeloid cells	- CXCL1 and CXCL2 are expressed by breast cancer cells and attract myeloid cells, that secrete chemokines to promote cancer cell survival	(16)
	CXCL5 (ENA-78) CXCL8 (IL-8)	MDSC	- CXCR2^{+} MDSC are recruited via CXCL5 and CXCL8 to RCC - targeting CXCR2 reduces MDSC numbers and increases T cell infiltration - Combination of CXCR2 blockade and immune-checkpoint inhibition leads to more pronounced tumor growth reduction in murine models	(17)
	CXCL5 (ENA-78)	TAN	- CXCR2^{+} TAN are recruited into PDAC along CXCL5 - CXCR2 blockade reduces TAN numbers and increases T cell numbers	(18)
	CXCL5 (ENA-78)	MDSC	- MDSC are attracted via CXCL5 in murine metastatic uveal melanoma models and enhance epithelial-mesenchymal transition (EMT) in tumor cells	(19)
	CXCL5 (ENA-78)	TAN	- Neutrophils were efficiently recruited by CXCL5 release from human melanoma cells in xenograft mouse models	(20)
	CXCL5 (ENA-78)	TAN	- CXCL5 can be induced by TGFb and Axl and promotes neutrophil recruitment toward HCC cells	(21)
	CXCL8 (IL-8)	MDSC	- MDSC are efficiently recruited to the tumor site via CXCL8 expression in genetically modified mice	(22)
	CXCL1 (GROα) CXCL3 (GROγ) CXCL5 (ENA-78) CXCL8 (IL-8)	TAN	- TANs are recruited to orthotopic pancreatic tumor sites via the CXCR2 axis; numbers of CXCR2^{+} neutrophils in pancreatic cancer patients correlate with prognosis - In an orthotopic PDAC model CXCR2 blockade prevents TAN mobilization from peripheral blood and increases effector T cell numbers in the tumor	(23)
	CXCL8 (IL-8)	NK	- Accumulation of highly cytotoxic NK cells in metastatic lymph nodes of melanoma patients	(24)
CXCR3	CXCL10 (IP-10)	Treg	- Treg recruitment via the CXCR3/CXCL10 axis increases HCC recurrence rate after liver transplantation	(25)
	CXCL11 (I-TAC)	Treg	- CXCL11 is highly expressed in colorectal cancer; similarly CXCR3^{+} regulatory T cells are abundant in CRC specimen and can be efficiently recruited *in vitro* by CXCL11	(26)
	CXCL9 (MIG) CXCL10 (IP-10)	TIL/NK	- CXCL9 and CXCL10 expression is associated with improved patient survival in advanced HGSC through recruitment of TIL (tumor-infiltrating lymphocytes)	(27)
	n.a.	Effector T cells	- Intratumoral CXCR3 expression was upregulated in patients with advanced gastric and was associated with increased CD4^{+}, CD8^{+} TILs infiltration and improved OS	(28)
	CXCL9 (MIG) CXCL10 (IP-10)	Effector T cells	- CXCL9, CXCL10 are important chemokines within the melanoma tumor microenvironment and are able to recruit CD8 effector T cells in a murine xenograft model	(29)
		Effector T cells	- CXCR3$^{-/-}$ melanoma mice show accelerated tumor growth and impaired T cell infiltration of tumor tissue	(30)
		Effector T cells	- CXCR3 is essential for effector T cell trafficking through tumor vessels, even in absence of its ligands	(31)
	CXCL9 (MIG) CXCL10 (IP-10)	Effector T cells NK cells	- Human colorectal cancer samples show high CXCL9 and CXCL10 expression that correlates with T cell, but not NK cell numbers	(32)
	CXCL10 (IP-10)	NK	- CD27high CXCR3^{+} NK cells infiltrate tumors in murine lymphoma and melanoma models in an CXCL10-dependent fashion and lead to improved survival NK cells from CXCR3$^{-/-}$ mice show impaired tumor infiltration	(33)

(Continued)

TABLE 1 | Continued

Chemokine receptor	Chemokine (systematic name/common name)	Cell type	Role within the tumor microenvironment	References
CXCR4	CXCL12 (SDF-1α/ß)	MDSC	- PGE2 increases CXCL12 levels in ascites of ovarian cancer patients - $CXCR4^+$ MDSC are recruited toward CXCL12	(34)
		Treg	- CXCL12 levels are elevated in NSCLC, which results in increased recruitment of $CD4^+CD69^+CXCR4^+$ T cells	(35)
		NK	- Genetically modified NK cells that overexpress CXCR4 lead to improved tumor eradication in a murine glioblastoma model	(36)

TABLE 2 | Clinical trials with modulators of chemokine functions within the tumor microenvironment.

Name	Mode of action	Clinical trial	Current status
CHEMOKINE RECEPTOR ANTAGONISM			
Reparixin	CXCR 1/2 inhibition	Phase IB (NCT02001974)	- Completed: 30% response rate in patients with metastatic breast cancer, well tolerated (42) - Combined with chemotherapy (paclitaxel)
AZD5069		Phase I/ II (NCT03177187)	- Recruiting patients with metastatic castrate-resistant prostate cancer - Combined with antiandrogen medication (enzalutamide)
SX-682		Phase I (NCT03161431)	- Recruiting patients with metastatic melanoma - Combined with immune checkpoint inhibitor (pembrolizumab)
AMD3100 (Plerixafor)	CXCL12/CXCR4 inhibition	Phase I (NCT03277209)	- Recruiting patients with pancreatic, ovarian and colorectal adenocarcinomas - Assess safety and impact on TME
		NCT02695966	- *Ex-Vivo* assessment of T lymphocyte function and localization in pancreatic cancer
Ulocuplumab (BMS-936564)		Phase I/II (NCT02472977)	- In combination with nivolumab - Terminated due to lack of efficacy
LY2510924		Phase II (NCT01439568)	- In combination with carboplatin and etoposide - No clinical benefit in patients with extensive-disease small cell lung carcinoma (43)
		Phase II (NCT01391130)	- In combination with sunitinib - Terminated due to insufficient efficacy in patients with metastatic clear cell renal cell carcinoma
		Phase 1 (NCT02737072)	- In combination with durvulumab for patients with advanced solid tumors - Terminated
USL 311		Phase I / II NCT02765165	- Recruiting patients with glioblastoma multiforme
Olaptesed (NOX-A12)		Phase I/II (NCT03168139)	- Olaptesed in combination with pembrolizumab - Recruiting patients with colorectal and pancreatic cancer
GENETICALLY MODIFIED EFFECTOR IMMUNE CELLS			
	CXCR2 + NGFR + T cells	Phase I/ II (NCT01740557)	- Recruiting patients with metastatic melanoma

effector and memory T cells (44). CXCR3 expression on Tregs, however, can hamper effector immune cell functions due to competitive recruitment. In HCC, Treg infiltration in the liver after liver transplantation was associated with higher rates of recurrence (25). Patients with higher numbers of circulating Tregs and increased levels of CXCL10 within the graft were more susceptible. Similarly, high expression of CXCL11 in a colorectal cancer model was shown to recruit $CXCR3^+$ Tregs (26). In contrast, in ovarian cancer, high CXCL9 and CXCL10 expression doubled the overall survival time due to improved recruitment of tumor-infiltrating lymphocytes (27). Enhanced effector T cell recruitment via the CXCR3 axis has also been confirmed in the case of gastric cancer and melanoma (28, 29). Tumor growth was accelerated in $CXCR3^{-/-}$ melanoma-bearing mice and T cell infiltration was severly impaired (30). Anti-programmed death receptor (Anti-PD1) therapy was not beneficial in $CXCR3^{-/-}$ tumor-bearing mice due to failure of efficient T cell recruitment (30). Importantly, CXCR3 has been shown to be indispensable

for $CD8^+$ effector T cell trafficking across tumor vasculature due to its role in intravascular adhesion, even in the absence of its ligands. CCR2 and CCR5, in contrast, promoted tumor site infiltration only in a chemokine ligand dependent manner (31). CXCR3 expression plays an important role in recruiting NK cells to the tumor site: We showed that CXCR3 expression on human NK cells increased during *ex vivo* culture (53). In xenograft mice models, these expanded NK cells could be efficiently recruited toward $CXCL10^+$ melanomas (53). However, the sole presentation of CXCR3 ligands within the TME does not always predict efficient effector cell recruitment. In a mouse model of uveal melanoma that leads to spontaneous metastasis into the skin and viscera, application of the chemotherapeutic drug temozolomide increased CXCL9 and CXCL10 levels within the metastatic sites (54). Nonetheless, increased T cell infiltration was only observed in the visceral sites and not in the cutaneous tumors due to altered matrix architecture and mode of CXCL9/10 presentation (54). Interestingly, high expression levels of CXCL9 and CXCL10 in colorectal cancer samples correlated with T cell infiltration, but not with NK cell infiltration that was scarce in the analyzed samples (32). The expression level of CXCR3 was not measured on NK cells versus T cells. In contrast, $CXCR3^+$ NK cells infiltrated tumor tissue in murine lymphoma and melanoma models in a CXCL10-dependent manner (33). CXCL10 was augmented via application of IFNγ (33). Several factors can modify CXCR3 expression on T cells and NK cells. For instance, elevated CXCR3 ligands in patients with cutanenous T cell lymphoma lead to CXCR3 downregulation on cytotoxic T cells (55). Soluble HLA-G was also shown to downregulate CXCR3 expression on cytotoxic T cells and inhibit migration along CXCL9 and CXCL10 gradients (56). In another study, STAT3 signaling in $CD8^+$ T cells was shown to downregulate IFNγ production, leading to decreased CXCL10 expression by tumor-associated macrophages. Additionally, STAT3 diminished CXCR3 expression on $CD8^+$ T cells (57). Collectively, these data underline not only the importance of the CXCR3 axis in recruitment of effector immune cells, but also reveal complex relationships of receptor-ligand interactions in a TME-specific context.

To enhance effector cell recruitment, efforts are made to increase CXCL9 and CXCL10 expression within the TME. Several enzymes can modulate CXCR3 ligands such as dipeptidyl peptidase-4 (DPP-4/CD26) (58, 59), furin (60) as well as certain peptidylarginine deaminases and matrix metalloproteinases (61). For instance, DPP-4 was shown to cleave CXCL9, 10 and 11, which in turn reduced their chemotactic activity on lymphocytes, while not affecting their antiangiogenic activities (59). In breast cancer cell lines, Prostaglandin E_2 (PGE_2) impaired IFN-γ mediated CXCL9 and CXCL10 release (62). Inhibition of the cyclooxygenase (COX) isoenzymes with indomethacin and acetylsalicylic acid suppressed the downregulatory functions of PGE_2 and increased CXCL9 and CXCL10 levels *in vitro* (62). Evidence for the role of CXCL9 in attracting NK and cytotoxic T cells was shown in a murine model of breast cancer (63). Gene transfer of CXCL10 by pCLNCX retroviral vectors in melanoma xenograft models decreased angiogenesis and tumor growth (64). Similarly, murine-leukemia virus (MLV)-derived replication-competent retroviruses were used to stably express CXCL10 in fibrosarcoma, melanoma and Lewis lung cancer models and were shown to inhibit tumor growth *in vivo* (65). However, the effect of CXCL10 on T or NK cell recruitment and functionality was not investigated in these early studies. Only recently, an oncolytic poxvirus was armed with CXCL11 in order to attract $CXCR3^+$ cytotoxic T cells and NK cells to the site of the malignancy in a murine mesothelioma model (66). Besides improving effector cell homing, the virus enhanced the systemic antitumor activity by inducing the proliferation of IFNγ-producing $CD8^+$ T cells.

Targeting the CXCR3 axis to improve efficient effector cell recruitment is hampered by the opposing role on tumor cells: CXCR3 expression can be found on tumor cells, especially at later stages of tumorigenesis and in patients with advanced disease, where it is positively correlated with the formation of metastasis (67–69). Thus, blocking CXCR3 on tumor cells might also impair the ability of CXCR3+ NK and T cells to efficiently kill tumor cells. Interestingly, ACKR3 (formerly CXCR7) is an atypical receptor of CXCL11 and CXCL12, that is not expressed on peripheral blood leukocytes but upregulated by various tumor types, including breast, esophageal and lung squamous cell cancer (70, 71). Targeting of ACKR3 with a monoclonal antibody in mice models of glioblastoma leads to increased tumor cell death via NK-cell mediated antibody-dependent cytotoxicity (ADCC) (72). Combination with temozolomide prolonged survival in tumor-bearing mice and resulted in enhanced infiltration of anti-tumorigenic M1 macrophages (72). CXCR3 and ACKR3 inhibitors are in preclinical testing for different solid tumors (72–74). Currently there are no registered clinical phase trials employing either CXCR3 or ACKR3 inhibitors in solid malignancies.

CXCR4 AND ITS LIGAND CXCL12

CXCR4 and its ligand CXCL12 are ubiquitously expressed under physiological conditions and are important for hematopoiesis, cardiogenesis, and neurogenesis. The CXCR4-CXCL12 axis is involved in HSC maintenance and homing within the bone marrow as well as during the development of B, T, and NK cells (75, 76). In the context of cancer, CXCR4 expression is found on tumor cells, where it promotes tumor cell growth, migration, and invasiveness (77, 78). Moreover, CXCL12 produced within the tumor can attract $CXCR4^+$ Treg, MDSC and plasmacytoid dendritic cells (pDC), potentiating the tumor-promoting effect (34, 79–81). High CXCR4 expression in biopsies of solid tumors is generally correlated with worse prognosis. In particular, CXCR4 expression in breast cancer was significantly associated with lymph node and distant metastasis and worse overall survival (82). Similar conclusions could be drawn for prostate cancer, melanoma and lung cancer (83–85).

The expression levels of CXCR4 on NK and T cells varies according to their maturation stage and subset, whereas their recruitment to the different organs is often dependent on the co-expression of other chemokines (86, 87). High CXCR4 expression on NK cells is associated with accumulation within

the bone marrow compartment, whereas CXCR4 desensitization is important to enable NK cells to leave the bone marrow (88, 89). Several factors can modulate the chemokine receptor repertoire on immune cells: For instance, conditioning human NK cells with TGFβ1, derived from neuroblastoma cells, significantly upregulated CXCR4 and CXCR3 expression and downregulated CX3CR1 on NK cells (90). This generated an NK cell phenotype that is retained in the bone marrow, rather than recruited to peripheral organs and tumor tissue (91). Another study suggested that PGE2 regulates CXCL12 levels in malignant ascites from ovarian cancer patients and CXCR4 expression on MDSC (34). Blockade of PGE2 abrogated migration of MDSC toward the malignant ascites. In line with this, non-small cell lung cancer (NSCLC) express high CXCL12 levels and especially recruits $CD4^+CD69^+CXCR4^+$ T cells with an increased ratio of regulatory T cells (35). Although the percentage of $CD8^+$ T cells was not altered, NK cell numbers within the tumor tissue decreased. In accordance, regulatory T cells are maintained within the bone marrow and can migrate along the CXCR4-CXCL12 axis (92). Regarding modulation of CXCR4 expression using pharmacological agents, tyrosine kinase inhibitors (TKIs) imatinib and nilotinib have been shown to selectively increase the cell surface of CXCR4 on NK cells and monocytes, *in vitro* experiments using NK cells derived from neuroblastoma patients (93).

Multiple approaches to target this axis have been explored, some of which have entered clinical trials with varying outcomes (summarized in **Table 2**). On a preclinical level, TN14003 and AMD3100 (Plerixafor), two anti-CXCR4 inhibitors, have been tested in patient-derived xenografts (PDX) of breast cancer showing antitumor activity in the HER2 subtype (94). Interestingly, in triple-negative PDX, both inhibitors appeared neither to control tumor growth nor to impede metastatic spread, which highlights the complexity of breast cancer subtypes and their respective TMEs. AMD3100 has also been tested in a murine model of human pancreatic cancer, alone or in combination with immunological checkpoint antagonists (95). In this study, AMD3100 was able to successfully block CXCR4 signaling and promote T-cell mobilization *in vivo*. More importantly, AMD3100 showed improved anticancer activity when combined with an anti-PD-L1 monoclonal antibody (96). CXCR4 is also highly expressed in colorectal cancer, building a therapeutic rationale for CXCR4 targeting (97). Blocking colon carcinoma cells with a CXCL12-KDEL retention protein *in vitro*, resulted in the inhibition of CXCR4-mediated signaling and a subsequent dramatic decrease in metastatic cancer outgrowth (98). AMD3100 has also been tested in the particular model, exhibiting similar promising preclinical results (99). Other means of modulating the CXCR4-CXCL12 axis include oncolytic viruses and gene-engineered NK cells. In particular, introducing an oncolytic virus equipped with a CXCR4 antagonist restored the pathologic signaling in a murine model of ovarian cancer, reduced metastatic spread and diminished regulatory T cell recruitment (100). On the other hand, NK cells engineered to co-express a chimeric antigen receptor (CAR) and the chemokine receptor CXCR4 enhanced NK cell infiltration and tumor cell killing in a glioblastoma tumor model (36). Last but not least, Spiegelmer aptamers, such as the CXCL12-targeting NOX-A12, hold great potential in modulating the TME of solid tumors. Although clinical trials are still ongoing (**Table 2**), NOX-A12 (Olaptesed pegol) is thought to increase immune cell infiltration, sensitize tumors to checkpoint inhibitors and obstruct tumor repair mechanisms in metastatic pancreatic and colorectal cancers (Noxxon Pharma). Examples of additional types of solid tumors that may benefit from inhibition of the CXCR4-CXCL12 axis are oesophageal (101)and gastric cancer (102).

CONCLUDING REMARKS

Although our current understanding of solid tumor microenvironment and its chemokine networks is more detailed, a lot remains unexplored. The future of chemokine modulation for therapeutic purposes is very much dependent on efforts to elucidate the complex pro-tumor and antitumor roles of chemokines in the TME. The current preclinical approaches have demonstrated some promising results and defined rational immunotherapeutic combinations. The results from the eagerly awaited clinical trials, in combination with investigations on new chemokine targets and advances in drug discovery, immunotherapy and cell therapy, are expected to shape the landscape of chemokine-based therapy further in the years to come.

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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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REVIEW
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Mechanisms and Functions of Chemerin in Cancer: Potential Roles in Therapeutic Intervention

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Chemerin [*RARRES2* [retinoic acid receptor responder 2], TIG2 [tazarotene induced gene 2 (TIG2)]] is a multifunctional cytokine initially described in skin cultures upon exposure to the synthetic retinoid tazarotene. Its secreted pro-form, prochemerin, is widely expressed, found systemically, and is readily converted into active chemerin by various proteases. Subsequent studies elucidated major roles of chemerin as both a leukocyte chemoattractant as well as an adipokine. Chemerin's main chemotactic receptor, the G-protein coupled receptor CMKLR1, is expressed on macrophages, dendritic, and NK cells. With respect to its role in immunology, chemerin mediates trafficking of these cells to sites of inflammation along its concentration gradient, and likely helps coordinate early responses, as it has been shown to have antimicrobial and angiogenic properties, as well. Recently, there has been mounting evidence that chemerin is an important factor in various cancers. As with its role in immune responses—where it can act as both a pro- and anti-inflammatory mediator—the potential functions or correlations chemerin has in or with cancer appears to be context dependent. Most studies, however, suggest a downregulation or loss of chemerin/*RARRES2* in malignancies compared to the normal tissue counterparts. Here, we perform a comprehensive review of the literature to date and summarize relevant findings in order to better define the roles of chemerin in the setting of the tumor microenvironment and tumor immune responses, with an ultimate focus on the potential for therapeutic intervention.

Keywords: chemerin, RARRES2, CMKLR1, CCRL2, GPR1, cytokine, chemoattractant, cancer

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INTRODUCTION

Chemerin [also known as retinoic acid receptor responder 2 (*RARRES2*) or tazarotene induced gene 2 (TIG2)] is a multifunctional, chemoattractant protein known for its roles in adipogenesis, angiogenesis, skin function, metabolic activity, and, recently, tumorigenesis. Initially secreted by the liver and white adipose tissue as prochemerin, the 163-amino acid precursor protein is readily cleaved by a specific set of serine proteases to become a chemotactically-active protein isoform of chemerin (1). Depending on the site of cleavage and subsequent interaction with its cognate heptahelical receptors, CMKLR1 (chemokine-like receptor 1), CCRL2 (C-C chemokine receptor-like 2), and GPR1 (G protein-coupled receptor 1), chemerin may exhibit varying degrees of bioactivity and elicit pro- or anti-inflammatory effects in different biological environments (1–3).

In the literature, CMKLR1 is usually discussed as the primary receptor for chemerin interaction, while CCRL2 is described to participate in various functions by binding and presenting chemerin in a non-signaling manner to establish concentration gradients (4, 5). On the other hand, GPR1 is less-well characterized and found in the central nervous system (5) and reproductive organs (6, 7) and may play a role in metabolism (8).

Since its initial discovery in skin cultures upon stimulation with anti-psoriatic synthetic retinoid tazarotene, chemerin has been further described in a number of biological settings (9). In human endothelial cells, chemerin has been found to mediate angiogenesis via interactions with CMKLR1 (10), while, in the epidermis, chemerin has been shown to engage in significant antimicrobial activity (11). Moreover, chemerin has been linked with conditions such as obesity and diabetes, where it may modulate metabolism and adipocyte development (12, 13). Most recently, chemerin has been shown to mediate the chemoattraction of various immunocytes in the tumor microenvironment, while expression of chemerin's receptors, CMKLR1 and CCRL2, has been identified on a number of leukocyte subsets, namely, dendritic cell subsets, natural killer cells, and macrophages (1, 2, 14, 15). With recent findings that chemerin's receptors are also expressed on malignant tumor cells and that chemerin's expression is often altered in different cancer types, newer studies have focused on chemerin's novel roles in immune surveillance and tumor progression.

As expected, these recent studies have confirmed the notion that chemerin's functions in cancer are context driven. In some cancer types [e.g., glioblastoma, mesothelioma, neuroblastoma, squamous cell carcinoma of the esophagus, and squamous cell carcinoma of the oral tongue (SCCOT)], chemerin is upregulated (16–22). In most cancer types [e.g., acute myeloid leukemia (AML), adrenocortical carcinoma (ACC), breast cancer, Ewing sarcoma, hepatocellular carcinoma, melanoma, non-small cell lung cancer (NSCLC), prostate cancer, and squamous cell carcinoma of the skin] chemerin is downregulated, likely via hypermethylation of *RARRES2* (15, 19, 23–28) (**Table 1**). Adding to this complexity, the ultimate effects on tumor growth are also context-dependent; both tumor suppression and accelerated growth have been observed as a result of altered chemerin expression levels (**Table 2**). Thus, in this review, we attempt to catalog and analyze chemerin's specific functions in each tumor type, with a focus on identifying patterns in its mechanism of action and suggesting ways in which the chemerin system may be utilized or manipulated for clinical benefit.

TABLE 1 | Alterations of chemerin expression profiles by cancer type.

Cancer type:	Tumor expression of chemerin	Serum levels of chemerin
Acute myeloid leukemia	↓	
Adrenocortical carcinoma	↓	↑
Breast cancer	↓	
Colorectal cancer		↑
Ewing sarcoma	↓	
Gastric cancer	↑	↑
Glioblastoma		
Hepatocellular carcinoma	↓	
Melanoma	↓	
Mesothelioma	↑	
Neuroblastoma	↑	
Non-small cell lung cancer	↓	↑
Prostate Cancer	↓	
Squamous cell carcinoma of the esophagus	↑	
Squamous cell carcinoma of the oral tongue	↑	↑
Squamous cell carcinoma of the skin	↓	

CANCERS

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is the most prevalent form of acute leukemia affecting adults, with a particularly high rate of incidence (12.2 cases per 100,000 people) for those above the age of 65 (32–34). Though recent advances in AML treatments have significantly improved prognosis for younger patients, those who are older have little chance of survival (35). In fact, a majority of AML patients over the age of 65 die within a year of diagnosis, underlying an urgent need for improved methods of detection that may enable earlier life-preserving treatment (36).

In light of this need, chemerin has recently been identified as a potential biomarker for diagnosis of AML. A study in 2017 revealed that chemerin was downregulated in the bone marrow mononuclear cells of AML patients compared to that of healthy controls, with ROC curve analysis showing a test specificity of 79% (true positive) and sensitivity of 54% (true negative) for chemerin expression as a marker for AML diagnosis. The same group performed a cohort study of 149 patients, 32 of whom had high chemerin expression and 117 of whom had low expression, with no significant variability in certain gene mutations, white blood cell count, platelets, and hemoglobin, and found that patients with low chemerin expression correlated with poorer overall survival. Moreover, multivariate analysis on parameters such as age, various gene mutations, chemerin expression, karyotypic classifications, and white blood cell count verified that chemerin was independently able to prognosticate AML patients, while univariate analysis of chemerin expression levels showed that high chemerin expression was associated with positive prognosis (22). In terms of chemerin receptor expression levels (independent of associations between chemerin expression and clinical outcome), a different group showed that CCRL2 was overexpressed in AML, identifying the non-classically signaling chemerin receptor as a potential therapeutic target, along with other GPCRs that were also overexpressed (37).

Adrenocortical Carcinoma

Adrenocortical carcinoma (ACC) is an extremely rare and aggressive tumor that is associated with poor prognosis in patients. The incidence rate for ACC is 0.7–2.0 cases per million people per year, and for patients that undergo first-line treatment (surgical resection), the 5-year median survival rate is 38.6%. For

patients ineligible or unwilling to undergo adrenalectomy, there is little chance of remission (38–40). In fact, the potential risk of ACC is considered the standard for adrenalectomy in patients with adrenal incidentalomas (38).

Recent research suggests that serum chemerin levels have prognostic value in ACC and that manipulating chemerin levels in ACC tumors may prove efficacious in ACC patients. Chemerin's role as a chemoattractant, in recruiting immune cells to sites of inflammation, has already been well documented; for instance, chemerin has been shown to suppress neoplasia by eliciting natural killer cells to the tumor site in melanoma (15). In line with those findings, a study by Chittenden et al. (28) featuring a group of 20 ACC patients, 53 benign tumor patients, and 21 healthy individuals reported that serum chemerin levels were elevated in ACC patients as compared to those with benign adrenocortical tumors, and the difference further increased when tested against healthy controls. They also discovered that higher serum chemerin levels were strongly associated with better overall survival. Though seemingly paradoxical, it was proposed that two important, proven factors may explicate this phenomenon. First, a mouse xenograft model showed that increased serum chemerin levels were not a result of secretions from the tumor itself but from the host environment (28). Second, as mentioned previously, chemerin has been shown to be an effective recruiter of immune cells to sites of inflammation (1, 14, 15, 41). Thus, it was suggested that ACC may downregulate chemerin as a method of immune evasion and that the host environment may increase secretion of chemerin in serum as a counteractive response (28).

Interestingly, in addition to the potential for immune-dependent methods of tumor suppression, a new study has found evidence that chemerin may directly alter malignant cells in an immune-independent manner. A study by Liu-Chittenden et al. (42) found that the RARRES2 gene was transcriptionally downregulated in ACC, in line with similar findings in other cancer types. Specifically, the silenced RARRES2 gene in ACC tumors was characterized by hypermethylation at five CpG sites. This was confirmed in three human ACC cell lines and HEK293 cells via treatment with a DNA methyltransferase inhibitor, which showed that such treatment reversed the effects of methylation at all CpG sites in a dose dependent manner (42, 43). [As an aside, in Ewing sarcoma, a rare, malignant tumor that grows inside the bones and in nearby soft tissues, RARRES2 showed a high rate of methylation and was one of only eight genes to have a frequency of silencing >20% (25). Thus, hypermethylation of RARRES2 is likely a common method of gene silencing in tumors where chemerin is downregulated].

When ACC cell lines were transfected to express chemerin, significant inhibition of tumor growth was observed in a dose dependent manner, as the cell lines that had higher expression of chemerin correlated to a more compelling reduction in tumorigenesis (42). Supporting these results, exogenous addition of recombinant chemerin *in vitro* showed no meaningful effect in affecting cell proliferation among various ACC cell lines, implying that the mechanism of growth inhibition was not mediated by the binding of chemerin to its receptors, such as CMKLR1 (42). In fact, chemerin's function in reducing ACC tumor growth was found to be mediated by two different, immune-independent mechanisms. First, chemerin overexpression could induce β-catenin phosphorylation, and thus, proteasome mediated degradation. In phosphokinase arrays of a chemerin overexpressing ACC cell line, significant reduction in total β-catenin levels was observed, whereas treatment with a proteasome inhibitor prevented proteasome mediated degradation, allowing for detection of elevated phosphorylated β-catenin levels (42). The phosphorylated sites were identified as Ser33, Ser37, and Thr41. Consequently, a decrease in Wnt/β-catenin pathway activity was observed and confirmed via a TCF/LEF luciferase reporter assay (42). Second, chemerin overexpression could inhibit p38 MAPK phosphorylation. The phosphokinase array, which showed a decrease in total β-catenin levels, also showed a reduction in phospho-p38 MAPK levels (42). *In vivo* xenograft studies in athymic nude mice and NSG mice, both immunodeficient mouse models, confirmed that chemerin overexpression resulted in lower β-catenin and phosphorylated p38 MAPK levels (42). Together, these results indicated that chemerin could promote tumor suppression through immune-independent pathways, both *in vitro* and *in vivo*.

These findings may have positive implications for ACC patients and other cancer patients alike. β-catenin (CTNNB1), a proto-oncogene, is frequently mutated in ACC, resulting in constitutive activation of the Wnt/β-catenin pathway (44). The occurrence of activating mutations of CTNNB1 is generally known to be a significant pathway for ACC tumorigenesis. Indeed, a majority of adrenocortical tumors exhibit activation of the Wnt/β-catenin pathway, which is correlated with poor outcomes in ACC patients (45, 46). Aberrant activation of the Wnt/β-catenin pathway is common in many other cancer types, such as breast cancer, lung cancer, hepatocellular carcinoma, and squamous cell carcinoma (47). Additionally, elevated phospho-p38 MAPK levels are found in a majority of adrenocortical tumors (42). In other cancer types such as lung cancer and colon cancer, abnormal activation of p38 signals are detected (48, 49). As such, it is reasonable to suspect that chemerin may have similar, tumor-suppressive effects in other cancer types.

Finally, chemerin may also serve as a prognostic marker for ACC. Among patients, tissue chemerin levels did not correspond to prognosis, though chemerin expression was downregulated in ACC tissue samples (28). However, serum chemerin levels did correspond to prognosis. A clinical survey showed that serum chemerin levels were significantly elevated in ACC patients in comparison to patients with benign tumors and even more so in comparison to healthy controls. Survival analysis of median serum chemerin levels in ACC patients determined that higher serum chemerin levels correlated with longer survival. Moreover, prognosis for patients with recurrent ACC could be stratified according to varying serum chemerin levels, further verifying its value as a marker for ACC prognosis (28).

TABLE 2 | Mechanisms of chemerin-mediated effects by cancer type.

Cancer type	Model	Technique/method	Results	Chemerin-mediated effect
Adrenocortical Carcinoma (28)	HEK293, H295R	BioCoat Matrigel invasion/migration assays	Transient overexpression of chemerin inhibited cell proliferation in HEK293, while inhibiting cellular invasion in KEK293, H295R	Inhibition of tumor cell proliferation and invasion
	H295R	Phosphokinase array +Western blot analysis	Phosphorylation and subsequent degradation of β-catenin and inhibition of p38 MAPK phosphorylation observed in stable, chemerin-overexpressing H295R ACC cell lines	Reduced β-cateinin and phosphorylated p38 MAPK levels
	H295R, HumanACC Tumor Samples	TCF/LEF Luciferase Reporter Assay + Immunohistochemistry	TCF/LEF reporter activity reduced in chemerin-overexpressing ACC cell lines compared to vector cell line; nuclear localized phosphorylated p38 signals detected in a majority of ACC tumor samples	Decreased activity of Wnt/ β-catenin and MAPK pathways
	Athymic Nude Mice, NSG Mice + H295R	Subcutaneous inoculation ofH295R in Athymic nude + NSG mice	Overexpression of chemerin significantly impaired tumor growth and resulted in lower tumor weight in both mouse models	Suppression of tumor growth
Gastric Cancer (29)	AGS, MKN28	Matrigel-coated Transwell assay	Increased invasion of AGS and MKN28 cells through Matrigel-coated Transwells at extremely low concentrations (0.0 I ng/ml)	Enhanced tumor invasion
	AGS, MKN28	Real time-PCR	Induction of mRNA expression of pro-invasive genes, IL-6, VEGF, via chemerin in AGS and MKN28, and also MMP-7 in MKN28	Increased expression of IL-6, VEGF, MMP-7
	AGS, MKN28	Western blot analysis	Increased phosphorylation of p38, ERKl/2 via chemerin in AGS, MKN28	Increased activity of MAPK pathways (MEK-ERK, MKK3/6-p38)
Glioblastoma (17)	U87MG	Calcium mobilization assay	Chemerin stimulation induced a transient, dose dependent increase of intracellular calcium in U87 MG cells	Induction of intracellular calcium
Hepatocellular Carcinoma	C57BL/6 Mice +Hepal-6	Implantation of HCC hepal-6 in C57BL/6 mice + subcutaneous model	WT mice with chemerin-expressing Hepa 1-6 tumors had a lower mortality rate and liver tumor growth/weight compared to Rarres2$^{-/-}$ mice with Hepa1-6 tumors	Inhibition of tumor growth
	C57BL/6 Mice +Hepa1-6	Intravenous injection of hepal-6 in C57BL/6 mice	WT mice with chemerin-expressing Hepa1-6 tumors had less metastatic nodules compared to Rarres2$^{-/-}$- mice with Hepal-6 tumors	Reduced lung metastasis
	C57BL/6 Mice +Hepa1−6	Flow cytometry	Rarres2$^{-/-}$ mice with Hepa1-6 tumors showed heightened proportions of MDSCs and TAMs and decreased levels ofCD4/CD8 T cells compared to WT mice with chemerin-expressing Hepal-6	Reduced induction of MDSCs and TAMs and increased accumulation of tumor infiltrating CD4/CD8 T cells
	C57BL/6 Mice +Hepal-6	Quantitative reverse transcriptase-PCR	Chemerin-expressing Hepal-6 tumor cells exhibited significantly decreased expression ofGM-CSF, IL-6	Reduced expression of GM-CSF + IL-6
Li et al. (30)	7404, HepG2	Boyden chamber + Transwell invasion assay	Overexpression of chemerin resulted in reduced migratory ability and invasiveness of 7404 cells, whereas chemerin knockdown enhanced these properties in 7404/che Hand Hep G2 cells	Reduced migratory ability and invasiveness
	BALB/c Mice +PVTT-1	Left ventricular + intrahepatic injection model	Prolonged survival and reduced/delayed appearance of metastatic foci was observed in mice injected with PVTT−1 che cells (chemerin overexpressing), compared to mice injected with PVTT−1 con cells in both injection models (Resuts were replicated by regularly injecting chemerin in mice with PVTT-1 tumors)	Inhibited metastasis and prolonged survival times (Reduced weight loss)

(Continued)

TABLE 2 | Continued

Cancer type	Model	Technique/method	Results	Chemerin-mediated effect
	HCCTMA	Immunohistochemistry	High levels of chemerin were correlated with low levels of p-Akt (Ser473) and high levels of PTEN in HCC TMA	Reduced Akt/MMP 1 as a result of positive regulation of PTEN via chemerin
	7404, PVTT-1, Hep3B, HepG2	Western blot analysis	In chemerin-overexpressing HCC cells (7404, Hep3B, and PVTT-1), reduced p-Akt(Ser473) levels were observed, while elevated p-Akt(Ser473) levels were observed in chemerin knockdown HCC cells (7404/che H, HepG2)	Downregulation ofp-Akt (Ser473)
Melanoma (15)	C57BL/6 Mice +Bl6	Flow cytometry	The average frequency of NK and T cells were increased in chemerin- expressing tumors	Enhanced infiltration of antitumor leukocytes
	C57BL/6 Mice C57BL/6 Mice	Subcutaneous inoculation ofB16 murine melanoma in C57BL/6 Mice	Chemerin-expressing melanomas exhibited delayed growth compared to control transfectants (measured by tumor size)	Impaired tumor growth
Neuroblastoma (21)	SK-N-SH	Confocal laser scanning microscopy	Fast, transient increase in intracellular calcium observed in SK-N-SH cells following stimulation with lOnM chemerin	Induction of intracellular calcium
	SK-N-AS, SK-N-BE(2)	Western blot analysis	Increased, dose dependent phosphorylation of Akt, MEKl/2, ERKl/2 in cell lines observed after stimulation via chemerin	Increased activity of MAPK and Akt pathways
	SK-N-AS, SK-N-BE(2)	Real-time zymography	Increased synthesis ofMMP-2 in cell types after 6, 12, 24, 48 h stimulation with chemerin	Increased expression of MMP-2
Non-Small Cell Lung Cancer (31)	LLCl	Flow cytometry	Splenocytes cultured with prochemerin-expressing Lewis Lung Cancer cells (LLC) exhibited decreased levels ofTNF-a, IL-12 p40 and slightly increased levels of IFN-*y*	Decreased expression of inflammatory cytokines, TNF-a, IL-12 p40
	C57BL/6 Mice +LLCl	Subcutaneous inoculation of Prochemerin-LLCl into C57BL/6 mice	C57BL/6 mice with prochemerin-expressing LLC tumors exhibited a lower incidence of tumor formation, but not a decrease in tumor growth rate	Prochemerin induced inhibition of tumor formation
Squamous Cell Carcinoma of the Esophagus (20)	OE21	Matrigel-coated boyden chamber invasion assay	Enhanced invasion of OE21 cells in Matrigel-coated Boyden chambers upon stimulation with chemerin (effects abrogated by CCX832, a CMKLRl inhibitor)	Enhanced tumor invasion
	OE21	Western blot analysis	Increased expression of MMP-1, MMP-2, MMP-3 in OE21 cells stimulated by myofibroblast-secreted chemerin (effect mediated by protein kinase C; MMP responses inhibited by Ro31822, a PKC inhibitor)	Increased expression of MMP-l, MMP-2,MMP-3
	OE21	Enzyme activity assay	Increased activity ofMMP-1, MMP-2, MMP-3 (measured in fluorescence intensity) in OE21 cell media, induced by chemerin	Increased activity of MMP-1, MMP-2, MMP-3
Squamous Cell Carcinoma of the Skin (19)	SCL-1, SCC-12B2, SCC-13, A431	Transwell Chamber Migration assay	Significant increase in cell migration (cells/HPF) induced by l 0, 20, 40 nM of recombinant human chemerin (SCL-1), 20,40 nM (SCC-12B2), 10, 20, 40 nM (SCC-13), 20 nM (A431)	Enhanced tumor migration
	SCL-1	GPCR Cignal Finder array	Enhanced MAPK activity of JNK and ERK signaling pathways after l hour of stimulation via recombinant human chemerin	Increased activity of MAPK pathways (JNK, ERK)
	SCL-1	Western blot analysis	Increased phosphorylation of ATF2, c-Jun, SEK1, ERKl/2 observed in chemerin treated SCL-1 cells compared to untreated SCL-1 cells	Increased activity of MAPK pathways (JNK, ERK)

Breast Cancer

Breast cancer is the most common malignancy in women, accounting for a quarter of all cancer cases in females (50). It is estimated that one in every eight women will develop breast cancer at some point in their lives (51). In this context, chemerin has not been deeply investigated; though, there are some preliminary pieces of evidence showing that chemerin is significantly downregulated in breast adenocarcinomas, one of the most common forms of breast cancer (15). Additionally, studies have detected CMKLR1 in breast tissue (52). Taken together, these facts raise the suspicion that chemerin may exhibit antitumor effects in breast cancer.

However, pro-tumor effects are also possible. Given the increased risk of breast cancer due to post-menopausal obesity and chemerin's known associations with obesity and obesity related parameters, such as blood pressure and BMI, some suspect that chemerin may be correlated to higher risk of breast cancer, albeit indirectly (51, 53). Moreover, it has been experimentally shown that some adipose-derived angiogenic factors may promote breast cancer growth, though this remains to be tested in the context of chemerin (54, 55). It has also been speculated that chemerin may play a role in instigating metastasis through its angiogenic functions (51). For prognosis, it is unclear whether chemerin can be used as a biomarker for breast cancer. A cross sectional study involving 117 breast cancer patients by Akin et al. (51) showed that serum chemerin levels could not be used in staging of breast cancer, as there were no differences in serum chemerin levels of patients with metastatic and non-metastatic cancer.

Colorectal Cancer

Colorectal cancer has the third highest incidence rate and fourth highest mortality rate of all cancers types, with 1.4 million new cases and 700,000 related deaths per year (56). Current treatment methods range from chemotherapy (e.g., single-agent 5-fluorouracil, oxaliplatin, and/or irinotecan) to other regimens consisting of newer, targeted substances, such as anti-vascular endothelial growth factor-A antibodies or anti-epidermal growth factor receptor antibodies (57).

Among the early diagnostic tools that have become available in recent years, serum chemerin levels have been identified as an effective biomarker for colorectal cancer. A study in 2018 compared 32 colorectal cancer patients with sex, BMI, and age matched healthy volunteers and reported that chemerin was significantly upregulated in serum of colorectal cancer patients, increasing with higher tumor stage progression (58). The area under the receiver operating characteristic (ROC) curve for serum chemerin levels was 1, with a sensitivity and specificity of 100% (58). The results clearly indicated that serum chemerin could be an effective biomarker for colorectal cancer and stage progression.

Additionally, a pilot study of 110 colorectal cancer survivors in Korea showed that there was an inverse relationship between serum chemerin levels and colorectal cancer-related quality of life, defined in terms of Functional Assessment of Cancer Therapy (FACT)-General, -Colorectal, and -Fatigue scores (59). Given that chemerin has been shown to have antimicrobial properties, it was proposed that chemerin may play a role in modulating gut microbial activity, which may play a role in the various bowel symptoms affecting colorectal cancer patients (11, 60). Supporting this hypothesis, colorectal cancer patients who have undergone resection surgery have consistently reported altered gut microbial activity, which has been linked to numerous bowel symptoms in these patients (61–63). Interestingly, the gut microbiome is somewhat altered in chemerin KO mice compared to WT controls, with an increase in Desulfovibrionaceae (64). Increases in this pathobiont have been reported as a component of IBD-associated gut dysbiosis (65).

Gastric Cancer

Gastric cancer is the fourth most common cancer and the second most common cause of cancer-related death in the world (66). Treatment of gastric cancer generally consists of a combination of surgery and chemotherapy (66). Though current clinical parameters for diagnosis are incapable of accurately predicting patient outcomes, recent evidence has revealed chemerin's prognostic value in gastric cancer, as well as its functions in tumor growth (67).

A 2014 study comparing 196 gastric cancer patients with 196 age- and sex-matched healthy controls showed that preoperative plasma chemerin levels were significantly elevated in cancer patients (67). Multivariate analysis of clinical parameters currently used for diagnosis and serum chemerin levels showed that serum chemerin could be used as an independent predictor of 5-year mortality, 5-year adverse event, overall survival, and 5-year disease free survival, though further research is needed to adjust for the possibility of postoperative alterations (67).

In terms of functional roles, studies have shown that chemerin exhibits pro-tumor effects in gastric cancer, confirming the notion that chemerin's pro/anti-tumor functions are context dependent. Indeed, elevated serum chemerin levels were observed in gastric cancer patients and were shown to be correlated with increased tumor invasiveness (29). Specifically, chemerin contributed to tumor growth by inducing phosphorylation of p38 and ERK 1/2 MAPKs and upregulating IL-6, MMP-7, and VEGF (29). Chemerin treatment did not alter the *in vitro* proliferation of two human gastric cancer cell lines, AGS and MKN28. However, chemerin increased the invasiveness of both cell types through Matrigel-coated transwells with only miniscule concentrations of chemerin. In fact, the number of cells invading through the membrane increased fivefold or more for AGS and MKN28 cells exposed to chemerin. Moreover, chemerin increased mRNA expression of VEGF and IL-6 in AGS and MKN28 cells, while also increasing expression of MMP-7 exclusively in MKN28 (29). VEGF, IL-6, and MMP-7 have all been associated with enhanced tumor invasiveness in gastric cancer (68–70), while high expression of VEGF and IL-6 have been shown to stimulate metastasis of malignant cells and indicate poor clinical outcomes in gastric cancer patients, suggesting a potential impact of chemerin in this setting (69–72).

In addition to increased expression of various pro-invasion genes, stimulation of AGS and MKN28 via chemerin resulted in elevated phosphorylation of p38 and ERK1/2 in both cancer lines, suggesting that chemerin could activate MKK3/6-p38

and MEK-ERK MAPK signaling cascades (29). Importantly, these are known mechanisms by which tumor cells modulate growth and invasion in gastric cancer (73–76). Elevation of phosphorylated ERK 1/2 levels have been associated with poor survival in patients, and p38 activation has been found to incite peritoneal dissemination in gastric cancer (75, 76). Given that these effects were abrogated by treatment with inhibitors of MEK-ERK signaling, it was clear that the MEK-ERK MAPK pathway was primarily associated with increased gastric cancer invasiveness (29). Together, the results of the studies indicated that chemerin may engender pro-tumor effects in gastric cancer, while also serving as a marker for patient prognosis.

Glioblastoma

Glioblastoma is the most malignant form of brain tumors affecting adults (77). Recent advances in glioblastoma research have made new treatment methods available to patients, such as tumor-treating fields and immunotherapy. However, the classical approach to treating glioblastoma remains a multipronged effort, using a combination of surgical resection, radiation, and chemotherapy (77). The incidence rate of glioblastoma hovers around 3.19 cases per 100,000 people in the United States and is very uncommon in children (78).

Though chemerin's role in glioblastoma is still unclear, studies have reported an altered chemerin profile in glioblastoma cases (17). First, one study found that chem158 K, a bioactive isoform of chemerin, was elevated in the cerebrospinal fluid of patients with malignant glioblastoma (79). A later study found that chemerin mRNA expression was significantly increased in grade 3 and 4 glioma, equivalent to malignant glioblastoma, compared to epilepsy and grade 2 glioma (17). In contrast, CMKLR1 and CCRL2 mRNA expression was unaltered in those cases. To further test whether chemerin could function as a signaling molecule, *in vitro* experiments were conducted using U-87 MG cells, a human glioblastoma cell line. Experimental results determined that the addition of chem157S, another bioactive isoform of chemerin, to U-87 MG cells resulted in a transient, dose-dependent increase of intracellular calcium, indicating that chemerin could instigate intracellular signaling in U-87 MG cells (17). Whether these results translate to experiments *in vivo* remains to be seen.

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in the world, with more than 500,000 new cases being diagnosed each year (80). It is mainly caused by chronic liver damage due to hepatitis B or hepatitis C and is associated with risk factors such as obesity and diabetes (80). Currently, a variety of treatment options are available to patients, including liver transplantation, curative resection, radiotherapy, radio/chemo-embolization, and systemic therapies (81).

In recent years, chemerin has been identified as a potential therapeutic agent for HCC. A study comparing chemerin expression levels in 124 HCC patient tumors and matched, normal tissues showed that chemerin was significantly downregulated in 72 of the patients' liver tissues and that tissue chemerin expression correlated with tumor size, grade, and infiltration of dendritic cells and natural killer cells. Additionally, chemerin was identified as an independent factor for prognosis via multivariate analysis, with lower chemerin expression corresponding to poorer overall survival (23).

A later study by Lin et al. (82) further reported that chemerin could inhibit HCC tumor growth. In an orthotopic mouse model of HCC, chemerin knockout mice showed aggressive tumor growth and metastasis. In contrast, overexpression of chemerin in mice resulted in delayed tumor growth, suggesting that chemerin may hamper tumor progression. Specifically, the inhibitory effect was mediated by suppressing pro-tumor inflammatory cytokines. When Hepa1-6 cells were treated with chemerin *in vitro*, cell survival and proliferation was unaffected. Moreover, chemerin knockout mice with accelerated tumor growth exhibited increased expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 and accumulation of myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). Neutralization of GM-CSF and IL-6 reversed the effects in the chemerin knockout mice, showing that these two cytokines were mainly responsible for the accelerated growth. Combined with the fact that serum chemerin levels were inversely correlated to GM-CSF and IL-6 expression levels in patients with HCC, the study suggested that chemerin may have a negative regulatory role for these two cytokines (82).

Further verifying these claims, chemerin was shown to inhibit nuclear factor-κB activation, an important factor for GM-CSF and IL-6 expression (83, 84). The data consistently showed that chemerin targeted tumor cells and tumor-associated endothelial cells, the major source of GM-CSF and IL-6, via interaction with CMKLR1 and CCRL2, reducing expression of inflammatory cytokines and inhibiting NF-κB activation (82). Downregulation of CMKLR1 and CCRL2 via siRNA in Hepa1-6 cells reestablished GM-CSF and IL-6 expression, indicating that decreased expression of the cytokines was mediated by chemerin-CMKLR1/CCRL2 interactions. The observed consequences of reduced GM-CSF and IL-6 expression were impaired MDSC accumulation, reestablishment of anti-tumor IFN-γ^+ T-cell activity, and hampered tumor angiogenesis (82).

The inhibitory function of chemerin in HCC was mediated by both T-cell dependent and independent mechanisms. Because chemerin showed less of an inhibitory effect in Rag1 knockout and CD8-T-cell depleted mice, it was determined that chemerin's function in HCC was not completely based on T-cell anti-tumor activity (82). It was postulated that chemerin's T-cell independent mechanism of inhibition was mediated by reduced accumulation of MDSCs, which are a major source of pro-angiogenic factors Bv8 and MMP-9. Supporting this hypothesis, overexpression of chemerin in mice resulted in a notable decrease in Bv8 and MMP-9 expression and, ultimately, tumor angiogenesis (82).

A recently published study showed a novel interaction of chemerin and the tumor suppressor PTEN in a mouse model of HCC (30). *In vitro*, overexpression of chemerin resulted in reduced migratory ability and invasiveness of human HCC line 7404. Chemerin knockdown, in turn, resulted in increased migratory ability and invasiveness. *In vivo*, overexpression of chemerin inhibited intra- and extrahepatic metastases of HCC cells in nude mice, lengthening survival times of HCC inoculated

mice. Nude mice with chemerin overexpressing tumors showed markedly less distant metastases and delayed appearance of metastatic foci using control or chemerin-expressing PVTT-1 HCC cells. Consistent observations were made in an intrahepatic injection model. Moreover, regular intraperitoneal injections or intrahepatical injections of chemerin in mice with PVTT-1 tumors replicated these results, with the additional benefit of reduced weight loss, showing that chemerin could potentially be therapeutically administered to suppress HCC metastases.

Underlying these tumor suppressive effects, the mechanism of reduced HCC cell migration and invasion via chemerin was shown to be mediated by negative regulation of p-Akt (Ser473) by PTEN. P-Akt and MMP-1 levels, as well as ubiquitination of PTEN, were reduced in chemerin-overexpressing HCC cell lines and elevated in chemerin-knockdown HCC cell lines. PTEN levels were upregulated in chemerin-overexpressing HCC cell lines and downregulated in chemerin-knockdown HCC cell lines. Immunohistochemistry of HCC tissues showed that upregulation of chemerin correlated with low p-Akt and high PTEN levels. Supporting these findings *in vivo*, data from their tumor models showed that overexpression of chemerin was associated with reduced tumor MMP-1 and p-Akt levels, elevated PTEN levels, and reduced metastases. Taken in aggregate, these studies provided strong support for therapeutic applications of chemerin in HCC.

Melanoma

Melanoma has one of the fastest growing incidence rates in the world, which in the US has risen from 8.2–9.4 cases per 100,000 people in 1975 to 24.2–35.4 cases per 100,000 people in 2010 (85). Unlike many malignancies, melanoma affects a diverse range of age groups, with a median age of diagnosis of 57 years (86). It is also widely recognized as the most aggressive form of skin cancer, accounting for the majority of skin cancer-related deaths vs. a fraction of total cases (86). Significantly, recent research has elucidated chemerin's ability to induce tumor suppression in melanoma, where chemerin was found to be downregulated (15).

Our group previously identified the mechanism of tumor suppression via chemerin in melanoma, showing that tumor expression of chemerin led to inhibited tumor growth *in vivo*. When chemerin-expressing B16F0 (hereafter, B16) cells were implanted into mice, the mouse melanoma showed significant delay in growth compared to control B16 cell lines. Importantly, the RARRES2-transfected B16 cells expressed bioactive chemerin, confirmed via chemotaxis, and both the transfected line and control B16 lines lacked expression of CMKLR1. When tested *in vitro*, chemerin treatment failed to alter B16 cell proliferation, suggesting that chemerin's inhibitory effects in melanoma was mediated by host immune responses (15). It was also noted that chemerin expressing tumors exhibited enhanced infiltration by NK and T cells; ratios of NK and T cells to MDSCs and/or plasmacytoid dendritic cells (pDC) were also increased (15, 87, 88). Given that certain subsets of pDCs and MDSCs have been reported to be tolerogenic, suppressing the body's antitumor immune responses, it was noted that increased ratios of antitumor immune cells to tolerogenic ones would translate to a more favorable environment for tumor suppression (89, 90). Moreover, chemerin's tumor suppressive effects in melanoma were mediated by NK cells, as their depletion abrogated the antitumor effects, while a lack of T cells and B cells did not alter the tumor growth-inhibition phenotype (15). Further experiments showed that host expression of CMKLR1 was necessary for inhibited tumor growth, since chemerin-expressing mouse melanoma grew faster than control B16 tumors in CMKLR1-negative mice. Finally, local administration of chemerin suppressed tumor growth *in vivo* (15).

Thus, in melanoma, chemerin was shown to inhibit tumor growth by eliciting antitumor responses and altering the tumor microenvironment in favor of growth inhibition. Whether by tumoral expression or local administration, these tumor-suppressive effects could be observed *in vivo* (15). Additionally, high expression of chemerin was shown to be associated with better outcomes for patients in two clinical studies, demonstrating chemerin's potential for therapeutic intervention in melanoma (15).

Mesothelioma

Mesothelioma is a tumor that grows in the linings of various organs, such as the lungs or the heart. Tumorigenesis is instigated by exposure to specific, carcinogenic mineral fibers, namely asbestos (63). Due to the commercial use of such materials, the incidence of mesothelioma has increased over the years, from near non-existence to several thousand cases per year (63). Though treatments are available, such as chemotherapy with cisplatin and pemetrexed, prognosis is still dismal and diagnosis very difficult, as it may take several decades for symptoms to appear (91). Regarding chemerin's role in mesothelioma, literature on the topic is scarce, though it was one of the first identified cancer types where RARRES2 expression was altered (significantly increased, compared to matched normal tissue) (16). It remains unclear if chemerin contributes in any way to mesothelioma progression.

Neuroblastoma

Neuroblastoma is a pediatric cancer affecting the sympathetic nervous system. With <50% probability of cure for high-risk cases, the prognosis for children with advanced stage neuroblastoma is bleak (92). For those with high-risk neuroblastoma, treatment is intensive, consisting of several modalities, such as chemotherapy, surgery, immunotherapy, stem cell rescue, and differentiation therapy (93).

A recent study by Tümmler et al. (21) showed that expression of chemerin receptors could successfully prognosticate neuroblastoma. Based on data from public datasets, the study found that high expression of GPR1 and CMKLR1 was associated with low survival rates. Furthermore, expression of CMKLR1 and CCRL2 was found to be upregulated in neuroblastoma cohorts in comparison to benign counterparts, while increased CCRL2 expression was correlated to poor prognosis. Increased chemerin expression was also found in neuroblastoma cohorts in comparison to neural crest, though no differences in expression levels were found between neuroblastoma and benign neurofibroma. Overall, CMKLR1 and

GPR1 expression was shown to be a potentially viable indicator of prognosis in neuroblastoma patients (21).

According to the same study, inhibition of the chemerin/CMKLR1 axis exhibited antitumor effects *in vitro* and *in vivo*, suggesting that the chemerin/CMKLR1 axis could be a potential therapeutic target for neuroblastoma. *In vitro*, expression of CMKLR1, chemerin mRNA/protein, and GPR1 was verified in neuroblastoma cell lines (as well as primary tumors) via real time-PCR and western blot analysis (21). Exposure to inflammatory cytokines TNF-α, IL-1β, and serum resulted in increased secretion of chemerin by neuroblastoma cells. Moreover, the mechanistic effects of added chemerin in neuroblastoma cell lines was the induction of intracellular calcium, activation of MAPK and Akt signaling, and synthesis of MMP-2. Specifically, in a human neuroblastoma cell line (SK-N-SH), chemerin induced a rapid increase in intracellular calcium. In SK-N-AS and SK-N-BE(2) cells, a dose-dependent increase in MEK 1/2, Akt, and ERK 1/2 phosphorylation was observed upon addition of chemerin, indicating the activation of Akt and MAPK pathways. In the same cell lines, a dose-dependent increase in MMP-2 synthesis was shown via real-time zymography, after cells had been stimulated via chemerin for various timepoints. Significantly, inhibition of CMKLR1 on four neuroblastoma cell lines via α-NETA, a recently described CMKLR1 inhibitor (94), dose-dependently reduced cell viability and clonogenicity (21). Similar results were achieved *in vivo*. In a SK-N-AS xenograft model, mice that were pretreated with α-NETA, before tumors reached a specified volume, showed longer survival and delayed tumor growth compared to control mice (21). Thus, the results of both *in vitro* and *in vivo* experiments indicated that targeting chemerin/CMKLR1 could potentially elicit antitumor effects in clinical settings.

Non-small Cell Lung Cancer

Lung cancer is the leading cause of cancer-related deaths in the world, having a 5-year survival rate of <15% and resulting in ∼1.4 million deaths per year (66, 95). This is despite the fact that various chemotherapy-based treatment methods are available to patients. For non-small cell lung cancer (NSCLC), which accounts for the majority (∼85%) of lung cancer cases, multiple studies indicate that chemerin may be of great diagnostic and prognostic value (24, 27, 96). Two independent studies, one in 2011 and another in 2016, reported that chemerin was downregulated in NSCLC (24, 27). Zhao et al. (24) compared 108 NSCLC tumor samples with corresponding disease-free tissues and found that 56 of the NSCLC patient tumors showed a lower chemerin expression profile. They also found a positive correlation between chemerin expression levels and infiltration of NK cells into tumor, as revealed by CD56 IHC staining of NSCLC patient tissue samples. Moreover, multivariate analysis of various parameters, such as age, smoking history, tumor size, and differentiation grade, and univariate analysis, together, revealed that lower levels of chemerin expression in tissue were significantly associated with tumor-node metastasis stage, degree of differentiation, and poorer survival rates (24). In line with these findings, a 2016 study by Cai et al. (27) comparing 20 NSCLC tumor samples with their corresponding non-tumor tissues found that 16 of the 20 tumor samples exhibited a significant downregulation of chemerin. Chemerin mRNA expression was also tested in 26 NSCLC tumor samples along with their healthy tissue counterparts, and the RT-qPCR results of these specimen showed that chemerin expression was significantly reduced in most (19) of the samples. Furthermore, chi-squared analysis of chemerin expression levels in NSCLC specimen, distinguished between low and high based on a mean score, found that tissue chemerin expression levels were associated with tumor-node metastasis stage, differentiation, and lymph node metastasis (24, 27). Both studies identified tissue chemerin levels as an independent prognostic factor for NSCLC and reported that, overall, higher chemerin expression was associated with positive prognosis (24, 27).

Unlike tissue expression levels, elevated serum chemerin levels were observed in NSCLC patients, which correlated to poorer overall survival, and was identified as an independent risk factor for poorer prognosis in NSCLC patients (97). In a study by Xu et al. (97), analysis of serum samples from 189 NSCLC patients and 120 healthy individuals revealed that serum chemerin levels were upregulated in NSCLC patients. Furthermore, both early stage and advanced stage NSCLC patients showed elevated levels of chemerin in serum, with a further increase for patients with advanced stage NSCLC. For diagnosis of NSCLC, serum chemerin levels had a test sensitivity and specificity of 62.4% and 67.5%, respectively. When combined with carcinoembryonic antigen tests, the sensitivity and specificity of the test increased to 78.3 and 84.2%, respectively. Thus, serum chemerin expression was also established as a viable biomarker for diagnosis and prognosis of NSCLC (97).

To elucidate chemerin's mechanistic roles in lung cancer, Unver et al. (31) examined chemerin's functions in Lewis lung carcinoma (LLC), a mouse model of lung cancer. LLC cells were genetically altered to secrete prochemerin at low levels insufficient to induce chemotaxis. Media from the modified LLC cells was used to culture splenic leukocytes and suppress their expression of inflammatory cytokines, TNF-α, and IL-12 p40 (31). Results were replicated *in vivo*, as expression of TNF-α and IL-12 p40 was also reduced in the prochemerin expressing LLC tumor tissues. These observations are notable because inflammation, though sometimes necessary for antitumor activity, is often exploited by tumors to establish a pro-tumor environment, particularly in cases of NSCLC (98, 99). In syngeneic C57BL6 mice implanted with prochemerin-expressing LLC grafts, tumor formation was impeded by prochemerin expression (31). However, in mice with successful tumor formation, control tumors and prochemerin expressing tumors showed no difference in growth rate, indicating that prochemerin expression may play a role in modulating tumorigenesis through reduced inflammation but not in tumor suppression of LLC (31).

Prostate Cancer

Prostate cancer is one of the most frequently diagnosed cancers in the United States, with more than 164,000 new cases per year (100). It is also the second leading cause of cancer-related mortality in the country (101). Treatment of prostate cancer mainly consists of surgery and radiotherapy for localized

cases and androgen deprivation therapy and chemotherapy for metastatic cases (102).

Studies have shown that chemerin is significantly downregulated in prostate cancer. Both analyses of public datasets and RT-qPCR of human prostate tumor samples in several studies demonstrated reduced expression of chemerin, indicating that prostate tumors may downregulate chemerin as a means of immune evasion (15, 26, 103, 104). Thus, it is possible that therapeutic application of chemerin in prostate cancer may enhance anti-tumor immunity and slow tumor progression.

Squamous Cell Carcinoma of the Esophagus

Esophageal cancer affects millions of people worldwide, with approximately half a million new cases being diagnosed each year (105). Of those cases, esophageal squamous cell carcinoma (ESCC) accounts for a dominant majority, 90% (105). The outlook for patients is bleak, as esophageal cancers are among the deadliest tumors in the world due to its fast progression and late diagnosis (106). Standard therapy for esophageal cancer patients consists of esophagectomy, radiotherapy, and/or chemotherapy (106).

Identifying chemerin's role in ESCC, kumar et al. (20) showed that chemerin was upregulated in ESCC myofibroblasts, which also expressed CMKLR1 (20). Moreover, chemerin was shown to increase invasiveness of ESCC *in vitro*, which was mediated by the accumulation of MMP-1, MMP-2, and MMP-3. In Boyden chambers, chemerin stimulated the migration and invasion of OE21 cells, a human ESCC cell line, and this effect was inhibited by antagonists of CMKLR1 and chemerin siRNA, supporting previous results. Analysis of the OE21 media showed that there was increased expression of MMP-1, MMP-2, and MMP-3, and this effect could be reduced by inhibiting p44/42 MAPK kinase and protein kinase C, showing that they were responsible for increased MMP expression levels (20). Combined with previous reports that MMP-1 expression is associated with poor prognosis in esophageal cancer, the study suggested that chemerin could elicit pro-tumor effects in ESCC (107–109). Thus, it was postulated that targeting chemerin/CMKLR1 may be a viable therapeutic approach for ESCC.

Squamous Cell Carcinoma of the Oral Tongue

Squamous cell carcinoma of the oral tongue (SCCOT) is the most common form of oral squamous cell carcinoma (OSCC), which has a worldwide incidence rate of ∼6.6 cases per 100,000 males and 2.9 cases per 100,000 females (110). Current treatment for SCCOT ranges from surgery alone, for stage 1 and 2 cases, to a combination of surgery and radiotherapy for later stages of disease (111). Though early diagnosis and treatment methods are currently available, the primary causes of mortality in SCCOT patients are lymph node metastasis and recurrence (112). Thus, biomarkers for those parameters may be of great prognostic value for SCCOT patients.

A study by Wang et al. (18), looking at chemerin mRNA expression in 19 SCCOT tumor tissues and matched adjacent tissues via qRT-PCR and IHC stains of 147 SCCOT specimen and their associated peritumoral, healthy tissues, determined that both chemerin mRNA and protein was upregulated in primary SCCOT specimens (18). Significantly, overexpression of chemerin was correlated with poor differentiation, high clinical stage, and lymph node metastasis. Multivariate survival analysis further showed that chemerin was an independent prognostic factor for SCCOT and that patients with overexpressed chemerin had a shorter cancer-related survival time (18). Though chemerin's role in SCCOT tumor progression is still unclear, the results indicate that chemerin may be a therapeutic target for inhibiting tumor growth.

Another study found that serum and salivary levels of chemerin were also elevated in OSCC patients, indicating that chemerin may be used to diagnose patients (113). The study included serum and salivary samples from 15 patients with early stage OSCC, 15 patients with oral premalignant lesions (OPML), and 15 healthy individuals, and analysis of these specimen showed that serum and salivary levels of chemerin, and MMP-9, were upregulated in OSCC compared to OPML, and in OPML compared to healthy patients. Analysis of the ROC curve revealed that serum and salivary chemerin levels had a test sensitivity and specificity of 100% for detecting early stage OSCC (113).

Squamous Cell Carcinoma of the Skin

Cutaneous Squamous Cell Carcinoma (CSCC) is the second most prevalent form of skin cancer in the world (114). Affecting the elderly at disproportionately higher rates, CSCC is mainly caused by chronic exposure to ultraviolet radiation (114, 115). Fortunately, advances in treatment have made new, targeted molecular therapies available to patients, in addition to the standard options of surgery and chemotherapy (116).

A recent study indicated that chemerin may contribute to CSCC cell migration and tumor growth. The study by Farsam et al. (19) found that chemerin was downregulated in CSCC but upregulated in senescent fibroblasts and skin samples of elderly patients (19, 117). Transcriptional analysis of CCRL2 in CSCC cell lines further revealed that CCRL2 expression was significantly increased on CSCC tumor cells *in vitro*, and enhanced tumor cell migration was observed as a result of increased levels of senescence-associated chemerin, which was abrogated by inhibition of chemerin in senescent fibroblasts. Moreover, CMKLR1, or the combination of CCRL2 and GPR1, was necessary for CSCC cell migration via chemerin, given that suppression of either one of the receptors reversed these effects. Finally, chemerin was shown to activate MAPK signaling in SCL-1 cells, a human CSCC cell line, with high expression of CCRL2 and low expression of GPR1. Specifically, the JNK and ERK 1/2 pathways were indicated as the primary mediators of chemerin-mediated effects in CSCC tumor cells, as inhibition of these pathways neutralized the previously observed migratory response. Taken together, these results showed that chemerin enhanced CSCC cell migration and promoted tumor growth through chemerin/CMKLR1/CCRL2/GPR1 interactions and subsequent activation of MAPK pathway subtypes (19).

CONCLUSION

Chemerin is a versatile protein with significant functions in modulating tumor growth. With altered expression profiles in a variety of cancer types, it can have context dependent effects on tumorigenesis and tumor progression. In some cases, cancers may silence RARRES2 via hypermethylation to evade immune surveillance. In others, host systems may increase chemerin expression as a defensive measure, recruiting antitumor immunocytes, reducing secretion of inflammatory cytokines, and/or modifying signaling pathways. Through both immune-independent and immune-dependent mechanisms, chemerin has been shown to elicit tumor-suppressive effects in various cancers, and there is a strong possibility that the mechanisms of tumor inhibition seen in these tumor types may be replicated in others as well. Importantly, for cases in which silencing of RARRES2 has been reported, the restoration and/or forced overexpression of chemerin in the microtumor environment may incite compelling antitumor effects, indicating new avenues of research for chemerin in cancer.

Here, we have comprehensively reviewed the current data on chemerin's functions in cancer, along with its underlying mechanisms when available. Concentrating on aspects that may lead to clinical applications, chemerin's diagnostic and/or prognostic value have also been evaluated in a few cancer types. Given the efficacy of chemerin-mediated anti-tumor responses seen now in several tumor settings, future research investigating chemerin's roles in additional tumor types is warranted, with a particular focus on manifesting its therapeutic potential for cancer treatment. Importantly, it should be noted that chemerin-based treatments may add to the efficacy of approved checkpoint inhibitors, as they likely act through independent mechanisms to diminish tumor growth.

AUTHOR CONTRIBUTIONS

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

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Chemerin Suppresses Breast Cancer Growth by Recruiting Immune Effector Cells Into the Tumor Microenvironment

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Infiltration of immune cells into the tumor microenvironment (TME) can regulate growth and survival of neoplastic cells, impacting tumorigenesis and tumor progression. Correlations between the number of effector immune cells present in a tumor and clinical outcomes in many human tumors, including breast, have been widely described. Current immunotherapies utilizing checkpoint inhibitors or co-stimulatory molecule agonists aim to activate effector immune cells. However, tumors often lack adequate effector cell numbers within the TME, resulting in suboptimal responses to these agents. Chemerin (*RARRES2*) is a leukocyte chemoattractant widely expressed in many tissues and is known to recruit innate leukocytes. CMKLR1 is a chemotactic cellular receptor for chemerin and is expressed on subsets of dendritic cells, NK cells, and macrophages. We have previously shown that chemerin acts as a tumor suppressive cytokine in mouse melanoma models by recruiting innate immune defenses into the TME. Chemerin/*RARRES2* is down-regulated in many tumors, including breast, compared to normal tissue counterparts. Here, using a syngeneic orthotopic EMT6 breast carcinoma model, we show that forced overexpression of chemerin by tumor cells results in significant recruitment of NK cells and T cells within the TME. While chemerin secretion by EMT6 cells did not alter their phenotypic behavior *in vitro*, it did significantly suppress tumor growth *in vivo*. To define the cellular effectors required for this anti-tumor phenotype, we depleted NK cells or CD8+ T cells and found that either cell type is required for chemerin-dependent suppression of EMT6 tumor growth. Finally, we show significantly reduced levels of *RARRES2* mRNA in human breast cancer

samples compared to matched normal tissues. Thus, for the first time we have shown that increasing chemerin expression within the breast carcinoma TME can suppress growth by recruitment of NK and T cells, thereby supporting this approach as a promising immunotherapeutic strategy.

Keywords: chemerin, *RARRES2*, breast cancer, leukocyte trafficking, immunotherapy, NK cells, T cells

INTRODUCTION

Breast cancer is one of the most common malignancies with an estimated ∼266,000 new cases in 2018, according to SEER estimates. The impact of infiltrating lymphocytes on breast cancer patient outcomes has been studied in several contexts, but in general is a favorable prognostic factor (1–3). The presence of pre-existing immune effectors cells within the tumor microenvironment (TME) within breast and other tumor types can not only predict response to traditional cytotoxic chemotherapy, but also immunotherapies (4–6). Compared with tumor types that are more responsive to checkpoint immunotherapy, however, there is a relative paucity of infiltrating lymphocytes in breast cancer (7). Thus, strategies to enhance recruitment of immune effector cells to the breast TME are highly desirable.

Chemerin (retinoic acid receptor responder 2; *RARRES2*) is a leukocyte chemoattractant initially discovered as being highly up-regulated in the skin by the synthetic retinoid tazarotene (8). Chemerin is widely expressed throughout tissues and has myriad roles including the chemoattraction of innate cells [e.g., NK cells, macrophages, dendritic cells (DCs)] (9–11), functioning as an important antimicrobial agent in the skin (12), and is able to induce angiogenesis in human endothelial cells (13), suggesting chemerin may be a key factor in early immune responses to infection, injury, and/or inflammation. Chemerin is initially secreted in an inactive pro-form, prochemerin, which is then cleaved by specific proteases to become bioactive (9, 10). Chemerin has three described serpentine cell membrane receptors: chemokine-like receptor 1 (CMKLR1; ChemR23), C-C chemokine receptor-like 2 (CCRL2), and G protein-coupled receptor 1 (GPR1) (9, 14, 15). CMKLR1 is a chemotactic cellular receptor, while, atypical chemoattractant receptor CCRL2 likely acts to sequester and concentrate chemerin at sites of CCRL2 expression, such as on activated endothelial cells (14–17). The function of GPR1 is poorly understood, though it is reported to be expressed in the CNS (18, 19).

Chemerin/*RARRES2* has been studied in the context of several different tumor types, with its dysregulation dependent on the specific context. While we and others have reported on several tumor types where chemerin/*RARRES2* is significantly down-regulated compared to normal tissue counterparts (e.g., melanoma, lung, prostate, liver, adrenal, etc.) (20–25), chemerin/*RARRES2* has been shown to be up-regulated in fewer tumor types (e.g., mesothelioma, squamous oral cancers) (26–28). Several groups have correlated chemerin/*RARRES2* expression levels in the TME with clinical outcomes, showing improved patient survival in those patients with higher expression levels (20–22, 24). Importantly, two of these studies also evaluated the tumor biopsies for infiltrating leukocytes, showing an increase and correlation between higher chemerin levels and infiltrating NK cells in those patients with improved overall survival (20, 21).

Our group was the first to show that in a mouse melanoma model, overexpression and secretion of chemerin protein by tumor cells increased total CD45+ tumor infiltrating leukocytes (TIL), resulting in significantly suppressed tumor growth. In this model, the effect was mediated by NK cells, as depletion via anti-asialo GM1 resulted in complete abrogation of chemerin's tumor suppressive effects (22). In contrast, T cells were dispensable, as RAG deficiency had no effect on the anti-melanoma effects of chemerin *in vivo* (22). Importantly, neither engineered chemerin expression nor incubation of mouse B16F0 melanoma cells with exogenous, recombinant chemerin affected *in vitro* growth or phenotype, suggesting chemerin's main anti-tumor activity was due primarily to its ability to recruit immune effector cells into the TME.

Here, we studied the effect of chemerin/*RARRES2* overexpression using the transplantable orthotopic syngeneic EMT6 breast carcinoma model, which has been shown to be responsive to immunomodulation in a variety of settings (29–31). Utilizing a similar approach as in the B16 model, we engineered EMT6 tumor cells to express and secrete functional chemerin within the TME and then assessed the impact on tumor growth and TIL. Chemerin overexpression significantly suppressed tumor growth, which correlated with an increase in TIL. Depletion studies identified NK and CD8+ T cells as key effector leukocytes mediating chemerin's anti-tumor activity, suggesting an interplay between innate and adaptive arms. In human breast tissue, chemerin/*RARRES2* RNA expression was significantly reduced in malignant samples compared to normal controls. Taken together, these data suggest that loss of chemerin/*RARRES2* expression occurs in breast cancer during tumorigenesis, potentially as an immune evasion mechanism, and that restoring or enhancing chemerin levels within the TME may prove efficacious in increasing TIL, thereby slowing or reversing tumor progression in the clinic.

MATERIALS AND METHODS

Microarray Analysis

Publicly available breast cancer studies were evaluated using the Oncomine database (www.oncomine.org), in which expression data has been curated using statistical methods and standardized normalization technique as previously described (32). The two largest breast cancer studies comparing normal to malignant

tissues were selected: Curtis et al. (http://www.ebi.ac.uk/ega/studies/EGAS00000000083) (33) and TCGA (http://tcga-data.nci.nih.gov/tcga) (34). The Curtis dataset contains 1,992 breast carcinoma samples and 144 paired normal breast samples which were analyzed for the METABRIC project using the Illumina HumanHT-12 V3.0 R2 Array. The TCGA data included 532 invasive breast carcinomas and 61 paired normal breast tissue samples using level 2 (processed) data from the TCGA portal. The *RARRES2* probe was selected for normal, invasive/infiltrating ductal carcinoma (IDC) and invasive/infiltrating lobular carcinoma (ILC) subsets, and gene expression (mRNA) data were shown as log2 transformed, median centered per array with *p*-values and fold change between subsets generate by Oncomine.

Mice and Cell Lines

All mice were used in experiments were purchased from The Jackson Laboratory. Wild type or Rag1 knockout (RAG KO; #003145, *Rag1*^{tm1Mom}) (35) female BALB/c mice were used as indicated. Mice were maintained in the facilities at Washington University under the direction and guidelines of the Division of Comparative Medicine and used at approximately 9–12 weeks of age. All animal experiments were conducted in accordance with approved Washington University and National Institutes of Health Institutional Animal Care and Use Committee guidelines under an approved protocol (#20140232). The EMT6 mouse mammary carcinoma cell line was purchased from ATCC (CRL-2755). L1.2 cells transfected to express mouse CMKLR1 were a kind gift from BA Zabel. Cell lines were grown in complete media consisting of RPMI 1640 or DMEM supplemented with 10% FBS, sodium pyruvate, penicillin/streptomycin, and beta-mercaptoethanol, with or without appropriate antibiotics for selection. EMT6 cell lines (wild type and transduced) were serially tested for mycoplasma and found to be negative using the MycoProbe Mycoplasma Detection Kit (R&D Systems).

EMT6 Clone Production

The full-length gene that encodes mouse active chemerin, mouse *RARRES2*, was inserted into the lentiviral transfer vector pCDH1-MSC1-EF1-Puro (System Biosciences) using the NheI and EcoRI restriction enzyme digestion sites. Empty vector pCDH1-MSC1-EF1-Puro was used to produce control lentivirus. 293T/17 cells were grown in DMEM complete media in 10 cm dishes for 16 h before transfected with packaging plasmid (Δ8.2), coat protein vector (pCMV-VSV-G) and transfer vector (pCDH-Puro-wt *RARRES2* or pCDH-Puro Empty vector) by using the FuGENE® HD Transfection Reagent (Promega) according to the manufacturer's protocol. The culture supernatants containing lentiviruses were collected at 48 and 72 h post transfection. The collected media were centrifuged at 300 × g to remove cell debris and followed by filtration with 0.45 μM filters. Viral supernatants were either used immediately for cell transduction or stored at −80°C. To create EMT6 cell lines with constitutive chemerin expression or control vector, viral supernatants added with polybrene were used to infect wild type EMT6 cells. Starting 24 h infection, cells were selected with media containing 2 μg/ml puromycin for 3 days. Culture media containing puromycin was replaced daily. Monoclonal cell populations were obtained by limiting dilution.

In vitro Cell Line Evaluation

EMT6-pCDH-VEC or EMT6-pCDH-*RARRES2* cells (1,000 cells/well) were plated in 96-well black walled plates (Corning). Cells were grown in a 5% CO_2 humidified incubator at 37°C for the indicated days. On each day, alamar blue reagent (ThermoFisher Scientific) was added directly to each well, the plates were incubated at 37°C for 1–4 h and the fluorescence signal was measured according to the manufacturer's protocol. Data were shown as relative fluorescence values compared with that of day 0, which was normalized to 1. Control and chemerin-expressing EMT6 lines were plated at 200 k/ml/well in 24 well plates and evaluated for chemerin secretion by using a mouse chemerin ELISA (R&D Systems) on 48 h conditioned media. Surface marker expression of control and chemerin-expressing clones was evaluated by flow cytometry with indicated monoclonal antibodies and appropriate isotype controls (Biolegend). The functionality of secreted chemerin was tested using conditioned media from control and chemerin-expressing clones in chemotaxis assays. Briefly, 96 well HTS Transwell Permeable Supports with 5 μm pores (Corning) were used according the manufacturers protocol; 250 k mCMKRL1+ L1.2 cells/75 μl were placed in the top chamber and 240 μl of complete media +/− 3 nM recombinant, active mouse chemerin (R&D Systems), or conditioned media in the bottom chamber. Assays were left at 37°C for ∼1–1.5 h. Migrated cells in the bottom chamber were counted and percent migration calculated.

Tumor Inoculation

To evaluate the effect of constitutive chemerin secretion on *in vivo* tumor growth, control or chemerin-expressing EMT6 breast tumor cells ($0.5–1 \times 10^6$) were inoculated subcutaneously into 9–12 weeks old female BALB/c mice (JAX). Prior to inoculation, EMT6 lines were grown to ∼60–80% confluence to ensure log-growth kinetics, and viability was tested using trypan blue and ensured to be ∼ >95% (or cells were not used). Tumor growth was measured every 2–4 d by calipers, and size was expressed either as the volume product of perpendicular length by width in square millimeters, or by tumor size as indicated by width × length (in square mm). Mice were euthanized when tumor size reached ∼400 mm^2 or when tumor sites ulcerated or at indicated time points for TIL analyses.

In vivo Leukocyte Depletion

Mice were injected i.p. with 100 μl of anti-asialo GM1 or control rabbit sera (Wako Chemicals) diluted 1:10 in PBS. Mice were treated with antibodies on day 1, day 0 and every 2–3 days after tumor inoculation. NK depletion efficiency was determined by staining blood cells collected from the venous sinus. Briefly, blood samples were isolated via retro-orbital bleed and washed once with PBS. After centrifugation at 300 × g for 5 min, cells were stained with CD45, CD3, and DX5 or its isotype control (Biolegend) and analyzed by FACS. For CD4+, CD8+ T cell depletion, mice were injected i.p. with 250 μg/500 μl PBS of anti-CD4 (clone GK1.5, BioXCell), anti-CD8β (Lyt 3.2) (clone

53-5.8, BioXCell) or both, and rat IgG (Sigma) for control. Antibodies were given weekly for 3 doses. Depletion efficiency was determined by staining blood cells collected via retro-orbital bleed with CD45, CD3, CD4, and CD8 antibodies (Biolegend) and analyzed by FACS.

Evaluation of Tumor Infiltrating Leukocytes

At indicated time points, whole subcutaneous tumors were resected en bloc including overlying skin and subcutaneous tissues. Tumors were then processed into single cell suspensions as previously described (22). Briefly, cells were counted using trypan blue, and samples were blocked with PBS/FBS containing 1% rat serum and Fc block (anti-CD16/32; Biolegend). Stained samples were analyzed on a BD Fortessa. For live/dead cell discrimination, AmCyan LIVE/DEAD Fixable Dead Cell Stain kit (Invitrogen) was used. Antibodies or appropriate isotype controls were purchased from Biolegend and FlowJo software (Tree Star) was used for analysis, with gating based on appropriate isotype control staining, and percentages expressed as shown of total live tumor cells or total live CD45+ cells, as indicated. FACS analyses was used to define the follow leukocyte subsets (all Live+CD45+): plasmacytoid DCs (pDCs; Lin-CD11c^{int}B220^{hi}), conventional DCs (cDCs; Lin- CD11c^{hi}B220^{low}), CD4 (CD3$^+$CD4$^+$) T cells, CD8 (CD3$^+$CD8a$^+$) T cells, total T cells (CD3$^+$CD4$^+$CD8$^+$), NK cells (CD3-DX5+), monocyte/macrophages (Lin-CD11b$^+$GR1$^-$), MDSCs (Lin-CD11b$^+$GR1$^+$), M1 (Lin-CD11c^{hi}F4/80+), and M2 (Lin-CD11c^{low}F4/80+) macrophages, CD19$^+$ B cells (CD3-CD19$^+$). CD8+ T cell subsets were based on staining with CD44 and CD62L: naïve (CD44^{low}CD62L^{hi}), effector (CD44^{int}CD62L^{low}), or memory (CD44^{hi}CD62L^{low}).

Breast Tissue Microarrays

Tissue microarray (TMA) Breast Tissue FFPE sections were collected from the St. Louis Breast Tissue Registry (funded by The Department of Surgery at Washington University School of Medicine, St. Louis, MO) under IRB-approved institutional protocols. All patient information was de-identified prior to sharing with investigators. Data and tissue was obtained in accordance with the guidelines established by the Washington University Institutional Review Board (IRB #201102394) and WAIVER of Elements of Consent per 45 CFR 46.116 (d). Each TMA core was 5 μm thick and 2 mm in diameter. Normal and Tumor tissue was confirmed by a Board-Certified Pathologist (Dr. Marshall Poger) using a stained Hematoxylin and Eosin (H&E) section. Breast Tumor TMA section contained 37 IDC cases and 8 ILC cases. Normal TMA Section contained 45 cases of Terminal Ductal-Lobular Unit (TDLU) with 1 Tonsil and 4 Liver cores for control and TMA positioning.

Quantitative Real-Time PCR

De-identified, paired RNA samples of malignant or non-malignant human breast tissues were from the Siteman Cancer Center Tissue Procurement Core, collected under an IRB-approved research protocol (#201106191). Quantitative Real-Time PCR was carried out using the SYBR® Green master mix (Bio-Rad) with the real-time PCR primers for human chemerin and the housekeeping gene GAPDH (sequence listed below). Measurements were standardized to GAPDH using delta-delta Ct methods. RNA from human liver was the positive control for chemerin expression. RNA from RAJI cells was the negative control. Data were expressed as fold expression levels of negative control (RAJI, normalized to 1). Data shown are mean ± SEM of two independent experiments using identical starting RNA. Significant outliers identified by Grubbs' test were removed. The primers used for human *RARRES2* have been previously described (36): Forward: 5′- TGGAAGAAACCCGAGTGCAAA-3′; Reverse: 5′- AGAACTTGGGTCTCTATGGGG-3′ Primers for human GAPDH: Forward: 5′- GAGTCAACGGTTTGGTCGTATTG-3′; Reverse: 5′- ATGTAGTTGAGGTCAATGAAGGGG-3′.

In situ Hybridization and Analysis

Manual chromogenic RNAScope (ACDBio) was performed using company protocols on TMA tissue sections to detect target RNA at single cell level. Tissue pre-treatment (Liver) included baking for 1 h at 60 degrees Celsius, deparaffinization using xylene and alcohol, RNAscope® Hydrogen Peroxide (ACD# 322335) treatment for 10 min at RT and protease treatment (RNAscope® Protease Plus ACD# 322331) for 30 min at 40 degrees Celsius using the HybEZ Oven. Pre-treatment of non-adherent cells (RAJI) included fixation by 10% NBF and dehydration in series of 50, 70, and 100% ethanol. Cells were treated with RNAscope® Hydrogen Peroxide for 10 min at RT (ACD# 322335) and treated with RNAscope® Protease III (ACD# 322337) for 30 min in 40 degrees Celsius using HybEZ oven. For all tissue sections and non-adherent cells, ACDBio pre-treatment protocol was used according to manufacturer's instructions. Detection of specific probe binding sites was with RNAScope 2.5 HD Reagent kit—brown from ACD (Cat. No. 322310). Single ISH detection for human *RARRES2* (ACD Probe: 457921), Positive Control Probe (PPIB - ACD Probe: 313901) and Negative Control Probe (Dapb—ACD Probe: 310043) was performed manually using RNAscope® 2.5 HD Reagent Kit-Brown (ACD, 322310). Target probes were hybridized for 2 h at 40 degrees Celsius using HybEZ oven and a series of 6 amplification steps followed. A DAB-based chromogenic reagent was used to detect the brown signal for the *RARRES2* probe expression. The experimental procedure followed the manufacturer's instructions for single plex assay. Positive staining was indicated by brown granular dots present in the nucleus and/or cytoplasm.

Quantitative analysis was completed using regions of interest (ROIs) and by random sampling. The ROIs for Normal and Tumor breast tissue were manually selected by a Board-Certified Pathologist (Dr. Marshall Poger) for imaging. Random sampling was done by numbering each core on the TMA section and using a random number generator to select which TMA core was to be selected for analysis. HALO Software by Indica Labs was used, specifically with the RNAScope ISH Module per recommendation by ACD, with user-defined thresholds. This module allowed the user to teach HALO software to recognize hematoxylin (blue) and positive signal

(brown granular dots). Positive signal is reported by number of RNA copies. The Cytonuclear Module was used to teach HALO Software to recognize hematoxylin (blue) to identify nuclei. This generated a contrasted image allowing the user to count the number of nuclei in the region of interest. ISH module provided the user the number of RNA copies and the Cytonuclear module provided the user the number of cells. Thus, RNA copies per nuclei was determined allowing analysis to be normalized to each nuclei. Slides were imaged using a Nikon eclipse 50i microscope at 40x resolution. Three comparable regions of interest for tumor (IDC and ILC) and normal breast (TDLU) were subject to HALO Software for image analysis.

Statistical Analysis

In vitro and *in vivo* tumor data was plotted using Prism software v7 and further analyzed with InStat (GraphPad Software). Differences between groups were evaluated by applying unpaired Student's *t*-test or non-parametric Mann-Whitney test, as indicated. Paired human RNA samples were evaluated by a paired student's *t*-test. $p < 0.05$ were considered significant.

RESULTS

Reduced *RARRES2* Expression in Human Tumors and Associated Poor Survival Outcome

We and others previously showed that chemerin/*RARRES2* expression is commonly down-regulated in multiple tumor types, including breast cancer, compared to normal tissue controls *RARRES2* (22). Our published expression analysis was limited to the publicly available GEO microarray datasets, thus, to confirm reproducibility we sought to further investigate chemerin expression in larger datasets. Here, we analyzed the two largest breast cancer datasets with data for *RARRES2* that were curated within the Oncomine database (32). Chemerin/*RARRES2* expression in both Curtis (**Figure 1A**) and TCGA (**Figure 1B**) datasets was significantly decreased by approximately 2.6- to 3.4-fold in tumor specimens compared to normal (33, 34). Subsequently, to examine the association between reduced *RARRES2* expression and patient survival outcome, we analyzed two sets of mRNA microarray data with cohort sizes of 33 breast cancer patients and 135 early-stage breast cancer patients, respectively, and found that low chemerin levels significantly correlated with poor survival outcomes in both groups (**Figures 1C,D**). By *in situ* hybridization (ISH) comparing normal tissues to both invasive ductal carcinomas (IDC) as well as invasive lobular carcinoma (ILC)—the two most common histologic subtypes (37)—chemerin/*RARRES2* was also down-regulated in the tumor samples (**Figure 2**). Thus, across multiple datasets and analytical expression methods, chemerin/*RARRES2* was consistently down-regulated in malignant breast cancer samples vs. controls, and reduced chemerin/*RARRES2* expression was correlated with poor survival outcome.

Reduced Chemerin Expression in Human Invasive Breast Cancers

Next, we wanted to independently validate the findings of our public microarray analyses (**Figure 1**). We collected human breast tissues from two different sources and, using two different modalities, evaluated chemerin expression via measurement of *RARRES2* mRNA. Matched total RNA from normal and malignant breast tissues ($n = 13$ patients with IDC) were obtained from the Siteman Cancer Center Tissue Procurement Core. De-identified frozen samples were collected under approved consents, pathologically reviewed, and processed into RNA per established protocols. RNA quantity and quality (i.e., RIN) was assured and validated primers for human *RARRES2* (36) were used in real-time quantitative PCR. Expression of *RARRES2* mRNA in malignant breast tissues was significantly reduced compared to patient matched, normal tissue (**Figure 2**). Group mean/SEM of individually matched samples (**Figure 2A**) are shown. Next, using samples collected from the St. Louis Breast Tissue Registry under IRB-approved institutional protocols, we then constructed normal ($n = 45$ cases) and malignant breast tissue microarrays (TMA) incorporating both IDC ($n = 37$ cases) and ILC ($n = 8$ cases). Utilizing ACDBio RNAscope, RNA *in situ* hybridization (ISH) was performed. *RARRES2* was undetectable in both IDC and ILC samples compared to low but significant *RARRES2* signal in normal tissues (**Figure 2B**). Duplicate TMA slides were used with positive and negative probes (ACDBio), in parallel with human liver (*RARRES2*-positive) and Raji cells (*RARRES2*-negative) as controls (**Supplemental Figure 1**). Representative images are shown in **Figure 2C**, with the majority of staining localized to epithelial components of the normal breast tissue. Taken together, our data confirms significant down-regulation of *RARRES2* mRNA expression in both IDC and ILC compared to normal breast tissues.

Forced Expression of Chemerin by EMT6 Breast Carcinoma

After confirming down-regulation of *RARRES2* mRNA in additional human studies, we then set out to favorably modulate chemerin expression in the EMT6 mammary carcinoma model. The EMT6 tumor line is a clonal isolate from a mouse mammary carcinoma that arose from an implanted hyperplastic alveolar nodule (38), and has been shown to be responsive to immunomodulation (31, 39, 40). In order to test our hypothesis that forced overexpression of chemerin by tumor cells would act to recruit anti-tumor leukocytes and suppress tumor growth, we used lentiviral transduction to introduce the mouse *RARRES2* gene into EMT6 tumor cells. The pCDH1-MSC1-EF1-Puro (System Biosciences) vector was used to produce either control (empty vector) or *RARRES2* viral particles for transduction. Control and chemerin-expressing EMT6 clonal lines were generated by limiting dilution plating. Evaluation of tumor-secreted chemerin was assessed by mouse chemerin ELISA (R&D Systems). Both wild type and control-transduced EMT6 lines showed no detectable chemerin by ELISA (not shown), while *RARRES2*-transduced clones showed significant production of secreted chemerin in the ng/ml range (**Figure 3A**).

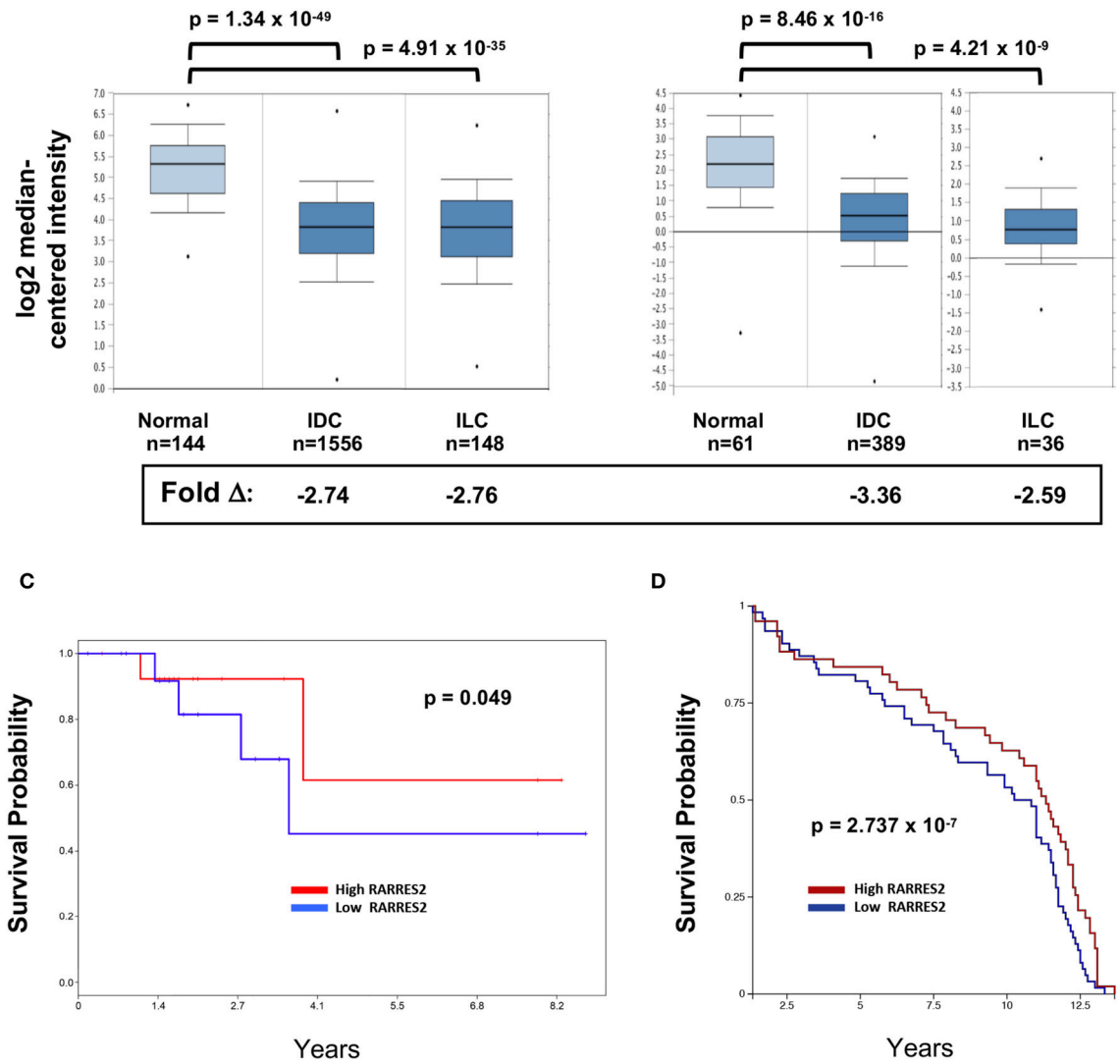

FIGURE 1 | *RARRES2* microarray expression in breast tissues. The two largest mRNA studies comparing normal and malignant breast tissues were selected in Oncomine (www.oncomine.org) for analysis. **(A)** Curtis, $n = 2{,}136$ total, **(B)** TCGA, $n = 593$ total. Both infiltrating ductal carcinoma (IDC) and infiltrating lobular carcinoma (ILC) subsets show significantly lower expression of *RARRES2* mRNA when compared to normal breast tissue. *RARRES2* probes were selected and relative expression by log2 median-centered intensity plotted for normal, IDC, and ILC subsets within each study. Oncomine calculated *p*-values and fold change compared with normal subset are shown. Down-regulation of chemerin in breast cancer can be associated with poor survival outcomes. **(C)** mRNA microarray data (accession # GSE6130-GPL887) from a cohort of 33 patients with breast cancer. mRNA microarray data was visualized using PROGgeneV2. The patients were stratified according to chemerin expression (divided at 50th percentile), and survival plotted for each group. Hazard ratio: 0.42 (0.18–1.00), *p*-value: 0.049, indicating that low chemerin levels significantly correlated with poor survival in this group. **(D)** mRNA microarray data (Caldas, Naderi Gene Exp 2007) from a cohort of 135 early-stage breast cancer, visualized using the UCSC Xena Browser. The patients were stratified according to chemerin expression (divided at 50th percentile), and survival plotted for each group. *p*-value: 2.737×10^{-7}, indicating that low chemerin levels significantly correlated with poor survival in this group.

From these, two clones, one with low (LC) and one with high (HC) chemerin expression, were then selected for further evaluation. In order to determine if the tumor-secreted chemerin was functional and active, we utilized standard chemotaxis assays using 5 um pore transwell chambers. Conditioned media from both control and chemerin-expressing tumor lines was evaluated. The mouse pre-B lymphocyte cell line L1.2 engineered to express high levels of mouse CMKLR1 (10) was used to assess chemerin-dependent migration. Conditioned media from control transduced lines was unable to induce CMKLR1+ L1.2 cell chemotaxis, while conditioned media from chemerin-expressing tumor lines triggered robust migration comparable to recombinant, active chemerin (3 nM, R&D Systems). Chemotaxis of CMKRL1+ L1.2 cells in the HC clone was ∼2-fold compared

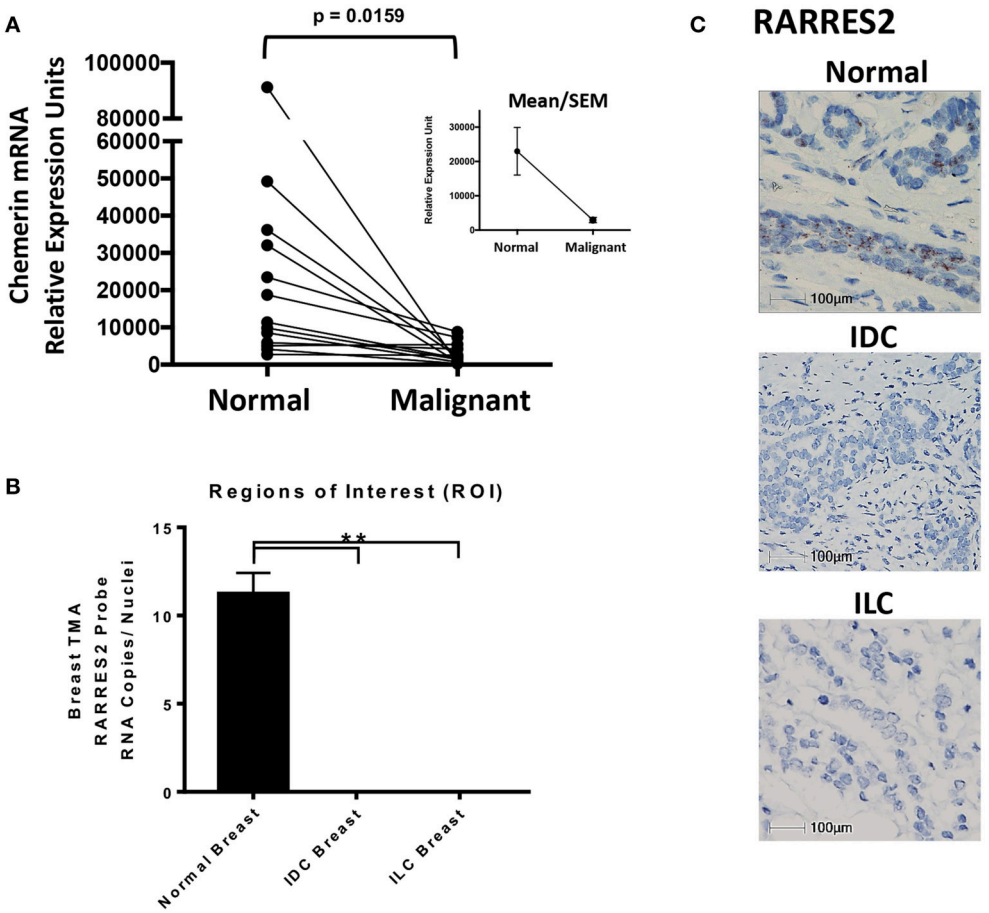

FIGURE 2 | Chemerin RNA expression in human breast tissue. **(A)** Real-time quantitative PCR (RT-qPCR) of chemerin mRNA expression in matched normal and malignant breast tissue. RNA was isolated from paraffin embedded tissues and assessed for concentration, purity, and integrity. Chemerin expression was normalized to GAPDH loading control for each sample (**$p = 0.0159$ compared to normal breast tissue, $n = 13$ for each subset using two tailed paired *t*-test). Inset shows group means/SEM. **(B)** Quantified *RARRES2* RNA expression in normal breast, IDC breast, and ILC breast tumor tissue microarrays (TMA) using RNAScope *in-situ* hybridization (ISH). Three comparable regions of interest (ROI) from each case of normal breast ($n = 7$) or tumor (IDC and ILC; $n = 7$ each) were subject to HALO Software for image analysis. The ROIs for normal and tumor breast tissue were manually selected for imaging/analysis. RNA expression is normalized to the number of nuclei in each image to determine RNA copies per nuclei. (**$p = 0.0001$ compared to normal breast using a one sample *t*-test). Results are representative of two TMAs containing (1) 45 normal cases and (2) 37 IDC cases and 8 ILC cases. **(C)** Representative ISH images for *RARRES2* RNA expression in normal, IDC breast, and ILC breast tissue. Slides were imaged using a Nikon eclipse 50i microscope at 40X resolution; 100 mm bar shown. Positive staining is indicated by brown granular dots present in the cell nucleus and/or cytoplasm.

to the LC clone, in line with measured secreted chemerin levels (**Figure 3B**). In order to assess the effects of chemerin production and secretion on *in vitro* tumor cell proliferation, we utilized an alamar blue assay (ThermoFisher) and measured growth as a function of fluorescence signal over several days. There were no consistent differences between control or chemerin-expressing EMT6 clones (**Figure 3C**). Next, we looked at expression of several common surface markers involved in tumor-immune recognition (MHC class I, CD1d, PD-L1) as well as tumor cell migration and invasion (CD44) (41). While CMKLR1 has been reported to be expressed on some human tumors (42), we did not see detectable surface levels of CMKLR1 above isotype control, in line with our prior studies of the mouse melanoma line B16F0 (22). **Figure 3D** shows comparable phenotypic expression of these markers between control and chemerin-expressing tumor lines. These data show that transduction with *RARRES2* and expression/secretion of chemerin by EMT6 tumor cells does not appear to meaningfully impact *in vitro* growth or the immunophenotype of key surface proteins, and that secreted chemerin is functionally active and can induce migration of CMKLR1+ cells.

Chemerin Overexpression Suppresses EMT6 Tumor Growth *in vivo*

Given that chemerin-overexpression failed to impact EMT6 proliferation *in vitro* or expression of MHC class I, CD1d, CD44, or PD-L1, we next wanted to study the impact of chemerin expression in the TME on *in vivo* growth. Using WT

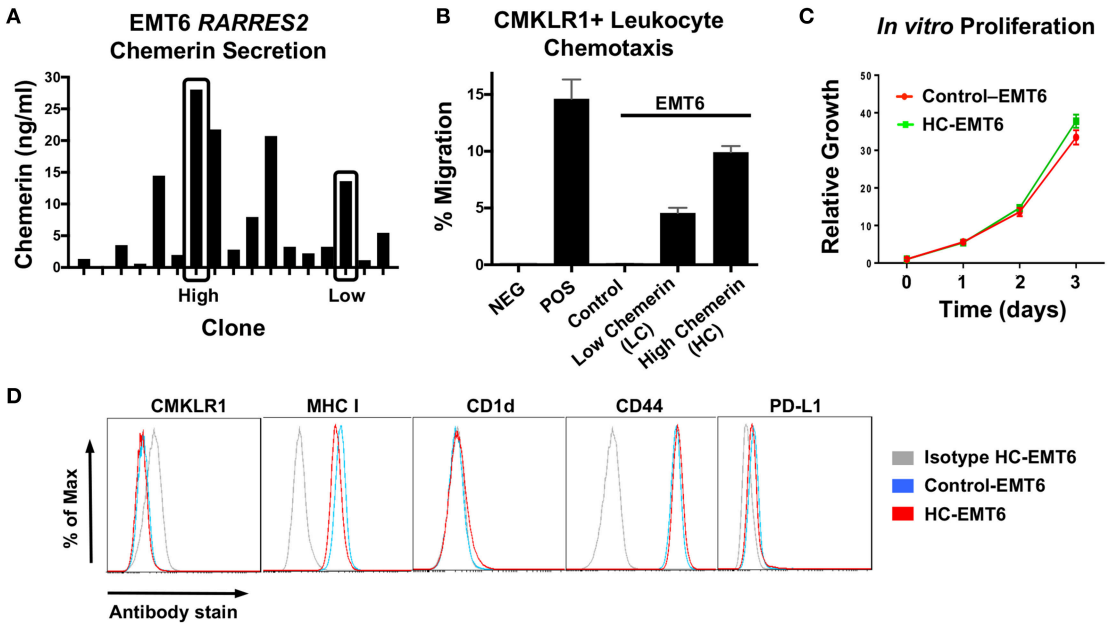

FIGURE 3 | Evaluation of chemerin-expressing EMT6 tumor lines. The mouse breast carcinoma line EMT6 was used to study the impact of forced chemerin expression on tumor growth. Wild type EMT6 cells were transduced using the lentiviral transfer vector pCDH1-MSC1-EF1-Puro, with the full length mouse *RARRES2* gene inserted (*RARRES2*) or not (control). **(A)** Clonal cell lines ($n = 17$) were generated and chemerin protein secretion in clonal conditioned media samples was quantified by ELISA. Clones with low (LC) and high chemerin (HC) secretion (indicated by boxes) were selected for subsequent *in vitro* and *in vivo* analysis. **(B)** Chemotaxis assays using CMKLR1+L1.2 transfectants and 5 micron transwell chambers were performed to confirm functionality of EMT6-secreted chemerin. Conditioned media was used from control and chemerin-expressing tumor lines (LC, HC). Media alone (NEG) and 3 nM recombinant mouse chemerin (POS; R&D Systems) were used as controls. The normalized "percent of input" migration is shown; mean/SEM plotted for duplicate wells for each condition. Representative proliferation and chemotaxis assays are shown, and were each performed several times prior to tumor inoculation in mice. **(C)** Control and high chemerin-expressing lines were evaluated for *in vitro* cell proliferation using an alamar blue assay; relative fluorescence values normalized to 1 on day 0 are shown. **(D)** Surface expression of CMKLR1, MHC class I, CD-1d, CD44, and PD-L1 was determined by FACS for control and high chemerin-expressing EMT6 tumor lines. For each marker indicated, the appropriate isotype control antibody (gray) is shown.

female BALB/c recipients, control or chemerin-expressing EMT6 tumor cells were orthotopically implanted into the mammary fat pad as described (22). To determine if the level of chemerin secretion from transduced clones affected *in vivo* tumor growth, we implanted low-chemerin (LC) and high-chemerin (HC)-secreting clones. The *in vivo* growth of HC EMT6 tumors was significantly suppressed compared to LC- or control-EMT6 cells (**Figure 4A**), with some mice showing complete suppression of *in vivo* tumorigenesis. To confirm this was not an effect of clonality, we utilized completely independent, bulk transduced EMT6 tumor cell lines (i.e., polyclonal) and saw a similar significant reduction in *in vivo* tumor growth (**Figures 4A,B**). This might suggest that an adequate concentration gradient of chemerin within the TME needs to be established to recruit anti-tumor leukocytes and suppress tumor growth. Indeed, there was an approximately 2-fold increase in the total CD45+ tumor infiltrating leukocytes (TIL) relative to tumor cells in HC-EMT6 tumors compared to LC- or control-EMT6 tumors at time of euthanasia (**Figures 4C,D**). We next looked at the composition of infiltrating leukocyte subsets in the TME by flow cytometry and identified significant increases in the relative percentages of total T cells, CD4+ T cells, and NK cells among total CD45+ cells in HC-EMT6 tumors compared with controls by day 35 of tumor growth (**Figures 4E,F**; **Supplemental Figure 2**). CD8+ T cells were also enriched among the total CD45+ cells in the HC-EMT6 tumors by day 35, compared to day 14 (**Figure 4G**). However, no significant differences in percentages of total T cells, CD4+ or CD8+ T cell subsets, B cells, NK cells, cDCs, pDCs, MDSCs, or macrophages among CD45+ TILs were detected between the two groups at an earlier time point in tumor growth (day 14, not shown), potentially suggesting that sufficient time is needed to establish an adequate concentration gradient of chemerin and resultant chemoattraction of effector cells. No significant differences were seen in either CD4+ or CD8+ regulatory T cells (CD25+FoxP3+) between the groups at either early or late time points (**Figures 4H,I**). Taken together, these data show high-chemerin expression within the EMT6 TME results in significant tumor growth suppression and a favorable anti-tumor skewing of both NK cells and T cells, as a percentage of total TIL.

The Anti-Tumor Effects of Chemerin Are Mediated by NK Cells and T Cells

Our initial *in vivo* EMT6 tumor data identified a correlation among high-chemerin expression by EMT6 tumors, increased NK cells and T cells in the TIL population and suppressed tumor growth. (**Figure 4**). To further define the cellular mechanism

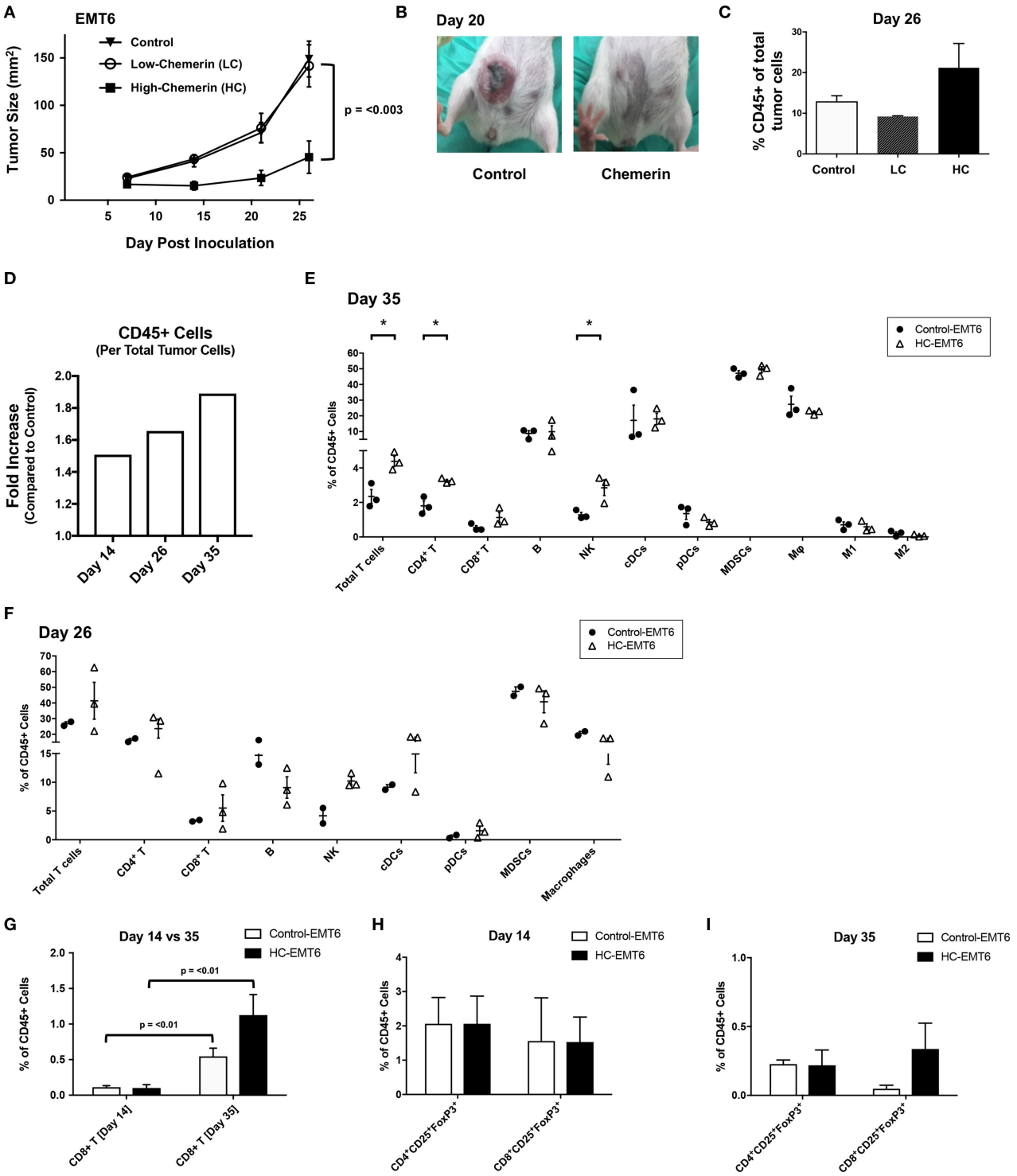

FIGURE 4 | High chemerin expression by EMT6 tumors suppresses *in vivo* growth and results in altered TIL makeup. **(A)** High and low chemerin-expressing EMT6 clones were used in parallel with a control transduced EMT6 cells. 0.5–1 × 10^6 tumor cells were inoculated subcutaneously into 9–12 weeks old female BALB/c mice. Tumor growth was serially assessed using calipers. Mean/SEM shown with groups $n = 10$ per indicated line. Two-way ANOVA with Tukey's multiple comparisons test show no significant differences between control and LC groups, with p-value as indicated for differences compared to HC group. **(B)** Representative images of mice from control and chemerin-expressing groups showing significant *in vivo* suppression of tumorigenesis, 20 days post-inoculation. **(C)** Percent CD45 positive of total tumor cells by FACS analysis within the tumor microenvironment (TME) shown for **(A)** tumors ($n = 2$–3/group) resected at time of euthanasia (day 26).

(Continued)

FIGURE 4 | (D) Graph of fold increase showing CD45+ cells per total tumor cells, comparing HC-EMT6 tumors to control-EMT6 tumors resected at time of euthanasia (day 14, $n = 6$/group; day 26, $n = 2$–3/group; day 35, $n = 3$/group) and analyzed via FACS. Graph depicts two independent experiments; tumors collected on day 14 and day 35 are derived from the same experiment. **(E,G–I)** In a separate cohort of mice, we euthanized animals at pre-defined timepoints for TIL analysis. Of the mice that were initially inoculated; six mice per group were euthanized on day 14 for FACS analysis, and an additional three mice per group were euthanized on day 35 for further FACS analysis. Graphs show mean/SEM values; statistical significance (defined in Methods) was determined between groups using a 2-sided unpaired *t*-test. **(E)** FACS analysis of TIL from control-EMT6 or HC-EMT6 tumors ($n = 3$/group) resected on day 35. **(F)** FACS analysis of TIL from control-EMT6 or HC-EMT6 tumors resected on day 26 ($n = 2$–3/group). **(G)** FACS analysis of TIL from HC-EMT6 tumors ($n = 6$) resected on day 14 compared to TIL from HC-EMT6 tumors resected on day 35 ($n = 3$), specifically showing the CD8+ T cell population. **(H)** FACS analysis of TIL from control-EMT6 or HC-EMT6 tumors resected on day 14 ($n = 6$/group), specifically showing CD4+ and CD8+ regulatory T cell populations (CD25+FoxP3+). **(I)** FACS analysis of TIL from control-EMT6 or HC-EMT6 tumors resected on day 35 ($n = 3$/group), specifically showing CD4+ and CD8+ regulatory T cell populations (CD25+FoxP3+). *$P < 0.05$.

of action of chemerin-dependent tumor growth suppression, we selectively depleted candidate lymphocyte subsets (or used genetically-modified subset-deficient animals) and evaluated HC-EMT6 tumor growth. We first used anti-asialo GM1 to deplete NK cells. Control and chemerin-expressing lines were inoculated into mice treated with control sera or anti-asialo GM1 (Wako Chemicals) sera. Anti-asialo GM1 treatment had no effect on control EMT6 tumor growth *in vivo*, while similar treatment resulted in the complete abrogation of tumor suppression in the chemerin-expressing tumors (**Figure 5A**). There were no significant differences noted between the growth of control-EMT6 tumors (+/- anti-asialo GM1) and NK cell-depleted chemerin-expressing tumors (**Figure 5A**). The extent of NK cell depletion was confirmed by analysis of peripheral blood prior to tumor inoculation (**Figure 5B**; **Supplemental Figure 3A**). Next, to explore the potential role of adaptive immunity in chemerin-dependent EMT6-tumor growth suppression, we used Rag1 KO mice, which lack mature T and B cells (35). Growth suppression by tumor-secreted chemerin was only seen in wild type mice and was completely abrogated in RAG KO mice (**Figure 5C**), suggesting a requirement of the adaptive immune response in this model. Given the lack of change in B cells and the significant increase in T cells in the TIL population in chemerin-expressing tumors, we then set out to define specific T cell subsets responsible for the chemerin-dependent anti-tumor effect. We used specific antibodies to deplete CD4+ and/or CD8+ T cells as indicated. Control antibody treatment did not affect suppression of tumor growth in chemerin-expressing tumors. However, depletion of CD8+ T cells in chemerin-expressing tumors—either alone or in combination with CD4+ T cell depletion—resulted in growth comparable to control tumors (**Figure 5D**). T cell subset depletion was confirmed by analysis of peripheral blood, which was essentially complete (**Figure 5E**; **Supplemental Figure 3B**). Interestingly, CD4+ T cell depletion alone in chemerin-expressing tumors resulted in improved tumor growth suppression (**Figure 5D**). Recently published data show that CD4+ T cell depletion in the EMT6 model results in a significant increase in CD45+ TIL, with a ∼3-fold increase in IFNγ+CD8+ T cells in the draining lymph nodes compared to controls. CD4+ T cell depletion—as in our model—resulted in significantly reduced tumor growth, hypothesized to be due to a reduction in immunosuppressive regulatory CD4+ T cells (40). In line with this data, analysis of our control and T cell depleted cohorts showed a significant increase (∼3-fold) in total CD45+ TIL only in the CD4+ T cell depleted mice (not shown). Taken together, these data suggest critical roles for both NK and CD8+ T cells in mediating chemerin tumor suppression.

DISCUSSION

Chemerin is a multifunctional protein with wide tissue expression and myriad roles in host defense, implicated in antibiosis, angiogenesis, as well as chemoattraction of leukocytes (43). Several groups have described its dysregulation in the context of tumorigenesis, with the majority—but not all—showing decreased chemerin/*RARRES2* expression within malignant tissues (20–22, 42, 44–48). Our group was the first to show tumor suppression via therapeutic modulation of chemerin in a mouse tumor model, with now several studies confirming the role of chemerin as a tumor suppressor in various settings (22, 45, 46, 48, 49). Importantly, two independent studies showed not only improved patient survival but also increased immune effector cell infiltrates in tumor samples with higher chemerin expression (20, 21). Our prior studies in the B16F0 mouse melanoma model showed increases in tumor-infiltrating NK and T cells with forced overexpression of chemerin by tumor cells, with suppression mediated by NK cells in that model (22). This led us to hypothesize that chemerin may play a key role in tumor immune surveillance and, further, that malignant tissues may selectively down-regulate chemerin/*RARRES2* as a means of immune escape (**Figure 6**).

Human breast cancers have variable levels of infiltrating immune cells, with ER/PR+HER2- subtypes typically showing the lowest (2). Breast cancer subtypes with high TILs may also show higher expression of checkpoint molecules such as PD-1 and CTLA-4 (50), which may play a role in higher response rates to checkpoint inhibitors in these tumor subtypes (e.g., ER/PR-HER2-, HER2+) (51). Decreased levels of TIL have been described in metastatic breast tumors compared to matched primary tumors (52), suggesting a role for immune escape in breast cancer progression. Thus, strategies to increase TIL and improve immunosurveillance in breast cancer are attractive from a therapeutic standpoint.

Here, we present—for the first time to our knowledge—studies focused on the expression and role of chemerin/*RARRES2* in human breast tissues and a mouse model of breast cancer. Using the fully immune competent mouse EMT6 breast tumor model, we have shown that overexpression and secretion of chemerin by tumor cells significantly suppressed tumor growth *in vivo*. As in our melanoma model, chemerin appears to have no

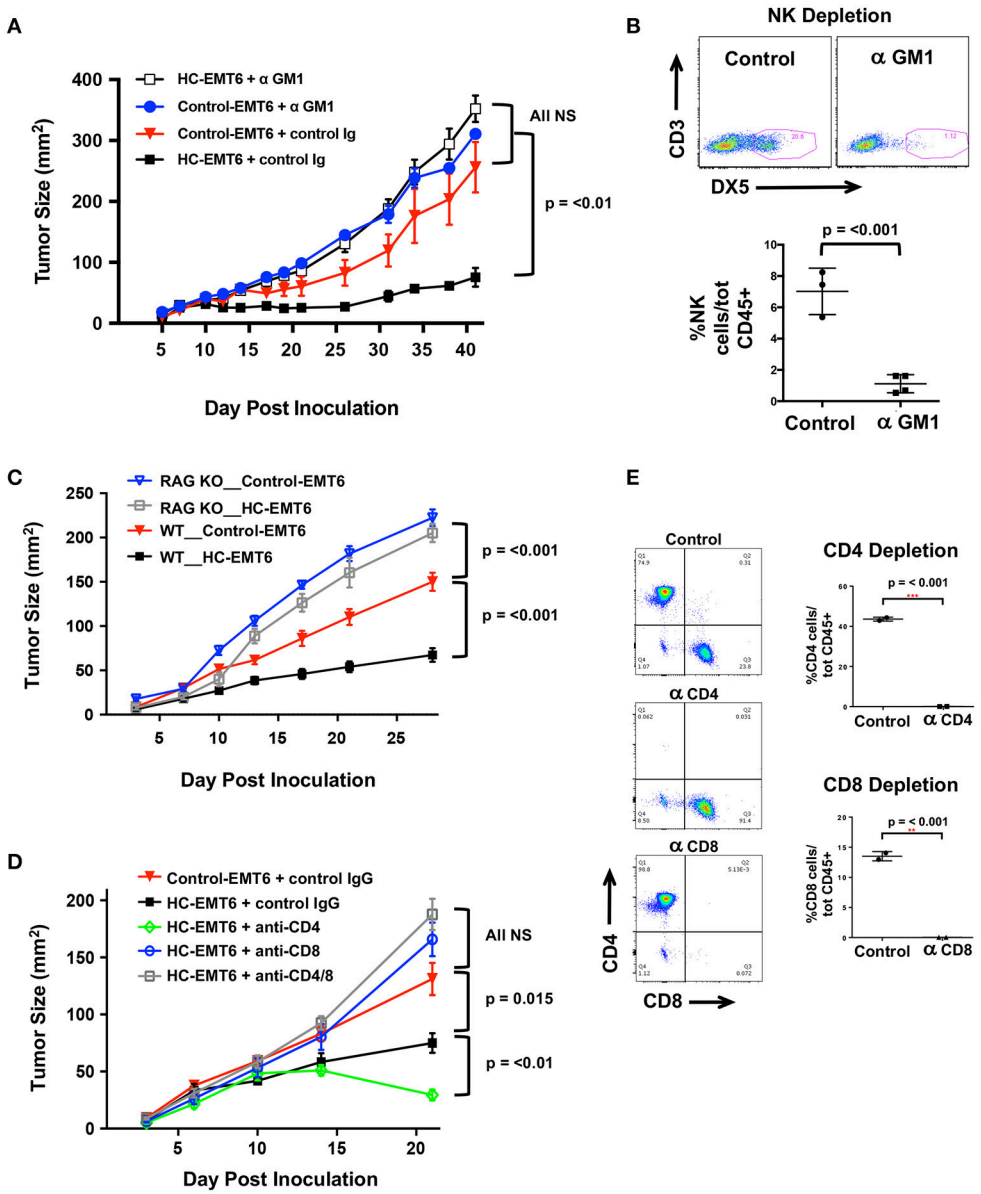

FIGURE 5 | Depletion of NK, T cells abolishes chemerin-induced tumor suppression. **(A)** Control or high chemerin-expressing (HC) EMT6 cells were inoculated in wild type BALB/c mice ($n = 4$–5/group). NK cell depletion was accomplished using anti-asialo GM1 (control rabbit sera was used as a negative control) (Wako Chemicals). **(B)** Depletion of blood NK cells was confirmed in each experiment at either day −1 or day 0 (time of inoculation). **(C)** Control and chemerin-expressing lines were inoculated in both wild type (WT; $n = 10$/group) and Rag-1 knockout (RAG KO, Jackson Labs; $n = 7$/group). **(D)** Antibody depletion of T cell subsets (CD4, CD8) was accomplished using i.p., injection of 250 ug/500 ul PBS of anti-CD4 (clone GK1.5, BioXCell), anti-CD8β (Lyt 3.2) (clone 53-5.8, BioXCell) or both, or control rat IgG (Sigma). Control or depleting antibodies were used in both control and chemerin-expressing (HC) tumors as indicated ($n = 5$–6/group). **(E)** Depletion of blood T cell subsets was confirmed in each experiment at either day−1 or day 0 (time of inoculation). Graphs show mean/SEM from representative experiments with similar results ($n = 4$ experiments for NK depletion, $n = 2$ experiments each for T cell depletion, RAG KO studies). *P*-values are indicated from 2-tailed unpaired *t*-tests between indicated groups at time of euthanization.

significant effect on tumor intrinsic proliferation or phenotype *in vitro*, though this may be a function of specific tumor types as well as the presence or absence of chemerin receptors on tumor cells, as others have shown direct effects of chemerin on tumor cells (45, 46, 48, 49). *RARRES2*-transduced EMT6 clones with lower expression of chemerin grew similarly to control cells *in vivo*, suggesting that in this model adequate expression and secretion of chemerin within the TME is necessary to successfully establish the concentration gradient necessary to recruit leukocytes.

The EMT6 mouse tumor model has recently been shown to recapitulate an "immune excluded" tumor phenotype with

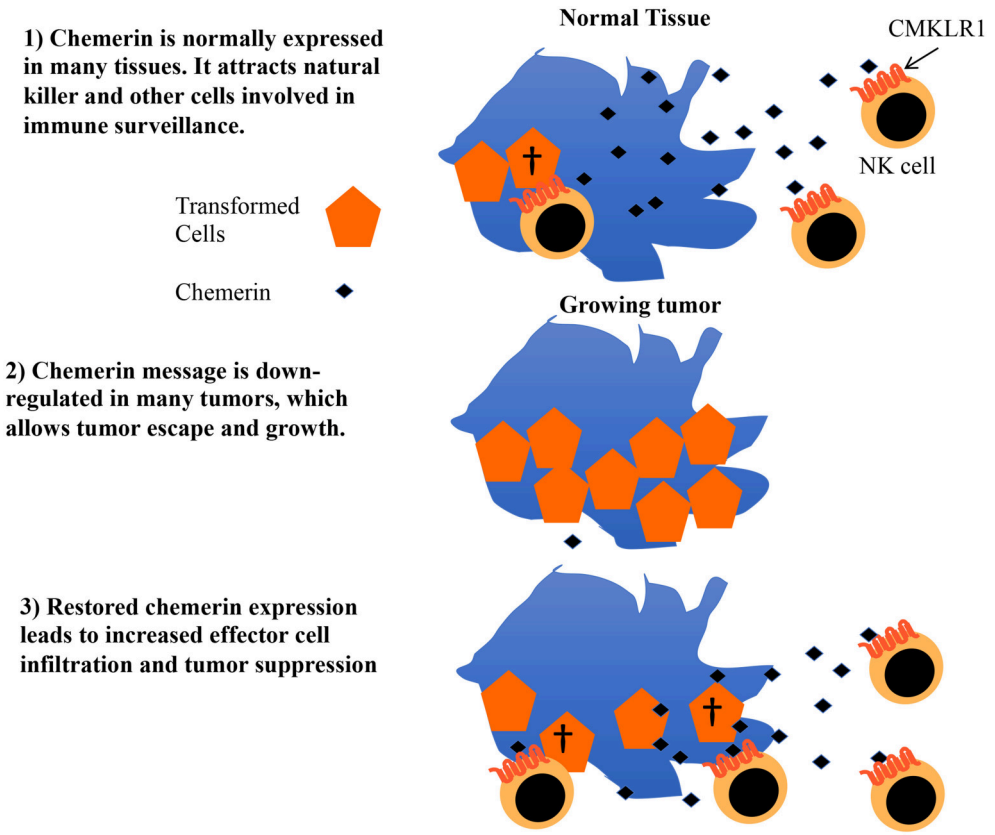

FIGURE 6 | Proposed model for role of chemerin in tumor immune surveillance and suppression. Chemerin/RARRES2 is down-regulated in breast and other tumors compared to their normal tissue counterparts. Data from our mouse tumor models show that forced over-expression of chemerin within the tumor microenvironment (TME) results in an increase in tumor-infiltrating leukocyte effector subsets (NK cells, CD8+ T cells) that are required to suppress tumor growth. Strategies focused on augmenting chemerin in the TME may represent an attractive strategy to increase effector cell composition within the tumor and potentially favorably impact the effect of existing immunotherapies such as checkpoint inhibitors in otherwise treatment-refractory tumors.

exclusion of CD8+ T cells from the tumor parenchyma, often seen in human tumors such as urothelial cancers (31). Importantly, we found meaningful increases in both NK and T cells within chemerin-expressing tumors compared to controls, similar to our findings in the melanoma model. Depletion studies indicate important roles for NK and CD8+ T cells in mediating the tumor suppressive effects of chemerin in this model, not surprisingly as supportive roles of NK cells in T cell function and the adaptive immune response are well described (53–56). Though chemerin does not seem to directly recruit CD8+ T cells via CMKLR1 interactions in this model, there is compelling evidence in the literature to suggest that NK cells mediate various functions that enhance CD8+ T cell cytolytic activity; for example, NK cells have been shown to moderate CD8+ T cell priming during influenza A viral infection and activate CD8+ T cell anti-tumor activity in the YAC-1 mouse lymphoma model (57, 58). Other studies have found that intratumor NK cell recruitment induces further leukocyte infiltration into the tumor (59), together articulating the point that chemerin may not need to act directly on CD8+ T cells to play a role in chemerin-dependent tumor growth inhibition. Additionally, ongoing studies include the impact of chemerin expression on the establishment of immune memory as well as the development of metastatic disease in this model.

Our *de novo* studies of human breast tissues using two independent cohorts of normal, IDC, and ILC samples across two assay platforms confirm large publicly available microarray datasets showing RARRES2 is significantly down-regulated in breast malignancies. Additionally, analysis of two mRNA microarray datasets showed that reduced chemerin levels significantly correlated with poor survival outcomes. In our *in vivo* experiments, we did not directly assess the effects of chemerin down-regulation/silencing during tumorigenesis in the EMT6 model. Rather, we focused on studying the potential therapeutic activity of restoring and/or overexpressing chemerin in the TME. Additional tumor studies are needed in animals with spontaneous carcinomas to determine whether chemerin down-regulation in the TME correlates with poor survival and thus models the clinical results we described in **Figures 1**, **2**. Given the variability within and across tumor types, evaluation of chemerin/RARRES2 and receptor expression will

be important prior to pursuing human translational studies. Importantly, recently published data provides a mechanistic link between chemerin and PTEN expression and function in hepatocellular carcinoma (48), suggesting chemerin may have other tumor suppressive mechanisms of action in addition to the recruitment of immune effector cells into the TME. Taken together our data elucidate mechanistic insights into the role of chemerin in breast tumor suppression and provide rationale for translational studies in human breast cancer.

ETHICS STATEMENT

This study was carried out in compliance with the ethics policies of Washington University School of Medicine. All animal studies were carried out under institutional IACUC-approved protocol, and human tissues were obtained from the St. Louis Breast Tissue Registry (funded by The Department of Surgery at Washington University School of Medicine, St. Louis, MO) in accordance with the guidelines established by the Washington University Institutional Review Board (IRB #201102394) and WAIVER of Elements of Consent per 45 CFR 46.116 (d). All patient information was de-identified prior to sharing with investigators. All of the human research activities and all activities of the IRBs designated in the Washington University (WU) Federal Wide Assurance (FWA), regardless of sponsorship, are guided by the ethical principles in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects Research of the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research.

AUTHOR CONTRIBUTIONS

RP, EB, and BZ conceived and designed experiments. RP, W-IL, PW, GV, KR, JR, YZ, LN, and NS performed experiments. RP, PW, GV, KR, WS, VN, and BZ analyzed data. RP, PW, WS, GV, KR, and BZ 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.2019.00983/full#supplementary-material

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REVIEW
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Targeting Chemokines and Chemokine Receptors in Melanoma and Other Cancers

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The tumor microenvironment is highly heterogeneous. It is composed of a diverse array of immune cells that are recruited continuously into lesions. They are guided into the tumor through interactions between chemokines and their receptors. A variety of chemokine receptors are expressed on the surface of both tumor and immune cells rendering them sensitive to multiple stimuli that can subsequently influence their migration and function. These features significantly impact tumor fate and are critical in melanoma control and progression. Indeed, particular chemokine receptors expressed on tumor and immune cells are strongly associated with patient prognosis. Thus, potential targeting of chemokine receptors is highly attractive as a means to quench or eliminate unconstrained tumor cell growth.

Keywords: chemokine, chemokine receptor, melanoma, immune cell trafficking, cell migration

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INTRODUCTION

Patient outcome is dictated by the capacity of immune cells to mount an effective anti-tumor response. Migration to, and infiltration of, tumors by immune cells is critical for achieving this goal. Elevated tumor immune infiltration is often associated with a favorable prognosis in many malignancies (1–3) including melanoma (4–6). Although fundamental in the anti-tumor immune response, tumor infiltration by immune cells is a challenging process. Immune cells are guided from the circulation to the tumor microenvironment by an evolutionarily conserved and sophisticated system in the form of the chemokine network. Chemokines are cytokines with chemotactic properties. This superfamily consists of 48 proteins classified into 4 groups (XCL, CCL, CXCL, and CX3CL) based on the position of two cysteine residues in their sequence. They bind to 19 G protein-coupled seven transmembrane receptors that form either homodimers or heterodimers (7–11). Similar to their ligands, chemokine receptors are classified into 4 groups, namely XCR, CCR, CXCR, and CX3CR. Each receptor can bind to several ligands of the same family and vice versa (**Figure 1**). Beyond this, atypical chemokine receptors also exist and most act as decoy receptors that compete for ligand binding but are unable to deliver normal chemokine receptor signals. They serve as negative regulators during inflammatory responses (12). The expression of these receptors and ligands is finely regulated, both spatially and temporally, revealing distinct functions at steady-state and during inflammatory responses. Many chemokines are constantly expressed and participate in the maintenance of tissue integrity, while some chemokines are transiently overexpressed or specifically induced in certain conditions (i.e., during inflammatory

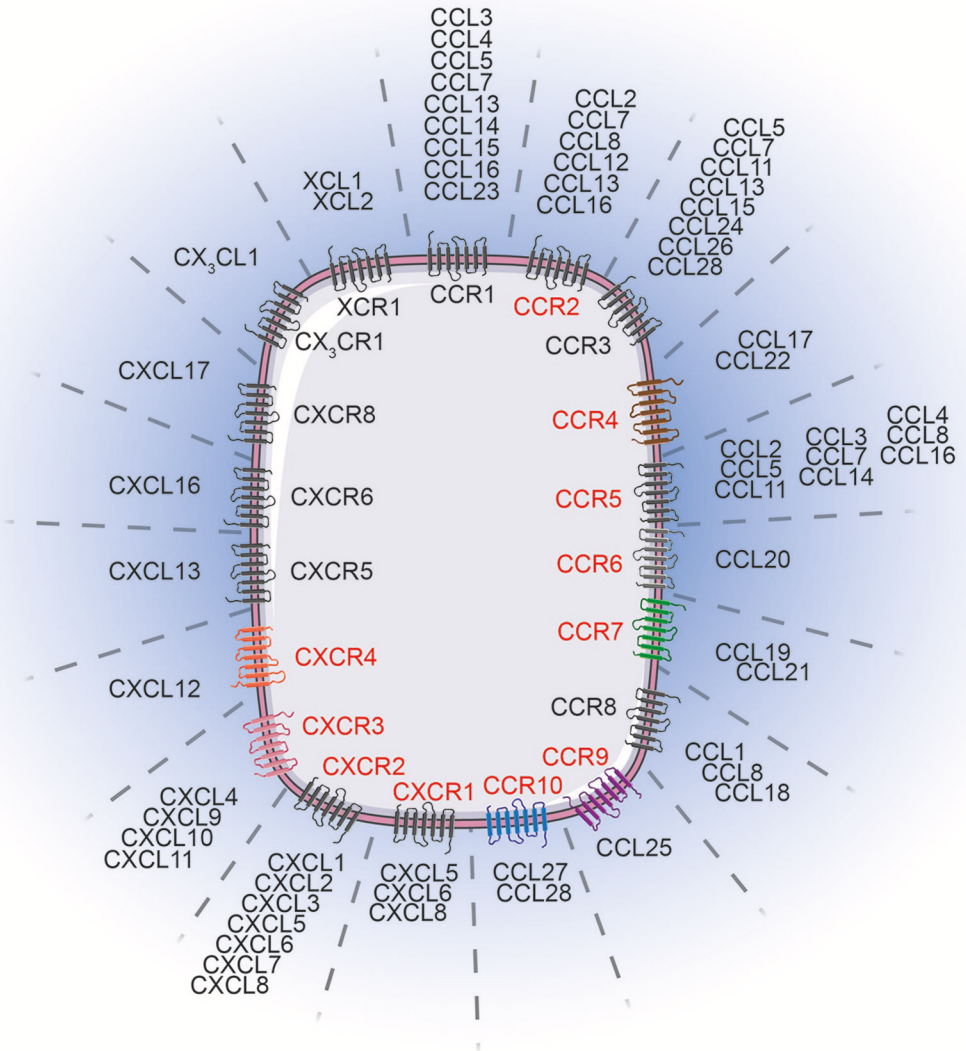

FIGURE 1 | Chemokine receptors and their corresponding ligands. Chemokine receptors (red) influence melanoma tumor cell migration/invasion or immune cell trafficking to the tumor lesions. The chemokine receptor associated color code is conserved between **Figures 1**, **2**. Images were taken from Servier Medical Art (https://smart.servier.com) and modified by the authors under the following terms: Creative Commons Attribution 3.0 Unported License.

processes) where they are involved in critical biological functions (i.e., immune cell migration, tissue repair, cell proliferation and angiogenesis) (10, 13, 14). Both immune and non-immune cells express these receptors and ligands, and the impact of this expression differs according to cell types. On one hand, selective expression drives the recruitment of specific immune cells into tumors, subsequently influencing patient prognosis. On the other hand, overexpression of chemokine receptors on cancer cells facilitates tumor dissemination. Collectively, dysregulation of this tightly regulated system contributes to tumor escape, and therefore, appears to be an attractive target in melanoma and other cancers.

Here, we review the expression of chemokines and chemokine receptors critically involved in skin migration, their expression on immune and tumor cells and consequences on dictating patient prognosis and, finally, their potential of targeting in melanoma and other cancers.

MIGRATION TO THE SKIN

The skin forms a physical barrier between an organism and the environment. It is mainly composed of melanin-producing cells, melanocytes, epithelial cells, keratinocytes, stromal cells, and immune cells that play critical roles both in maintaining homeostasis with commensals and in rapidly detecting and limiting pathogen infection and dissemination. Several immune cell types reside in the skin and act as essential sentinels (15). These include memory T cells, Langerhans cells and other types of dendritic cells (DC), macrophages, mast cells and innate lymphoid cells that collectively form a dense network that underlies the entire skin surface (15, 16). Localized at the frontline, keratinocytes are fundamental in protecting us against infections. They express different receptors, called pattern recognition receptors, specialized in the identification of conserved motifs across microorganisms (17). Upon detection of an infection or even after injury, activated keratinocytes start to secrete antimicrobial peptides, pro-inflammatory cytokines and chemokines (14, 15, 18, 19). In response to this local accumulation of chemokines and particularly to CXCL8, CXCL1, CCL2, CCL3 and CCL5, CXCR2-expressing monocytes and neutrophils are attracted to the inflammatory site and amplify this initial response (10, 15). Moreover, neutrophils are also attracted to the skin via binding of surface expressed formyl peptide receptor 1, to formylated peptides released by pathogens or dead or dying cells (20). In parallel, skin-resident DC drive immune responses through their potential to take up antigens. This process induces DC maturation and activation leading to membrane expression of CCR7 and CXCR4. In addition, this expression provokes their migration from the skin to the closest skin-draining lymph node (10, 21). Antigen-specific T cells are imprinted with skin-homing molecules following their engagement with, and activation by, primed DC. These homing molecules include CCR3, CCR4, CCR5, CCR10, CXCR3, and Cutaneous Lymphocyte associated Antigen (CLA), a ligand for E-selectin (22–25). The expression of these receptors facilitates T cell migration to the skin through binding of E-selectin that is expressed selectively on activated skin endothelial cells (22, 26). Moreover, together with skin-resident cells, these endothelial cells also secrete specific chemokines such as CCL17, CCL20, CCL22 and CCL27, ligand of CCR4, CCR6, and CCR10, respectively, that guide these antigen-specific T cells specifically to the inflamed skin lesion (15, 27–31). This migratory pathway is essential for wound healing after skin injury and for efficient elimination of infections. In addition, these chemokine—chemokine receptor interactions are also of extreme importance in melanoma immunity. Primary tumors localized in the skin are continuously evolving as a result of the constant infiltration to, and egress of cells from, the microenvironment. This is facilitated by the presence of blood and lymphatic vessels that guide immune cells to the tumor bed but also enable cancer cells to disseminate to various organs. Chemokines and their receptors are critically involved in these migratory processes and actively control the specific metastatic melanoma landscape.

SPECIFIC CHEMOKINE RECEPTOR EXPRESSION ON MELANOMA CELLS IS ASSOCIATED WITH DISTINCT METASTATIC DISSEMINATION

The formation of secondary lesions involves two major steps. First, tumor cells are guided from the circulation to their final location in response to a chemokine gradient expressed in different organs and then, these newly seeded tumor cells must survive and proliferate in these specific environments subsequently forming distant metastases (9, 32). In cutaneous melanoma, as a result of a specific chemokine receptor expression pattern, melanoma cells disseminate in an organ-specific manner that forms secondary lesions preferentially in draining lymph nodes, lung, liver, gut and brain (**Figure 2**) (33, 34). To determine the role of key chemokine receptors in tumor cell migration in melanoma, many of the mouse studies described here have used the prototypic mouse melanoma model, B16, or its highly metastatic subclone B16F10 (35, 36). The combination of preclinical studies and retrospective assessment of human melanoma samples for chemokine receptor expression have shed light on a finely controlled process that notably involves CCR4, CCR6, CCR7, CCR9, CCR10, CXCR3, CXCR4, and CXCR7 expression.

CCR4–CCL17/CCL22 Axis

Several lines of evidence evoked by Klein et al. (37) tend to associate CCR4 expression with increased brain melanoma metastases (37). Endothelial cells, astrocytes and microglia cells were shown to express high levels of CCR4 ligands, CCL17 and CCL22 (37) that likely attract $CCR4^{+}$ cells. *In vitro* incubation of microglia cells with conditioned media from brain metastasizing melanoma cells increased CCR4 ligand secretion. Furthermore, CCR4 is more highly expressed on melanoma brain metastases than on paired-primary melanoma tumors (37) (**Figure 2**). Klein et al. (37) have further studied whether CCR4 overexpression in melanoma cells favor brain metastasis formation. *In vitro*, CCR4 overexpression enhanced cell viability and migration in response to astrocyte-conditioned media and to recombinant CCL17. This migration is partially abrogated by the concomitant use of an anti-CCL17 antibody. *In vivo*, CCR4 overexpression promoted primary tumor growth and enhanced brain metastases formation in immunocompromised nude mice. Importantly, mice inoculated with $CCR4^{high}$ expressing tumor cells and treated with a CCR4 antagonist had a significant reduction of primary tumor growth associated with a decrease of the presence of brain micrometastases (37). Collectively these results suggest that CCR4 overexpression on melanoma tumors might enhance their potential to metastasize to the brain (**Table 1**, **Figure 2**).

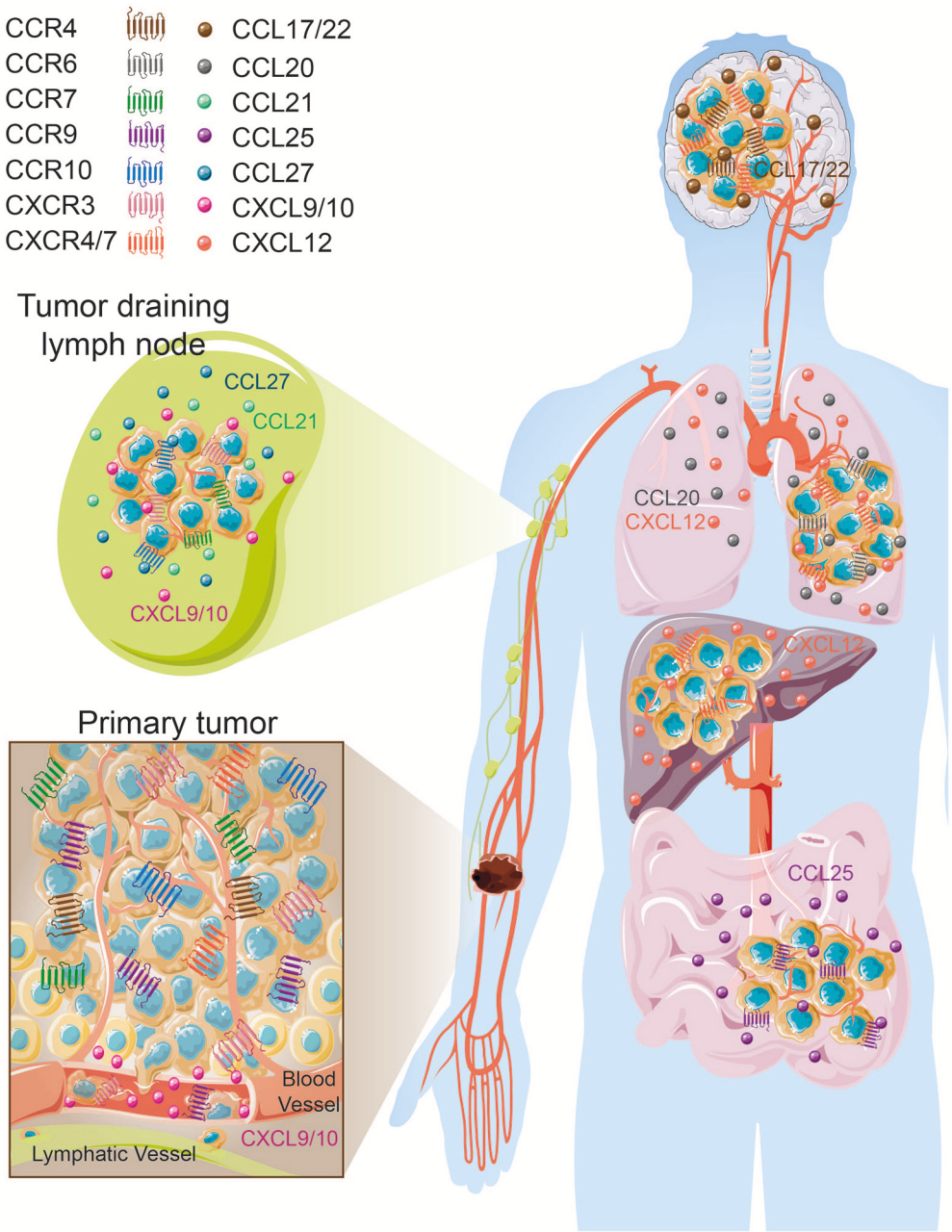

FIGURE 2 | Organ-specific melanoma metastases according to tissue/melanoma specific chemokine/chemokine receptor expression. Images were taken from Servier Medical Art (https://smart.servier.com) and modified by the authors under the following terms: Creative Commons Attribution 3.0 Unported License.

CCR6–CCL20 Axis

CCR6 is expressed on melanoma cell lines and enhances their migration and proliferation in response to stimulation by its ligand, CCL20 (38). Importantly, CCR6 expression is detected on tumor cells from primary melanomas, lymph node, skin, colon, and brain metastases. Despite high expression on tumor cells, CCR6 positivity is not associated with patient outcome. However, CCL20 administration in CCR6^{+} tumor bearing mice increased tumor weight and numbers of spontaneous lung metastases (38) (**Table 1**, **Figure 2**) suggesting the potential involvement of CCR6 in lung metastasis formation. Interestingly, Fusi et al. (53) have evaluated the presence of CCR6 expression on circulating tumor cells collected from metastatic carcinoma ($N = 28$) and melanoma ($N = 21$) patients. Positive CCR6 expression on circulating tumor cells, evaluated on the whole cohort, was not found to be associated with the presence of lung metastases (53). However, this chemokine receptor might be regulated differently according to tumor type. Thus, further studies are required to

TABLE 1 | Expression of chemokine receptors at the surface of melanoma cells involved in tumor progression.

Chemokine Receptor	Roles in tumor development/progression	Clinical association	Cohort details	Statistical analyses	References
CCR4	Favor tumor cell viability, migration, primary tumor growth, and brain metastases formation	Not known	*In vitro* and preclinical models		(37)
CCR6	Enhanced tumor cell migration, proliferation, tumor growth, and lung metastasis formation	Not associated with patient outcome*	40 primary melanomas	Log-rank and Cox regression	(38)
CCR7	Associated with regional lymph node metastases	Poor prognosis	Preclinical model and 38 primary human samples	Log rank test—$P = 0.009$	(39, 40)
CCR9	Expressed on tumor cells localized in the small intestine–Sensitive to CCL25 stimulation	Not associated with patient outcome* or not assessed	38 primary samples	Log rank test	(40–42)
CCR10	Associated with an increase of regional lymph node metastases, metastatic sentinel lymph node, thickening of primary lesions and poor T cell density	Shorter progression free survival	40 primary lesions and 38 primary melanoma samples	Spearman correlation and Log rank test–$P = 0.002$	(40, 43, 44)
CXCR3	Associated with thick primary lesions, the absence of lymphocytic infiltration and the presence of distant metastases—Increase in cell adhesion, migration, and invasion of CXCR3 expressing melanoma cells lines upon stimulation.	Not associated with patient outcome*	Primary melanomas and 9 Lymph node metastases	χ^2, Mann-Whitney U and Kruskal Wallis tests—Log-rank test and Cox regression	(45–48)
CXCR4	Associated with the presence of ulceration, thicker lesions—Induce tumor cell proliferation, migration, and invasion—Associated with liver and lung metastases	Reduced disease-free and overall survival	Primary melanomas and metastatic samples	χ^2 2-sided test—Log-rank test and Cox regression	(47, 49–52)

**Complementary analyses on larger cohorts are warranted.*

understand the impact of tumoral CCR6 expression in metastatic dissemination and how this chemokine receptor might influence melanoma outcome.

CCR7–CCL19/CCL21 Axis

Kuhnelt-Leddihn et al. have shown that 6 out of 38 primary melanoma tumors evaluated presented with high CCR7 expression (40), a chemokine receptor involved in leukocyte trafficking to secondary lymphoid organs in response to the local production of CCL19 and CCL21 (**Table 1**, **Figure 2**). CCR7 has also been found on circulating tumor cells and human metastatic melanoma cell lines (51, 53). Treatment of metastatic melanoma-derived cell lines with histone deacetylase inhibitor and demethylating agents demonstrated that this increase in CCR7 expression is associated with the enhanced migratory responses to CCL21 stimulation (54). Interestingly, CCL21 expression is decreased in invaded lymph node compared to non-invaded lymph node (55) that may suggest an escape mechanism to avoid tumor immune infiltration, specifically by CCR7 expressing T cells and DC (10, 56). In mice, overexpression of CCR7 in B16 melanoma cells increased metastasis to the lymph node and neutralizing its ligand, CCL21, using a specific antibody blocked this metastatic process (39), highlighting the importance of this CCR7/CCL21 axis in the metastasis to the regional lymph node. Overexpression of CCL21 in tumor cells induce a tolerogenic microenvironment associated with a production of Transforming Growth Factor-β (TGF-β) that favors the recruitment of regulatory T cells (Tregs) and myeloid deriving suppressor cells (MDSC) (57). More importantly, high expression of CCR7 by melanoma cells is associated with a worse patient outcome (40) (**Table 1**).

CCR9–CCL25 Axis

CCR9 is a chemokine receptor involved in the migration of T cells and other immune cells to its ligand, CCL25, which is highly expressed in the small intestine (58). Melanoma tumor cells that have metastasized to the small intestine have been shown to express CCR9 (41, 42) (**Table 1**, **Figure 2**). Importantly, $CCR9^+$ melanoma cell lines derived from small intestinal metastases are responsive to CCL25 (41, 42). CCR9 expression has been also reported on circulating tumor cells (53). Unfortunately, the association between CCR9 expression on circulating tumor cells and small intestine metastases has not been assessed. Moreover, after screening a panel of 38 primary melanoma tumors, CCR9 expression was not found to be associated with patient's prognosis despite being highly expressed in one third of lesions (40). Collectively, these results suggest that CCR9 expression at the surface of melanoma cells may be essential for the migratory process to the gut (**Figure 2**).

CCR10–CCL27 Axis

CCR10 is expressed on melanoma cells in primary tumor lesions (40, 43). Using a preclinical model of melanoma, overexpression of CCR10 in B16 tumor cells protected them from the host

immune responses leading to an increase in tumor size and increased regional lymph node metastases (43). Incubating tumor cells with a neutralizing antibody for CCL27, one of the ligands of CCR10, prevented tumor formation (43). These results indicate that CCR10 may play an important role in sustaining tumor viability, protecting cells from immune responses and favoring metastases formation to the regional draining lymph node in response to CCL27. In humans, high CCR10 expression may be associated with a shorter progression free survival (40) (**Table 1**). Strikingly, patients with metastatic sentinel lymph nodes had higher levels of CCR10 expression on primary tumor cells than patients with negative sentinel lymph node (44). This observation further supports the probable role of this chemokine receptor in regional lymph node dissemination (**Figure 2**). Moreover, high CCR10 expression was associated with thick primary lesions and negatively correlated with intratumoral T cell density (44) (**Table 1**). Altogether, CCR10 overexpression on melanoma cells is associated with the possible presence of regional lymph node metastases (**Figure 2**) accompanied by an immune negative climate.

CXCR3–CXCL9/CXCL10 Axis

CXCR3 expression on primary lesion tumor cells is positively associated with deleterious clinical parameters including thickening of primary lesions, absence of lymphocytic infiltration, and presence of distant metastases (47, 48) but, surprisingly, is not correlated with patient outcomes (48). Nonetheless, high CXCR3 expression evaluated on 40 primary melanoma tumors tended to be associated with poor disease-free and overall survivals (48). CXCR3 positive tumor cells are also found in invaded lymph nodes (**Figure 2**) and together with other metastatic locations including the kidney, ovary and pleura (45, 59). Interestingly, tumor endothelial cells facilitate melanoma migration through their production of CXCL9 (and CXCL10). This results in endothelial barrier disruption and transendothelial migration (59) (**Figure 2**). In addition, *in vitro* stimulation of melanoma cell lines with CXCL9 induced cytoskeletal rearrangements, cell adhesion and migration (45), that favor cell trafficking and metastasis. Similarly, *in vitro* incubation of the mouse melanoma cell line B16F10 with CXCR3 ligands significantly enhanced migration and invasion of these cells (46). Conversely, specific downregulation of CXCR3 in subcutaneous injected B16F10 tumor cells reduced their metastatic capabilities to invade the tumor draining lymph node (46). Mouse melanoma tumor cells incubated with the CXCR3 ligand, CXCL9, exhibited greater viability than the control cells (**Table 1**), thus demonstrating that CXCR3 imparts a selective advantage to tumor cells most likely allowing them to compete more effectively for oxygen and nutrient availability in the competitive tumor microenvironment (60–62).

CXCR4/CXCR7–CXCL12 Axis

In primary skin tumors, cancer cells express CXCR4, a chemokine receptor involved in bone marrow homing and cell retention (10). Importantly, high CXCR4 expression is associated with the presence of tumor ulceration and thicker lesions, as well as shorter disease-free survival, time to metastasis and overall survival (47, 63) (**Table 1**). Tumoral CXCR4 expression has also been detected on circulating tumor cells (53) as well as in liver, lung, and nodal metastases (49, 51). Using melanoma cell lines, Scala et al. demonstrated that these cells express functional CXCR4, as *in vitro* stimulation with CXCL12 in serum free media increased their proliferation that was abrogated with the concomitant use of a CXCR4 inhibitor, AMD3100 (51). The B16 mouse melanoma cell line constitutively expresses CXCR4. This increased the cell migration, invasion and proliferation in response to the binding its ligand, CXCL12 (52). Importantly, CXCL12 stimulation induced cell adhesion to liver sinusoidal endothelial cells and *in vivo*, B16 liver metastases are often localized to CXCL12 expressing liver sinusoidal endothelial cells. Mendt and Cardier (52) have shown that stimulation of B16 cells with CXCL12 prior *in vivo* injection increased the number of liver metastases (52). Several lines of evidence tend to also involve the CXCR4-CXCL12 pathway in lung metastasis formation. Firstly, high CXCL12 concentrations are found in lungs (64). Secondly, overexpression of CXCR4 in B16 cells enhanced lung nodules formation (49, 50, 65) (**Table 1**). Thirdly, the use of specific CXCR4 inhibitors, T22 or a dimeric form of CXCL12, reduced lung metastases formation and inhibited the growth of primary melanoma tumors (49, 66, 67). However, CXCR4 expression on circulating tumor cells was not found preferentially associated with liver metastases or with lung metastases in metastatic carcinoma or melanoma patients (53).

CXCL12 also binds to its high-affinity receptor CXCR7, an atypical chemokine receptor also known as ACKR3. CXCR7 is expressed on normal human epidermal melanocytes (68) and primary melanoma tumors (63, 69). The role and functions of CXCR7 in cell migration/chemotaxis is still controversial (70). In neuroblastoma cell lines, overexpression of CXCR7 was shown to limit cell growth and CXCR4/CXCL12-mediated chemotaxis (71). In contrast, some studies have demonstrated that CXCR7 expression favors hepatocellular carcinoma cell proliferation, migration and VEGF production (72), transendothelial migration of cancer cells (73, 74), and tumor cell migration by forming heterodimers with CXCR4 (75). Using the M14 melanoma cell line that expresses functional CXCR7, Li et al. have demonstrated that *in vitro* incubation of M14 cells with CXCL12 induced cell migration, which was specifically reduced following abrogation of CXCR7 expression (69). Furthermore, downregulation of CXCR7 expression in the melanoma cell line decreased the growth of the xenotransplanted tumor. However, the expression of CXCR4 was not reported in this study. The full deletion of CXCR4 in M14 cells together with the modulation of CXCR7 expression are warranted in order to definitively determine the impact of this atypical chemokine receptor on M14 cell growth and migration. Furthermore, its expression on melanoma metastases and its association with patient prognosis remain to be determined. Altogether, CXCR4 is involved in the metastatic spreading of melanoma cells and therefore may influence patient outcomes. Based on pre-clinical results, it is also tempting to say that tumoral CXCR4 expression is more preferentially associated with lung and liver metastases (**Table 1**, **Figure 2**). However, additional studies are warranted

to determine the involvement of the CXCR4/CXCR7 -CXCL12 axis in favoring organ-specific metastasis formation as reported in breast or colorectal cancer (76–79).

In the past 20 years, numerous studies have demonstrated the pivotal role of these chemokine receptors in melanoma dissemination and how this coordinated chemokine receptor expression on the surface of melanoma cells is preferentially associated with specific organ metastases (9, 50, 80). CCR10, CCR7, and CXCR3 are found mainly involved in regional metastases formation while CCR9 is often associated with the intestine, CCR6 or CXCR4 are preferentially implicated in the formation of lung and liver lesions. CCR4 does however seem to be associated with brain metastases, which considerably impacts patient prognosis (81) (**Table 1**, **Figure 2**). Collectively, tumor cells eventually use these chemokines and chemokine receptors to their own advantage to be guided through the body to invade distant organs and create secondary lesions.

CHEMOKINE RECEPTOR EXPRESSION ON IMMUNE CELLS – DECISIVE ROLES IN MELANOMA LESION INFILTRATION AND TUMOR FATE

Tumor immune cell infiltration is critical in dictating melanoma patient outcome (82–84). Specific expression of chemokine/chemokine receptors and integrins is fundamental to this process and is involved in the guidance and tissue retention of immune cells. Transcriptomic analyses of 569 cutaneous samples and 120 melanoma metastases have demonstrated the positive association of 12 chemokines (CCL2, CCL3, CCL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, and CXCL13) with the presence of tertiary lymphoid structures, ectopic lymph node-like structures containing antigen presenting cells, B cells and T cells (85). This chemokine signature was associated with a favorable prognosis irrespective of tumor localization. This has been further validated in patients harboring primary tumors that contain peritumoral matured DC in combination with activated T lymphocytes (86). Furthermore, Harlin et al. found that a restricted signature of six chemokines, CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10, were preferentially expressed in melanoma metastases that were highly infiltrated by T cells (87). Importantly, high gene expression of *Cxcl2*, *Cxcl9*, *Cxcl10,* and *Ccl5* together with *Ifnγ*, *Stat1,* and *Irf1* expression have been associated with the efficacy of MAGE-A3 vaccination (88) and with clinical responses to CTLA-4 blockade (89). Collectively, chemokines profoundly affect tumor immune cell composition and melanoma responses irrespective of tumor location. To date, the evaluation of these chemokines are not yet considered in daily clinical practice but they are likely to be essential to more accurately evaluate the prognosis of melanoma patients and/or therapeutic responses. Immune cell trafficking occurs after specific interactions between chemokines with their receptors that guide the immune cells to their final location. Thus, this expression is extremely important and dictates the tumor microenvironment diversity, considerably influencing melanoma evolution.

CCR4–CCL2 Axis

In human and mouse melanomas, the presence of $Foxp3^+$ cells, mainly Tregs, in primary and metastatic tumors was associated with a poor prognosis (83, 90–93). Effector and regulatory T cells both express CCR4 but $Foxp3^+$ Tregs expressed higher levels of CCR4 than their $Foxp3^-$ effector T cell counterparts. Salerno et al. (94) have described the accumulation of $CCR4^+$ effector $CD4^+$ T cells, but not $CD8^+$ T cells, in skin and bowel melanoma metastases (94). Given the large proportion of Tregs within the $CD4^+$ population in tumor lesions, it is tempting to associate the presence of $CCR4^+$ effector cells to Tregs. These cells migrated to the tumor bed in response to CCL2 accumulation (95–97). The use of an anti-CCR4 antibody *in vitro* efficiently reduced Tregs numbers enabling the induction of cancer/testis antigen-specific T cell responses (97) (**Table 2**). In pre-clinical models, the use of an anti-CD25 antibody, or Foxp3DTR (Diphtheria Toxin Receptor) mice where Foxp3-expressing cells can be inducibly deleted following diphtheria toxin injection, delayed tumor growth (100). However, in transgenic mouse melanoma models, the removal of Tregs was not sufficient to induce clinical improvements (96) suggesting that other immunosuppressive pathways are acting in concert to suppress anti-tumor immune functions. Moreover, in a therapeutic setting, anti-CD25 antibody injection did not reduce Treg proportions in tumors (96) potentially explaining the absence of clinical activity from the treatment.

CCR5–CCL3/CCL4/CCL5 Axis

The relationship between CCR5 expression on immune cells and tumor fate is not clear. In humans, little is known about the impact of CCR5 expression on immune cells and its association with patient outcomes. High CCR5 expression has been found on the surface of tumor infiltrating T cells (94). Interestingly, stage IV melanoma patients carrying a 32-bp – deletion polymorphism in the *Ccr5* gene, rendering this protein non-functional, have decreased survival following interferon treatment, interleukin-2 administration, or vaccination (101) suggesting a potential benefit of CCR5 expression in these specific settings. However, the use of CCR5-deficient mice, blockade antibody or CCR5-Ig fusion protein that acts as a decoy receptor neutralizing the CCR5 ligands, led to delayed tumor growth and increased the survival of these animals compared with control groups (102–104). Thus, CCR5 expression appears to be deleterious in pre-clinical models. CCR5 is highly expressed on tumor infiltrating $CD8^+$ T cells, conventional and regulatory $CD4^+$ T cells (102), and on the surface of MDSC (104). Importantly, $CCR5^+$ MDSC displayed a more suppressive phenotype than their $CCR5^-$ counterparts, expressing higher levels of Arginase 1 and producing more reactive oxygen species. The CCR5 ligands, CCL3, CCL4, and CCL5, are produced by intratumoral and circulating MDSC (102), acting in an autocrine manner on $CCR5^+$ cells. Clinical improvements observed in CCR5-deficient mice or using CCR5 blockade were associated with a reduction of Tregs (102) and MDSC infiltration (103) together with a decrease of their immunosuppressive activities (104). In these models, conventional $CD4^+$ and $CD8^+$ T cell infiltration were maintained suggesting that CCR5

TABLE 2 | Expression of chemokine and chemokine receptors by immune cells associated with melanoma control or progression.

Chemokine receptor	Immune cell expression	Roles in melanoma development/progression	Cohort details	Statistical analyses	References
CCR2	Tumor macrophages and MDSC	Neutralization decreased tumor macrophage accumulations associated with a reduction of tumor angiogenesis and tumor growth	Preclinical studies		(98, 99)
CCR4	Blood and tumor Tregs	Depletion enhanced anti tumor immune responses. Controversial using the spontaneous *Ret* melanoma model.	*In vitro* and preclinical studies		(96, 97, 100)
CCR5	Blood and tumor Tregs and MDSC	$CCR5^{\Delta 32}$ polymorphism in patients receiving immunotherapy associated with decreased survival Immunosuppression -Neutralization resulted in increased survival of tumor bearing mice	139 stage IV patients Preclinical studies	Log-rank test and Cox regression– $P = 0.002$	(101–104)
CCR6	Blood and tumor pDC—Blood $CD8^+$ T cells	Higher expression in melanoma patients—circulating effector $CCR6^+CD8^+$ T cells and CCL20 expressed by tumor-associated macrophages conveyed a dismal prognosis	40 primary melanomas—57 stage III-IV patients	Log rank test and Cox regression	(38, 105, 106)
CCR9	Blood $CD8^+$ T_{Naive}	Associated with increased overall survival	57 stage III-IV patients	Log-rank test and Cox regression– $P = 0.0036$ (Stage-adjusted)	(106)
CCR10	Blood $CD4^+$ T_{EM}	Associated with worse survival	57 stage III-IV patients	Log-rank test and Cox regression– $P = 0.0189$ (Stage-adjusted)	(106)
CXCR2	Tumor MDSC and neutrophils	Accumulation of tumor $CXCR2^+$ MDSC and neutrophils. CXCR2 neutralization reduced tumor growth	Preclinical studies		(107, 108)
CXCR3	Blood and tumor $CD4^+$ and $CD8^+$ T_{EM}	Critical in intratumoral T cell trafficking—Associated with clinical benefit	Preclinical studies–Stage III-IV patients	Log-rank test, χ^2 and Cox regression	(87, 106, 109, 110)
CXCR4	Blood $CD45RA^+CD4^+$ T cells	Associated with prolonged disease free survival	195 stage I-III patients	Log-rank test and Cox regression– $P = 0.0091$	(111)

T_{EM}: Effector memory T cells.

expression on the surface of these cells is not required for tumor infiltration (102). This observation has been confirmed by Mikucki et al. (110). Indeed, they demonstrated that the presence of CCR5 on $CD8^+$ T cells was not essential for tumor infiltration despite high CCR5 ligand levels found in the tumor microenvironment (110). However, it remains unclear why MDSCs needs CCR5 expression for tumor infiltration, whereas T cells do not. In humans, both circulating monocytic ($CD14^+$) and polymorphonuclear MDSC ($CD15^+CD11b^+HLA\text{-}DR^{lo/-}$) express higher amounts of CCR5 on their membrane, compared to levels observed in healthy volunteers (104). Interestingly, CCR5 is more highly expressed on tumor infiltrating monocytic MDSC than on peripheral cells and high concentrations of CCL3, CCL4, and CCL5 are found in melanoma lesions, potentially explaining the enrichment of $CCR5^+$ MDSC in tumors (104). Collectively, CCR5 expression sustains MDSC suppression activities, intratumoral Treg infiltration, and melanoma tumor growth (**Table 2**). Further studies in patients are needed to investigate the impact of CCR5 expression on immune cells and its association with prognosis in melanoma. Given the role of CCR5 in T cell costimulation (112), it would be interesting to understand the relationship between CCR5 expression on T cells and patient outcomes.

CCR6–CCL20 Axis

In melanoma patients, CCR6 was found to be more highly expressed on circulating plasmacytoid DC (pDC) than on pDC found in healthy volunteer controls (105). CCR6-expressing pDC migrated in response to CCL20 stimulation. The presence of $CCR6^+$ pDC have been detected in primary melanoma tumors. This infiltration might be in part due to the presence of high concentrations of CCL20, often detected within these primary tumor lesions (105) and mainly produced by tumor-associated macrophages (38). Interestingly, high CCL20 expression is associated with a shorter disease-free period and overall survival of melanoma patients (38). Moreover, given the

negative prognostic value conveyed by tumor-infiltrating pDC in melanoma (113), CCR6 is likely to also be associated with poor patient outcome. However, this needs to be explored further and to validated the prognostic value of $CCR6^+$pDC in the melanoma tumor microenvironment. We have found that a low proportion of circulating effector memory $CD8^+CCR6^+$ T cells was associated with a better overall survival in stage IV melanoma (106). Collectively, it seems that both CCL20 and CCR6 immune cell expression in multiple cell types are associated with a poor patient outcome (**Table 2**).

CCR9–CCL25 Axis

CCR9 is expressed at the membrane of several immune cell subsets and is mostly associated with gut homing with the exception of immature T cells in transit from the bone marrow to the thymus (114). Further $CCR9^+$ cell populations include intestinal infiltrating T cells (115), gut pDC (116), and small intestinal IgA producing plasma cells (117). Unfortunately, to date, the role of CCR9 expression on immune cells in melanoma and other cancers is poorly understood. We have investigated the impact of CCR9 expression on the membrane of circulating T cells in stage IV melanoma patients. Interestingly, high CCR9 expression on naïve circulating CD8 T cells is associated with a favorable prognosis (106) (**Table 2**). In mice, we have found tumor infiltrating T cells that express CCR9 and importantly, blockade of its ligand, CCL25, in a sarcoma model, led to increased tumor growth. This is associated with a reduction of $CD4^+$ T cell infiltration. Moreover, in this tumor model, high levels of CCL25 were found in the tumor microenvironment and these levels were much higher than the levels found in the gut (106) providing a possible explanation for the recruitment of these $CCR9^+$ T cells to the tumor bed. Further studies are warranted to validate this positive impact of CCR9 expression on T cells in this pathology.

CCR10–CCL27 Axis

CCR10 is one of the chemokine receptors that specifically guide the migration of immune cells to the skin in response to the local production and accumulation of CCL27. In contrast to benign lesions where CCL27 is expressed at low levels, many primary melanoma lesions express substantial amounts of this chemokine (44). CCL27 expression is correlated with T lymphocyte density, but unexpectedly, higher chemokine expression is associated with lower T cell infiltrate (44). This suggests that despite the local accumulation of CCL27, CCR10-expressing T cells are unable to infiltrate CCL27-expressing melanoma lesions and these T cells are therefore restricted to circulate in the periphery. Supporting this hypothesis, in our own work we have shown that in stage IV patients, the accumulation of circulating effector memory CCR10 expressing $CD4^+$ T cells was associated with shorter overall survival (106). With the exception of these two studies, little is known about the impact of CCR10 expression on immune cells and prognosis. However, it seems that CCL27 tumor concentration was not associated with T cell accumulation and thus their peripheral increase was associated with a poor prognosis (**Table 2**).

CXCR3–CXCL9/CXCL10 Axis

High expression of CXCR3, on melanoma infiltrating T cells together with the recruitment of effector memory $CD8^+$ T cells has been associated with a better patient outcome (87, 89, 109, 118) (**Table 2**). Mullins et al. (109) reported that high CXCR3 expression on antigen specific $CD8^+CD45RO^+$ T cells is associated with a favorable prognosis in stage III patients but fail to do so in patients with distant metastases (109). We have found that high CXCR3 expression on circulating effector memory $CD4^+$ T cells is associated with an enhancement of stage III-IV patient survival, irrespective of tumor lesion location and patient stages (106). Mikucki et al. (110) have demonstrated the critical requirement of CXCR3 expression on mouse $CD8^+$ T cells for cell adhesion to, and migration through, the endothelial barrier to infiltrate tumor lesions (110). Furthermore, CXCR3 is associated with Th1/Tc1 polarization and anti-tumor functions (119, 120). Interestingly, therapy such as peptide vaccination in Montanide Adjuvant led to the upregulation of CXCR3 expression on circulating tumor antigen-specific T cells (121) but Hailemichael et al. have shown that most of these $CXCR3^+$ T cells induced by the vaccination are retained to the site of vaccine administration (122). Despite this potential induction of CXCR3 expression, $CXCR3^+$ T cells are unlikely to reach melanoma lesions in this context. Furthermore, we have found that in stage III/IV patients, CXCR3 is poorly expressed on T cells compared with expression levels observed in healthy volunteers (106). This last observation suggests that (i) CXCR3 is potentially downregulated due to a negative feedback loop of cell regulation following STAT3 activation or (ii) these $CXCR3^+$ T cells, which are underrepresented in the periphery, are actually localized to melanoma lesions. Currently, there is little evidence to support either of these two hypotheses. In favor of CXCR3-regulated expression, Yue et al. (123) found that STAT3 expression and signaling mediated CXCR3 downregulation on $CD8^+$ T cells thus inhibiting intratumoral $CD8^+$ T cell accumulation and impacting anti-tumor functions (123). At steady-state, CXCR3 is tightly regulated at the surface of T cells and downregulation of its expression with or without ligand binding is finely controlled by a regulatory feedback mechanism to preserve cells from over activation (124) and this may even be exacerbated in a pro-inflammatory context. Moreover, we have previously found an enrichment of CXCR3-expressing $CD4^+$ T cells in metastatic lymph nodes compared with circulating T cells (106) perhaps explaining the differences found in the blood between melanoma patients and healthy volunteers. In tumor lesions, CXCR3 expression might be sustained by the presence of pro-inflammatory molecules such as IFNγ that has been shown to sustain *Tbx21* expression and subsequently TBET to positively regulate CXCR3 expression at the surface of T cells (125, 126). Together, these studies highlight that the expression of CXCR3 on the surface of T cells is finely regulated and is essential to melanoma infiltration and tumor control. Furthermore, high tumor expression of CXCR3 ligands together with high expression of CXCR3 on T cells are both associated with a favorable prognosis in melanoma (**Table 2**). Thus, strategies enhancing CXCR3 ligand production or CXCR3 expression on

effector and memory T cells, but not melanoma cells, is highly desirable.

CXCR4–CXCL12 Axis

The CXCR4-CXCL12 axis is required for the development and survival of mice as complete deletion of CXCR4 is embryonically lethal (127, 128). This axis plays an essential role in haematopoiesis and cerebellar development, bone marrow immune cell retention and thymic homing (10, 127, 128). To study the role of CXCR4 expression on non-tumor cells and its association with melanoma progression, D'alterio et al. (64) have used CXCR4 heterozygous mice where they intravenously injected CXCR4 expressing B16 melanoma cells. The partial loss of host-CXCR4 expression reduced lung metastases formation that is accompanied by a decrease of CXCL12 concentration together with $Ly6G^+$ cell accumulation in lung tissues (64). Similar results have been found in wild type mice treated with a CXCR4 antagonist, Plerixafor (AMD3100) (64). In stage I-III melanoma patients, high expression of CXCR4 in circulating $CD4^+CD45RA^+$ was associated with prolonged disease free survival (**Table 2**). Moreover, the presence of CXCR4 expressing $CD4^+CD45RA^+$ T cells correlated with absence of primary tumor ulceration (111).

DO CHEMOKINE RECEPTOR EXPRESSION ON IMMUNE CELLS REFLECT THE METASTATIC DISSEMINATION OF MELANOMA?

This question was first raised by Salerno et al. (94). They studied whether the expression of organ-specific chemokine receptors and integrins on the surface of T cells differs according to the metastatic site (94). This included the evaluation of CCR4, CCR5, CCR7, CCR9, CXCR3, CLA, and tissue retention integrins on the surface of $CD4^+$ and $CD8^+$ T cells by flow cytometry. This group found limited evidence that tissue site-specific chemokine receptor expression was associated with the site of metastatic location with the exception of CCR9, which was found to be preferentially expressed on T cells that infiltrate small intestine metastases. Expectedly, the expression of tissue retention integrins was higher on tumor infiltrating T cells than on circulating T cells suggesting a specific maintenance of a pool of intratumoral effector and memory T cells in melanoma lesions (94). This lack of site-specific expression of chemokine receptors on infiltrating T cells might be due in part by an absence of infiltration of these site-specific chemokine receptor-expressing cells. Thus, these cells may be maintained in the circulation. Salerno et al. (94) found that CCR4, CCR5, and CLA are highly expressed on circulating T cells (94). However, how this expression differs from healthy volunteers and to what extent this peripheral expression correlates with site-specific metastases and dictates patient's prognosis were, at this stage, unknown. With this in mind, we retrospectively evaluated the surface expression of nine chemokine receptors and integrins on circulating and tumor infiltrating T cells collected from stage III-IV patients (106). These included the expression of CCR6, CCR7, CCR9, CCR10, CXCR3, CXCR4, CXCR5, CLA, and CD103. Moreover, we studied the expression of the chemoattractant receptor-homologous molecule expressed on Th2 cells, CRTH2, known for its involvement in Th2 polarization and responses (129, 130). When comparing these expression levels to those found on circulating T cells from healthy volunteers, patients with a lower expression of CXCR3 and CCR6 on effector/memory circulating T cells had preferential metastases to the skin and lymph nodes and a decrease of CCR9, together with CXCR4 and CXCR5 expression on both $CD4^+$ and $CD8^+$ T cells, which was an indicator of the presence of pulmonary lesions (**Table 3**). In addition, multi-metastatic patients with a broad dissemination of disease displayed an increase of chemokine receptor/integrin expression on naïve T lymphocytes, specifically CCR10, CD103, and CRTH2 (**Table 3**). This disseminated localization was also associated with a loss of CXCR3 on effector/memory T cells and a decrease in CXCR4 and CCR9 expression on CD4 effector and terminal effector T cells (**Table 3**). Collectively, these results indicated that the expression pattern of chemokine receptors/integrins on the surface of circulating T cells potentially mirror the metastatic spreading in melanoma patients (106).

Interestingly, CD103 expression on naïve T cells was strongly associated with liver metastases (106) suggesting that this integrin might play a role in binding T cells to this organ. CD103 expression is a feature of tissue resident memory T lymphocytes (134) and many T lymphocytes that reside in the gut (115) or the liver (135) express this integrin. Its ligand, E-cadherin, is naturally expressed on hepatocytes (136), and notably in the interlobular bile duct epithelia (137). Shimizu et al. have demonstrated that CD103-expressing $CD4^+$ and $CD8^+$ T cells accumulated in the liver and these cells harbored a particular phenotype with a decrease of TCRαβ expression (135). As observed in hepatocellular carcinoma (136, 138), a decrease of E-cadherin expression during epithelial-mesenchymal transition of liver metastasis on the surface of hepatocytes is associated with an increase of its soluble form in the serum (139) potentially favoring the circulation of $CD103^+$ T cells and their accumulation in the blood of melanoma patients harboring liver metastases (**Table 3**).

Further retrospective and prospective investigations are warranted to support the clinical relevance of differences in expression of chemokines and chemokine receptors in melanoma. Their evaluation would likely benefit patients in the early detection of metastases and in targeting specific subsets of T cells to favor their migration to desired organs and to target these metastases. Strategies to modulate their expression and functions are needed in order to ameliorate patient prognosis and therapeutic outcomes.

POTENTIAL FOR TARGETING

Chemokines and their receptors have dual roles in melanoma and other cancers. On one hand, they promote immune cell recruitment necessary for tumor control (e.g., CXCL9/10/11 and CXCR3). On the other hand, they are involved in tumor escape and metastases formation by (i) selectively guiding tumor

TABLE 3 | Chemokine receptors expression at the surface of peripheral immune T cells mirrors the melanoma metastatic dissemination.

Melanoma Stage	Tumor lesion localization	Chemokine receptors and integrins involved
Stage III	Regional cutaneous and lymph node metastases	Decrease of CCR6 and CXCR3 expressions on effector/memory peripheral T cells
Stage IV	Regional cutaneous and lymph node metastases + lung metastases	Reduction of CCR9, CXCR4, and CXCR5 expression on circulating T cells
Stage IV	Multi-disseminated disease with or without lung involvement	Increase expression of CCR10, CD103*, and CRTH2 on naïve peripheral T cells—Loss of CXCR3 and CCR6 expression on effector and memory circulating T cells—Decrease of CXCR4 and CCR9 expression on effector and terminal effector blood T cells

Chemokine receptors expression was retrospectively evaluated on circulating blood T cells collected from 57 stage III–IV melanoma patients (131–133).
**Elevated expression of CD103 on naïve T cells is correlated with the presence of liver metastases.*

cells toward specific organs, which subsequently form secondary lesions (e.g., CCR7 or CXCR4), (ii) favoring the recruitment of immunosuppressive cells (e.g., CCR5) and, (iii) influencing tumor vasculature associated with tumor dissemination (e.g., CXCL10 and CXCR3) (140, 141). Thus, targeting these molecules is of particular interest in melanoma and other cancers as an approach to limit tumor development and to considerably reduce its metastatic spreading. However, the design of selective drugs will need to specifically target tumor cells, the immune system, or both compartments.

Many small molecule antagonists and therapeutic antibodies have been developed (142) but so far, this has led to only a moderate improvement in various diseases. As a consequence, only 3 targeting agents have been approved to treat patients, or are in phase III clinical trials. These include a blocking CCR4 antibody, Mogamulizumab, approved in Japan to treat refractory adult T-cell leukemia, peripheral T cell lymphoma and cutaneous T cell lymphoma (142), an anti-CCR5 antibody tested in graft-vs.-host disease and human immunodeficiency virus-1 (143) and an anti-CXCR4 antibody evaluated in lymphoma and multiple myeloma (144). Thirty-seven additional compounds are currently being tested targeting CCR1, CCR2, CCR3, CCR4, CCR5, CCR9, CXCR1, CXCR2, CXCR4, and CX3C1 (142, 145, 146). In a small study (147), metastatic colorectal cancer patients with $CCR5^+$ liver metastases were treated with a small molecule that antagonizes CCR5, Maraviroc, with encouraging results. Therefore, further evaluation in a larger cohort is warranted to determine the benefits and toxicity of this approach.

In melanoma, CXCR4 inhibition with AMD11070 abrogated tumor cell migration in response to CXCL12 stimulation (148). Similarly, the CXCR4 antagonist, AMD3100, prevents the development of squamous cell carcinomas under chronic UV exposure. Mechanistically, UV radiation induced CXCL12 expression in the skin and this was responsible for attracting $CXCR4^+$ mast cells. Thus, blocking the CXCR4-CXCL12 pathway using this antagonist reduced mast cell infiltration into the skin, tumors and draining lymph nodes, and this subsequently prevents immune suppression and tumor development (149). Given the involvement of CXCR4 in tumor cell migration to many different organs, oral administration of CXCR4 inhibitors could be particularly efficient. Moreover, CXCR4 is also involved in the recruitment of suppressive immune cells, such as mast cells in the tumor microenvironment.

CCR9 blockade using an antibody significantly reduced the tumor cell migration in response to CCL25 stimulation (42). Interestingly, a new mouse anti-human CCR9 antibody was developed by Somovilla-Crespo et al. showing promising results in blocking the growth of human $CCR9^+$ leukemia cells in NSG mice (150). Similarly, the use of the CCR9 antagonist CCX8037 could also specifically interfere with small intestinal dissemination. However, we have shown that the blockade of CCL25 in a sarcoma model inoculated in immunocompetent mice was detrimental and notably, resulted in increasing the tumor growth (106). Further investigations are required to determine the impact of such drugs on both leukocyte trafficking and tumor cell spreading (151) to avoid unexpected off-target effects.

Neonatal skin exposed to UVB induced an IFNγ gene signature response from melanocytes including CCL8 expression (99). Thus accumulation of CCL8 drives the recruitment of $CCR2^+$ macrophages that were shown to promote melanomagenesis. The blockade of IFNγ using a specific antibody or the use of CCR2 deficient mice, which were subjected to UVB exposure, have decreased of macrophages infiltration in the skin and reduced tumor volume (99). Similarly, the overexpression of a dominant negative version of CCL2, a non-functional protein that competes with the native form for binding to CCR2, in melanoma tumor bearing mice specifically reduced tumor associated macrophage infiltration that is associated with a decrease of tumor angiogenesis and tumor growth (98). Interestingly, mice inoculated with B16F10 tumors engineered to express GM-CSF harbored an accumulation of monocytic $CCR2^+$ MDSC compared to non-GM-CSF expressing tumors. This accumulation of MDSC in melanoma lesions was associated with a reduction of $CD8^+$ T cell infiltration and an increase in tumor burden (152). Although vaccination with irradiated B16 cells producing GM-CSF was shown to favor immune responses to immunotherapies in preclinical melanoma models (153, 154), in this setting, this cytokine seemed to play a negative role in antitumor immune surveillance. CCR2 appears to be an attractive target in melanoma and potentially in other tumor types and a CCR2 antibody, plozalizumab, is currently being tested in phase I clinical trial (NCT02723006) in combination with an immune checkpoint blocker, nivolumab.

CRTH2 associated with Th2 responses would be an attractive target in melanoma as this chemokine expression

is increased in patients with a multi-metastatic disease (**Table 3**). CRTH2 is also expressed on eosinophils, basophils, and some monocytes/macrophages (155), immune subsets which all convey a distinct prognosis in melanoma (84, 156). Initially designed for targeting CRTH2$^+$ T cells involved in respiratory diseases (157, 158), CRTH2 antagonists could be indicated in multi-metastatic melanoma patients with high CRTH2 expression.

SX-682 (Syntrix Biosystems, Inc) is a selective and potent CXCR1/2 antagonist. CXCR1/2 is expressed on melanoma cells, MDSC and neutrophils and sustains tumor immunosuppression, tumor growth, angiogenesis and tumor dissemination in response to CXCL1, CXCL2 or CXCL8 (107, 108, 159–164) (**Tables 1**, **2**). In melanoma, MDSC accumulated both in tumor lesions and in periphery, correlating with tumor stage. This feature has been associated with a negative prognostic value (84). Furthermore, this compound has been evaluated in combination to anti-CTLA-4 and anti-PD-1 co-blockade in an elegant mouse model of prostate cancer (165). In this model, the authors demonstrated the crucial role of MDSC in sustaining cancer progression. The combination of immune checkpoint inhibitors and SX-682 resulted in decreased prostate mass, lymph node and lung metastases (165). This inhibitor is currently being evaluated in stage III/IV melanoma patients in combination with an anti-PD1 antibody, Pembrolizumab (NCT03161431). This phase I study aims to evaluate the tolerability and safety profile of SX-682 together with the response rate, tumor response duration, progression free and overall survival of the combination. Interestingly, another CXCR1/2 inhibitor, Ladarixin, was shown to significantly reduce human melanoma cell motility and to induce apoptosis *in vitro*. *In vivo* treatment of melanoma xenografts with Ladarixin reduced tumor growth, polarized intratumoral macrophages to M1 phenotype, and inhibited angiogenesis (166). Inhibition of CXCR1/2 appears to be very promising as it targets both melanoma and immune cells, reducing tumor burden alone or in combination with immune checkpoint blockers.

Modulation of chemokine receptor expression on the surface of chimeric antigen receptor (CAR) T or NK cells prior to infusion is promising as this would enhance their tumor infiltration and potentially improve therapeutic results. CX3CR1 genetically modified T cells transferred into CX3CL1 producing colorectal adenocarcinoma tumor bearing mice displayed enhanced tumor infiltration and anti-tumor responses (167). Moreover, significant reduction in tumor size and complete remission have been observed with CCR2b-GD2-CAR T cells and CXCR4-EGFRvIII-CAR NK cells infused in mice bearing CCL2 producing GD2 neuroblastoma or CXCL12 secreting EGFRvIII glioblastoma cells, respectively (168, 169). Similarly, genetically engineered CCR2 expression on CAR T cells directed to the tumor antigen mesothelin increased tumor cell infiltration and anti-tumor responses against large and established tumors inoculated in severe immunodeficient mice (170). To date, CAR specific cells genetically engineered to express particular chemokine receptor have only been tested in preclinical models. Despite having shown impressive anti-tumor responses against primary tumors, it will be challenging to find a chemokine that is highly, specifically and commonly expressed across different tumor microenvironments, found in multi metastatic patients in order to efficiently eradicate all disseminated lesions.

CONCLUSION AND PERSPECTIVES

Chemokines and chemokine receptors are key molecules involved in cell migration, proliferation and survival that are critical in maintaining tissue homeostasis. Melanoma cells overexpress many chemokine receptors that are likely involved in cancer progression and metastasis. Thus, modulation of chemokines and chemokine receptors appears to be an attractive target in cancer therapy. However, targeting them is a double edged sword, as treatments will not only affect immune cell migration to tumor lesions or tumor dissemination but also in the long term, impact immune cell development and polarization (e.g., CXCR4). This may partly explain why there is low number of approved drugs targeting chemokines and their receptors in treating chronic diseases, such as cancer. How can we overcome this? In the era of personalized medicine, designing bispecific antibodies that can specifically target a chemokine receptor and a tumor antigen, which are both expressed on the surface of cancer cells is highly attractive. However, antigen escape due to the emergence of tumor variants, which do not express the targeted antigen, are likely to emerge, rendering the treatment ineffective. Another promising area of research is to combine chemokine receptor blockers with anti-PD-1 or anti-CTLA-4 antibodies to further improve the clinical activity of these antibodies and thus further increase patient survival (171). Together, this would lead to reduced tumor infiltration by immunosuppressive cells as Tregs or MDSCs and subsequently, induce anti-tumor immunity by releasing the immunosuppressive brakes. Another approach would be to use engineered antibodies to target privileged metastatic sites. The therapeutic management of brain metastases in melanoma and other cancers is challenging, as the brain is protected by a highly selective blood-brain barrier impermeable to many cells, in particular, immune cells. In melanoma, a bispecific antibody could be designed to target CCR4 and a nanobody, that selectively binds to human cerebromicrovascular endothelial cells. This attached nanobody is then internalized and able to transmigrate across the endothelial barrier (146). As a proof of principle, a bispecific antibody specific for the metabotropic glutamate receptor 1, expressed in the brain, and also carrying a specific nanobody was able to translocate across the endothelial layer into the brain and regulate physiological functions (172).

Given the association between the accumulation of certain chemokines in tumor lesions and the presence of tertiary lymphoid structures, it would be interesting to reinstate chemokine expression in "cold" tumors to favor the emergence of ectopic-like lymphoid organs that are positively associated with immune cell activation and patient survival. Several strategies are currently being tested, aiming to modulate anti-tumor responses through the induction of tertiary lymphoid structures (173).

Collectively, chemokine and chemokine receptors are essential for guiding immune cells to tumor lesions, however

melanoma cells often harness these molecules to disseminate to distant organs. Given their broad expression profile and potential side effects, drugs targeting these molecules must be carefully designed. Novel technologies have now rendered this challenge possible with the development of compounds that specifically affect a desired target (145, 146). Many chemokine receptor antagonists are currently being tested in melanoma and other malignancies, if successful, these treatments will diversify the oncologic armamentarium currently available therefore increasing possible therapeutic combinations and ultimately improving patient outcome.

AUTHOR CONTRIBUTIONS

NJ wrote the initial draft. CD, GB, and LZ made substantial contributions to discussions of the content. All authors reviewed and/or edited the manuscript prior submission.

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Notch-Mediated Tumor-Stroma-Inflammation Networks Promote Invasive Properties and CXCL8 Expression in Triple-Negative Breast Cancer

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Stromal cells and pro-inflammatory cytokines play key roles in promoting the aggressiveness of triple-negative breast cancers (TNBC; Basal/Basal-like). In our previous study we demonstrated that stimulation of TNBC and mesenchymal stem cells (MSCs) co-cultures by the pro-inflammatory cytokine tumor necrosis factor α (TNFα) has led to increased metastasis-related properties *in vitro* and *in vivo*. In this context, elevated release of the pro-metastatic chemokines CXCL8 (IL-8) and CCL5 (RANTES) was noted in TNFα- and interleukin-1β (IL-1β)-stimulated TNBC:MSC co-cultures; the process was partly (CXCL8) and entirely (CCL5) dependent on physical contacts between the two cell types. Here, we demonstrate that DAPT, inhibitor of γ-secretase that participates in activation of Notch receptors, inhibited the migration and invasion of TNBC cells that were grown in "Contact" co-cultures with MSCs or with patient-derived cancer-associated fibroblasts (CAFs), in the presence of TNFα. DAPT also inhibited the contact-dependent induction of CXCL8, but not of CCL5, in TNFα- and IL-1β-stimulated TNBC:MSC/CAF co-cultures; some level of heterogeneity between the responses of different TNBC cell lines was noted, with MDA-MB-231:MSC/CAF co-cultures being the most sensitive to DAPT. Patient dataset studies comparing basal tumors to luminal-A tumors, and mRNA analyses of Notch receptors in TNBC and luminal-A cells pointed at Notch1 as possible mediator of CXCL8 increase in TNFα-stimulated TNBC:stroma "Contact" co-cultures. Accordingly, down-regulation of Notch1 in TNBC cells by siRNA has substantially reduced the contact-dependent elevation in CXCL8 in TNFα- and also in IL-1β-stimulated TNBC:MSC "Contact" co-cultures. Then, studies in which CXCL8 or p65 (NF-κB pathway) were down-regulated (siRNAs; CRISPR/Cas9) in TNBC cells and/or MSCs, indicated that upon TNFα stimulation of "Contact" co-cultures, p65 was activated and led to CXCL8 production mainly in TNBC cells. Moreover, our findings indicated that when tumor cells interacted with stromal cells in the presence of pro-inflammatory stimuli, TNFα-induced p65 activation has led to elevated Notch1 expression and activation,

which then gave rise to elevated production of CXCL8. Overall, tumor:stroma interactions set the stage for Notch1 activation by pro-inflammatory signals, leading to CXCL8 induction and consequently to pro-metastatic activities. These observations may have important clinical implications in designing novel therapy combinations in TNBC.

Keywords: cancer-associated fibroblasts, CXCL8, interleukin 1β, mesenchymal stem cells, notch1, p65, triple-negative breast cancer, tumor necrosis factor α

INTRODUCTION

The triple-negative subtype of breast cancer (TNBC), which in gene signature studies is often used as a surrogate for the "Basal/Basal-like" subgroup (e.g., in PAM50 analyses), accounts for ∼15% of breast cancers. TNBC cells are negative for the expression of type α estrogen receptors, progesterone receptors or amplified HER2; thus, TNBC tumors do not respond to receptor-targeted therapies, and following chemotherapy they are most likely to recur (1–3). These clinical parameters emphasize the ultimate need for improved understanding of the mechanisms leading to tumor progression in this aggressive subtype of disease.

Key roles in regulating tumor progression in TNBC are attributed to elements of their surrounding tumor microenvironment (TME) (4, 5). We and others have investigated the interactions of TNBC cells with TME elements which promote tumor development and metastasis-related functions in TNBC: (1) Stromal cells such as mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs) that enhance TNBC progression by releasing pro-angiogenic factors and additional tumor-promoting mediators (6–15); (2) Pro-inflammatory cytokines, particularly tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β) - or their signaling-related components which are expressed in TNBC tumors - and promote the aggressiveness profile of TNBC cells (16–25).

Because information is lacking on the outcomes of TNBC interactions with stromal cells in the context of pro-inflammatory signals, we have studied in a companion research (26) the effects of tumor-stroma-inflammation networks on pro-metastatic processes in TNBC. We demonstrated that TNFα stimulation of TNBC:MSC "Contact" co-cultures has led to enhanced migration and invasion of TNBC cells, to increased angiogenesis and to higher metastatic potential of the tumor cells *in vivo*. Moreover, TNFα- and IL-1β-stimulated TNBC:MSC/CAF co-cultures released elevated levels of CXCL8 and CCL5, identified as pro-metastatic chemokines in TNBC (27–36). The release of CXCL8 by TNFα- and IL-1β-stimulated TNBC cells grown with MSCs/CAFs was partly dependent on the exchange of soluble factors between the TNBC cells and the stromal cells, but also required direct physical contacts between these two cell types. In contrast, induction of CCL5 in TNFα- and IL-1β-stimulated TNBC:MSC co-cultures was entirely dependent on cell-to-cell contacts. Of importance, CXCL8 induction in the context of TNFα-stimulated TNBC:MSC "Contact" co-cultures was significantly involved in mediating the increased metastasis-related phenotypes of TNBC cells; this included induction of angiogenesis, as well as of the migratory and invasive properties of tumor cells.

These findings provided novel insights to processes controlling TNBC aggressiveness, and have led us to investigate in the current study the mechanisms involved in such tumor-stroma-inflammation networks. Here, we were specifically out to unravel the regulation of processes that necessitated physical contacts between the tumor cells and stromal cells. Along these lines, we focused on the potential roles of the Notch pathway in controlling the tumor-stroma-inflammation networks we have identified in TNBC (26).

The Notch pathway regulates differentiation, proliferation, and cell death through direct cell-to-cell signaling (37–40). Following receptor-ligand interactions, a series of proteolytic cleavages in the Notch receptor lead to the γ-secretase-dependent release of the Notch intracellular domain (NICD); NICD then translocates to the nucleus, where it forms an activator complex that regulates the transcription of target genes controlling various regulatory and functional programs (37–40). In breast cancer, particularly in TNBC, increasing evidence indicates that Notch family members are ultimate contributors to cancer stem cell maintenance, invasion, angiogenesis and recurrence (41–45). However, the Notch pathway was not explored so far for its involvement in regulating inflammation-driven TNBC-stroma interactions.

Thus, in this study we investigated the roles of Notch receptors in regulating such cross-talks using the research system we have described in our accompanying study (26). We now demonstrate that the Notch pathway is a prime regulator of tumor cell invasiveness in the tumor-stroma-inflammation setting. Also, our findings indicate that NF-κB-induced Notch1 activation is a key regulator of inflammation-driven TNBC-stromal contacts that lead to elevated release of the pro-metastatic chemokine CXCL8; as we have shown before, CXCL8 then contributed to elevated angiogenesis, tumor cell migration and tumor cell invasion in the tumor-stroma-inflammation network in TNBC (26). Together, our findings point at complex control mechanisms that are governed by the NF-κB and Notch pathways in the setting of TNBC-stroma-inflammation triage that promotes TNBC progression, and may have clinical implications.

MATERIALS AND METHODS

Breast Tumor Cell Lines and Stromal Cells

The TNBC human MDA-MB-231, MDA-MB-468 and BT-549 cells (ATCC), and human luminal-A MCF-7 cells (ATCC) were

grown as previously described (26). Human bone marrow-derived MSCs from three different healthy donors were purchased from Lonza (#PT-2501; Walkersville, MD) and were grown for up to 10 passages, as previously described (26). CAFs that were isolated from patients' breast tumors (from a primary tumor in ELISA studies, and from a lung metastasis in tumor cell invasion studies), were immortalized and grown as described in (6) (Kindly provided by Dr. Bar, Sheba Medical Center, Ramat Gan, Israel).

TNFα and IL-1β Concentrations Used in Different Analyses

Titration analyses of cytokine stimulation were performed as described in the accompanying study (26). To follow up on those studies, recombinant human (rh) TNFα (#300-01A, PeproTech, Rocky Hill, NJ) and rhIL-1β (#200-01B, PeproTech) were used in the following concentrations: MDA-MB-231 cells, MCF-7 cells, MSCs and CAFs: TNFα 10 ng/ml, IL-1β 350 pg/ml; MDA-MB-468 cells: TNFα 50 ng/ml, IL-1β 500 pg/ml; BT-549 cells: TNFα 25 ng/ml, IL-1β 350 pg/ml.

Tumor Cell Migration and Invasion

In migration assays, mCherry-expressing MDA-MB-231 cells were added to the upper part of transwells (8-μm pore membranes; #3422, Corning, NY) together with MSCs (ratio 10:1). The cells were stimulated by TNFα, and were treated by DAPT [10 μM; (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester]; #565770; Calbiochem, Merck, Darmstadt, Germany) or by its vehicle control (Dimethylsulfoxide, DMSO; #D5879; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Migration assays toward medium with 10% FBS were performed for 12 h. Then, cells at the upper side of the membranes were removed, the membranes were fixed in ice-cold methanol and stained with Hemacolor (#1.11661; Merck, Darmstadt, Germany). Photos of multiple high power fields were taken in bright fields and in fluorescent fields at ×100 magnification. After verifying that the transmigrating cells expressed mCherry (and thus were tumor cells), the cells at the lower side of the membranes were counted in multiple Hemacolor fields.

In invasion assays, 3D multicellular spheroids of defined size and cell number were formed by co-culturing of mCherry-expressing MDA-MB-231 and MSCs/CAFs (ratio 10:1) in hanging drops [as in Korff et al. (46), with minor modifications] for 72 h. These experiments were performed in the presence of DAPT (10 μM) or control DMSO. The spheroids were then embedded in matrigel (9–10.5 mg/ml; #356234, Corning, Bedford, MA) and stimulated by TNFα (10 ng/ml) with fresh DAPT (10 μM) or control DMSO. Invasion of mCherry-tumor cells out of spheroids that were formed with MSCs was determined after 48 h or 96 h with CAFs. Multiple MDA-MB-231:MSC spheroids were photographed in fluorescent fields at ×40 magnification, and the invaded areas were determined by mCherry signals of cells that invaded out of spheroid cores, quantified by ImageJ. In parallel, many MDA-MB-231:CAF spheroids were photographed in fluorescent fields and in bright fields at ×100 magnification. Quantification by ImageJ was performed in fluorescent fields at ×40 magnification.

Cell Stimulation for ELISA Assays

TNBC cells were grown together with MSCs/CAFs (10:1 ratio) in "Contact" conditions (in which the two cell types could form physical contacts) in 6-well plates (#3516, Corning, Kennebunk, ME); in parallel, similar cell concentrations were used to generate "Transwell" conditions (in which the two cell types could only exchange soluble materials between them) in 6-well plates, with an insert of 0.4 μm permeable polycarbonate membrane (#3412, Corning). In the studies demonstrated in **Supplementary Figures 2A,B**, separate cultures of TNBC cells and MSCs were also included, grown individually in the same cell numbers as in co-cultures. Co-cultured cells, and individual cell types (when appropriate) were grown in media containing 10% FBS for 12 h and were then stimulated by TNFα or IL-1β in media containing 0.5% FBS for 7 h. Following removal of cytokine stimulation, cytokine-free media supplemented with 0.5% FBS were added for additional 60 h. Then, conditioned media (CM) were removed and cleared by centrifugation, and CXCL8 and CCL5 extracellular levels were determined by ELISA, using standard curves at the linear range of absorbance. To this end, rhCXCL8 (#200-8M, PeproTech), and antibodies (Abs) to CXCL8 were used (Coating Abs: #500-P28. Detecting Abs: #500-P28Bt; PeproTech). In parallel, CCL5 levels were detected by using rhCCL5 (#300-06; PeproTech) and Abs to CCL5 (Coating Abs: #500-M75; PeproTech. Detecting Abs: #BAF278; R&D Systems, Inc., Minneapolis, MN). Following the addition of HRP-conjugated Streptavidin (#016-030-084; Jackson Immunoresearch laboratories, PA) and substrate TMB/E solution (#ES001; Millipore, Temecula, CA), the reaction was stopped by addition of 0.18 M H_2SO_4. Absorbance was measured at 450 nm.

When indicated, cell cultures were treated by DAPT (10 μM) or by its vehicle control (DMSO); Down-regulation of CXCL8 and NOTCH1 expression by siRNA was introduced in other experiments, as detailed below. CM that were collected from such co-cultures were analyzed for CXCL8 and/or CCL5 expression by ELISA, as detailed above.

Analyses of Patient Datasets

The cancer genome atlas (TCGA) dataset of breast cancer patient (47) was used for RNAseq-based gene expression analyses. The TCGA dataset contained samples of 821 patients: Basal (often overlapping the term TNBC): 141 patients; Luminal-A: 421 patients; Luminal-B: 192 patients; HER2+: 67 patients. The PAM50 annotation file provided within the dataset was used to define disease subtypes. Log2-transformed expression values of NOTCH1, NOTCH2, NOTCH3 and NOTCH4 by subtypes, were presented as boxplots. Statistical analyses were performed based on Shapiro-Wilk normality test, for each gene by subtype. Kruskal-Wallis test with correction for multiple comparisons using the Benjamini-Hochberg procedure controlling the false discovery rate (FDR), was used for comparison of expression levels between the different subtypes. Log2-transformed co-expression levels of NOTCH1 or NOTCH2 with genes of interest

were determined in basal patients and in luminal-A patients, and were outlined as scatter plots. In NOTCH1 studies, the centroid of the scatter plot (determined by the average values of NOTCH1 and of the second analyzed gene) in luminal-A patients was used to set rectangles demonstrating the shift in basal patients.

Quantitative Real-Time PCR Analyses

MDA-MB-231 and MCF-7 cells were co-cultured with MSCs (10:1 ratio) under "Contact" conditions; in parallel, when appropriate, each of the cell types was grown alone. Then, the co-cultures/cells were stimulated by TNFα or IL-1β for 7 h in media containing 0.5% FBS. Similar procedures were performed using MDA-MB-231 cells that were subjected to gene down-regulation by siRNA and/or CRISPR/Cas9, as detailed below. Total RNA was isolated using the EZ-RNA kit (#20-400; Biological Industries, Beit Ha'emek, Israel) for quantitative real-time PCR (qRT-PCR) analyses. The M-MLV reverse transcriptase (#AM2044; Ambion, Austin, TX or #95047; Quantabio, Beverly, MA) was used to generate first-strand cDNA from RNA samples. cDNA targets were quantified on Rotor Gene 6000 (Corbett Life Science, Concorde, NSW, Australia) or on CFX Connect real time PCR Detection System (Bio-Rad, Hercules, CA). To detect transcripts, absolute Blue qPCR SYBR Green ROX mix (#AB-4163/A; Thermo Fisher Scientific, Waltham, MA) was used, according to manufacturer's instructions. The housekeeping gene GAPDH was used for data normalization. For each primer set, dissociation curves indicated a single product and "no-template" controls were negative after 40–45 cycles used for analysis. Analyses were performed by standard curves, within the linear range of quantification. The sequences of the primers are provided in **Supplementary Table 1**.

Western Blot Analyses

MDA-MB-231:MSC "Contact" co-cultures (ratio 10:1), or each of the cell types alone (when appropriate) were stimulated for 15 min or 7 h by TNFα, IL-1β or vehicle control, in media containing 0.5% FBS. Similar procedures were taken when the co-cultures were subjected to gene down-regulation by siRNA or CRISPR/Cas9, as detailed below. Following lysis in RIPA buffer, conventional Western blot (WB) procedures were taken. To detect p65 expression and activation, the following Abs were used: Total (T)-p65: #8242 [Cell Signaling Technology (CST), Danvers, MA]; Phosphorylated (P)-p65: #3033 (CST). To detect Notch1 expression and activation, the following Abs were used: Full length Notch1: #3608 (CST); Notch1 intracellular domain (N1-ICD; N1-ICD appeared as single band or two bands, probably due to technical reasons): #4147, reacting specifically with cleaved Notch 1 (directed to Val1744; CST). GAPDH (#ab9485, Abcam, Cambridge, UK), served as a loading control. The membranes were reacted with streptavidin-horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (#111-035-003) or HRP-conjugated goat anti-mouse IgG (#115-035-071), as appropriate (both from Jackson ImmunoResearch Laboratories). The membranes were subjected to enhanced chemiluminescence (#20-500, Biological Industries).

Knocking-Down and Knocking-Out Target Genes

Knock-down (KD) of CXCL8, p65 (RELA) and NOTCH1 by transient siRNA transfections was performed in MDA-MB-231 cells and/or MSCs, as appropriate, using the Lipofectamine RNAiMAX transfection reagent (#56531; Invitrogen, Grand Island, NY) according to manufacturer's instructions. The following ON-TARGET plus siRNA SMART pools were used (all from Dharmacon, Lafayette, CO): Human CXCL8: #L-004756-00; p65: #L-003533-00; NOTCH1: #L-007771-00. siRNA control was introduced by ON-TARGET plus non-targeting control siRNA pool (#D-001810-10). After 24 h, the cells were used in assays, as necessary. Down-regulation of CXCL8 was validated by ELISA or qRT-PCR, as appropriate; p65 and Notch1/N1-ICD down-regulation was validated by WB.

Knock-out (KO) of p65 (RELA) in MDA-MB-231 cells was introduced by lentiviral infection using the clustered regularly-interspaced short palindromic repeats associated protein endonuclease 9 (CRISPR/Cas9) system [as in Danziger et al. (48)]. Two different single guide RNAs targeting p65 were used (sgRNA1: 5′-AGCGCCCCTCGCACTTGTAG-3′; sgRNA2: 5′-CAAGTGCGAGGGGCGCTCCG-3′). Validation of p65 KO in single clones was determined by WB; then, 3 different single cell clones (2 clones expressing sgRNA1 and 1 expressing sgRNA2) were selected to generate a KO-p65-MDA-MB-231 cell pool. In parallel, strand targeting green fluorescent protein (GFP) (5′-GGGCGAGGAGCTGTTCACCG-3′) was used to generate control KO-GFP-MDA-MB-231 cell pool, as above. Efficiency of p65 KO of pooled cells was determined by WB.

Statistical Analyses

The statistical analyses of the TCGA patient dataset were described in their respective section. *In vitro* experiments were performed in $n \geq 3$ independent experimental repeats, with MSCs from ≥2 different donors, as indicated in respective figure legends. The results of ELISA, qRT-PCR, WB, migration and invasion assays were compared by two-tailed unpaired Student's *t*-test. Values of $p \leq 0.05$ were considered statistically significant. Adjustment for multiplicity of comparisons was done using the Benjamini-Hochberg procedure controlling the FDR at 0.05. All the significant results remained statistically significant after correcting for their multiplicity, except for some of the WB results in **Figure 9**. In these latter cases lack of significance was due to high variance between the intensities of effects of the experimental repeats of the test, despite the fact that they all demonstrated the same trend.

RESULTS

DAPT Inhibits the High Migratory and Invasive Properties Acquired by TNBC Cells Following Their Interaction With Stromal Cells in the Context of Pro-inflammatory Stimulation

In our previous study, we demonstrated that MDA-MB-231 TNBC cells acquired an increased migratory and invasive

potential following their interactions with MSCs and CAFs, in the presence of TNFα (26). To determine if the Notch pathway regulates these processes, TNFα-stimulated MDA-MB-231:MSC and MDA-MB-231:CAF co-cultures were established and migration and/or invasion assays were performed in the presence or absence (control DMSO-treated cells) of DAPT, a potent inhibitor of γ-secretase that participates in the activation of all Notch receptors (49–51).

The findings of **Figure 1A** indicate that the migration of mCherry-MDA-MB-231 cells that interacted with MSCs in the presence of TNFα was markedly inhibited by DAPT (mCherry signals, showing that the migrating cells were tumor cells, are demonstrated in **Supplementary Figure 1**). Moreover, much of the invasive advantages that were endowed to the tumor cells by their co-culturing with MSCs in the context of TNFα stimulation (26), were inhibited by DAPT (**Figure 1B**). In parallel, in TNFα-stimulated spheroids of co-cultured MDA-MB-231 cells with breast cancer patient-derived CAFs, reduced ability to invade was revealed upon DAPT treatment (**Figure 1C2**); in addition, a marked change in the invasion pattern was noted after inhibition of the Notch pathway: The organized and directional motility of control cells (untreated by DAPT) has diverted into a disordered and non-orchestrated phenotype in the presence of DAPT (**Figure 1C1**).

DAPT Inhibits the Contact-Dependent Induction of CXCL8, but Not of CCL5 in TNBC:Stroma Co-cultures Stimulated by Pro-inflammatory Cytokines

In our companion study (26) we demonstrated that TNFα and IL-1β stimulation of TNBC:MSC "Contact" co-cultures has led to exacerbated release of CXCL8 and CCL5, more than in non-stimulated "Contact" co-cultures, in cytokine-stimulated/non-stimulated individual cells and in "Transwell" co-cultures (for readers' convenience, **Supplementary Figures 2A,B** demonstrate the entire panel of cells and stimulations that was provided in our previous study for CXCL8 and CCL5, respectively; different experiments are demonstrated in the two papers). We also found that the induction of CXCL8 was mediated by physical contacts between the two cell types as well as by exchange of soluble factors between them, whereas the induction of CCL5 was entirely contact-dependent [(26); **Supplementary Figures 2A,B**].

To investigate the roles of the Notch pathway in regulating the contact-dependent process of CXCL8 and CCL5 induction in our system, "Contact" and "Transwell" TNBC:MSC co-cultures were stimulated by TNFα in the presence of DAPT or its DMSO control; then, CXCL8 and CCL5 levels in TNFα-free CM were determined (as described in "Materials and methods"). Here, we focused on the ability of DAPT to inhibit the amount of CXCL8 and CCL5 added to "Contact" conditions compared to "Transwell" conditions, as this increment in chemokine release (**Figure 2A** and **Supplementary Figure 2A**; for CXCL8: above the dashed lines) was due to physical contacts that were formed between the two cell types.

The results of **Figure 2A1** indicate that DAPT caused pronounced inhibition of the contact-dependent increase in CXCL8 (above the dashed line), when MDA-MB-231 cells interacted with MSCs in the presence of TNFα stimulation, and also without TNFα stimulation. To follow up on reports on high heterogeneity of TNBC cells (3), our analyses of two additional TNBC cell lines (**Supplementary Table 2**) demonstrated less pronounced effects of DAPT on TNFα-stimulated MDA-MB-468:MSC "Contact" co-cultures, and no consistent effects of DAPT on the responses of BT-549:MSC "Contact" co-cultures stimulated by TNFα.

Additional experiments have further supported the roles of the Notch pathway in up-regulating CXCL8 expression by TNBC-stroma-inflammation networks: First, similar to MDA-MB-231:MSC co-cultures stimulated by TNFα (**Figure 2A1**), DAPT has inhibited CXCL8 elevations in TNFα-stimulated co-cultures of MDA-MB-231 cells with patient-derived CAFs (**Figure 2A2**). Second, analyses that were performed on IL-1β-stimulated co-cultures of TNBC cells (MDA-MB-231, MDA-MB-468, BT-549) with MSCs and/or CAFs (**Supplementary Figure 3A**; **Supplementary Table 2**) demonstrated generally a similar pattern to the findings obtained by TNFα stimulation.

In contrast to this mode of regulation in CXCL8, the contact-dependent process of CCL5 induction in TNFα-stimulated MDA-MB-231:MSC and in MDA-MB-231:CAF co-cultures, was not affected by DAPT treatment (**Figure 2B**). Of note, parallel experiments that were performed with IL-1β-stimulated MDA-MB-231:MSC and MDA-MB-231:CAF co-cultures have shown similar findings to those with TNFα (**Supplementary Figure 3B**).

In TNFα-Stimulated TNBC:MSC Co-cultures, Mainly TNBC Cells but Also MSCs, Contribute to Elevations in CXCL8 Expression, Through a p65-Depenent Process

Following the above observations, we set to determine the molecular mechanisms regulating CXCL8 expression in the tumor-stroma-inflammation network, and to reveal the roles and regulation of Notch receptors in this setting. We began this part of the study by asking which of the two cell types, the MSCs and/or the tumor cells, contribute/s to CXCL8 expression in TNFα-stimulated MDA-MB-231:MSC "Contact" co-cultures. In addition, in view of our previous observations on p65 activation by TNFα and IL-1β in both cell types [(26); **Supplementary Figure 2C**] we asked if p65 controls CXCL8 transcription, and in which of the two cell types this regulation takes place.

To determine the cellular source of CXCL8, the expression of the chemokine was knocked-down by siRNA in MDA-MB-231 cells, in MSCs or in both cell types together during the co-culture process, in "Contact" conditions. Then, we determined the levels of CXCL8 produced with and without TNFα stimulation (**Supplementary Figure 4A** demonstrates high efficacy of CXCL8 down-regulation in both cell types). When MDA-MB-231:MSC co-cultures were established without TNFα stimulation, the tumor cells were almost the exclusive

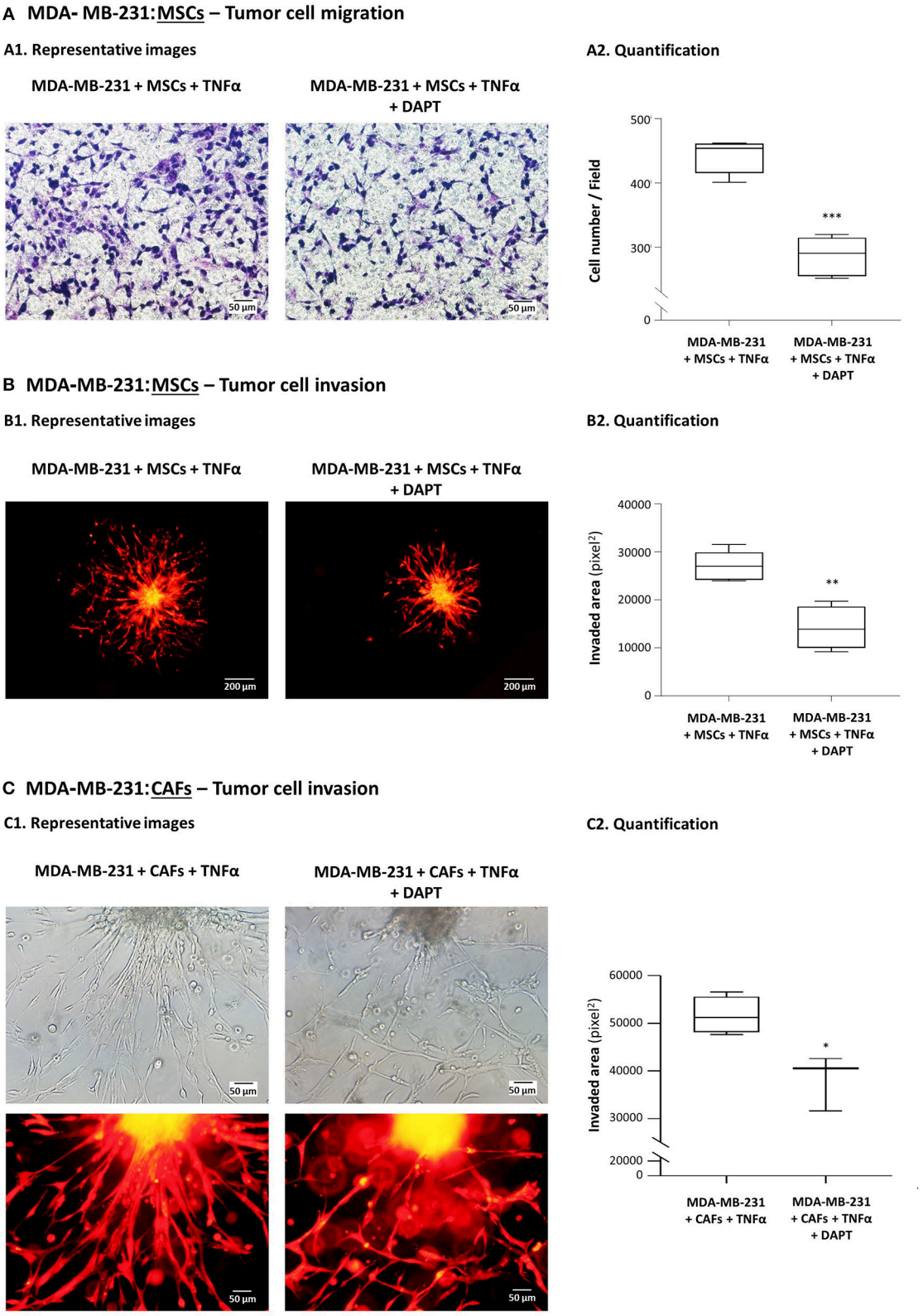

FIGURE 1 | DAPT inhibits the migratory and invasive properties gained by TNBC cells following their interactions with MSCs in the presence of TNFα stimulation. **(A)** Tumor cell migration. mCherry-MDA-MB-231 cells and MSCs were cultured together in migration transwells in the presence of TNFα (10 ng/ml), with DAPT (10 μM) or with its vehicle control (DMSO) in serum-free media. Tumor cell migration was determined toward medium containing 10% FBS, after 12 h.

(Continued)

FIGURE 1 | Comparisons of migration of MDA-MB-231 cells following interactions with MSCs and TNFα stimulation to migration of the tumor cells grown in control conditions (without MSCs and TNFα) were presented in our previous study (26). In the current Figure: **(A1)** Representative photos (Bar, 50 μm) and **(A2)** quantifications of multiple photos by ImageJ are provided. ***$p < 0.001$. The photos and their quantifications are representatives of $n = 3$ independent experiments, performed with MSCs of 2 different donors. Parallel photos taken by fluorescence microscope indicated that migrating cells expressed mCherry, and thus consisted of tumor cells (**Supplementary Figure 1**). **(B,C)** Tumor cell invasion out of matrigel-embedded 3D spheroids. Spheroids containing mCherry-expressing MDA-MB-231 cells together with MSCs **(B)** or with breast cancer patient-derived CAFs **(C)** were formed in the presence of DAPT (10 μM) or its vehicle (DMSO). Then, spheroids were embedded in matrigel, were stimulated by TNFα (10 ng/ml) and supplemented with fresh DAPT (10 μM) or DMSO. Comparisons of invasion of MDA-MB-231 cells following interactions with MSCs and TNFα stimulation to invasion of the tumor cells grown in control conditions (without MSCs and TNFα) were presented in our previous study (26). In the current Figure: **(B1,C1)** Representative photos (Bar: 200 μm in **B1**, 50 μm in **C1**) and **(B2,C2)** quantifications of multiple photos by ImageJ are provided. **$p < 0.01$, *$p < 0.05$. The photos and their quantifications are representatives of $n > 3$ independent experiments, in Part **(B)** performed with MSCs of 2 different donors.

source for the chemokine (**Figure 3A1**). However, following stimulation by TNFα, the equilibrium between the two cell types was changed: not only the tumor cells but also MSCs - although at lower levels - contributed to the elevation in CXCL8 expression by the cytokine-stimulated co-cultures (**Figure 3A2**). Similar findings were noted following IL-1β-induced TNBC:MSC stimulation, as demonstrated in **Supplementary Figure 5**.

Next, p65 was knocked-down in the MSCs by siRNA and was knocked-out in the tumor cells by CRISPR/Cas9, leading to efficient reduction in p65 expression and activation in both cell types (**Supplementary Figures 4B,C**; although p65 activation was not down-regulated completely in MSCs, it was sufficient to clearly reveal the mechanistic roles of p65 in the studied processes, as shown below). The data of **Figure 3B** indicate that in MDA-MB-231:MSC "Contact" co-cultures that had not been stimulated by TNFα, p65 basal activation in the tumor cells was the sole inducer of CXCL8 expression. However, in TNFα-stimulated co-cultures, p65-regulated CXCL8 expression was partially contributed by MSCs, even though most of the p65-induced CXCL8 expression was contributed by the tumor cells (**Figure 3B2**). Most importantly, when p65 was down-regulated in both cell types together, almost no CXCL8 was produced by the "Contact" co-cultures indicating that p65 was the master regulator of CXCL8 expression in this setting of the tumor-stroma-inflammation network.

Analyses of Notch Receptors Point at Notch1 as Possible Regulator of CXCL8 Induction in the Tumor-Stroma-Inflammation Networks in TNBC

To provide additional molecular insights into processes that possibly connect Notch receptors to CXCL8 induction in the TNBC-stroma-inflammation network, we next assessed the potential relevance of each of the four human Notch receptors to the research systems of our study. First, in view of our previous findings demonstrating that the tumor-stroma-inflammation network is more effective in TNBC than in the less aggressive subtype of breast cancer, luminal-A (26), we asked if the expression of any specific Notch receptor is significantly elevated in TNBC tumors compared to the luminal-A tumors. We also compared the expression of Notch receptors in basal tumors to luminal-B and HER2+ tumors, in order to identify Notch receptors whose elevated expression signifies more clearly the basal subtype of breast cancer. Using the TCGA breast cancer dataset, we found that the expression of NOTCH1 was significantly higher in basal tumors than in all other subtypes of disease (**Figure 4A1**). In contrast, NOTCH2 expression levels were similar in basal and luminal-A tumors (**Figure 4A2**), and NOTCH3 was similarly expressed in basal tumors and HER2+ tumors (**Figure 4A3**). Of note, NOTCH4 expression was lower in basal tumors than in tumors of the less aggressive luminal-A subtype and of the HER2+ subtype (**Figure 4A4**).

We next determined in TNFα-stimulated MDA-MB-231:MSC "Contact" co-cultures the levels of Notch receptors. Here, we found that NOTCH1 and NOTCH2 mRNA levels were significantly elevated by TNFα stimulation, whereas the levels of NOTCH3 were somewhat reduced (**Figure 4B1**; Based on the TCGA results, NOTCH4 was not analyzed). Unlike these findings on NOTCH1 and NOTCH2 regulation by TNFα in TNBC:MSC co-cultures, TNFα stimulation of luminal-A MCF-7:MSC "Contact" co-cultures did not lead to NOTCH1 and NOTCH2 elevations (**Figure 4B2**). Cell-specific analyses complemented these results by indicating that NOTCH1, NOTCH2 and NOTCH3 were up-regulated by TNFα in the tumor cells but not in the MSCs (**Figure 5**). Of note, the findings described above - mainly those on NOTCH1 up-regulation following TNFα stimulation of MDA-MB-231:MSC "Contact" co-cultures and of each cell type alone - were in general similarly produced by IL-1β stimulation (**Supplementary Figure S6**).

To follow up on these findings, suggesting that Notch1, and possibly also Notch2 may be connected to the cytokine-stimulated integrative system that we study in TNBC, we next used the TCGA dataset to analyze the relevance of Notch1 and Notch2 to the tumor-stroma-inflammation network. To this end, we determined the co-expression patterns of NOTCH1/NOTCH2 with two major players in this setting: TNFα and its target, CXCL8. Specifically, we asked which of the Notch receptor-related co-expression patterns would dissociate the basal subtype from the luminal-A subtype. To this end, the expression levels of NOTCH1 and TNFα in each individual patient tumor were plotted, in both groups of patients. The findings of **Figure 6A1** indicate that in general, basal tumors co-expressed higher levels of NOTCH1 and TNFα than luminal-A tumors as demonstrated by an upward-right shift compared to luminal-A patients (marked by blue rectangle, set as described in "Materials and methods"), thus dissociating the basal patients from the luminal-A patients. In contrast, the pattern of NOTCH2-TNFα co-expression in basal patients overlapped with the co-expression pattern in luminal-A patients, and thus did not dissociate between these

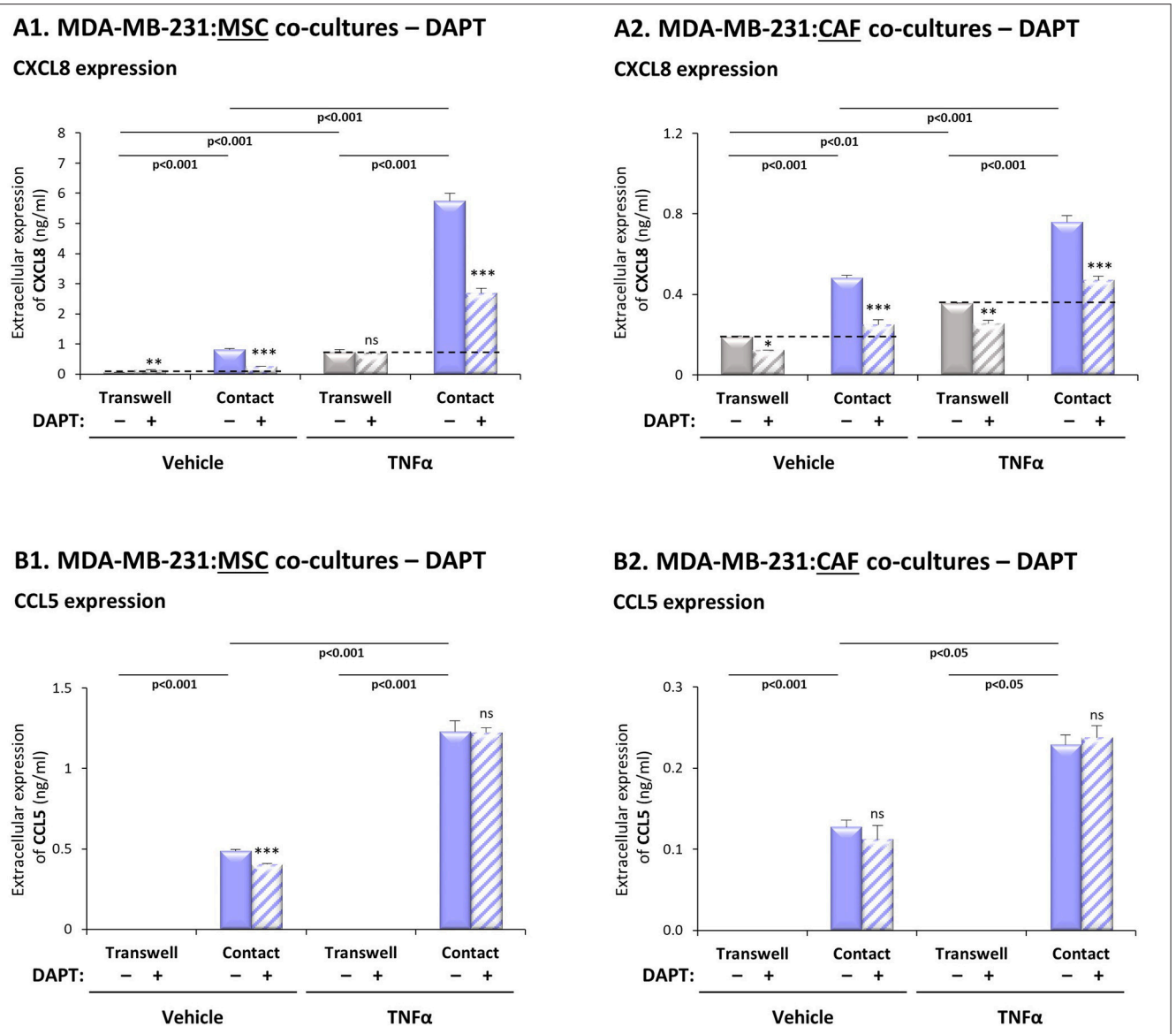

FIGURE 2 | DAPT inhibits the contact-dependent induction of CXCL8, but not of CCL5, in TNFα-stimulated TNBC:stroma co-cultures. Co-cultures of MDA-MB-231 cells with MSCs **(A1,B1)** and co-cultures of MDA-MB-231 cells with breast cancer patient-derived CAFs **(A2,B2)** were established under "Transwell" conditions and "Contact" conditions. Co-cultures were stimulated by TNFα (10 ng/ml) or vehicle control for 7 h; then, TNFα stimulation was removed and the cells were grown in TNFα-free media with DAPT (10 μM) for additional 60 h. CM were collected and the extracellular expression of CXCL8 **(A1,A2)** and CCL5 **(B1,B2)** was determined by ELISA. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns=non-significant for differences between DAPT- and DMSO-treated cells, within each group. The results are of a representative experiment of $n > 3$ independent experiments, performed with MSCs of 2 different donors, and of $n = 3$ independent experiments performed with patient-derived CAFs. To provide the readers with the entire setup of co-culture conditions compared to separate cells, as we have demonstrated in our previous study (26), **Supplementary Figures 2A,B**; demonstrate the entire panel of cells/stimulations relevant to CXCL8 and CCL5 induction (without DAPT treatment). The results presented in **Supplementary Figure 2** were derived from a different experiment than those presented in our previous study.

two groups of patients (**Figure 6A2**). As with TNFα, the co-expression pattern of NOTCH1 but not of NOTCH2, with CXCL8, has differentiated the basal patients from the luminal-A patients (**Figure 6B**). The higher relevance of Notch1 than of Notch2 to the tumor-stroma-inflammation networks in TNBC was corroborated by similar findings that were obtained with NOTCH1 *vs.* NOTCH2 co-expression analyses performed with IL-1β (**Supplementary Figure 7**).

Notch1 Activation Is Required for the Contact-Dependent Induction of CXCL8 in the Tumor-Stroma-Inflammation Network in TNBC

Based on the above findings, pointing at Notch1 as a potential regulator of the TNBC-stroma-inflammation networks, we determined the roles of Notch1 in regulating

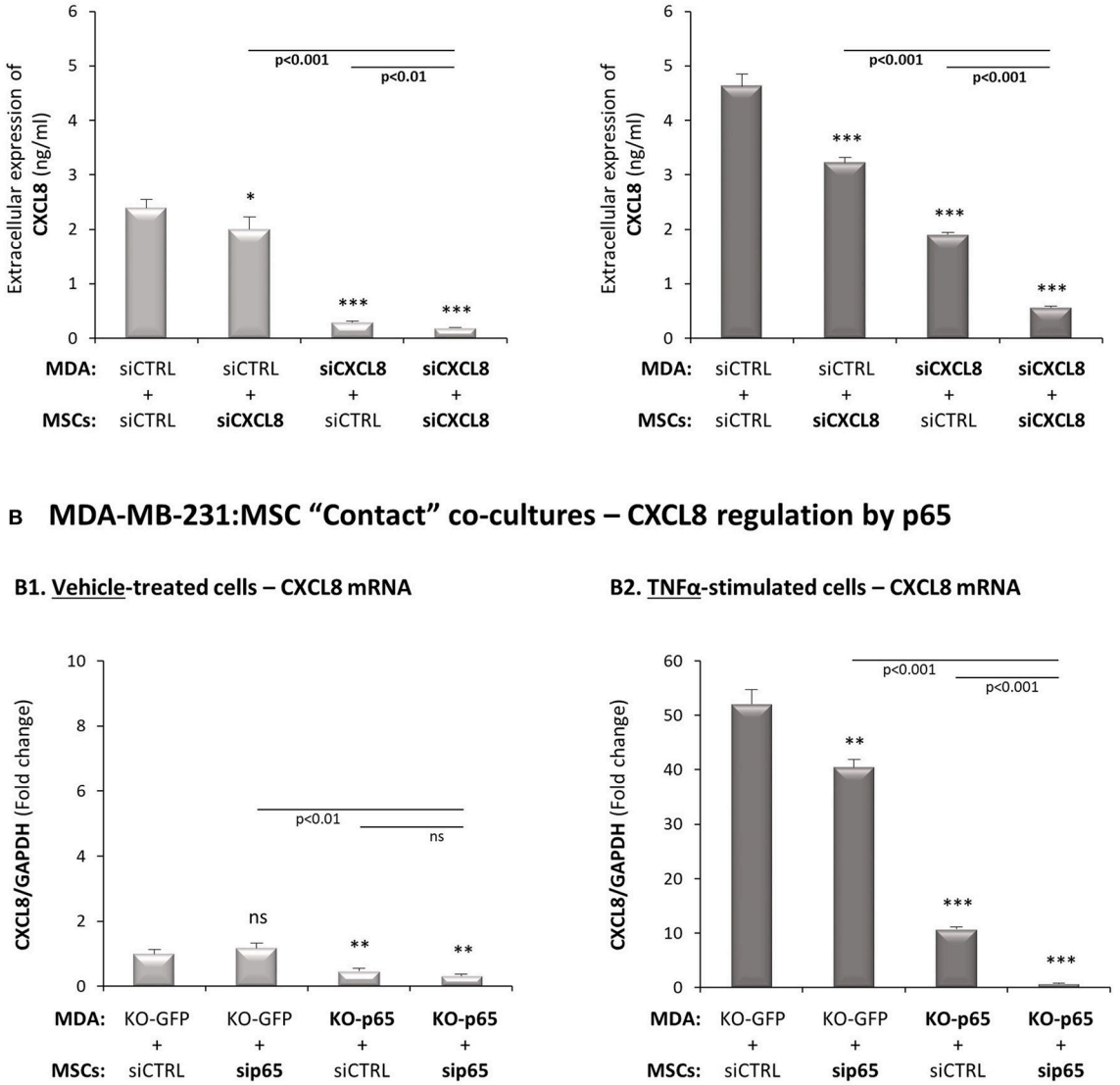

FIGURE 3 | In TNFα-stimulated TNBC:MSC "Contact" co-cultures, mainly TNBC cells but also MSCs contribute to elevated levels of CXCL8, through a p65-dependent process. **(A)** The cellular source of CXCL8. siRNA to CXCL8 was expressed in MDA-MB-231 cells ("MDA"), in MSCs or both cell types together (validation of CXCL8 down-regulation is demonstrated in **Supplementary Figure 4A**), and "Contact" co-cultures were established in the presence of vehicle **(A1)** or TNFα (10 ng/ml) **(A2)**. CXCL8 levels in cell supernatants were determined by ELISA, as described in **Figure 2**. $^{***}p < 0.001$, $^{*}p < 0.05$ for differences between co-culture combinations that included siCXCL8-expressing cells, compared to co-cultures in which both cell types expressed siCTRL. The results are of a representative experiment of $n = 3$ independent experiments, performed with MSCs of 2 different donors. **(B)** Regulation of CXCL8 expression by p65. p65 was down-regulated in MDA-MB-231 cells ("MDA"), MSCs or both cell types together (validations of p65 down-regulation are demonstrated in **Supplementary Figures 4B,C**), and "Contact" co-cultures were established and were exposed to vehicle **(B1)** or to TNFα (10 ng/ml) **(B2)**. CXCL8 mRNA levels were determined by qRT-PCR. $^{***}p < 0.001$, $^{**}p < 0.01$, ns=non-significant for differences between different sip65/siCTRL and KO-GFP/KO-p65 cell combinations compared to siCTRL/KO-GFP control groups. The results are of representative experiment of $n > 3$ independent experiments, performed with MSCs of 3 different donors.

the contact-dependent process of CXCL8 induction in TNFα-stimulated MDA-MB-231:MSC co-cultures. First, we asked if MDA-MB-231 cells and/or MSCs respond to TNFα stimulation by Notch1 activation. Using Abs that specifically recognize the N1-ICD, the cleaved and activated form of Notch1 (directed to Val1744, which is exposed in Notch1

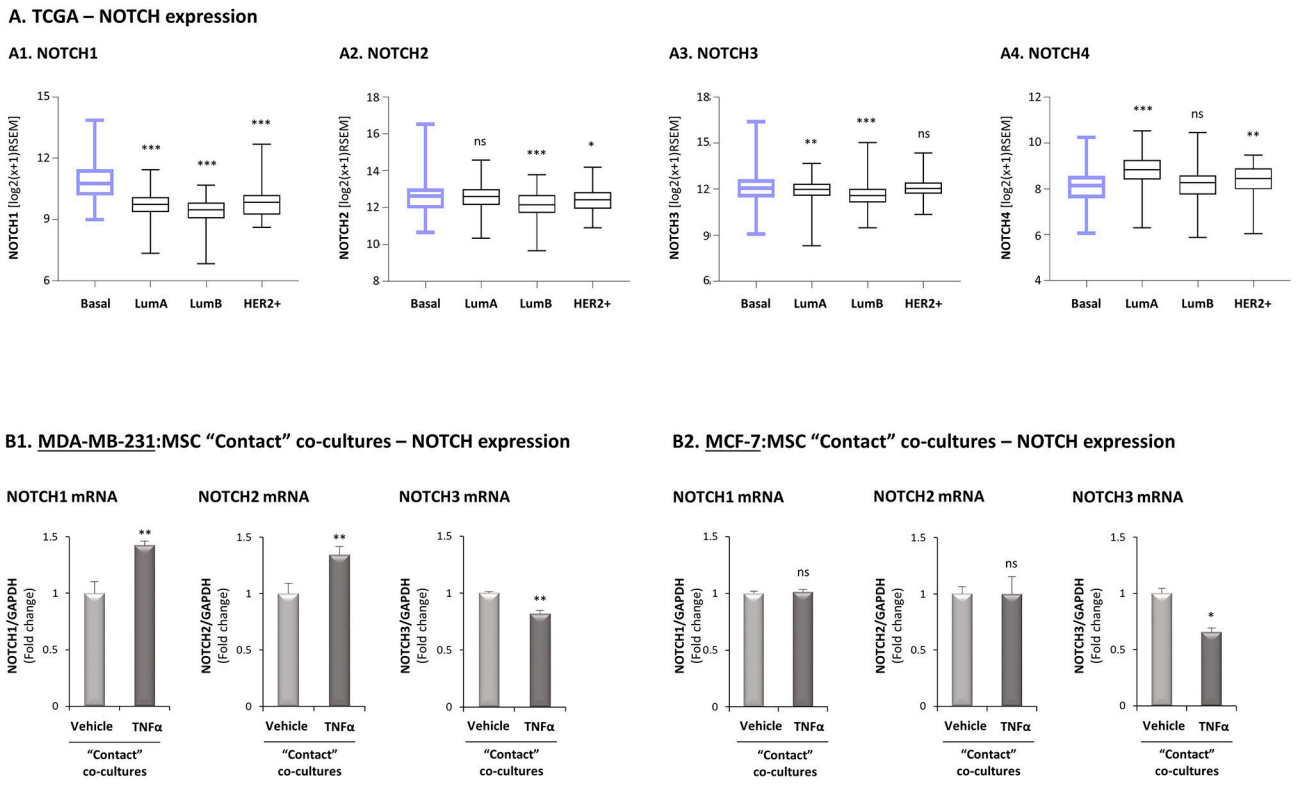

FIGURE 4 | Studies of the TCGA breast cancer patient dataset and of transcriptional regulation demonstrate high relevance of Notch1 to the tumor-stroma-inflammation network in TNBC. **(A)** The Figure demonstrates gene expression boxplot analyses, performed using the TCGA breast cancer patient dataset. **(A1)** NOTCH1. **(A2)** NOTCH2. **(A3)** NOTCH3. **(A4)** NOTCH4. ***$p < 0.001$, **$p < 0.01$ *$p < 0.05$, ns=non-significant for differences between the expression of NOTCH receptors in luminal-A, luminal-B and HER2+ tumors compared to basal tumors. **(B)** mRNA expression levels of NOTCH1, NOTCH2 and NOTCH3 were determined by qRT-PCR in MDA-MB-231:MSC **(B1)** or MCF-7:MSC **(B2)** "Contact" co-cultures stimulated by TNFα (10 ng/ml) or vehicle control. **$p < 0.01$, *$p < 0.05$, ns=non-significant for differences between cytokine-stimulated and non-stimulated co-cultures. The results are of a representative experiment of $n \geq 3$ independent experiments, performed with MSCs of 2 different donors.

following γ-secretase-mediated cleavage), we found that Notch1 underwent basal process of activation in MDA-MB-231 cells (**Figures 7A,B**). Then, we noticed that the N1-ICD levels in MDA-MB-231 cells were significantly elevated upon 7 h but not after 15 min of TNFα stimulation (**Figures 7A,B**, respectively). Thus, a time-dependent TNFα-induced process of Notch1 activation was revealed in MDA-MB-231 cells. In contrast, no evidence for basal Notch1 activation or for its induction by TNFα stimulation was observed in MSCs at any of the time points (**Figures 7A,B**). Similar results were observed following IL-1β stimulation, demonstrating that 7-h stimulation, but not 15-min stimulation by IL-1β has led to Notch1 activation in the tumor cells, but not in the MSCs (**Supplementary Figures 8A,B**). Furthermore, we noted that the levels of activated N1-ICD were much increased in MDA-MB-231:MSC "Contact" co-cultures compared to those of the tumor cells or MSCs when grown individually; the high N1-ICD levels in "Contact" co-cultures were slightly elevated by TNFα stimulation (**Figure 7C**).

To follow up on the findings described above we asked if Notch1 knock-down in MDA-MB-231 cells by siRNA would reduce the contact-dependent induction of CXCL8 in TNFα-stimulated MDA-MB-231:MSC co-cultures. To this end, we have analyzed the expression of CXCL8 in TNFα-stimulated and non-stimulated MDA-MB-231:MSC co-cultures in which Notch1 was down-regulated by siRNA in the tumor cells. After validating that Notch1 expression and activation was significantly down-regulated by the siRNA (**Supplementary Figure 4D**), we found that the contact-dependent induction of CXCL8 upon TNFα stimulation - noted by CXCL8 incremental expression in "Contact" *vs.* "Transwell" conditions - was markedly reduced by siRNA to Notch1 (**Figure 7D**). Although some levels of Notch1 expression remained after its knock-down by siRNA (**Supplementary Figure 4D**), the degree of Notch1 down-regulation in this setting gave rise to CXCL8 inhibition levels which were similar to those obtained by DAPT, following TNFα stimulation of "Contact" co-cultures (siRNA Notch1: 73 ± 21% in all experimental repeats; DAPT: 70 ± 13% as in **Figure 2A1**). Moreover, induction of CXCL8 under "Contact" conditions, in the absence of TNFα stimulation, was also partly dependent on Notch1 activation, as in DAPT studies. Of importance, similar findings on Notch1-regulated, contact-dependent CXCL8 induction were

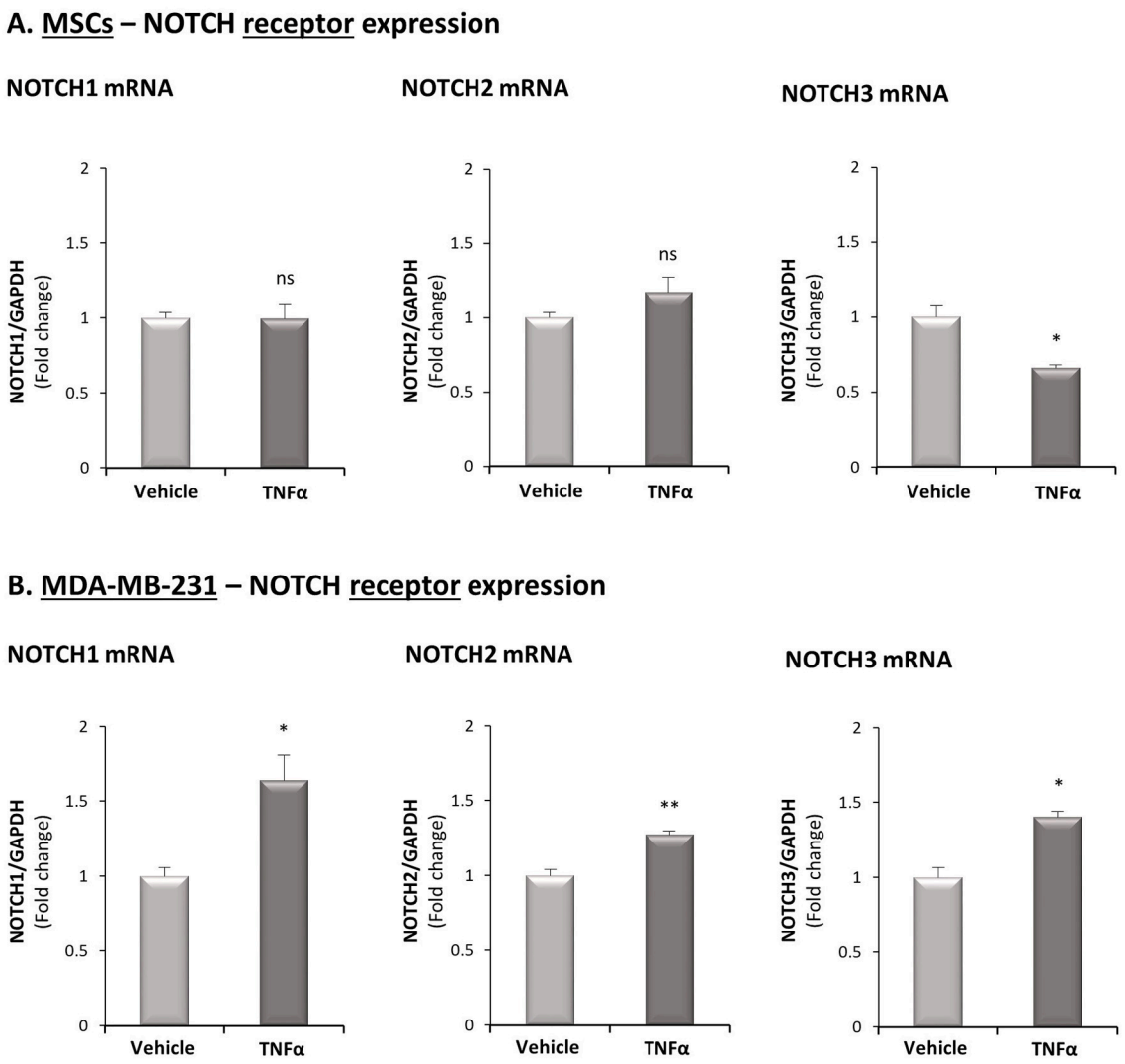

FIGURE 5 | TNFα up-regulates the expression of NOTCH1, NOTCH2 and NOTCH3 in TNBC cells but not in MSCs. The Figure demonstrates mRNA expression of NOTCH1, NOTCH2 and NOTCH3 in MSCs **(A)** and in MDA-MB-231 cells **(B)**, following stimulation by TNFα (10 ng/ml) or vehicle control. $^{**}p < 0.01$, $^{*}p < 0.05$, ns=non-significant for differences between cytokine-stimulated and vehicle-treated cells. The results are of a representative experiment of $n \geq 3$ independent experiments, performed with MSCs of 2 different donors.

also observed following IL-1β stimulation when siRNA to Notch1 was used (75 ± 25%; **Supplementary Figure 8C**), in similar levels to those obtained upon treatment by DAPT (65 ± 23%; **Supplementary Figure 3A1**).

To complement the above analyses, clearly indicating that Notch1 activation takes place only in the tumor cells and is required for CXCL8 induction in the TNBC-stroma-inflammation setting, we asked which of the Notch ligands may be a candidate partner that is expressed by MSCs. The analyses presented in **Figure 8** indicated that of the different Notch ligands, Delta-like 1 (DLL1) was the only ligand that was up-regulated by TNFα (**Figure 8A1**); DLL1 expression was also elevated by IL-1β stimulation in the MSCs (**Supplementary Figure 6A**). It was also interesting to note that following TNFα stimulation, p65 activation was involved in DLL1 up-regulation when MSCs interacted with MDA-MB-231 "Contact" co-culture conditions (**Figure 8B**). Of note, parallel analyses demonstrated that DLL1 was also up-regulated by p65 activation in MDA-MB-231 cells that interacted with MSCs in the presence of TNFα (**Figure 8B2**), despite the fact that DLL1 was not up-regulated by TNFα in MDA-MB-231 at all (**Figure 8A2**).

In TNFα-Stimulated TNBC:MSC Co-cultures, p65 Activation Mainly in TNBC Cells but Also in MSCs, Induces Notch1 Activation

Our above findings indicated that p65, as well as Notch1, were involved in CXCL8 up-regulation in the TNBC-stroma-inflammation network (**Figures 3B**, **7D**, respectively). Moreover,

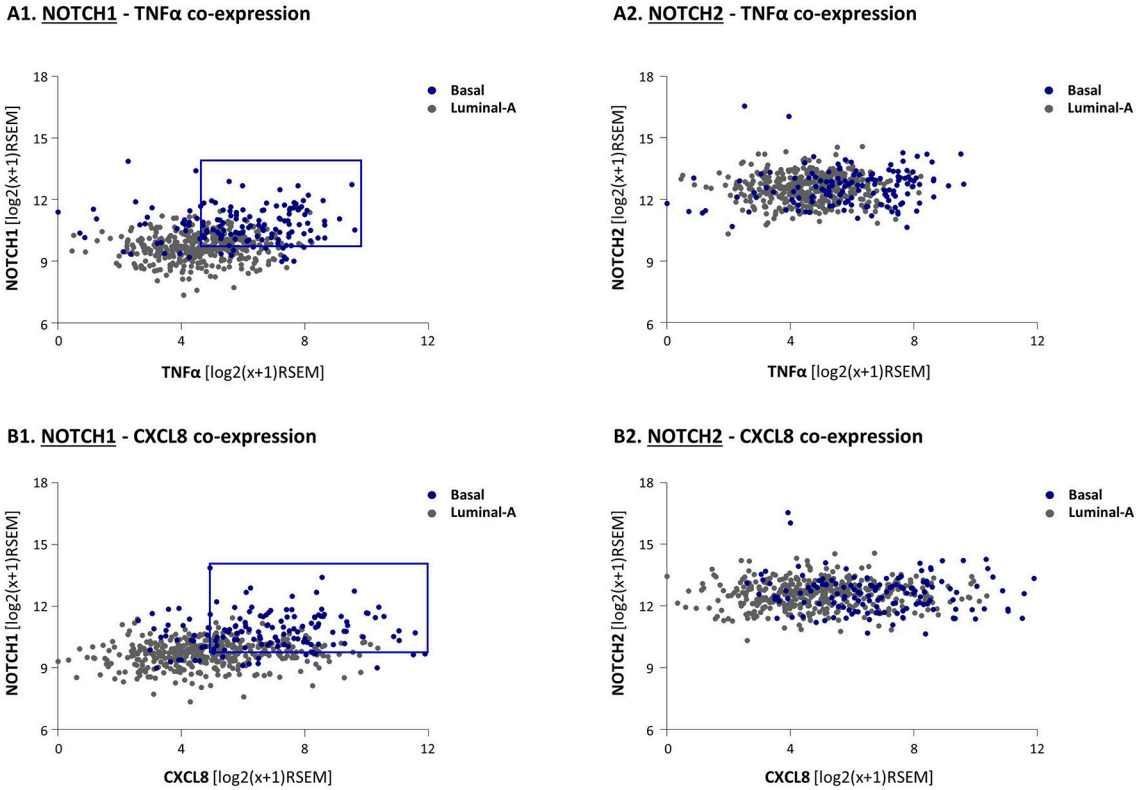

FIGURE 6 | High co-expression levels of NOTCH1 - but not of NOTCH2 - with TNFα and CXCL8 differentiate basal patients from luminal-A breast cancer patients. **(A,B)** The Figure demonstrates gene expression analyses, performed using the TCGA breast cancer dataset. The analyses demonstrate in individual patient tumor (dots) the co-expression of NOTCH1 with TNFα **(A1)**; NOTCH2 with TNFα **(A2)**; NOTCH1 with CXCL8 **(B1)**; NOTCH2 with CXCL8 **(B2)**. The blue rectangle illustrates the upwards-right shift observed in basal patients compared to luminal-A patients in NOTCH1 co-expression analyses (the rectangle was set as described in "Materials and methods").

we demonstrated that p65 was quickly activated by TNFα [15 min; **Supplementary Figure 2C** and (26)] but TNFα-induced Notch1 activation was slower (close to 7 h; **Figures 7A,B**). Thus, we determined the possibility that in TNFα-stimulated MDA-MB-231:MSC "Contact" co-cultures, p65 activation had up-regulated Notch1 expression and/or Notch1 activation.

By down-regulating p65 in the tumor cells and/or in the MSCs, we demonstrated that in the absence of p65 activation in both cell types together, the expression of Notch1 (FL-Notch1) was significantly reduced, in the presence of TNFα stimulation (**Figures 9A,B1**; lanes 5 *vs.* 8) and in its absence (**Figures 9A,B1**; lanes 1 *vs.* 4). Most importantly, based on the N1-ICD bands remaining, we found that the activation of Notch1 was almost completely abrogated following p65 down-regulation in both cell types together when TNBC:MSC interactions took place in the context of TNFα stimulation (**Figures 9A,C2**; lanes 8 *vs.* 5). In comparison, some degree of Notch1 activation remained upon p65 down-regulation in both cell types together, when similar co-cultures where formed in the absence of TNFα (**Figures 9A,C1**; lanes 4 *vs.* 1).

Detailed analysis has demonstrated that in the setting that lacked TNFα stimulation, p65 activation mainly in the tumor cells (**Figures 9A1,C1**; lanes 3 *vs.* 1) but also to some extent in MSCs (**Figures 9A1,C1**; lanes 2 *vs.* 1) has contributed to Notch1 activation in co-cultures [please note that the differences in this case (lanes 3 *vs.* 1, and lanes 2 *vs.* 1) were detected in all experiments but did not come out statistically significant because of differences in the extent of reduction noted in each of the experimental repeats]. Upon TNFα stimulation of MDA-MB-231:MSC "Contact" co-cultures, it became evident that down-regulation of p65 in the tumor cells has led to marked inhibition of Notch1 activation (**Figures 9A2,C2**; lanes 7 *vs.* 5), and siRNA to p65 in the MSCs also reduced Notch1 activation, although to much lower extent than in the tumor cells (**Figures 9A2,C2**; lanes 6 *vs.* 5). Together, these findings indicate that p65 is a most important inducer of Notch1 activation in MDA-MB-231:MSC co-cultures, primarily in the presence of TNFα stimulation.

DISCUSSION

TNBC tumors are characterized by high aggressiveness, necessitating improved understanding of the mechanisms that promote their progression. In a recent study we identified a tumor-stroma-inflammation network potentiating multiple

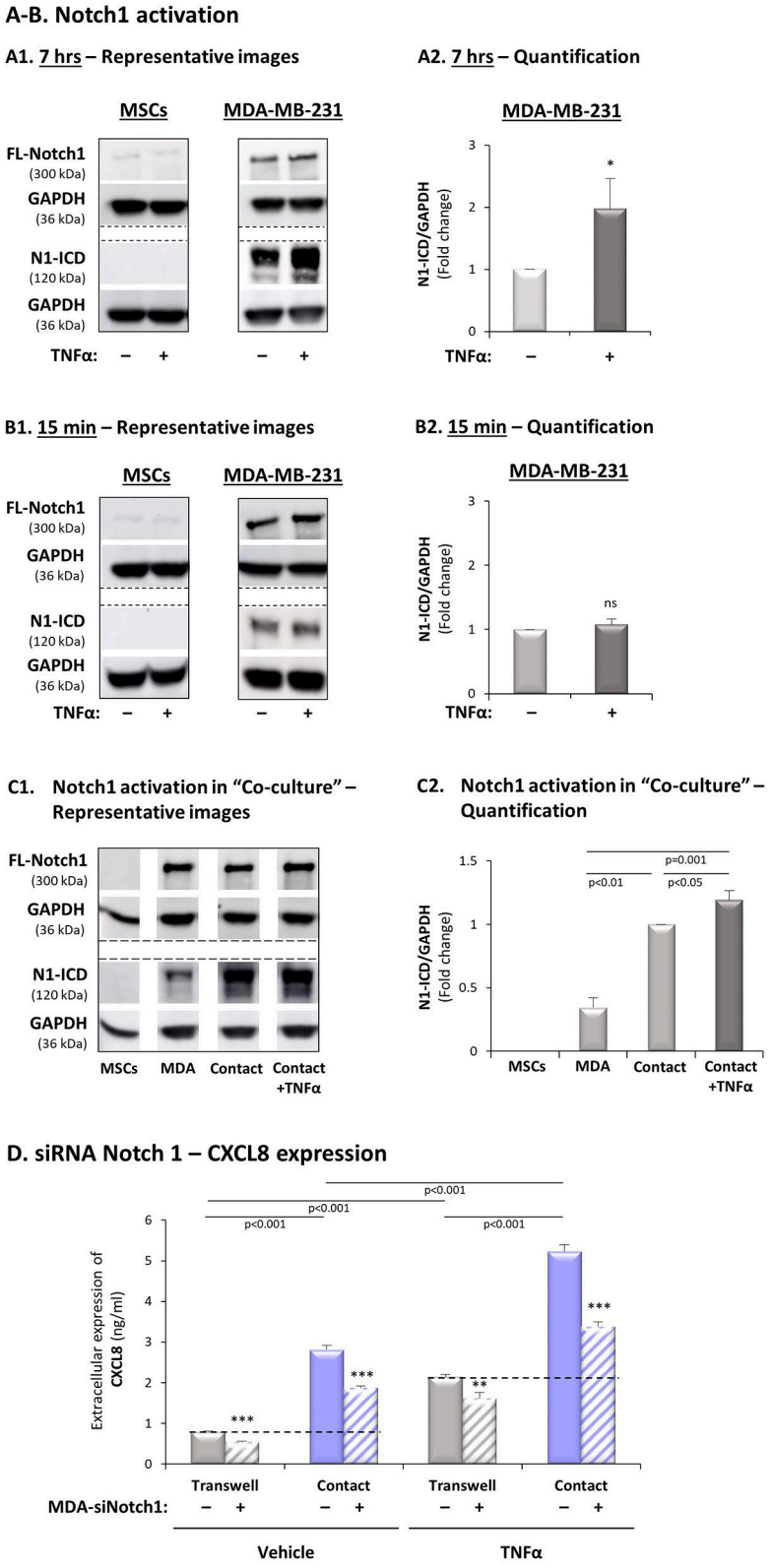

FIGURE 7 | Expression of Notch1 siRNA in TNBC cells inhibits the contact-dependent induction of CXCL8, in TNFα-stimulated TNBC:MSC co-cultures. **(A,B)** Notch1 activation by TNFα stimulation in MSCs and MDA-MB-231 cells. Each of the two cell types was stimulated by TNFα ("+"; 10 ng/ml) or vehicle control ("−") for 7 h **(A)** or

(Continued)

FIGURE 7 | 15 min **(B)**. **(A1,B1)** Representative experiments and **(A2,B2)** averages ± SD values of Notch1 activation in $n \geq 3$ independent experiments, performed with MSCs of 2 different donors. Notch1 activation levels were determined by WB analyses of N1-ICD=Notch1 intracellular domain. FL-Notch1=Full-length Notch1. GAPDH was used as a loading control. $^{*}p < 0.05$, ns=non-significant for differences between cytokine-stimulated cells and control non-stimulated MDA-MB-231 cells (MSCs are not presented in the graphs because no N1-ICD signals were detected). **(C)** Notch1 activation was determined in MDA-MB-231 ("MDA") cells, MSCs and "Contact" co-cultures that were stimulated by TNFα (10 ng/ml), or exposed to vehicle for 7 h. **(C1)** A representative experiment and **(C2)** averages ± SD of Notch1 activation in $n = 3$ independent experiments, performed with MSCs of 3 different donors. WB analyses were performed as described in Part **(A)** above. **(D)** The effects of siRNA Notch1 on CXCL8 expression (validation of Notch1 down-regulation is demonstrated in **Supplementary Figure 4D**). MDA-MB-231 cells ("MDA") transfected by Notch1 siRNA ("+") or siCTRL ("–") and were co-cultured with MSCs. Then, co-cultures were stimulated by TNFα (10 ng/ml) or by vehicle control, and extracellular expression of CXCL8 was determined by ELISA, as described in **Figure 2**. $^{***}p < 0.001$, $^{**}p < 0.01$ for differences between groups containing siNotch1-expressing MDA-MB-231 cells and groups of siCTRL-expressing MDA-MB-231 cells. The results are of a representative experiment of $n = 3$ independent experiments, performed with MSCs of 2 different donors.

processes that contribute to increased metastasis in TNBC; they included expression of pro-metastatic chemokines such as CXCL8 and CCL5, angiogenesis and tumor cell migration and invasion. Eventually, this interactive network was shown to give rise to increased metastasis of TNBC cells *in vivo* (26).

Our findings further indicated that cell-to-cell contacts played key roles in potentiating pro-metastatic activities in the tumor-stroma-inflammation setting, leading us to ask if the Notch pathway is involved in promoting pro-metastatic functions when TNBC cells interacted with MSCs in the presence of pro-inflammatory stimulation. Indeed, the findings presented in the current study indicate that the elevated levels of migration and invasion of TNBC cells following their interactions with MSCs/CAFs in the presence of TNFα were mediated by Notch signaling, as these tumor cell functions were prominently inhibited by DAPT. In parallel, we demonstrated that the contact-dependent induction of CXCL8 in cytokine-stimulated "Contact" co-cultures was inhibited by DAPT. Although CCL5 elevation in cytokine-stimulated TNBC:MSC co-cultures was entirely based on cell-to-cell contacts that were established between the two cell types (26), this effect was not inhibited by DAPT. Thus, our findings provide evidence to a novel, Notch-dependent mechanism, which regulates CXCL8 in TNBC, and indicate that this Notch-mediated regulatory mechanism is not shared by all pro-metastatic chemokines, like CCL5.

Moreover, the findings obtained with DAPT were recapitulated when Notch1 was knocked-down in the tumor cells by siRNA; in these experiments, Notch1 siRNA has led to similar reduction in TNFα-induced contact-dependent induction of CXCL8 as did DAPT. Moreover, Notch1 was up-regulated in the tumor cells at the mRNA and protein levels and was activated at the protein level in the tumor cells by TNFα stimulation. These findings and similar results obtained by IL-1β stimulation indicated that Notch1 is the actual Notch receptor involved in the up-regulation of CXCL8, and is activated only in the tumor cells upon TNFα/IL-1β stimulation of TNBC:MSC co-cultures. In further studies it will be interesting to identify the ligands that bind Notch1 in the tumor cells. Based on our findings at the mRNA levels, it is possible that DLL1, whose expression was elevated in MSCs by TNFα and IL-1β stimulation (through a p65-mediated pathway, as analyzed for TNFα stimulation), is a partner of tumor cell-expressed Notch1. However, our findings demonstrating that DLL1 is regulated by p65 also in the cancer cells suggests that reciprocal interactions take place between the two cell types, in a complex manner that requires further investigation.

In line with our observations, published studies indicate that of the four Notch receptors (Notch1-4), Notch1 is strongly linked to disease progression in TNBC. Notch1 was found to be over-expressed and hyper-activated in TNBC patients, and high Notch1 levels were associated with reduced overall survival in TNBC/basal breast cancer patients (45, 52–54). Moreover, meta-analysis of breast cancer studies revealed a significant association between high Notch1 expression and TNBC progression (55). Studies in breast cancer, particularly in the TNBC subtype, demonstrated that the activation of Notch1 promoted stemness, drug resistance, invasion and migration (41–45, 52–58).

Moreover, several studies connected Notch1 to stromal cells and inflammatory processes in TNBC by demonstrating Notch-mediated regulation of CXCL8 in this disease subtype (51, 56, 59–62). However, to date, our study provides the first mechanistic information on the regulation of CXCL8 by Notch1 in the tumor-stroma-inflammation network. Our data indicate that p65 is the prime regulator of CXCL8 expression and of Notch1 activation in this setting, and provide evidence to a molecular shift that takes place in TNBC:stroma co-cultures when they are stimulated by pro-inflammatory mediators such as TNFα.

Specifically, when tumor cells interacted with MSCs in "Contact" conditions in the absence of TNFα stimulation, they exchanged soluble materials and formed physical contacts that together led to CXCL8 induction, beyond the levels produced by each cell type alone and above the levels obtained in "Transwell" conditions in the absence of TNFα [(26); **Supplementary Figure 2A1**]. Our data indicate that without TNFα stimulation, CXCL8 produced in TNBC:MSC co-cultures was released exclusively by the tumor cells and resulted from basal p65 activation in the tumor cells. Moreover, in the absence of TNFα stimulation, basal p65 activation mainly in the tumor cells has induced Notch1 activation (**Figures 9A1,C1**), which then contributed to a contact-dependent induction of CXCL8 (**Figure 7D**).

However, the balance between the two cell types in their contribution to CXCL8 production was changed in the presence of TNFα stimulation, that led to further increase in CXCL8 release in the "Contact" co-cultures. The contacts between the tumor cells and the MSCs set the stage for the activities of TNFα which increased CXCL8 production, in a process that

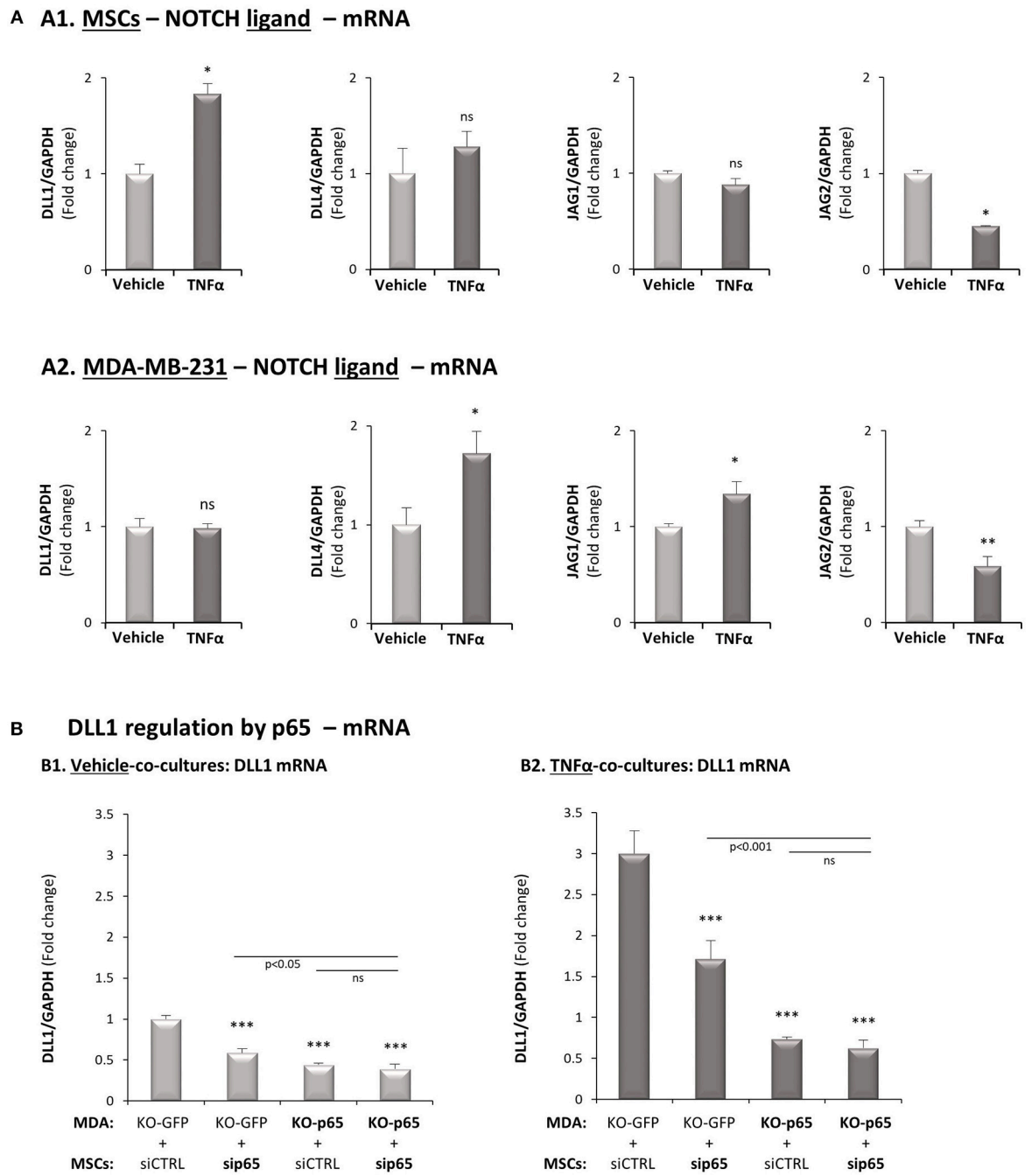

FIGURE 8 | DLL1 is up-regulated in MSCs following TNFα stimulation and is controlled by p65 activation. **(A)** The Figure demonstrates mRNA expression levels of Notch ligands, determined by qRT-PCR in MSCs **(A1)** or in MDA-MB-231 cells **(A2)**, stimulated by TNFα (10 ng/ml) or vehicle control for 7 h. $^{**}p < 0.01$, $^{*}p < 0.05$, ns=non-significant for differences between cytokine-stimulated and vehicle-treated co-cultures. **(B)** DLL1 regulation by p65. p65 was down-regulated in MDA-MB-231 cells ("MDA"), MSCs or both cell types together (as in **Figure 3B**) and both cell types were co-cultured in "Contact" conditions. Then, co-cultures were stimulated by TNFα (10 mg/ml) or exposed to vehicle control, and DLL1 mRNA was determined by qRT-PCR. $^{***}p < 0.001$, ns=non-significant. In all parts of the Figure, the results are of a representative experiment of $n \geq 3$ independent experiments, performed with MSCs of 2 different donors.

was entirely dependent on p65 activation (**Figure 3B2**). Now, in the presence of TNFα-induced signals, activation of p65 in the tumor cells has contributed much to CXCL8 release, and p65 activation in the MSCs provided its share as well. Also, in the context of TNFα stimulation, p65 activation that led to elevated Notch1 activation took place mainly in the tumor cells but also in the MSCs. Thus, a shift in regulatory pathways was induced by TNFα, eventually amplifying CXCL8 release to

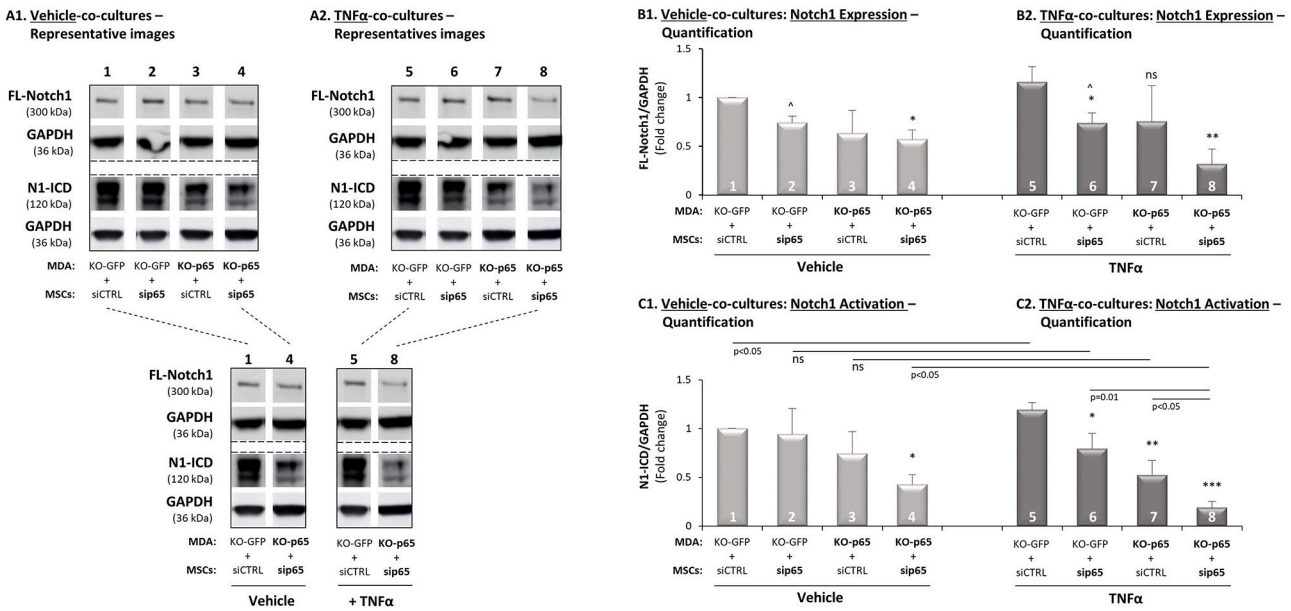

FIGURE 9 | In TNFα-stimulated TNBC:MSC co-cultures, p65 activation up-regulates Notch1 expression and activation. The Figure demonstrates studies performed following p65 down-regulation in MDA-MB-231 cells ("MDA"), MSCs or both cell types together, as described in **Figure 3B** (validations of p65 down-regulation are demonstrated in **Figures S4B,C**). **(A)** A representative experiment, **(B)** averages ± SD of Notch1 expression and **(C)** averages ± SD of Notch1 activation in $n = 3$ independent experiments (∧ In these groups, densitometry was performed in $n = 2$), performed with MSCs of 3 different donors. The two cell types were co-cultured in "Contact" conditions in different combinations, and were exposed to vehicle control **(A1,B1,C1)** or to TNFα stimulation (**A2,B2,C2**; 10 ng/ml). Notch1 activation levels were determined by WB analyses of N1-ICD=Notch1 intracellular domain. FL-Notch1=Full-length Notch1. GAPDH was used as a loading control. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.01$, ns=non-significant for differences between different sip65/siCTRL and KO-GFP/KO-p65 cell combinations compared to siCTRL/KO-GFP control groups. For readers' convenience, Panel A also provides another view of the comparisons between specific lanes that were shown in **(A1,A2)**. This approach was taken in order to clearly demonstrate the stronger reduction in Notch1 activation in TNFα-stimulated co-cultures following down-regulation of p65 in both cell types, compared to similar analyses performed without TNFα stimulation or without p65 down-regulation.

highest levels, when TNBC cells and MSCs interacted in the presence of TNFα stimulation.

Our observations also indicate that when the tumor cells and the stromal cells did not form physical contacts but exchanged soluble factors, TNFα stimulation gave rise to production of soluble factors; it is possible that these factors, together with TNFα itself have directly activated p65. Then, p65 which is well-known to be a strong inducer of CXCL8 transcription in other systems [e.g., (63)], has contributed to increased transcription of CXCL8 [as we have shown in our previous study (26)] in the tumor-stroma-inflammation network established herein.

In parallel, in the contact-dependent process, TNFα-driven activation of p65 - mainly in the tumor cells but also in the MSCs - has given rise to elevated Notch1 expression and activation. These effects could have been induced by processes of direct binding of p65 to Notch1 promoter, leading to increased NICD levels, as has been reported before (64, 65); in addition, Notch1 activation could have been induced by the TNFα-IKK pathway that was found to modify the function of molecules that participate in regulating Notch activation (66). The activation of Notch1 following TNFα stimulation has led to CXCL8 induction (**Figure 7**), possibly through the activity of elements that participate in Notch-induced transcription of target genes, such as p300 (67). Indeed, CXCL8 was found to be up-regulated in lung epithelial cells by p300 (68). Published studies indicate that the p300-mediated process of CXCL8 induction reflected interaction with the NF-κB pathway (68), further supporting our findings on p65-Notch1 cross-talk that regulates CXCL8 induction in our tumor-stroma-inflammation network in TNBC.

The overall outcome, therefore, was elevation in CXCL8 production that has reached its outmost levels only when TNBC cells interacted with stromal cells, and were stimulated by pro-inflammatory signals delivered by TNFα. Induction of CXCL8 in contact-dependent setting was driven partly by NF-κB-induced Notch1 activation. Moreover, since Notch1 activation following TNFα stimulation depended almost entirely on p65 activation, it is highly possible that Notch-mediated regulation of tumor cell migration and invasion was also induced by p65 activation, as result of TNFα activation.

To conclude, in our current study we have deciphered intricate mechanisms that control through p65 and Notch activation the interactions between TNBC cells and stromal cells in the context of the pro-inflammatory TME. We demonstrated

key roles for Notch family members not only in inducing the expression of pro-metastatic chemokines such as CXCL8, but also in activating migratory and invasive capacities in the tumor cells upon their interactions with stromal cells in the presence of pro-inflammatory signals. In view of the fact that CXCL8 was revealed in our accompanying study as key regulator of many of the pro-metastatic activities of this network (26), the "take home message" of this study is that the interactions between the TNBC cells and the stromal cells have set the conditions that enabled TNFα to bring its effects to maximum, partly through p65-induced Notch1 activation that has led to CXCL8 induction, and consequently to other tumor-promoting activities.

Obviously, further elucidation of the roles of the different players of the TNBC-stroma-inflammation network is required; preferably, such investigations should be performed in syngeneic TNBC systems where the effects of Notch1 activation on the expression of the murine counterparts of CXCL8 could be investigated in the context of the *in vivo* TME. Here, it is important to indicate that analyses with TNBC cells that were manipulated to express lower Notch1 levels may be complicated by compensation mechanisms that lead to activation of other Notch receptors (as suggested by our preliminary results; Data not shown). In parallel, inhibitory modalities that target the Notch pathway could be used; however they are not specific for one particular Notch receptor and suffer from toxic side effects (39, 41).

Thus, we propose that Notch1-TNFα-CXCL8 studies in TNBC patients (e.g., patterns of expression and localization in the tumors) and the design of combined Notch + inflammation targeting modalities may be of great value when improved therapeutics for TNBC are looked for. Indeed, it is possible that by using Notch-targeting treatments together with inhibitors of pro-inflammatory mediators such as TNFα and CXCL8 receptors, the doses of Notch inhibitors could be reduced and their toxicities would be alleviated. This may be a realistic option, because inhibitors of TNFα for example have been successfully introduced to the clinical setting for the treatment of pro-inflammatory diseases (69, 70). Such combined modalities could be used in animal model systems and if they provide promising results, they could be considered as treatment options in TNBC patients. This newly-introduced approach may offer novel and promising treatments that would halt or limit disease progression in the most aggressive subtype of TNBC.

AUTHOR CONTRIBUTIONS

YL generated all data and was the leading scientist involved in all steps of research conduct. She was also extensively involved in manuscript preparation. SL contributed to establishment of research systems. TM established the CRISPR/Cas9 system. DM and LR-A participated in ELISA studies of Notch1 effects. DS provided insights to Notch-related aspects. SW participated in conception of this research in its initial stages. CK contributed to TCGA analyses. ME contributed to introduction of the Notch pathway to the study. AB-B, the principal investigator, was responsible for research conception, study design, data analysis and manuscript preparation.

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SUPPLEMENTARY MATERIAL

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Tumor-Stroma-Inflammation Networks Promote Pro-metastatic Chemokines and Aggressiveness Characteristics in Triple-Negative Breast Cancer

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The tumor microenvironment (TME) plays key roles in promoting disease progression in the aggressive triple-negative subtype of breast cancer (TNBC; Basal/Basal-like). Here, we took an integrative approach and determined the impact of tumor-stroma-inflammation networks on pro-metastatic phenotypes in TNBC. With the TCGA dataset we found that the pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β), as well as their target pro-metastatic chemokines CXCL8 (IL-8), CCL2 (MCP-1), and CCL5 (RANTES) were expressed at significantly higher levels in basal patients than luminal-A patients. Then, we found that TNFα- or IL-1β-stimulated co-cultures of TNBC cells (MDA-MB-231, MDA-MB-468, BT-549) with mesenchymal stem cells (MSCs) expressed significantly higher levels of CXCL8 compared to non-stimulated co-cultures or each cell type alone, with or without cytokine stimulation. CXCL8 was also up-regulated in TNBC co-cultures with breast cancer-associated fibroblasts (CAFs) derived from patients. CCL2 and CCL5 also reached the highest expression levels in TNFα/IL-1β-stimulated TNBC:MSC/CAF co-cultures. The elevations in CXCL8 and CCL2 expression partly depended on direct physical contacts between the tumor cells and the MSCs/CAFs, whereas CCL5 up-regulation was entirely dependent on cell-to-cell contacts. Supernatants of TNFα-stimulated TNBC:MSC "Contact" co-cultures induced robust endothelial cell migration and sprouting. TNBC cells co-cultured with MSCs and TNFα gained migration-related morphology and potent migratory properties; they also became more invasive when co-cultured with MSCs/CAFs in the presence of TNFα. Using siRNA to CXCL8, we found that CXCL8 was significantly involved in mediating the pro-metastatic activities gained by TNFα-stimulated TNBC:MSC "Contact" co-cultures: angiogenesis, migration-related morphology of the tumor cells, as well as cancer cell migration and invasion. Importantly, TNFα stimulation of TNBC:MSC "Contact" co-cultures *in vitro* has increased the aggressiveness of the tumor cells *in vivo*, leading to higher incidence of mice with lung metastases than non-stimulated TNBC:MSC co-cultures. Similar tumor-stromal-inflammation networks established in-culture with luminal-A cells

demonstrated less effective or differently-active pro-metastatic functions than those of TNBC cells. Overall, our studies identify novel tumor-stroma-inflammation networks that may promote TNBC aggressiveness by increasing the pro-malignancy potential of the TME and of the tumor cells themselves, and reveal key roles for CXCL8 in mediating these metastasis-promoting activities.

Keywords: cancer-associated fibroblasts, CCL2, CCL5, CXCL8, interleukin 1β, mesenchymal stem cells, triple-negative breast cancer, tumor necrosis factor α

INTRODUCTION

Breast cancer is a common malignant disease classified into several subtypes that differ in their markers, molecular characteristics and prognosis. Tumors of the triple-negative subtype of breast cancer (TNBC; generally corresponding to the "Basal/Basal-like" subtype of patient datasets, determined by PAM50 gene signatures) lack the expression of estrogen receptor α, progesterone receptor and HER2, are highly aggressive and are most likely to recur. Unlike the luminal-A tumors that are characterized by better survival, or the HER2+ tumors, TNBC/basal tumors cannot be treated by receptor-targeted therapies and demonstrate high relapse rates following chemotherapy (1–4).

As with other malignancies, breast tumors develop and progress within an intimate tumor microenvironment (TME) (5–8). Recent studies indicate that stromal cells, including mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs) are key regulators of tumor progression in cancer (9, 10). In general, MSCs enrich the TME with tumor-promoting factors, and endow the tumor cells with improved abilities to invade and generate metastases; MSCs also undergo transition to CAFs that promote breast cancer/TNBC progression (11–18). Particularly in TNBC, MSCs contribute to higher aggressiveness by promoting the expression of angiogenic factors and pro-metastatic chemokines such as the pro-inflammatory chemokines CXCL8, CCL2 and CCL5 (or their murine counterparts) (19–26). The axes established by these chemokines and their receptors are well-known for their pro-tumorigenic roles, including in TNBC [e.g., (26–41)].

Tumor-educated MSCs evolve within a TME enriched with inflammatory cells and pro-inflammatory cytokines, which generally promote breast cancer progression (42–44). Two such pro-inflammatory cytokines are tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β). It was demonstrated that despite its potential anti-tumor cytotoxic activities, chronic presence of TNFα in tumors has led to tumor progression. Joined with the pro-angiogenic and pro-inflammatory activities of IL-1β, the two cytokines were identified as pro-metastatic factors in many tumor types (45–49). In TNBC, sequencing, serum-profiling and immunohistochemistry studies of small patient cohorts provided initial evidence to relevance of high expression of the two cytokines or of their signaling components to metastasis in TNBC patients (50–53); in parallel, studies in animal models have demonstrated causative tumor-promoting roles for TNFα and IL-1β in TNBC (52, 54–59). TNFα and IL-1β were also connected with pro-malignancy activities of MSCs and CAFs in TNBC (21, 60–65). Specifically with regards to chemokines, we and others have shown that TNFα and IL-1β elevated the expression of CXCL8, CCL2 and CCL5 (or their murine counterparts) by MSCs [(65–69); the degree of increase depended on assay conditions], thus promoting the pro-inflammatory and pro-malignancy phenotype of these stromal cells.

However, to date, we still lack understanding of the interactions that are established between TNBC cells, stromal cells and their intimate pro-inflammatory TME, and we do not have enough information on the way such interactions affect disease course. Specifically, it is not known whether the pro-metastatic characteristics of the TME and of the tumor cells are increased when TNBC cells interact with MSCs/CAFs in the presence of pro-inflammatory stimuli. Thus, the aim of our present study was to identify the influence of the tumor-stroma-inflammation triage on the content of pro-metastatic chemokines as proxies for potential pro-malignancy activities that enrich the TME of TNBC tumors, as well as on angiogenesis and on the migratory, invasive and metastatic properties of TNBC cells.

In view of their high relevance to tumor progression in TNBC, TNFα and IL-1β were selected as representatives of the pro-inflammatory TME in our study. Here, we demonstrate that stimulation of TNBC:stroma co-cultures by these two cytokines has led to increased pro-metastatic activities at multiple levels, including: expression levels of the chemokines CXCL8, CCL2 and CCL5, angiogenesis, cancer cell morphology, tumor cell migration and tumor cell invasion. Importantly, we found that CXCL8 was a key regulator of the pro-metastatic activities that came into play in the TNBC-stroma-inflammation networks, including angiogenesis, metastasis-related morphology, tumor cell migration and invasion of TNBC cells. Moreover, the tumor-stroma-inflammation network has promoted the metastatic potential of TNBC cells and has led to elevated metastasis *in vivo*. Parallel in-culture studies that were performed with tumor-stroma-inflammation networks established with luminal-A cells demonstrated that in general they were less potent or differently active than those established with TNBC cells.

Thus, our findings set the pro-inflammatory inputs acting at the tumor:stroma interface, and their pro-metastatic outputs, as targets for improved therapy in TNBC. Since inhibitors of TNFα and IL-1β are used vastly in the treatment of inflammatory diseases (70–72), our findings suggest that these two cytokines may be considered as novel therapeutic modalities in TNBC. Such an approach, combined with the use of chemokine receptor inhibitors [e.g., of the CXCL8 receptors CXCR1 or CXCR2;

(39, 73)], may prevent tumor-stroma interactions that increase metastasis in TNBC.

MATERIALS AND METHODS

Analyses of Patient Datasets

RNAseq-based gene expression analyses of breast cancer patient data were performed with the TCGA dataset (74). Subtypes were defined based on the PAM50 annotation file provided within the dataset: Basal (often overlapping the term TNBC): 141 patients; Luminal-A: 421 patients. Gene expression levels of TNFα, IL-1β, CXCL8, CCL2 and CCL5 were determined. In all analyses, log2-transformed expression values of the genes were presented. Statistical analyses were performed following Shapiro-Wilk test, determining the normality of distribution for each gene by individual subtype. Comparisons of gene expression levels between the two clinical subtypes were presented in boxplots. *p*-values were determined by two-tailed Mann-Whitney test. The distribution of gene expression levels in basal and luminal-A patients was presented in histograms, where statistical analyses were performed by two-tailed Mann-Whitney test. In studies correlating the expression levels of the different genes, correlation coefficients and *p*-values were analyzed using Spearman correlation. Values of $p \leq 0.05$ were considered statistically significant.

Breast Tumor Cell Lines and Stromal Cells

The human TNBC cell lines (all from ATCC) included: MDA-MB-231 and MDA-MB-468 cells that were grown in DMEM (Gibco, Life technologies, Grand island, NY); BT-549 cells that were grown in RPMI 1640 medium (Biological Industries, Beit Ha'emek, Israel). Media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Biological Industries); for BT-549 cells, recombinant human (rh) insulin (10 mg/ml; #I9278; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to the medium. The human luminal-A cell lines MCF-7 (from ATCC) and T47D [provided by Dr. Keydar who generated the cell line (75)] were grown in culture in the same medium as MDA-MB-231 cells. Human pulmonary microvascular endothelial ST1.6R cells (HPMEC) were kindly provided by Dr. Unger and Dr. Kirkpatrick, Institute of Pathology, Johannes-Gutenberg University, Mainz, Germany. These cells were grown as described in Krump-Konvalinkova et al. (76), with minor modifications.

Human bone marrow-derived MSCs were purchased from Lonza (#PT-2501; Walkersville, MD), which validated them as MSCs based on cell markers and differentiation potential. Routine growth of MSCs took place in mesenchymal stem cell growth medium (#PT-3001; Lonza) or in MesenCult (#05411; Stemcell Technologies Inc., Vancouver, BC, Canada) and they were used for up to 10 passages. In this study, MSCs of four different healthy donors were used. Patient-derived CAFs from a primary breast tumor (used in ELISA and their accompanying signaling experiments) and from a lung metastasis (used in tumor cell invasion assays) were kindly provided by Dr. Bar, Sheba Medical Center, Ramat Gan, Israel). The cells were grown, identified and immortalized as described in Katanov et al. (67).

TNFα and IL-1β Concentrations Used in Different Analyses

Titration studies were initiated by determining the ability of rhTNFα (#300-01A, PeproTech, Rocky Hill, NJ), and rhIL-1β (#200-01B, PeproTech) to elevate in MDA-MB-231 cells and/or MSCs/CAFs the expression of CXCL8, CCL2 and/or CCL5 to levels that enabled us to perform the required comparisons between different cell combinations in ELISA studies (concentrations studied - TNFα: 100 pg/ml, 1 ng/ml, 10 ng/ml; IL-1β: 20, 100, 250, 350, 500, 750 pg/ml). The selected concentrations of 10 ng/ml TNFα and 350 pg/ml IL-1β were appropriate also for MSC and CAF experiments. Therefore, in all MDA-MB-231 studies, alone or with MSC/CAF, these selected concentrations were used in *in vitro* and *in vivo* experiments.

In parallel, titration studies indicated that the above selected concentrations were not optimal for ELISA responses of BT-549 and MDA-MB-468 cells; thus, based on additional analyses, the concentrations of cytokines were raised in these two cell types: MDA-MB-468 cells - 50 ng/ml TNFα and 500 pg/ml IL-1β; BT-549 cells - 25 ng/ml TNFα and 350 pg/ml IL-1β. These selected cytokine concentrations were used in all studies of MDA-MB-468 and BT-549 cells, alone or with MSCs.

The effects of TNFα and IL-1β on morphological changes, angiogenesis, migration and invasion with MCF-7 cells were determined in the same concentrations as used for MDA-MB-231 cells (10 ng/ml TNFα and 350 pg/ml IL-1β). In ELISA studies (and their accompanying signaling experiments) in MCF-7 and T47D cells cytokine concentrations were raised to 50 ng/ml TNFα and 500 pg/ml IL-1β. Although published data [e.g., (77, 78)] and our past studies indicated that lower TNFα and IL-1β concentrations (as in MDA-MB-231 cells) induce signaling and up-regulate the levels of CXCL8, CCL2 and CCL5 in MCF-7 and T47D cells, we expected that these cytokine concentrations will not enable us to clearly distinguish "intermediate" levels of chemokine induction in ELISA assays. Thus, cytokine concentrations were increased, as described above.

Western Blot Studies

Based on kinetics analyses (Data not shown), cells were stimulated for 15 min by TNFα or IL-1β (concentrations as described above) or their vehicle (similar for both cytokines), in medium containing 0.5% FBS. The cells were lysed in RIPA lysis buffer, followed by conventional Western blot (WB) procedures. The following antibodies (Abs) were used: Phosphorylated (P)-p65 [#3033; Cell Signaling Technology (CST), Danvers, MA]; Total (T)-p65 (#8242; CST); P-JNK (#4668; CST); T-JNK (#9258; CST). Abs directed against GAPDH (#ab9485; Abcam, Cambridge, UK) served for loading controls. The membranes were reacted with streptavidin-horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (#111-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA), and were subjected to enhanced chemiluminescence (#20-500, Biological Industries).

Cell Stimulation in Co-culture Experiments

TNBC or luminal-A cells were grown in "Contact" conditions with MSCs/CAFs (10:1 ratio) in 6-well plates (#3516, Corning,

Kennebunk, ME). When relevant, "Transwell" co-cultures were grown in similar plates, in which the two cell types were separated by an insert of 0.4 μm permeable polycarbonate membrane (#3412, Corning). In parallel, each cell type was grown individually in the same cell numbers as in co-cultures, in similar plates. The cells were grown in medium containing 10% FBS for 12 h, and were then treated by TNFα or IL-1β (concentrations as described above) or their vehicle for 7 h in medium supplemented with 0.5% FBS. Then, media were replaced by cytokine-free media (with 0.5% FBS) for additional 60 h. Cell conditioned media (CM) were removed and taken for ELISA assays. When indicated, CM and cell lysates were produced from cells that were subjected to gene down-regulation by siRNA, as detailed below. Cell lysates were produced from cells grown in larger vessels 7 h or 15 min after the beginning of cytokine stimulation and were used in quantitative real-time PCR analyses (qRT-PCR) or in WB studies.

ELISA Assays

CM obtained from different stimulatory conditions were cleared by centrifugation, followed by determining the expression levels of CXCL8, CCL2 and CCL5 by ELISA. Standard curves at the linear range of absorbance were produced with rhCXCL8, rhCCL2 and rhCCL5 (#200-8M, #300-04 and #300-06, respectively; PeproTech). The following Abs were used (all from PeproTech, unless otherwise indicated): For CXCL8 - Coating Abs: #500-P28; Detecting Abs: #500-P28Bt. For CCL2 - Coating Abs: #500-M71; Detecting Abs: #500-P34Bt. For CCL5 - Coating Abs: #500-M75; Detecting Abs: #BAF278 (R&D Systems, Inc., Minneapolis, MN). HRP-conjugated Streptavidin (#016-030-084; Jackson Immunoresearch laboratories) was added, followed by the substrate TMB/E solution (#ES001; Millipore, Temecula, CA); then, the reaction was stopped by addition of 0.18 M H_2SO_4 and absorbance was measured at 450 nm. To provide data on the contents of chemokines generated in each of the treatments and to clearly denote the differences in chemokine production between the different conditions, the findings are presented in ng/ml.

In reversibility experiments, MDA-MB-231:MSC "Contact" co-cultures were stimulated by 10 ng/ml TNFα for 67 h or exposed to vehicle control. Media were replaced and cell growth was continued for 10–14 days in TNFα-free media. CM that were collected following 67 h of stimulation, and 10–14 days after cytokine removal, were subjected to ELISA analysis of CXCL8 expression as described above.

qRT-PCR Analyses

Total RNA was extracted using the EZ-RNA kit (#20-400; Biological Industries). Using the M-MLV reverse transcriptase (#AM2044; Ambion, Austin, TX), first-strand cDNA was generated from RNA samples. cDNA targets were quantified by qRT-PCR on Rotor Gene 6000 (Corbett Life Science, Concorde, NSW, Australia). Absolute Blue qPCR SYBR Green ROX mix (#AB-4163/A; Thermo Fisher Scientific, Waltham, MA) was used to detect transcripts, according to manufacturer's instructions. Two pairs of specific primers were used (**Supplementary Table 1**), designed to span different exons. Data were normalized to the housekeeping gene GAPDH. Dissociation curves for each primer set indicated a single product, and "no-template" controls were negative after the 40 cycles used for analysis. Quantification was performed by standard curves, within the linear range of quantification.

Endothelial Cell Migration and Sprouting

To generate CM for functional *in vitro* angiogenesis assays, MDA-MB-231:MSC and MCF-7:MSC "Contact" co-cultures (10:1 cell ratio in each) were stimulated by TNFα for 7 h; in parallel, CM were produced from tumor cells that were treated by vehicle, from tumor cells stimulated by TNFα, or from tumor cells grown with MSCs only. Media were removed and the cells were cultured for additional 60 h in TNFα-free medium (with 0.5% FBS). CM were collected, cleared by filtration through a 0.45 μm membrane, and loaded in the lower part of migration transwells (#3422, Corning) with 8-μm pore membranes. The migration of HPMEC cells in direction of the different CM was determined after up to 90 min. At the end of the experiments, cells were removed from the upper side of the membranes, the membranes were fixed in ice-cold methanol and stained by Hemacolor (#1.11661; Merck). Cells that transmigrated to the lower side of the membranes in multiple bright fields were counted.

In parallel, CM were also used in sprouting assays of mCherry-expressing HPMECs out of 3D multicellular spheroids (all generated by the same cell number) that were formed in hanging drops for 24 h (79) and embedded into collagen type I (1.3 mg/ml; #354236; Corning). Sprouting of endothelial cells in response to CM was visualized by fluorescent microscopy after 10 days, and was determined quantitatively in multiple spheroids by mCherry signals of cells that sprouted out of spheroid core, quantified by ImageJ.

Tumor Cell Morphology, Migration and Invasion

In morphology assays, "Contact" co-cultures of mCherry-expressing tumor cells - MDA-MB-231 or MCF-7 - and MSCs (ratio 10:1) were stimulated by TNFα for 67 h; in parallel, mCherry-tumor cells were grown with vehicle, with TNFα only or with MSCs only. Then, tumor cell morphology was determined by fluorescent microscopy at x100 magnification, in multiple fields. In reversibility experiments of MDA-MB-231 cells, media were replaced in "Contact" co-cultures after 67 h, and cell growth was continued for 10–14 days in cytokine-free medium. Morphology was determined following 67 h of stimulation, and 10–14 days after cytokine removal.

Migration assays of MDA-MB-231 cells were performed in transwells with 8-μm pore membranes (#3422, Corning). In these assays, mCherry-MDA-MB-231 cells and MSCs (ratio 10:1) were added to the upper part of the chambers, in the presence of TNFα (10 ng/ml) in serum-free medium. The same number of mCherry-MDA-MB-231 cells were cultured in parallel transwells, in the presence of vehicle control or of TNFα alone. Migration

was performed for 12 h toward medium containing 10% FBS [containing TNFα (10 ng/ml) or vehicle control, as appropriate]. The numbers of migrating tumor cells were determined by staining with rabbit Abs to RPF (recognizing mCherry; #PM005; MBL, Woburn, MA), followed by DyLight 550-conjugated Donkey Abs recognizing rabbit IgG (#ab96920, Abcam). Cells were removed from the upper side of the membranes and fixed in ice-cold methanol. Based on DyLight 550 signals and Hemacolor staining used in preliminary analyses (Data not shown), we validated that close to 100% of migrating cells were the tumor cells. Photos of multiple high power fluorescent fields were taken at ×100 magnification. Cells that transmigrated to the lower side of the membranes in multiple fluorescence fields were counted.

Migration of Hoechst-labeled MCF-7 cells was determined by using the same experimental groups as in TNBC studies. MCF-7 cells are known as having a relatively low basal migratory potential, thus appropriate conditions were set, based on published studies and preliminary analyses in our lab. Thus, the membranes were coated with fibronectin for 1 h (20 μg/ml; #03-090-1, Biological Industries), cells were loaded to the upper part and after 21 h photos were taken. Hoechst-expressing tumor cells that migrated to the lower side of the membranes were counted in multiple fields.

Tumor cell invasion assays were performed using 3D multicellular spheroids that were generated for 72 h in hanging-drops [(79), with minor modifications]. Spheroids of "Contact" co-cultures, consisting of mCherry-MDA-MB-231 cells with MSCs/CAFs (ratio 10:1) were embedded into matrigel (9–10.5 mg/ml; #356234, Corning) and were stimulated by TNFα (10 ng/ml) or vehicle. Spheroids were also formed with mCherry-MDA-MB-231 cells alone, and treated by TNFα or by vehicle (same number of cells as in co-cultures). Invasion of mCherry-MDA-MB-231 cells was determined 48 h after the addition of cytokine stimulation (or vehicle). Multiple spheroids were photographed in fluorescent fields at x40 magnification. The invaded area was determined by the mCherry signals of cells that invaded out of spheroid core, quantified by ImageJ. Invasion of MCF-7 cells was determined in a similar manner after 96 h, using mCherry-MCF-7 cells and patient-derived CAFs, in the presence of TNFα (10 ng/ml) or its vehicle control. Spheroids were photographed in fluorescent fields 96 h after the addition of stimulation, at ×40 magnification.

CXCL8 Down-Regulation by siRNA

Knock-down of CXCL8 expression by transient siRNA transfections was performed in both MDA-MB-231 cells and MSCs, using the Lipofectamine RNAiMAX transfection reagent (#56531; Invitrogen, Grand Island, NY) according to manufacturer's instructions. ON-TARGET plus siRNA CXCL8 SMART pool and non-targeting control siRNA pool (siCTRL) were used (#L-004756-00 and #D-001810-10, respectively; Dharmacon, Lafayette, CO). The efficacy of CXCL8 down-regulation was validated by qRT-PCR or ELISA [Data not shown; 80–90% as in (80)] and the cells were used in assays, as necessary.

Tumor Growth and Metastasis

"Contact" co-cultures of mCherry-MDA-MB-231 cells with MSCs (10:1 ratio) were stimulated by TNFα or vehicle control, in 0.5% FBS-containing medium for 67 h. In each group (Group 1: Co-culture with TNFα; Group 2: Co-culture with vehicle), the same amount of live co-cultured cells was mixed 1:1 with matrigel (final concentration 4.5 mg/ml; #356234; Corning). The cells were administered orthotopically to the mammary fat pads of female athymic nude mice (#NUDE242; Envigo RMS, Jerusalem, Israel). During the experiment, tumors of Group 1 were supplemented every 3 days by CM taken from MDA-MB-231:MSC "Contact" co-cultures; these CM were generated as follows: The co-cultures were stimulated for 7 h with TNFα (10 ng/ml), after which the cytokine was removed, and CM were collected after additional 60 h of growth in cytokine-free and serum-free medium. These CM were filtered, concentrated ×10, and administered in proximity to tumors. Cytokine- and serum-free media that were put in parallel flasks have undergone similar procedures, and were administered to tumors of Group 2. At the endpoint of experiments (when tumors reached detectable sizes and had to be removed prior to necrosis), approximately 30 days after tumor cell inoculation, mice were sacrificed and primary tumors and lungs were excised. Tumor weights were determined, and tumor volumes were calculated based on caliper measurements. Lung metastases were determined *ex vivo* by the CRi Maestro non-invasive intravital imaging system. The total number of mice that were included in two biological repeats were 12 in Group 1, and 11 in Group 2. Statistical analyses of primary tumor weight and volume were performed by two-tailed unpaired Student's *t*-test. The proportions of mice bearing lung metastases were compared by Fisher's exact test. Procedures involving experimental animals were approved by Tel Aviv University Ethics Committee, and were performed in compliance with local animal welfare laws, guidelines and policies.

Data Presentation and Statistical Analyses

The statistical analyses of TCGA analyses and *in vivo* experiments were described in their respective sections. *In vitro* experiments were performed in $n \geq 3$ independent experimental repeats, with MSCs from ≥2 different donors, as indicated in respective figure legends. The results of ELISA, qRT-PCR, WB, HPMEC sprouting and tumor cell migration and invasion assays were compared by two-tailed unpaired Student's *t*-tests. Values of $p \leq 0.05$ were considered statistically significant. Adjustment for multiplicity of comparisons was done using the Benjamini-Hochberg procedure controlling the FDR at 0.05. All the significant results remained statistically significant after correcting for their multiplicity, except for some of the WB results. It these latter cases lack of significance was due to high variance between the intensities of effects of the experimental repeats of the test, despite the fact that they all demonstrated the same trend. Thus, in presentation of WB analyses we demonstrate not only the average and standard deviations (SD) of the experimental repeats but also the level of effect in each experiment.

RESULTS

High Expression Levels of TNFα and IL-1β Are Noted in Tumors of Basal Patients and Are Significantly Coordinated With High Expression Levels of CXCL8, CCL2 and CCL5

To identify the roles of TNFα and IL-1β in regulating tumor-stroma interactions in TNBC, we have extended currently-available studies on TNFα and IL-1β in TNBC patients (50–52) and compared the expression levels of TNFα and IL-1β in two subtypes of breast tumors: (1) Basal tumors, corresponding to the TNBC subtype, which has a most aggressive phenotype; (2) Luminal-A tumors having the best prognosis of all breast cancer subtypes.

Here, by using the TCGA breast cancer dataset we found that TNFα and IL-1β were expressed in significantly higher levels in basal tumors than in luminal-A tumors (**Figures 1A1,B1**). Distribution analyses (**Figures 1A2,B2**) demonstrated a larger proportion of basal patients with high TNFα and IL-1β expression levels, than luminal-A patients. In parallel, we analyzed the expression of pro-inflammatory and pro-metastatic chemokines CXCL8, CCL2 and CCL5, chosen as proxies for pro-tumorigenic factors that may be enriched in basal patients due to pro-inflammatory signals. These studies demonstrated significantly higher levels of the three chemokines in basal patients than in luminal-A patients (**Figures 1C–E**).

Moreover, as TNFα and IL-1β are key inducers of CXCL8, CCL2 and CCL5 expression [e.g., (81)] we also determined the correlation between the expression levels of TNFα and IL-1β and each of the three chemokines in basal patients. The findings of **Figure 2** indicate that the expression levels of TNFα and IL-1β were significantly correlated and coordinated with the presence of CXCL8, CCL2, and CCL5 in basal tumors.

Pro-metastatic Chemokines Reach Their Highest Expression Levels When TNBC:Stroma "Contact" Co-cultures Are Stimulated by TNFα or IL-1β

To follow up on the above findings, we asked how the cytokines TNFα and IL-1β regulate TNBC:MSC interactions that may lead to elevated release of the chemokines CXCL8, CCL2 and CCL5. First, we validated that the two cytokines could activate transcription pathways that typically induce the expression of these chemokines, namely NF-κB/p65 and JNK/AP-1 (30, 67, 81, 82). Indeed, these pathways were rapidly activated by a brief TNFα and IL-1β stimulation of 15 min (time point and cytokine concentrations were determined by preliminary analyses) in the TNBC MDA-MB-231 cells and in MSCs (**Figure 3A**).

Then, to determine if CXCL8 is regulated by tumor-stroma-inflammation networks, MDA-MB-231:MSC co-cultures were plated in "Contact" conditions that enabled direct physical contacts between the two cell types or in "Transwell" conditions that allowed only for the exchange of soluble factors between tumor cells and stromal cells. These co-cultures and each cell type alone were stimulated by TNFα and IL-1β (or their vehicle) for 7 h (Cytokine concentrations were selected based on titration assays, as described in "Materials and methods"); the cytokines were removed and cytokine-free CM were collected 60 h later. These experiments revealed that the highest levels of CXCL8 were produced when "Contact" co-cultures were stimulated by TNFα and IL-1β (**Figure 3B1**), and that they were higher than in all other conditions including non-stimulated co-cultures and individual cell types treated with either cytokine. CXCL8 protein levels were elevated also in cytokine-stimulated "Transwell" conditions; however, their total levels (ng/ml) were significantly lower than in cytokine-stimulated "Contact" conditions (**Figure 3B1**). Accompanying qRT-PCR analyses indicated that CXCL8 induction was regulated at the transcription level (**Supplementary Figure 1A**), and that increased CXCL8 levels in cytokine-stimulated co-cultures did not result of elevated proliferation of the cells under these conditions (**Supplementary Figure 2**). Accordingly, we did not detect any substantial cell death or proliferation of any of the cell types due to cellular interactions or cytokine stimulation (Data not shown).

Additional experiments revealed similar regulatory modes for CCL2, demonstrating its highest expression levels in TNFα- and IL-1β-stimulated MDA-MB-231:MSC co-cultures, obtained partly in a contact-dependent process (**Figure 3B2**). With CCL5, absolute dependence on TNBC:MSC contacts was revealed, and its expression was further elevated when "Contact" co-cultures were stimulated by TNFα or IL-1β (**Figure 3B3**). As with CXCL8, TNFα and IL-1β induced the expression of CCL2 and CCL5 by elevating their mRNA levels (**Supplementary Figures 1B,C**).

Additional analyses performed with TNBC cells that interacted with breast cancer patient-derived CAFs revealed similar regulatory patterns to those described above with MDA-MB-231:MSC co-cultures: TNFα and IL-1β induced p65 and JNK activation in CAFs (**Figure 4A**) and elevated all three pro-metastatic chemokines to their highest levels of expression when tumor-stroma-inflammation interactions took place (**Figure 4B**); moreover, CXCL8 and CCL2 up-regulation depended partly on cell-to-cell contacts whereas induction of CCL5 was fully dependent on direct physical contacts between the tumor cells and the CAFs, and was further induced by stimulation with TNFα and IL-1β.

In view of the high heterogeneity of TNBC tumors (4), we asked if similar regulatory patterns exist in co-cultures of other human TNBC cells - MDA-MB-468 and BT-549 - with MSCs. The findings of **Figure 5A** indicate that in both cell lines TNFα and IL-1β induced p65 and JNK activation and that the highest CXCL8 expression levels were produced when these TNBC cells physically interacted with MSCs in the presence of TNFα and IL-1β (**Figure 5B**). Of interest, the elevation in CXCL8 levels following cytokine stimulation of MDA-MB-468:MSC co-cultures partly depended on physical contacts between the two cell types (**Figure 5B1**), as was seen in MDA-MB-231:MSC co-cultures (**Figure 3B1**). In parallel, in BT-549:MSC co-cultures, CXCL8 elevation in IL-1β-stimulated cells depended on cell-to-cell contacts, while TNFα did not have much of an impact under "Contact" conditions (**Figure 5B2**).

To follow up on the data of **Figure 1**, indicating that the expression levels of the pro-inflammatory cytokines TNFα and IL-1β and of their targets - CXCL8, CCL2 and CCL5 - were

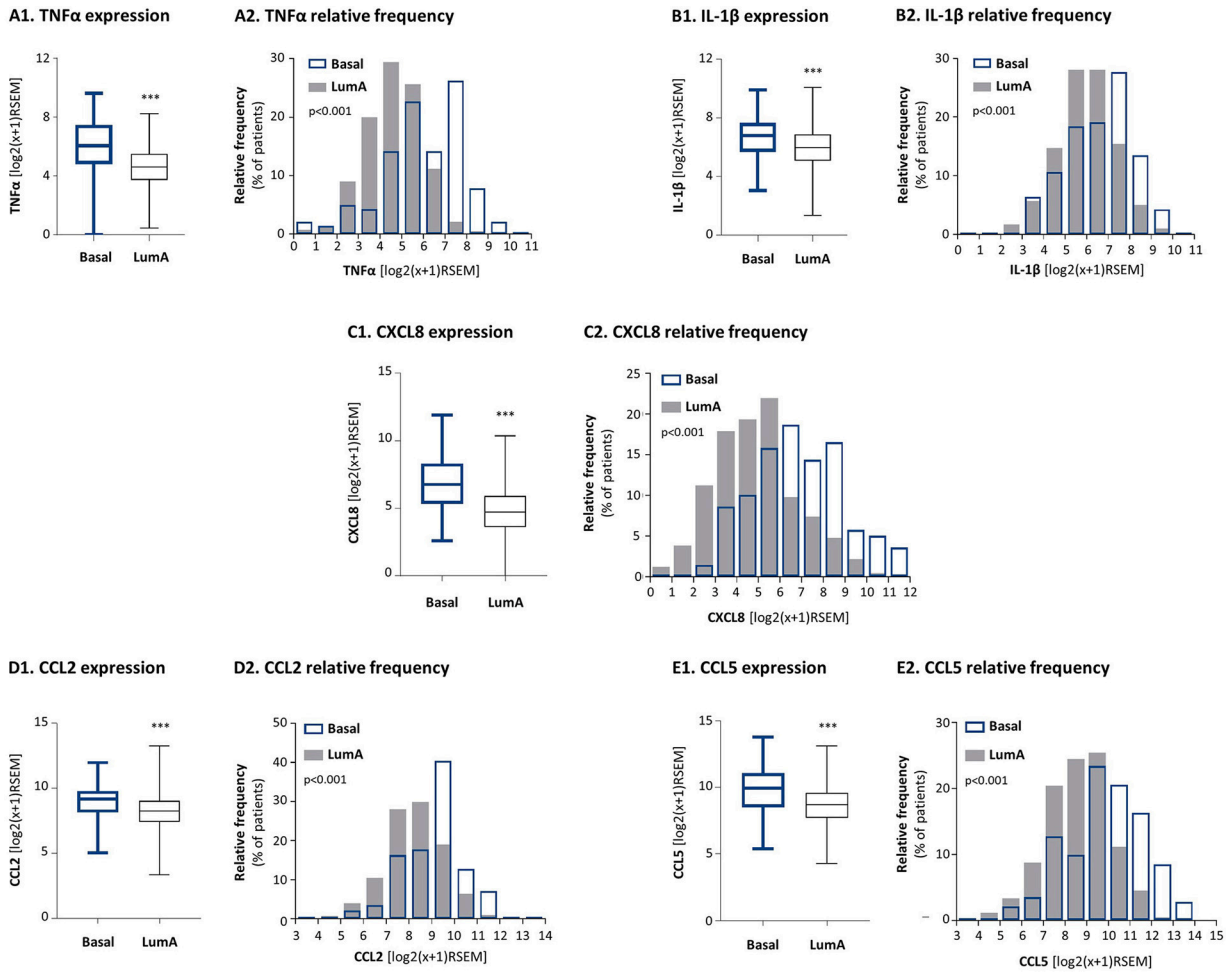

FIGURE 1 | The expression levels of the pro-inflammatory cytokines TNFα and IL-1β and of their chemokine targets are significantly higher in basal patients than in luminal-A patients. The Figure demonstrates gene expression analyses, performed with the TCGA breast cancer dataset. **(A)** TNFα, **(B)** IL-1β, **(C)** CXCL8, **(D)** CCL2, **(E)** CCL5. **(A1–E1)** Boxplots comparing expression levels in basal patients and luminal-A patients. ***$p < 0.001$. **(A2–E2)** Histograms demonstrating the distribution of expression levels of each of the factors in basal and luminal-A patient tumors. RSEM, RNAseq by expectation-maximization.

significantly lower in luminal-A patients than in basal patients, we determined how these three chemokines are affected by TNFα and IL-1β stimulation of luminal-A:MSC co-cultures. Despite the fact that TNFα and IL-1β induced potent p65 and JNK activation in T47D and MCF-7 luminal-A cells (**Figures 6A**, **7A**), CXCL8 levels were not increased but rather were decreased when the tumor cells interacted with MSCs in the presence of TNFα and IL-1β (**Figures 6B1**, **7B**). In parallel, CCL2 and CCL5 levels were increased by TNFα- and IL-1β-stimulated T47D:MSC "Contact" co-cultures (**Figures 6B2,B3**), but their expression levels were, in general, much lower than those obtained by TNFα- and IL-1β-stimulated TNBC:MSC co-cultures (**Figures 3B2,B3**). Further studies with MCF-7 luminal-A cells demonstrated elevations in the expression of CCL2 and CCL5 in some of the assays under "Contact" co-culture conditions following TNFα and IL-1β stimulation; however, the expression levels of these chemokines were often too low to provide clear-cut results (Data not shown).

These studies were followed by analyses of additional pro-metastatic effects, including tumor cell migration, invasion and angiogenesis. To this end, in the TNBC part we focused on the MDA-MB-231 cells because of their high metastatic potential. In luminal-A studies we chose to investigate MCF-7 cells because of our previous research indicating that they expressed more robust metastasis-related properties than T47D cells when stimulated by pro-inflammatory signals; additional investigations by our group also indicated that MCF-7 cells responded vigorously to TNFα-containing TME signals in *in vivo* metastasis studies (83–86).

Tumor-Stroma-Inflammation Networks Established With TNBC Cells Lead to Elevated Angiogenesis

To determine the functional consequences of tumor-stroma-inflammation networks, we first determined the ability of factors released by TNBC:MSC co-cultures stimulated by TNFα to promote processes involved in angiogenesis. Here, endothelial cells (HPMEC) sprouting assays demonstrated conclusive

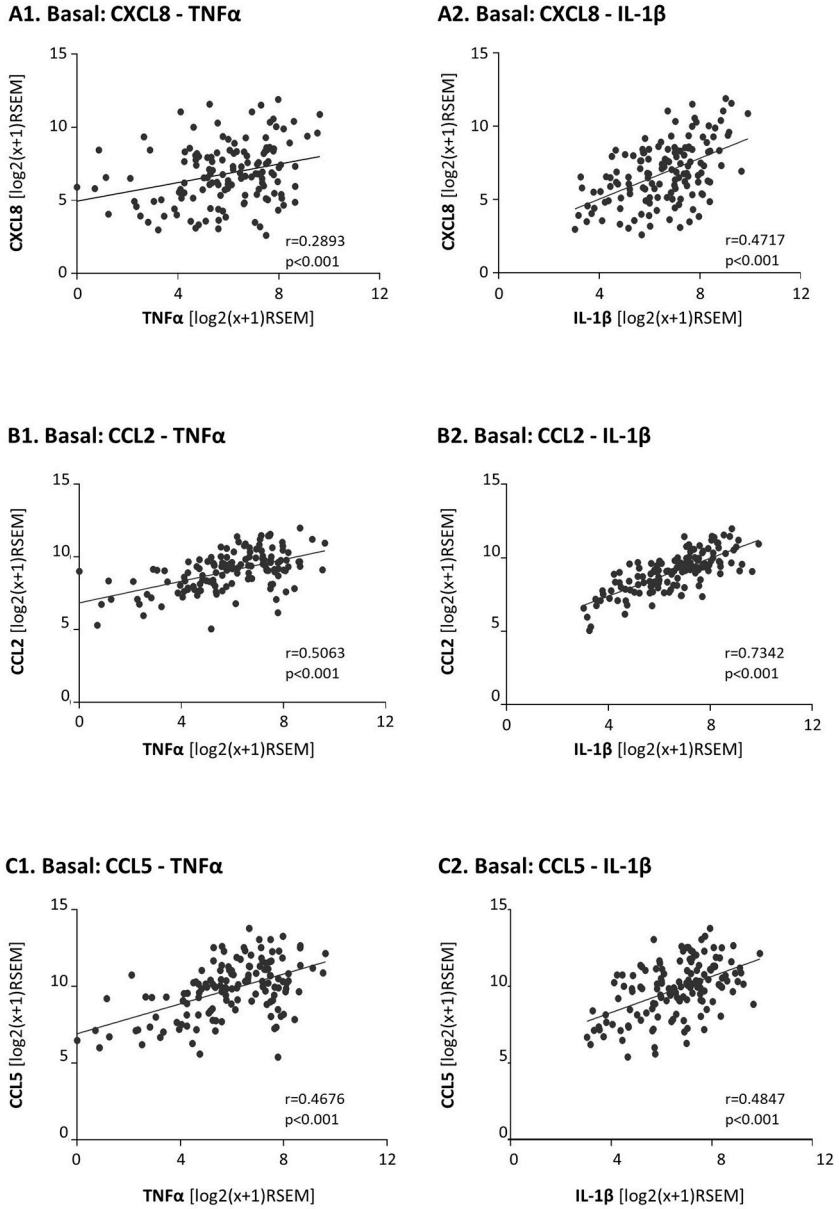

FIGURE 2 | In basal patients, the expression levels of the pro-inflammatory cytokines TNFα and IL-1β are significantly coordinated with the expression levels of their chemokine targets. The Figure demonstrates correlation analyses of gene expression in basal patients, performed with the TCGA breast cancer dataset. **(A)** CXCL8 correlations with TNFα **(A1)** and IL-1β **(A2)**. **(B)** CCL2 correlations with TNFα **(B1)** and IL-1β **(B2)**. **(C)** CCL5 correlations with TNFα **(C1)** and IL-1β **(C2)**. RSEM, RNAseq by expectation-maximization.

evidence to higher angiogenesis-supporting potential of TNFα-free CM derived from TNFα-stimulated "Contact" MDA-MB-231:MSC (**Supplementary Figure 3**) compared to CM obtained from control MDA-MB-231 cells. However, these studies did not reveal concrete information on the angiogenic potential of CM obtained from tumor cells + MSCs or from tumor cells + TNFα. Thus, additional studies were designed to provide another level of information on the ability of CM derived from the different groups to induce the migration of HPMEC in response to CM derived from different conditions. This assay enabled us to clearly demonstrate that the highest levels of endothelial cell migration were achieved when MDA-MB-231 cells interacted with MSCs in the presence of TNFα (**Figure 8A**). Moreover, our findings emphasized the contribution of TNFα to the angiogenic potential revealed by tumor cells grown in the presence of stromal cells.

We then performed parallel studies with luminal-A MCF-7 cells and found that CM of "Contact" MCF-7:MSC co-cultures had strong angiogenic activities as with TNBC cells (**Figure 8B**); however, in contrast to our studies with MDA-MB-231 cells (**Figure 8A**), in studies of MCF-7 cells TNFα did not push the

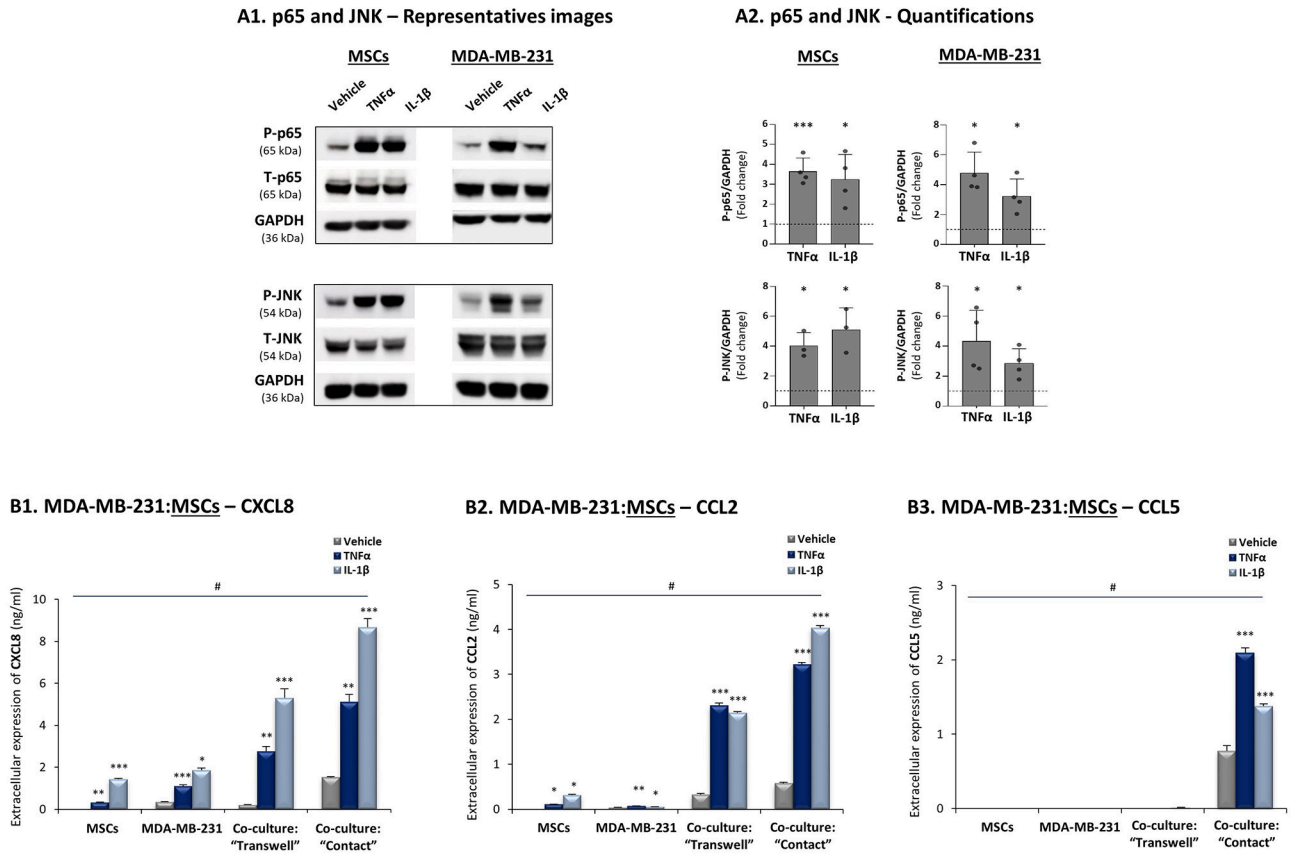

FIGURE 3 | CXCL8, CCL2 and CCL5 reach their highest expression levels when TNBC:MSC "Contact" co-cultures are stimulated by TNFα or IL-1β. **(A)** Activation of p65 and JNK, determined by WB in MSCs and in human TNBC MDA-MB-231 cells upon 15 min stimulation by TNFα, IL-1β or vehicle control (based on kinetics and titration studies. Information on cytokine concentrations is given in "Materials and methods"). GAPDH was used as a loading control. **(A1)** A representative experiment and **(A2)** averages ± SD of p65 and JNK activation (fold induction) in $n \geq 3$ independent experiments, performed with MSCs of 2 different donors. $^{***}p < 0.001$, $^{*}p \leq 0.05$ for differences between the values obtained for cytokine-stimulated cells and control non-stimulated cells. Dashed line stands for the value of 1 given to control cells. **(B)** CXCL8 **(B1)**, CCL2 **(B2)** and CCL5 **(B3)** expression levels were determined in TNBC:MSC co-cultures or each of the cell types grown alone, with or without TNFα or IL-1β stimulation. Co-cultures of MDA-MB-231 cells with MSCs were generated in "Transwell" and "Contact" conditions and were stimulated by TNFα (10 ng/ml), IL-1β (350 pg/ml) or vehicle control (similar for both cytokines) for 7 h. After change of media and growth for additional 60 h, the extracellular expression of CXCL8 in TNFα/IL-1β-free CM was determined by ELISA. $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ for differences between TNFα- or IL-1β-stimulated cells and vehicle-treated cells, within each group. #*p*-values were <0.01 or <0.001 (in most cases) in comparisons of "Contact" co-cultures with all other treatments, as well as in comparisons of "Transwell" co-cultures with all other treatments. The results are of a representative experiment of $n \geq 3$ independent experiments, performed with MSCs of 3 different donors.

angiogenic response induced by CM of "Contact" co-cultures any further.

Tumor-Stroma-Inflammation Networks Promote the Migratory and Invasive Properties of TNBC Cells

Next, we determined the effects of the tumor-stroma-inflammation network on tumor cell morphology, migration and invasion. First, we found that in TNFα-stimulated MDA-MB-231:MSC "Contact" co-cultures, the tumor cells acquired very elongated morphology (**Figure 9A1**) which is typical of cells that express high motility capabilities (87, 88). The morphology of the tumor cells under this condition was robustly different from the morphology of tumor cells grown alone, of tumor cells stimulated by TNFα and of tumor cells grown with MSCs only (**Figure 9A1**). These studies were followed by migration assays of MDA-MB-231 cells, known as having an aggressive phenotype which is manifested by a relatively high basal migratory potential. Despite their high basal motility, the interactions of MDA-MB-231 cells with MSCs in the presence of TNFα have led to significantly higher migratory capacity of the tumor cells compared to tumor cells grown alone (**Figure 9A2**). Of note, MDA-MB-231 cells grown in the presence of MSCs only or with TNFα alone did not migrate as well as tumor cells grown with MSCs and TNFα (**Figure 9A2**). **Supplementary Figure 4** demonstrates representative photos of MDA-MB-231 cells that migrated in the different study groups, identified by fluorescent staining.

In parallel, experiments performed with MCF-7 cells indicated that their morphology was modified by TNFα stimulation

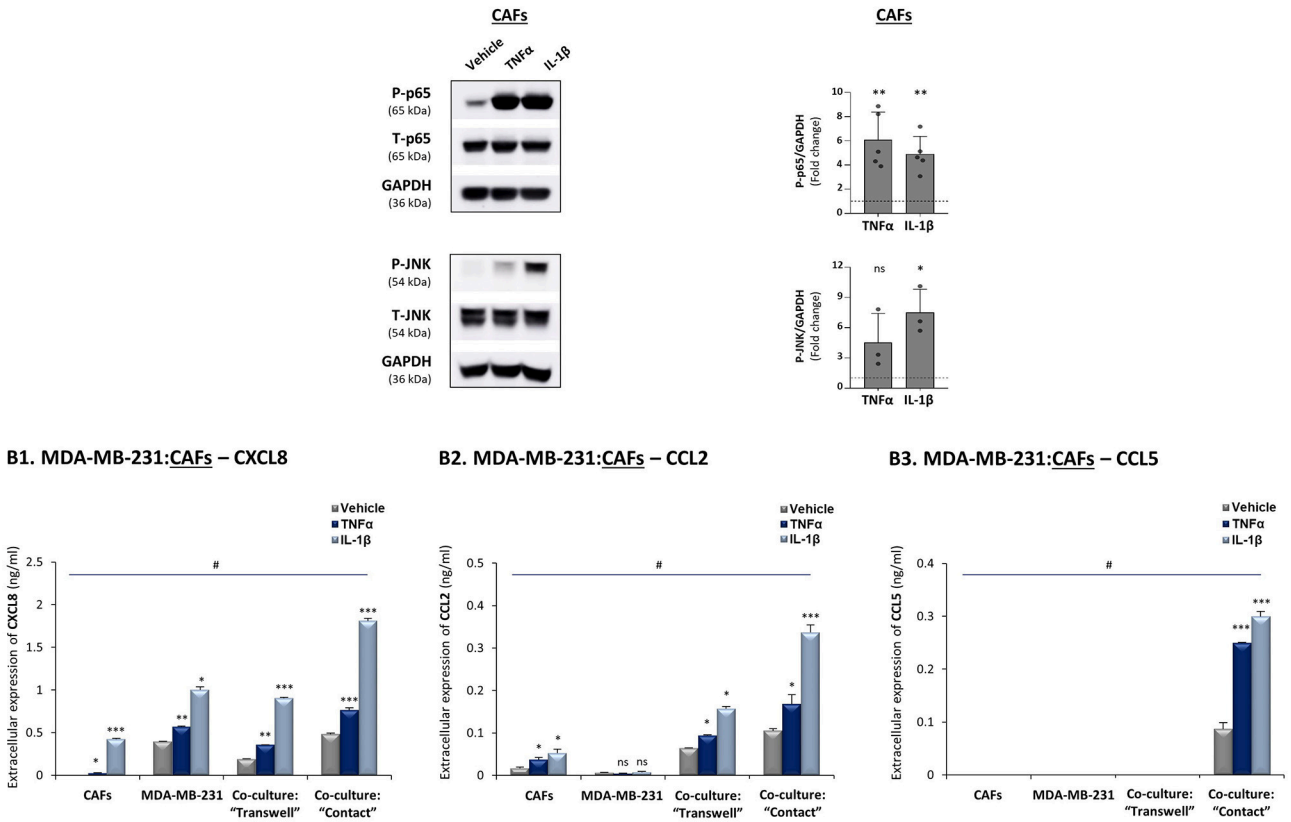

FIGURE 4 | CXCL8, CCL2 and CCL5 reach their highest expression levels when TNBC:CAF "Contact" co-cultures are stimulated by TNFα and IL-1β. The Figure demonstrates similar experiments and statistical analyses as those of **Figure 3**, performed herein with MDA-MB-231 cells and CAFs, using similar cytokine concentrations. **(A)** Activation of p65 and JNK. **(B)** CXCL8 **(B1)**, CCL2 **(B2)** and CCL5 **(B3)** expression levels. In all parts of the Figure, the results are of a representative experiment of $n \geq 3$ independent experiments. *, **, ***, # - As described in **Figure 3**. ns=non-significant.

(**Figure 9B1**) toward a metastasis-relevant phenotype [in line with our findings in (83–85)]. MCF-7 cells that grew in contact with MSCs also demonstrated modifications in their morphology, different than those induced by TNFα stimulation. However, in contrast to our findings with MDA-MB-231 cells (**Figure 9A1**), when TNFα was added to MCF-7:MSC "Contact" co-cultures, no additivity was found between TNFα and the MSCs in inducing more robust morphological changes in the tumor cells (**Figure 9B1**). Of note, the TNFα-stimulated MCF-7 cells that grew in co-culture with MSCs acquired elevated migratory capacity compared to control cells (**Figure 9B2**; **Supplementary Figure 5**). However, the overall migratory potential of MCF-7 cells at the tumor-stroma-inflammation setting (**Figure 9B2**) was much lower of MDA-MB-231 cells (**Figure 9A2**), although the two cell types were plated in same numbers in migration transwells. The relatively low migratory capacities of MCF-7 cells were noted despite the fact that they were given the proper conditions to support their migration (fibronectin coating of membranes and longer migration time than MDA-MB-231 cells).

To follow up on these findings, we investigated the ability of TNBC cells to invade out of 3D spheroids, a process requiring migration and invasion through extracellular proteins. We noted significantly increased invasion of MDA-MB-231 cells when they interacted with MSCs in the presence of TNFα stimulation (**Figure 10**), compared to all other cell combinations. Moreover, MDA-MB-231 cells exerted significantly increased invasion also when they interacted with patient-derived CAFs in the context of TNFα (**Figure 11A**; Please see "Note" in the legend of **Figure 11A**). In contrast to the TNBC cells, MCF-7 luminal-A cells that interacted with patient-derived CAFs in the presence of TNFα demonstrated very minor, if any, invasive properties (**Figure 11B**), even after longer invasion time compared to MDA-MB-231 cells (96 h for MCF-7 cells; 48 h for MDA-MB-231 cells).

TNBC-Stroma-Inflammation Networks Lead Through CXCL8 Activities to Increased Angiogenesis, as Well as to Elevated Migration and Invasion of TNBC Cells

The findings demonstrated so far indicated that tumor-stroma-inflammation networks can lead in TNBC to (1) increased production of pro-metastatic chemokines such as CXCL8 and

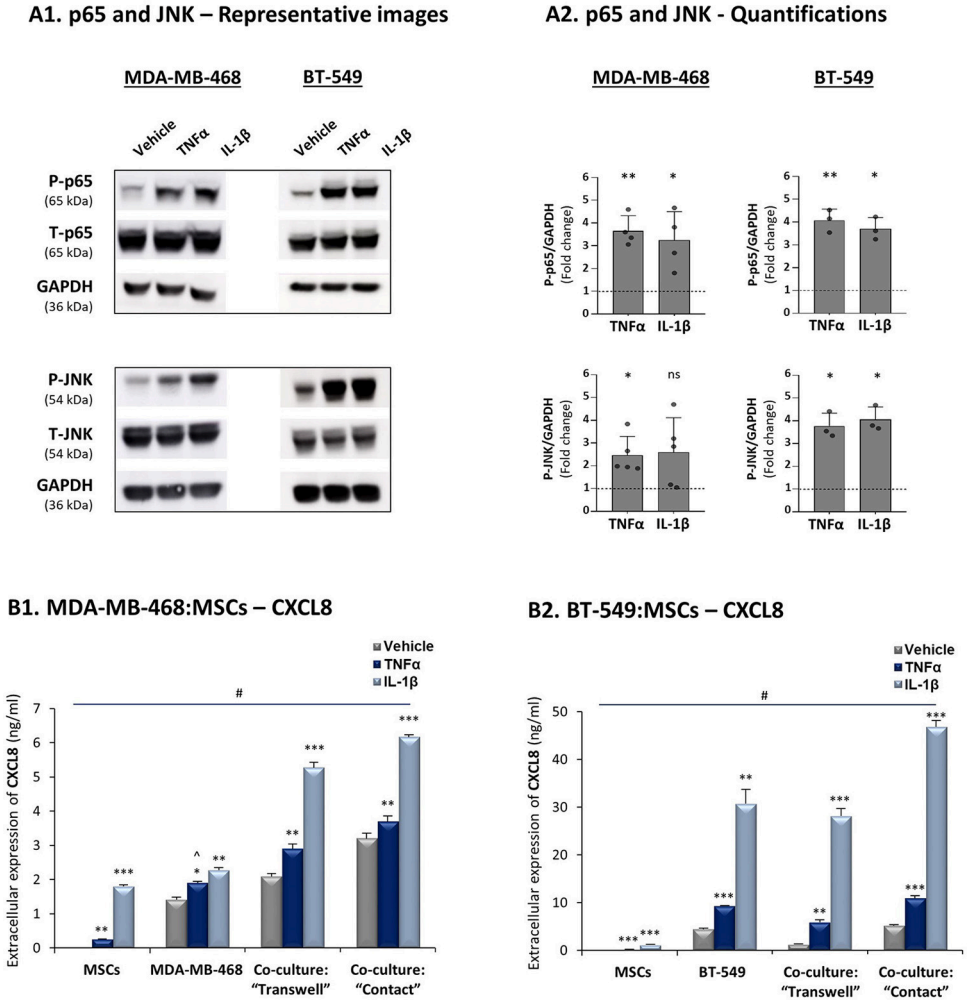

FIGURE 5 | Increased production of CXCL8 is a general characteristic of tumor-stroma-inflammation networks established with TNBC cells. The Figure demonstrates similar experiments and statistical analyses as those of **Figure 3**, performed herein with human TNBC MDA-MB-468, BT-549 cells and MSCs. Cytokine concentrations: MDA-MB-468 cells - 50 ng/ml TNFα and 500 pg/ml IL-1β; BT-549 cells - 25 ng/ml TNFα and 350 pg/ml IL-1β. **(A)** Activation of p65 and JNK. **(B)** CXCL8 expression levels. In all parts of the Figure, the results are of a representative experiment of $n \geq 3$ independent experiments, performed with MSCs of 2 different donors. ^In panel 5B1, this value was significant in 2 out of 3 experiments. *, **, ***, # - As described in **Figure 3**. ns=non-significant.

(2) elevated angiogenesis, tumor cell migration and invasion. To connect between these two processes, we asked if CXCL8 - selected because of its robust pro-angiogenic activities and pro-metastatic effects at the levels of TME and the tumor cells alike - was involved in mediating the functional properties of TNBC cells when the tumor-stroma-inflammation network was established.

To this end, we generated TNFα-stimulated MDA-MB-231:MSC "Contact" co-cultures in which CXCL8 was down-regulated in high efficiency by siRNA in the tumor cells and in the MSCs simultaneously [80–90% efficiency was found in CXCL8 down-regulation by the siRNA, similar to our findings in our parallel study (80); that study also demonstrates which of the cells contributed more to CXCL8 production when the tumor-stroma-inflammation network was established with MDA-MB-231 cells]. The findings of **Figure 12** clearly indicate that CXCL8 played significant roles in driving forward all metastasis-related alterations that were induced by the tumor-stroma-inflammation network in TNBC. Here, we found that in the absence of CXCL8 expression, endothelial cell migration in response to CM of TNFα-stimulated MDA-MB-231:MSC co-cultures was significantly reduced (**Figure 12A**). Moreover, upon CXCL8 down-regulation, the migration-relevant morphology of the tumor cells was partly reversed (**Figure 12B**), and the migration and particularly the invasion potentials of the tumor cells were significantly reduced (**Figures 12C,D**).

Tumor-Stroma-Inflammation Networks Promote the *in vivo* Pro-metastatic Properties of TNBC Cells

Many published studies have described the ability of MSCs and CAFs to promote the metastatic phenotype of TNBC cells (12, 16–18, 21, 23, 26, 65). Yet, they have not directly

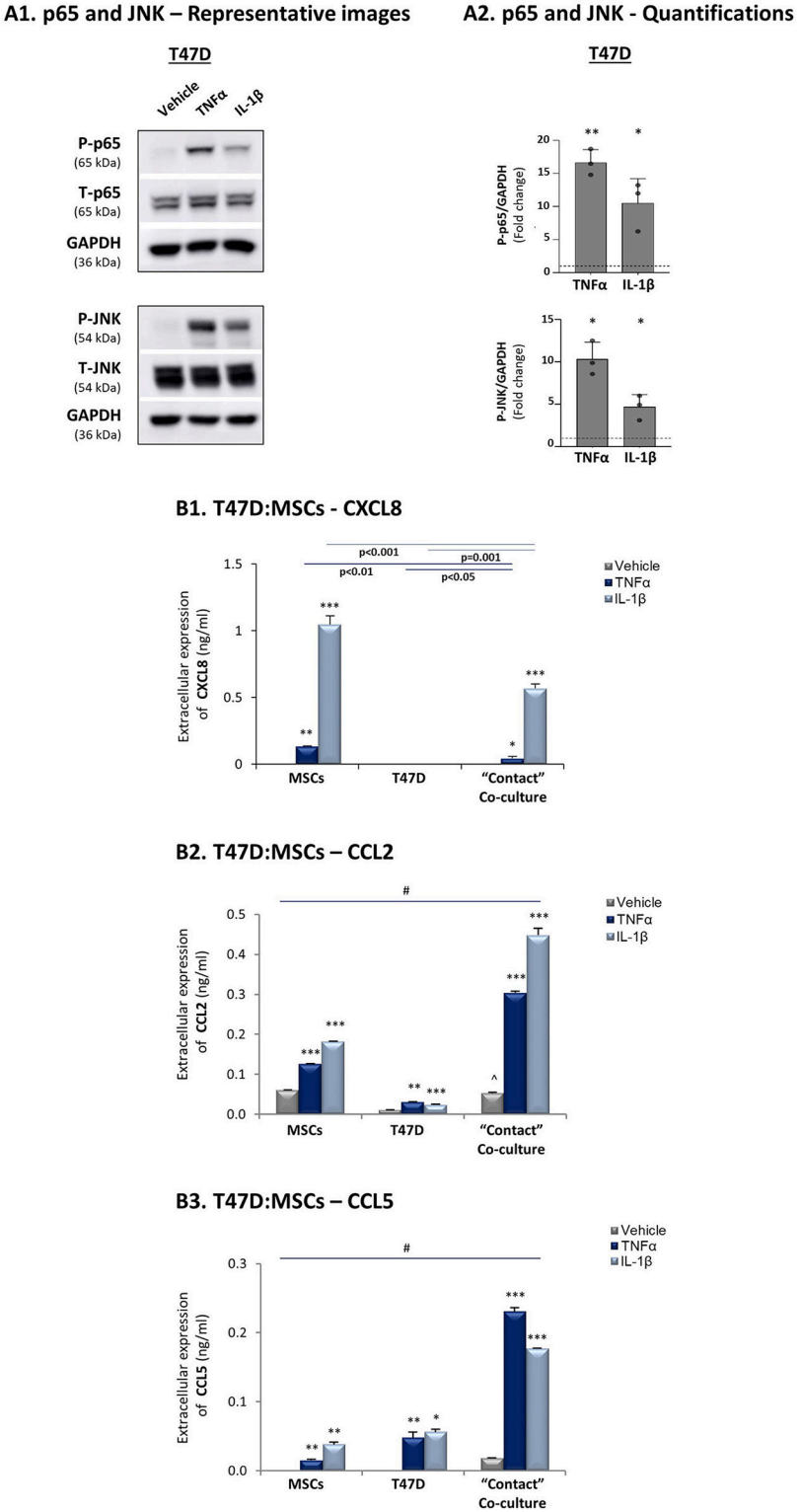

FIGURE 6 | CXCL8 expression is down-regulated in TNFα- and IL-1β-stimulated luminal-A (T47D): MSC "Contact" co-cultures. The Figure demonstrates similar experiments and statistical analyses as those of **Figure 3**, performed herein with T47D cells and MSCs. Cytokine concentrations: 50 ng/ml TNFα and 500 pg/ml IL-1β. **(A)** Activation of p65 and JNK. **(B)** CXCL8 **(B1)**, CCL2 **(B2)** and CCL5 **(B3)** expression levels. ^In panel B2, comparisons to non-stimulated MSCs were non-reproducible. In all parts of the Figure, the results are of a representative experiment of $n = 3$ independent experiments, performed with MSCs of ≥2 different donors. *, **, ***, # - As described in **Figure 3**.

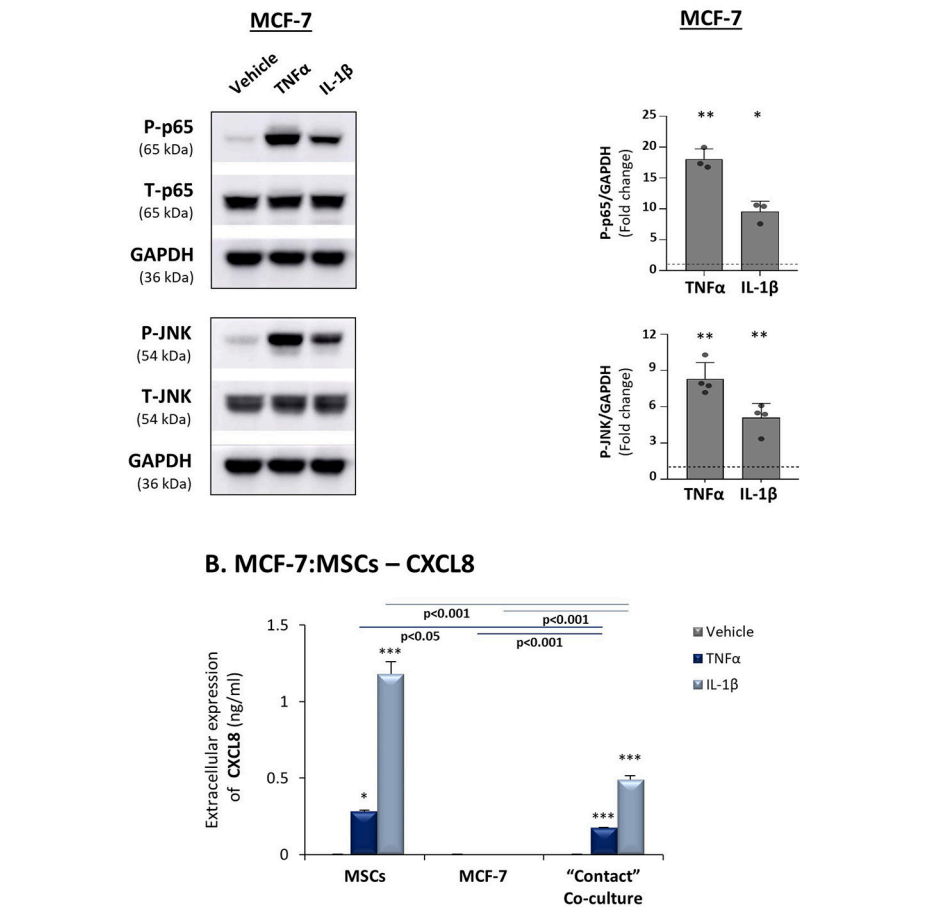

FIGURE 7 | CXCL8 expression is down-regulated in TNFα- and IL-1β-stimulated luminal-A (MCF-7): MSC "Contact" co-cultures. The Figure demonstrates similar experiments and statistical analyses as those of **Figure 3**, performed herein with MCF-7 cells and MSCs. Cytokine concentrations: 50 ng/ml TNFα and 500 pg/ml IL-1β. **(A)** Activation of p65 and JNK. **(B)** CXCL8 expression levels. In all parts of the Figure, the results are of a representative experiment of n≥3 independent experiments, performed with MSCs of 2 different donors. *, **, ***, # - As described in **Figure 3**.

determined the impact of the pro-inflammatory signals on tumor growth and metastasis when TNBC:stroma interactions are established. Our above findings motivated us to determine whether the pro-inflammatory signals delivered by TNFα to TNBC:MSC "Contact" co-cultures *in vitro* would potentiate tumor growth or metastasis in an animal model system.

The ability of TNFα to potentiate the *in vivo* aggressiveness of TNBC cells grown with MSCs requires that the *in vitro* advantages given to the tumor cells by their 3-day exposure to MSCs and to the cytokine, will persist *in vivo* (the cytokine is removed prior to injection to mice); Thus, we first determined whether the increased pro-metastatic capabilities endowed on the tumor cells by their co-culturing with stromal cells in the presence of TNFα withhold when TNFα is removed. To this end, TNBC:MSC "Contact" co-cultures were established for 67 h with TNFα stimulation, leading to high CXCL8 levels and clear changes in tumor cell morphology (**Figures 13A,B**); then, TNFα was removed and the growth of TNBC:MSC "Contact" co-cultures was continued in TNFα-deprived medium for ∼2 weeks. The findings of **Figure 13** demonstrate that the effects of TNFα were reversible: ∼2 weeks after TNFα removal the elevation in CXCL8 was completely abolished (**Figure 13A**) and the elongated cell morphology was almost entirely diminished (**Figure 13B**).

Taking into account these findings on reversibility of TNFα-mediated effects, proposing that the benefits that were provided by TNFα stimulation to TNBC:MSC co-cultures may not persist *in vivo,* we proceeded to the animal system setting. Here, we introduced an experimental design that will enable us to directly assess the impacts of TNFα on the metastatic potential of TNBC cells when they interacted with stromal cells. To this end, MDA-MB-231 cells were grown in "Contact" co-cultures with MSCs in the presence of TNFα for 67 h, and were compared to vehicle-exposed MDA-MB-231:MSC cultures, serving as controls. The cells were then administered to the mammary fat pads of female mice. To strengthen *in vivo* the possible effects of TNFα when

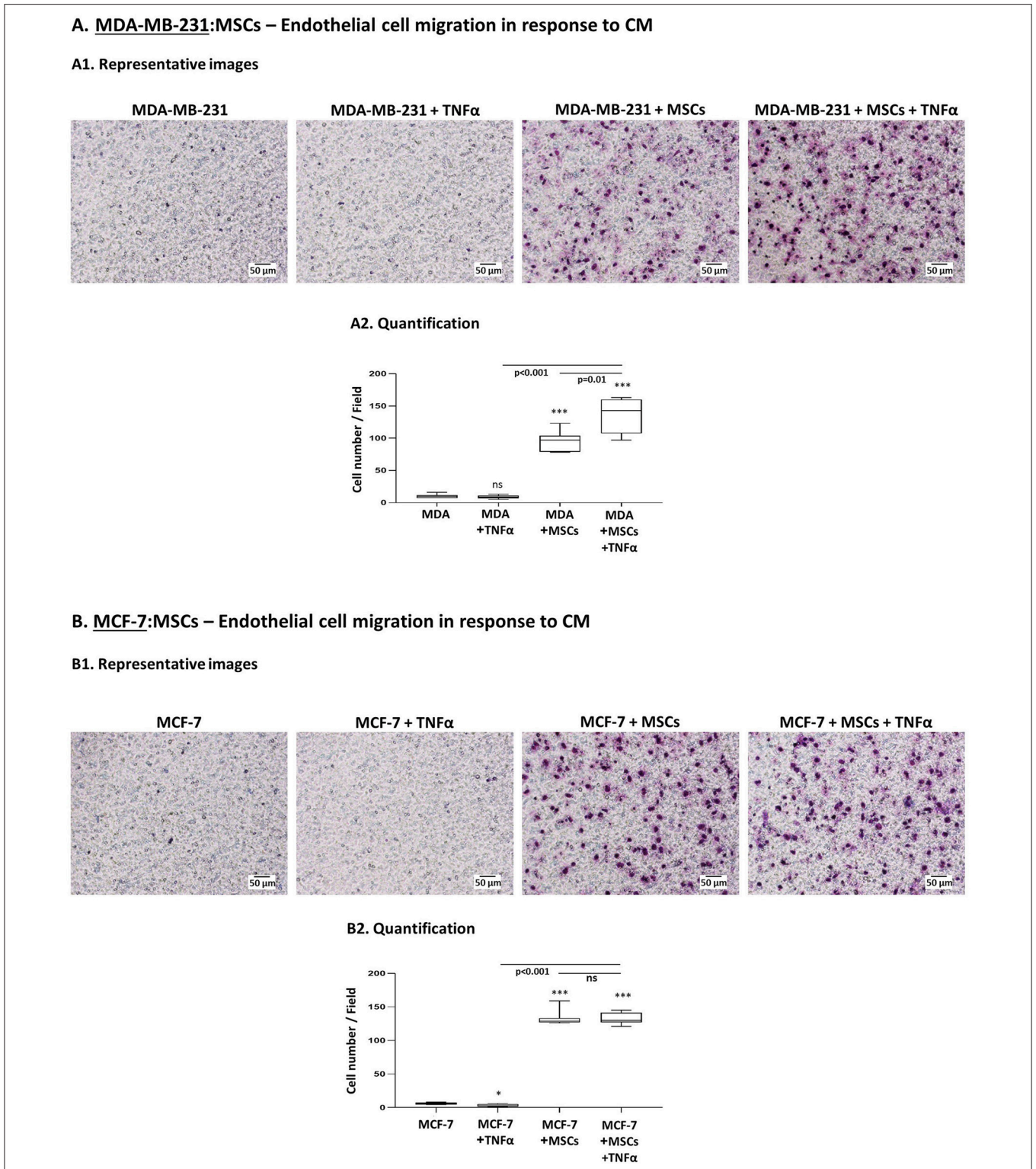

FIGURE 8 | The pro-angiogenic activities of factors released by tumor:MSC "Contact" co-cultures are promoted by TNFα in TNBC but not in luminal-A cells. Studies of endothelial cell (HPMEC) migration in response to CM derived from different cell combinations of MDA-MB-231 cells ("MDA") **(A)** and MCF-7 cells **(B)**. **(A1, B1)** Representative photos of HPMEC migration in response to TNFα-free CM derived from TNFα-stimulated tumor:MSC "Contact" co-cultures (10 ng/ml), from tumor cells alone, from tumor cells stimulated by TNFα alone and from tumor cells grown under "Contact" conditions with MSCs only. Bars, 50 µm. **(A2, B2)** Migrated HPMEC were counted in multiple photos per insert of the experiments presented in A1 and B1. ***$p < 0.001$, *$p < 0.05$, ns=non-significant for comparisons between CM of different cell combinations and CM of tumor cells treated by vehicle. Photos and their quantifications are representatives of $n \geq 3$ independent experiments, performed with MSCs of 3 different donors.

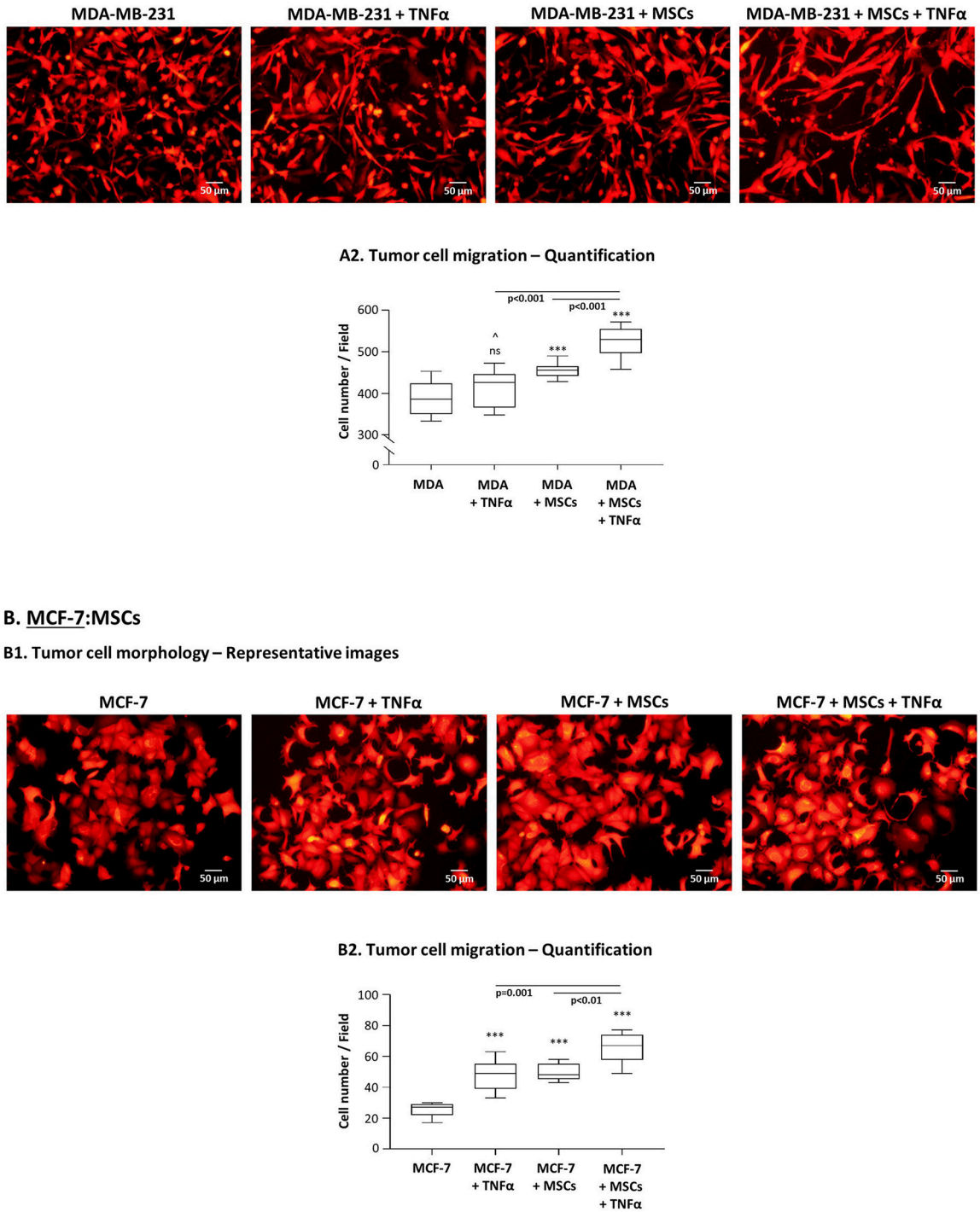

FIGURE 9 | TNBC cells, and less so luminal-A cells, acquire migration-related characteristics upon "Contact" co-culturing with MSCs in the presence of TNFα. **(A)** Morphology and migration phenotypes of MDA-MB-231 cells ("MDA"). **(A1)** Morphology of mCherry-MDA-MB-231 cells grown with MSCs in the presence of TNFα (10 ng/ml), compared to tumor cells treated by vehicle, tumor cells stimulated by TNFα or tumor cells grown with MSCs only. Bar, 50 µm. **(A2)** Migration of mCherry-MDA-MB-231 cells ("MDA") grown in "Contact" co-cultures with MSCs in the presence of TNFα (10 ng/ml) compared to migration of MDA-MB-231 cells treated by vehicle only, of MDA-MB-231 cells stimulated by TNFα or of MDA-MB-231 cells grown in co-culture with MSCs only. Migration assays were performed in response to medium containing 10% FBS, for 12 h. ***$p < 0.001$, ns=non-significant for differences between migration of tumor cells in different combinations,

(Continued)

FIGURE 9 | compared to the migration of non-stimulated TNBC cells. ^In panel **(A2)**, this value was significant in 1 out of 3 experiments. Representative fluorescent photos of migrating cells are presented in **Supplementary Figure 4**. In all sections of Part **(A)**, the Figures demonstrate representative experiments of $n = 3$ independent experiments of each type, performed with MSCs of ≥2 different donors. **(B)** Morphology and migration phenotypes of MCF-7 cells, determined as described in Part **(A)**, unless otherwise indicated. **(B1)** Morphology of mCherry-MCF-7 cells. Bar, 50 μm. **(B2)** Migration of Hoechst-loaded MCF-7 cells was performed in response to medium containing 10% FBS for 21 h through fibronectin-coated membranes, in similar combinations as of MDA-MB-231 cells in Part **(A)** (TNFα: 10 ng/ml). ***$p < 0.001$; Representative photos of migrating cells are presented in **Supplementary Figure 5**. In all sections of Part **(B)**, the Figures demonstrate representative experiments of $n \geq 3$ independent experiments, performed with MSCs of ≥2 different donors.

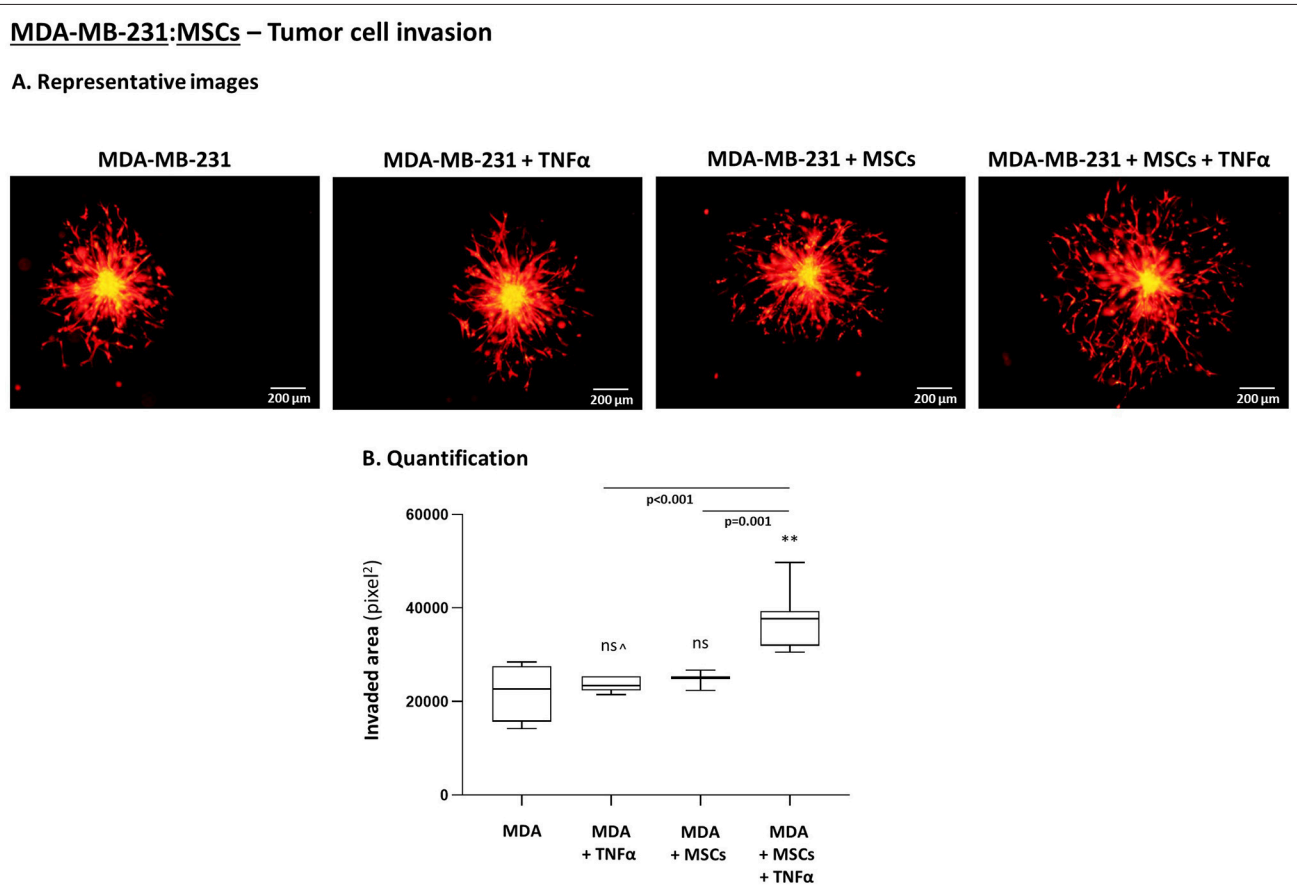

FIGURE 10 | TNBC cells acquire elevated invasive properties upon "Contact" co-culturing with MSCs in the presence of TNFα. The Figure demonstrates tumor cell invasion out of 3D spheroids that were formed by mCherry-MDA-MB-231 cells ("MDA") alone or by tumor cells in "Contact" co-culturing with MSCs. The spheroids were imbedded into matrigel and then stimulated by TNFα (10 ng/ml) or vehicle for 48 h. **(A)** Representative photos. Bar, 200 μm. **(B)** Invasion was quantified in multiple spheroids by ImageJ. **$p < 0.01$, ns=non-significant for differences between TNBC cell invaded out of spheroids in different combinations, compared to the invasion of non-stimulated TNBC-only spheroids. ^See "Note" in legend to **Figure 11**. Photos and their quantifications are representatives of $n > 3$ independent experiments, performed with MSCs of 2 different donors.

it acted on MDA-MB-231 and MSC "Contact" co-cultures, CM containing factors released by TNFα-stimulated MDA-MB-231:MSC "Contact" co-cultures (but deprived of TNFα itself) were injected in proximity to tumors generated by TNFα-stimulated co-cultures; in parallel, control media were administered to tumors arising from injection of vehicle-treated co-cultures. The findings of **Figure 13C** indicate that the sizes and weights of primary tumors were similar in the two groups of mice; however, most importantly, the metastatic potential of the tumor cells that interacted with MSCs was increased by in-culture TNFα stimulation (**Figure 13D**). This effect was revealed by the elevated incidence of mice carrying lung metastases following MDA-MB-231 co-culturing with MSC under the influence of TNFα *in vitro*, compared to the control group in which the co-cultured cells were not exposed to TNFα (**Figure 13D**).

DISCUSSION

The fundamental roles of the TME in promoting cancer progression are now well-appreciated, with stromal cells and pro-inflammatory elements being key contributors to disease

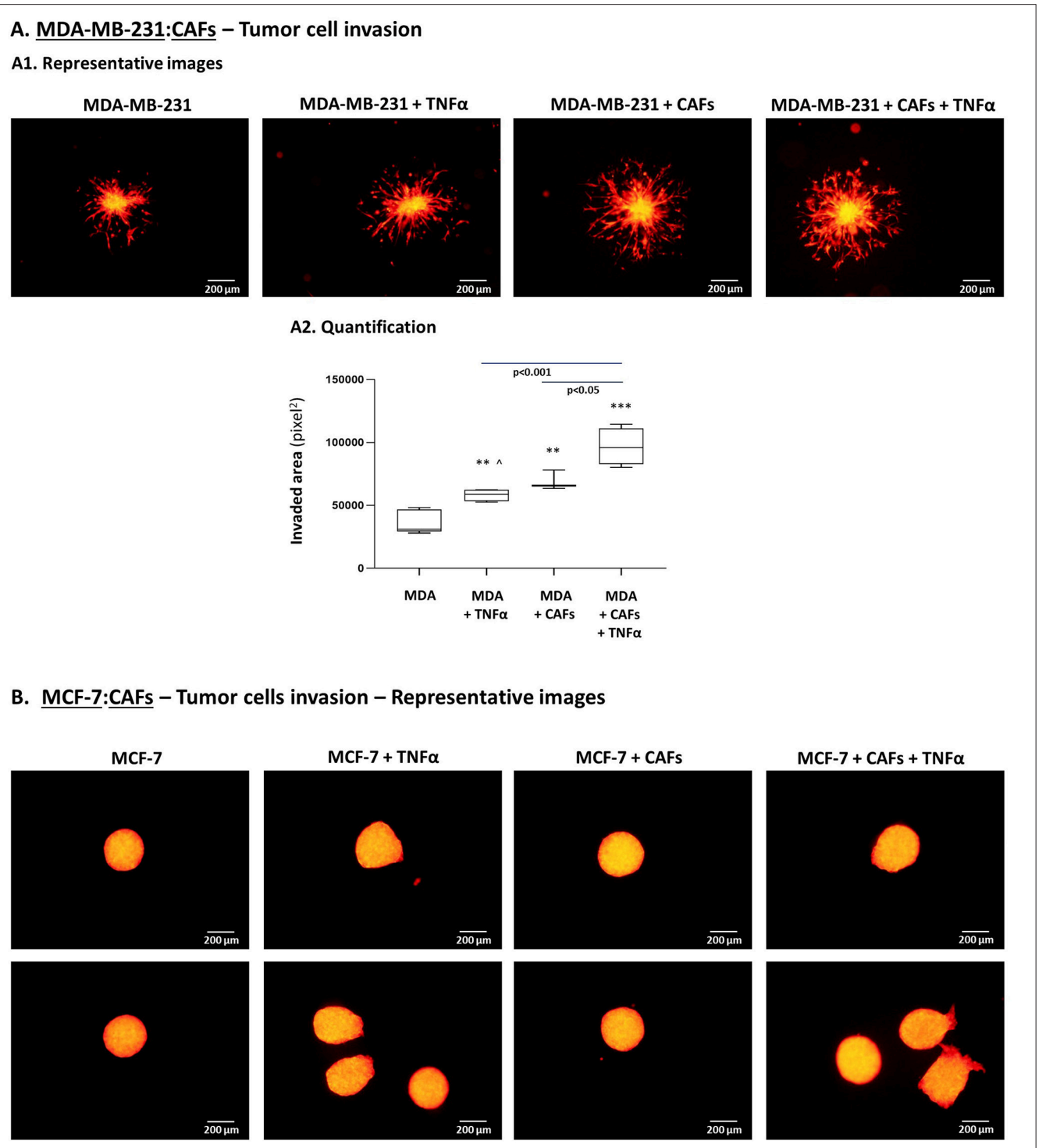

FIGURE 11 | TNBC cells but not luminal-A cells acquire elevated invasive properties upon "Contact" co-culturing with patient-derived CAFs in the presence of TNFα. **(A)** The Figure demonstrates similar experimental setup as in **Figure 10**, performed herein with mCherry-MDA-MB-231 cells ("MDA") and CAFs, using similar cytokine concentrations. [^Note: Possibly due to technical reasons (different matrigel batches) TNFα stimulation elevated tumor cell invasion in this setting but not in **Figure 10**]. **(A1)** Representative photos. Bar, 200 µm. **(A2)** Invasion was quantified in multiple spheroids by ImageJ. ***$p < 0.001$, **$p < 0.01$ for differences between invasion of tumor cells in different combinations, compared to the invasion of TNBC cells grown alone in spheroids. Photos and their quantifications are representatives of $n > 3$ independent experiments. **(B)** MCF-7 cells have undergone similar procedures to those described in **Figure 10**, for 96 h (TNFα 10 ng/ml). Because invasion of MCF-7 cells out of the spheroids was minimal or absent, quantitation could not be performed. Instead, two representative photos out of many taken in $n > 3$ independent experiments, are provided for each treatment. Bar, 200 µm.

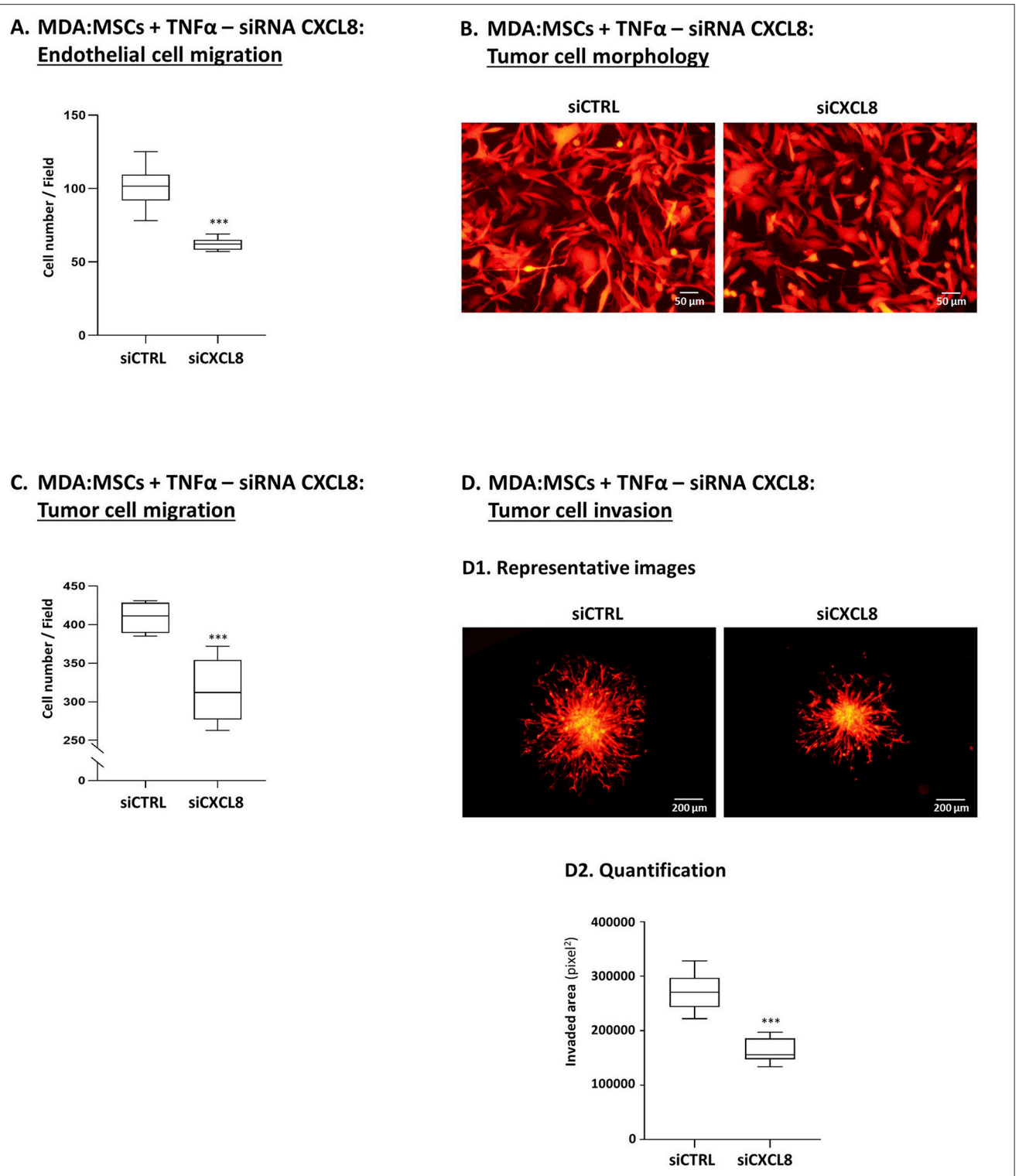

FIGURE 12 | Tumor-stroma-inflammation networks lead through CXCL8 activities to increased angiogenesis, invasion-related tumor cell morphology, tumor cell migration and invasion in TNBC cells. MDA-MB-231 cells ("MDA") and MSCs were both transfected by siCXCL8 or siCTRL. Parallel studies indicated that the efficiency of CXCL8 down-regulation was high [80–90% in most experiments, as in (80); Data not shown]. The cells were grown in "Contact" co-cultures and were stimulated by TNFα (10 ng/ml); then, the cells or their CM were assayed in the following tests: **(A)** Endothelial cell migration (Procedures as in **Figure 8A**). ***$p <$ 0.001; **(B)** Tumor cell morphology (Procedures as in **Figure 9A1**). Bar, 50 µm; **(C)** Tumor cell migration (Procedures as in **Figure 9A2**). ***$p < 0.001$; **(D)** Tumor cell invasion (Procedures as in **Figure 10**). **(D1)** Representative photos. Bar, 200 µm. **(D2)** Quantification. ***$p < 0.001$. In all parts of the Figure, photos and their quantifications are representatives of $n = 3$ independent experiments, performed with MSCs of 2 different donors.

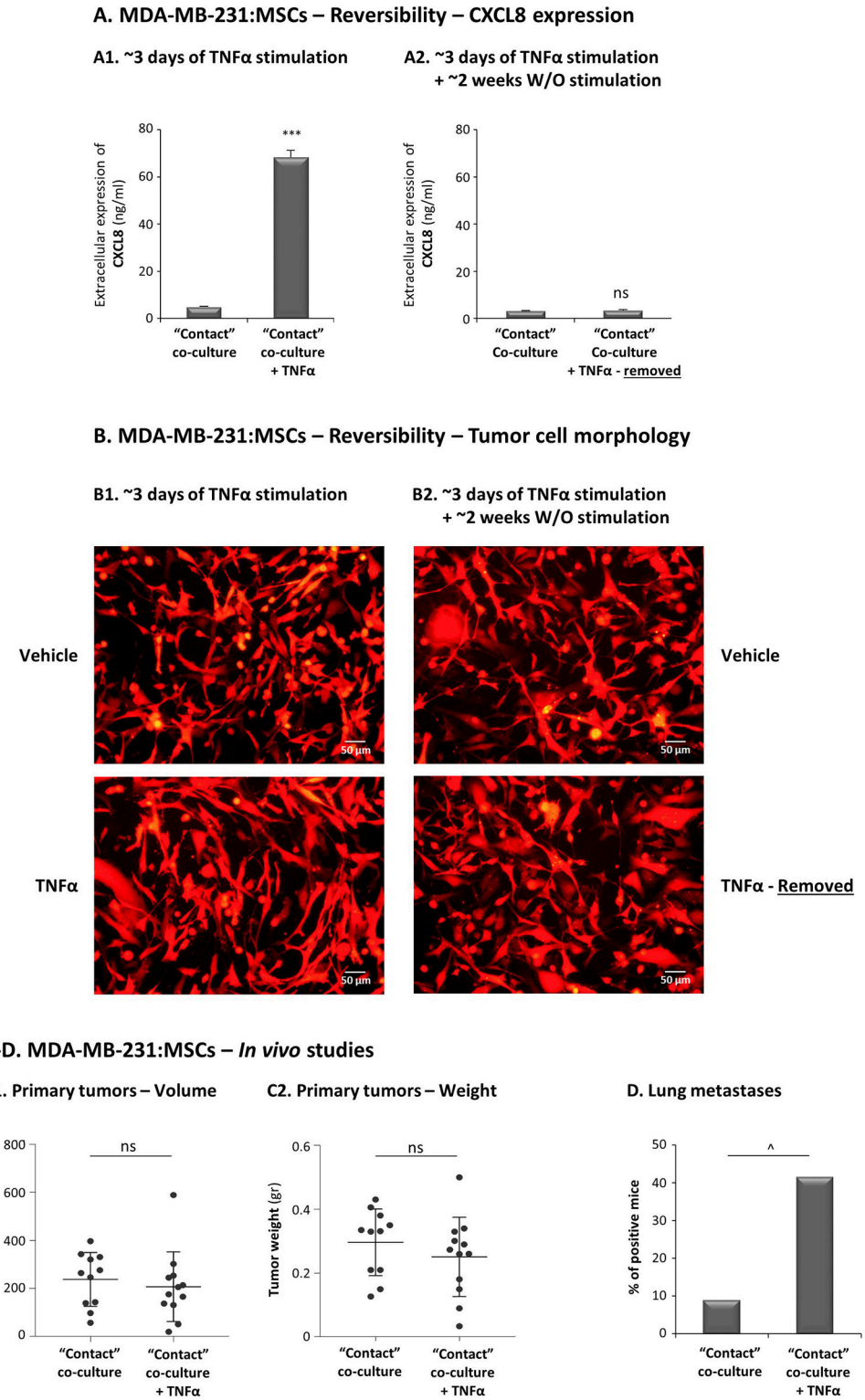

FIGURE 13 | TNFα promotes the metastatic potential of TNBC cells grown in contact with MSCs. **(A,B)** Reversibility of TNFα-induced tumor-promoting phenotypes, generated *in vitro* in "Contact" MDA-MB-231:MSC co-cultures following TNFα removal. mCherry-MDA-MB-231 cells were co-cultured in "Contact" conditions with MSCs in the presence of TNFα (10 ng/ml) or vehicle control for 67 h (termed "~3 days of TNFα stimulation") **(A1,B1)**. Then, vehicle-treated and TNFα-stimulated MDA-MB-231:MSCs co-cultures were re-cultured without further TNFα stimulation for additional 10–14 days (termed "~3 days of stimulation + ~2 weeks W/O TNFα stimulation) **(A2,B2)**. At both time points (~3 days and ~2 weeks), extracellular CXCL8 levels were determined in cell supernatants by ELISA **(A)** and tumor cell

(Continued)

FIGURE 13 | morphology was determined by fluorescent microscopy **(B)**. ***$p < 0.001$, ns, not significant. Bar, 50 μm. In all panels of section **(A,B)**, the results are representatives of $n = 3$ independent experiments, performed with MSCs of 2 different donors. **(C,D)** mCherry-MDA-MB-231 cells were grown in "Contact" co-cultures with MSCs in the presence of TNFα (10 ng/ml) or vehicle control, for 67 h (∼3 days). Then, co-cultured cells were injected to the mammary fat pad of nude mice in 2 independent experiments (For additional experimental details please see "Materials and methods"). Total mice numbers were: (1) In the group of mice administered with TNFα-stimulated co-cultures: $n = 12$; (2) In the group of mice administered with vehicle-exposed co-cultures: $n = 11$. At the end of the experiment (∼30 days post injection), primary tumor size was determined by volume **(C1)** and weight **(C2)**. ns=non-significant. **(D)** Metastases in lungs were detected by mCherry signals using the Cri Maestro fluorescence imaging system. ^$p = 0.095$.

development and metastasis. In the complex milieu that exists in tumors, cross-talks between the different TME players and the tumor cells eventually establish intricate networks whose roles in dictating disease course are still poorly defined and characterized.

In the present study, we were particularly interested in elucidating the roles of tumor-stroma-inflammation networks in regulating tumor progression in TNBC, a most aggressive subtype of breast cancer. In our study, we have used potent and most clinically relevant pro-inflammatory cytokines - TNFα and IL-1β - that are expressed in breast tumors and have pro-metastatic functions in TNBC. Our study provides novel findings indicating that interactions between TNBC cells and MSCs/CAFs in the presence of such pro-inflammatory cytokines can lead to significantly enhanced pro-metastatic phenotypes of the TME and of the tumor cells themselves. This was illustrated by increased expression of pro-metastatic chemokines, by elevated ability to induce angiogenesis, as well as by higher migratory and invasive capabilities of the tumor cells. Ultimately, the end result of the activities of the tumor-stroma-inflammation network was a higher metastatic potential of TNBC cells *in vivo*.

The tumor-stroma-inflammation network was found in our study to strongly induce the expression of the pro-metastatic chemokines CXCL8, CCL2 and CCL5. These chemokines are pro-inflammatory factors and as such contribute to cancer inflammation by recruiting myeloid inflammatory cells, as well as immune-suppressive cells, to tumors and metastases (27, 28, 31, 35, 89). In addition, of the three chemokines, particularly CXCL8 but also CCL2, are potent angiogenic factors that contribute to TNBC progression (90–93). Moreover, direct activities of the chemokines on tumor cells have led to increased invasion in TNBC cells (94–96).

As part of their pro-metastatic roles in TNBC, CXCL8, CCL2 and CCL5 and their receptors - for example, CXCR2 for CXCL8 and CCR2 for CCL2 - contributed to the pro-tumorigenic activities of stromal cells in TNBC mouse model systems (19–26). MSC/fibroblast-derived chemokines, including murine CXCL1 and CXCL2 (counterparts of human CXCL8), CCL2 and CCL5 were associated with recruitment of neutrophils, tumor-associated macrophages and myeloid-derived suppressor cells to TNBC tumors, where they promoted disease course (22, 25, 63, 65). CXCL8 and CCL5, produced by bone marrow- and adipose-derived MSCs were prime inducers of metastasis in TNBC, acting by elevating the proliferation and invasive properties of the tumor cells, and their resistance to chemotherapy (19, 20, 23, 24, 26, 97–99). Moreover, MSC-derived CCL2 has attracted macrophages to TNBC tumors, activating them to secrete CXCL8, thus leading to an overall increase in tumor-associated macrophages and endothelial cells (21).

The above studies strengthen the relevance and importance of our observations on the strong induction of CXCL8, CCL2 and CCL5 when TNBC cells interacted with MSCs/CAFs in the context of pro-inflammatory stimuli, introduced by TNFα and IL-1β. As noted above, both TNFα and IL-1β were found to be responsible for increased aggressiveness in TNBC, and in several studies were connected to increased pro-malignancy functions of MSCs/CAFs. For example, the findings by Shi and colleagues indicated that TNFα-activated MSCs promoted *via* CXCR2 and CCR2 ligands the metastatic ability of murine TNBC cells (63, 65). TNFα-primed MSCs were also found to reprogram neutrophils to acquire immunosuppressive functions (64). Other studies demonstrated that MDA-MB-231-derived CM elevated IL-1β release by MSCs, increasing their pro-inflammatory nature (100). In parallel, MSC-derived IL-1β increased the proliferation and chemoresistance of MDA-MB-231 TNBC cells (60).

However, these studies did not address the wider scope of the tumor-stroma-inflammation network, and did not identify the roles of pro-inflammatory cytokines such as TNFα and IL-1β in regulating TNBC-stroma interactions. Here, our current study provides novel findings, emphasizing the need for both TNBC:MSC cross-talk and pro-inflammatory signals delivered by TNFα and IL-1β, in order to achieve the most substantial levels of pro-metastatic activities: high levels of pro-metastatic chemokines, CXCL8, CCL2 and CCL5, angiogenesis, and tumor cell migration and invasion. Moreover, our findings suggest that previous studies on TNFα-treated MSCs that induced anti-tumor activities in TNBC tumors (101–104) may have overlooked the actual setting that takes place *in vivo*, when TNBC cells interact with MSCs in the presence of TNFα stimulation.

Of major importance in this context is the fact that CXCL8 was revealed in our current study as a key player in mediating the pro-metastatic functional effects of the inflammation-driven tumor-stroma networks in TNBC: angiogenesis, migration-related morphology of the tumor cells, as well as cancer cell migration and invasion. The effects of CXCL8 down-regulation on these pro-metastatic functions in TNBC was pronounced, and our results suggest that it can probably act in cooperativity with other factors that are produced under these network conditions to promote the aggressiveness of TNBC cells that interacted with stromal cells in the context of the pro-inflammatory TME.

Here, it is interesting to note that the tumor-stroma-inflammation networks established by luminal-A cells were less potent or differently active than those generated in TNBC, in all aspects: chemokine production, angiogenesis, and tumor cell morphology, migration and invasion. These findings may

reflect the fact that TNBC cells and luminal breast tumor cells interact differently with fibroblasts (105). They also agree well with our TCGA results demonstrating lower expression levels of TNFα, IL-1β and of the three chemokines in luminal-A patients compared to basal patients. Ultimately, these findings may provide a partial explanation to the more aggressive clinical course of TNBC tumors compared to luminal-A tumors.

Overall, our observations suggest that at the TME of TNBC tumors, which is enriched with TNFα and IL-1β, the two pro-inflammatory cytokines regulate tumor-stroma interactions that occur at the tumor site, and that under these conditions the *in vivo* aggressiveness of the tumor cells is increased. It would be interesting to establish similar systems with murine TNBC cells and investigate the possible effects of similar tumor-stroma-inflammation networks and of specific corresponding mouse chemokines on the immune and inflammatory contextures of mice tumors and metastases. Such systems may also enable further analyses that correlate the extent of stroma cell presence with the extent of expression of pro-inflammatory cytokines and chemokines, as well as with patterns of tumor cell migration and angiogenesis.

The tumor-stroma-inflammation network identified in our study suggests that inhibiting the activities of TNBC-typical pro-inflammatory cytokines, such as TNFα and IL-1β would halt tumor-stroma interactions that stand in the basis of TNBC progression. Indeed, inhibitors of TNFα and IL-1β are in clinical use in inflammatory diseases and were found to inhibit the aggressiveness phenotype of TNBC cells (54, 57). Obviously, implementation of inhibitory modalities to these cytokines in the *in vivo* and even more so in the clinical setting would require improved understanding of the entire context of their activities; for example, the activation and regulatory networks of TNFα and its TNFR1 and TNFR2 receptors take place at multiple regulatory levels that need improved understanding.

These considerations emphasize the relevance of the metastatic chemokines that are elevated due to the activity of the tumor-stroma-inflammation triage, particularly CXCL8, to therapy. Inhibitors of the axes of CXCL8, CCL2 and CCL5 and their receptors are also available (20, 106, 107), suggesting that treatments of TNBC cancers with combination therapies of chemokines and pro-inflammatory cytokines may provide novel treatment options for TNBC patients.

ETHICS STATEMENT

All procedures involving experimental animals were approved by Tel Aviv University Ethics Committee, and were performed in compliance with local animal welfare laws, guidelines, and policies.

AUTHOR CONTRIBUTIONS

YL generated all data, and was extensively engaged in study design and manuscript preparation. SL was involved in setting up the research systems. TM contributed to qRT-PCR studies. LR-A and DM participated in ELISA studies of luminal-A and TNBC cell lines. SW contributed to conception of research at its initial stages and CK participated in TGCA analyses. AB-B was the principal investigator, responsible for the entire study at all stages (conception, design and data accumulation), as well as manuscript preparation.

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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.2019.00757/full#supplementary-material

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REVIEW
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Support of Tumor Endothelial Cells by Chemokine Receptors

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Tumor-associated vascular endothelium comprises a specialized and diverse group of endothelial cells that, although not cancer themselves, are integral to cancer progression. Targeting the tumor vasculature can have significant efficacy in reducing tumor burden, although loss of efficacy due to acquisition of resistance mechanisms is common. Here we review mechanisms by which tumor endothelial cells (TEC) utilize chemokine receptors to support tumor progression. We illustrate how chemokine receptors support and may serve as functional markers of the diverse TEC population. We focus on ACKR1 (DARC), ACKR3 (CXCR7), CXCR4, and CCR2, as these are the best studied chemokine receptors in TEC; and suggest that targeting these receptors on the tumor vasculature may prove efficacious in slowing or reversing tumor growth. We also mention CXCR2 and CXCR3 as important mediators or tumor angiogenesis, given their distinct roles with angiogenic and angiostatic chemokines, respectively.

Keywords: chemokine receptor, chemoattractant, endothelial cell, tumor vasculature, tumor microenvironment

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INTRODUCTION

The endothelium consists of a network of endothelial cells (ECs) that form the inner lining of blood and lymphatic vessels. Endothelium is present only in vertebrates, including the hagfish, the oldest of extant vertebrates (1, 2). The endothelium is critical for the trafficking of leukocytes between the vasculature and the underlying tissues. The endothelium is also involved in most major pathologic conditions, from cancer, to cardiovascular disease, neuroinflammation, diabetes, and high blood pressure, either as a primary determinant of pathophysiology or as a victim of collateral damage(3).

In the context of cancer, endothelial cells form the inner lining of the blood vessels that make up part of a growing tumor. Compared to normal endothelial cells, tumor endothelial cells (TEC) become morphologically and phenotypically dysregulated at the cellular and molecular level, much like the tumor itself. TEC retain their ontological endothelial identity, remain distinct from cancer cells, and are not immortal. TEC support is, however, a major component of the tumor microenvironment, not only irrigating the tumor with nutrients, but also affecting the immune cell infiltrate and stromal composition of the tumor (3, 4). The dysregulation of ECs within the tumor leads to loss of proper vascular barrier function and gain of properties that provide tumors with survival advantages.

Misconceptions regarding the origin of TEC have been clarified in the last few years with the advent of improved technologies that have enabled a detailed compositional analysis of these cells and uncovered a vast repertoire of functional activities and associated markers. Key misconceptions included that (i) tumor endothelial cells were derived from the tumor, (ii) TECs were similar to their normal counterparts; and (iii) targeting only vascular endothelial growth factor (VEGF) would

be sufficient to destroy the tumor they were a part of. Interestingly, the heterogeneity of TECs is such that it is extremely challenging to pinpoint a single overall function or modus operandi that defines the TEC population. Tumors hijack otherwise normal homeostatic or developmental vascular endothelial processes, such as angiogenic sprouting or vasculogenesis; or engage in vessel co-option or other mechanisms, such as intussusception (splitting of pre-existing vessels to give rise to daughter vessels), and parasitize the host's vascular system to promote tumor survival, growth, and dissemination (5–8) (**Figure 1**).

Tumor angiogenesis results from growth factor and chemokine-dependent EC proliferation. Classic EC proliferation is stimulated by VEGFs (**Table 1**). Depending on the angiogenic cytokine, distinct characteristics evolve: for example, VEGF-B and placental growth factor (PlGF) bind and activate VEGF-R1, which is responsible for hematopoiesis, monocyte migration, EC metabolism, and arteriogenesis (9). VEGF-A and VEGF-C bind and activate VEGF-R2, which initiates and sustains the classic angiogenesis process, giving rise to blood endothelial vessels (BECs) (10–12). VEGF-C and VEGF-D bind and activate VEGF-R3, which gives rise to lymphatic endothelial cells (LEC). TEC derived from angiogenic processes are identifiable within the tumor as early as 3 days after tumor inoculation in preclinical models (13).

Given the conflicting evidence on whether or not there exists bone marrow-derived precursors that are bona fide endothelial progenitors cells (EPC), we adopt a more general definition of vasculogenesis as the process of new blood vessel formation assisted by pro-angiogenic bone marrow-derived precursors, if not actual EPC (14). Tumors utilize vasculogenic mechanisms to form new blood vessels (15, 16). EPCs are a subtype of stem-like cells with high proliferative potential that mobilize from the bone marrow and home to tumor sites in response to tumor-secreted cytokines/chemokines, where they continue the cycle to mature into ECs and secrete proangiogenic factors to facilitate vascularization of tumors (17). The main participants are VEGF-A/VEGFR-2, CXCL12/CXCR4/ACKR3, CXCL8/CXCR1, CXCL1,2,3,5,6,7,8/CXCR2, CCL2/CCR2, and CCL5/CCR5 (15, 17–19). An important driver of chemokine gradients in the tumor microenvironment are cancer cells under aerobic glycolysis, which produce lactic acid that activates NF-κB and induces CXCL8 expression in vascular endothelial cells, resulting in angiogenesis in breast and colon cancer (20, 21). CXCL8 also upregulates CXCR7/ACKR3, which is involved in stemness features of cancer cells suggesting it is also likely involved in EPC mobilization (22–24).

Vascular co-option is a process by which tumor cells surround host vessels and incorporate host–tissue capillaries within the tumor, thereby eliminating the need for new vessel formation. Vessel co-option occurs mainly in highly vascularized tissues, such as liver, lungs, and brain. Ronca et al. (16) explain that these tumors are considered non-angiogenic and are *less* likely to respond to antiangiogenic therapy. Ronca et al. (16) make the point that a critical limitation of studies is that most tumor endothelial cells are studied using known markers of ECs such as CD31, CD34, and/or vWF. However, these markers also stain co-opted host vessels. Smooth muscle cell actin (SMA), which stains pericytes that cover mature vessels, may better distinguish between co-opted and angiogenic vessels since the latter are less mature and often lack pericyte coverage (16, 25). Double immunostaining using an EC marker and an antibody against Ki67, BrdU or proliferating cell nuclear antigen (PCNA) that detects proliferating cells may also aid in the detection of ongoing angiogenesis vs. co-opted endothelium (16).

To date, therapeutic strategies to combat pathological angiogenesis primarily rely on vascular endothelial growth factor (VEGF) signaling blockade. Despite initial optimism, the efficacy of anti-angiogenic pharmaco-monotherapies is typically short-lived, and drugs such as Bevacizumab (anti-VEGF-A monoclonal antibody that blocks ligand binding to VEGF-R2) have failed to deliver the promise of a cure for cancer. Improvements in patient survival are limited by acquired refractoriness and drug resistance (26–28). Despite these challenges, there are currently hundreds of clinical trials focusing on anti-VEGF treatment or some combination thereof. Anti-angiogenic strategies in cancer have been designed largely upon the premise that the tumor vasculature is composed of a normal, genetically stable population of endothelial cells. However, recent studies indicate that tumor endothelial cells (TEC) are more complex and dynamic than expected. Future clinical trial design would benefit from personalized application of anti-angiogenic therapies, likely in combination with checkpoint-inhibitor immunotherapy, based on a patient's specific tumor and TEC profile, to enable optimal responses (28).

In this review, we aim to illustrate how chemokine receptors support and serve as functional markers of the TEC population. We also discuss several studies that demonstrate the role of chemokine receptors within the heterogeneous TEC population and how these receptors may be relevant and appropriate targets for the treatment of cancer.

CHEMOKINE RECEPTOR AND CHEMOKINE STRUCTURES AND ROLES IN CANCER

Chemokine receptors maintain a classic seven transmembrane (7-TM) family structure. An important feature of 7-TM receptors is that they have a conserved disulfide bond between two cysteines, and part of what determines the large diversity of these families is how many amino acids separate those two cysteines (29). 7-TM proteins, including chemokine receptors, are notoriously challenging to target because (i) the high homology shared among closely related family members and in some cases their overlapping ligand-binding profiles make specificity an obstacle; (ii) proper physiologic conformation requires receptor expression in a plasma membrane (when synthesized separately, the extracellular domains rarely mimic endogenous conformation); and (iii) the epitope space available on the cell surface for chemokine receptors is highly limited (the amino-terminus and extracellular loops 1 and 2 are typically small

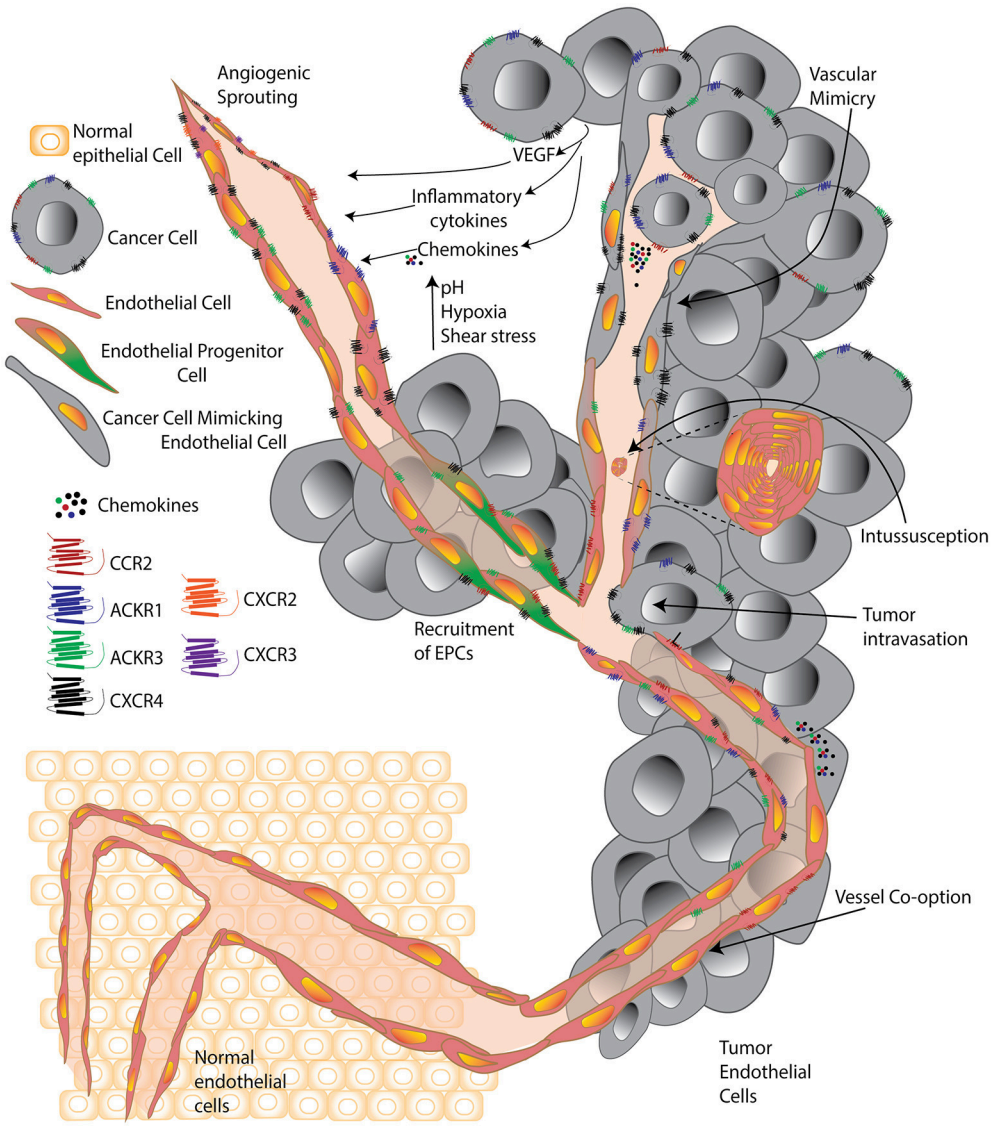

FIGURE 1 | A cell is known by the company it keeps. Simplified schematic comparing tumor endothelial cells surrounded by normal healthy cells and surrounded by cancer cells. Normal endothelial cells are significantly altered within the tumor environment and forced by tumors to undergo processes including classic angiogenic sprouting, vasculogenesis, intussusception, vessel co-option, vascular mimicry, and progenitor differentiation. Within the tumor, environmental factors including growth factors, cytokines, chemokines, high acidity, low oxygen, and high pressure, contribute to push individual tumor endothelial cells to acquire unique features and a vast diversity that supports the progression of the expanding tumor microenvironment.

in size, and chemokine binding occurs within a buried pocket lined by the 7-TM domains within the plasma membrane).

Chemokine receptors are well-known as leukocyte-expressed homing receptors that guide white blood cell localization throughout the body (30). Certain chemokine receptors are also overexpressed by cancer cells and tumor-associated stroma, the matrix that supports cancer cell proliferation and metastasis, and consists of infiltrating leukocytes, fibroblasts, pericytes, and endothelial cell populations. The role of chemokines and their receptors in the endothelium is particularly critical for tumor vascularization and metastatic spread.

TABLE 1 | Classic endothelial cell type determined by Vascular Endothelial Growth Factor (VEGF) type.

VEGFR-1	VEGF-B, PlGF	Arterial ECs
VEGFR-2	VEGF-A	MV, BECs
VEGFR-3	VEGF-C, VEGF-D	LECs

Classical, normal endothelial cell proliferation is stimulated by VEGFs. Depending on the angiogenic cytokine, distinct characteristics evolve for each endothelial cell type.

Chemokines are classified into 4 categories (CC, CXC, XC, and CX_3C) based on the position of conserved cysteine motifs (where X represents any non-cysteine residue). CXC

chemokines are further divided into two groups with defined biological activities. The first set contains the ELR motif that induces chemotactic activity, inducing selectin-dependent leukocyte rolling on activated endothelium, followed by integrin mediated firm adhesion and transendothelial migration to inflamed sites (31–33). The second chemokine set lacks the ELR motif and does not induce chemotaxis across endothelial cells (34). CXCL12/SDF-1 is the only CXC chemokine that does not have the ELR motif, but is chemotactic and pro-angiogenic (35).

Tumor microenvironment-derived chemokines can induce vascular permeability and enable efficient tumor cell extravasation, promoting tumor cell colonization of distant sites (i.e., metastasis) (36). Tumor microenvironment-derived chemokines can induce endothelial cell recruitment by attracting the cells overexpressing their receptors, turning on feedback loops to induce angiogenic support for tumors. For example, CCL2, along with other CXC chemokines including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8 promote recruitment, migration, and proliferation of endothelial cells (37).

In general, upon binding to their ligands, chemokine receptors undergo conformational changes that allow the binding of G proteins to intracellular loop epitopes and the carboxy terminal tail of the receptors. This initiates a signaling cascade of activated second messengers that lead to cell motility and multiple other functional effects in the target cells. Major cellular processes are influenced significantly by chemokines via their receptors. Cancer cells can hijack the chemokine receptor system to enhance their survival and proliferation. Epithelial cells, stromal cells, and normal cells secrete chemokines that attract tumor cells overexpressing chemokine receptors and thus increase metastatic dissemination. Increased chemokine receptor expression can also support angiogenesis that feeds tumor growth and gives tumor cells additional survival signals or a survival advantage.

FUNCTION OF KNOWN CHEMOKINE RECEPTORS FOUND IN TEC

Atypical Chemokine Receptors

ACKR1/DARC

Atypical chemokine receptor 1 (ACKR1), also known as Duffy antigen receptor for chemokines (DARC) binds more than 20 different chemokines, and is expressed in cerebellar neurons, venular ECs, and erythrocytes (38). ACKR1 is unique among vertebrate chemokine receptors because it can bind, with high affinity, both CC and CXC chemokines. ACKR1 is important for ligand transcytosis across endothelial layers, to enable presentation in the vascular lumen, as well as in buffering inflammatory chemokine levels in the circulation (39). DARC may serve as a marker to distinguish venular-ECs vs. non-venular-ECs (arterioles, capillaries) (40). In contrast to the other atypical receptors, ACKR1 is not believed to possess ligand-scavenging activity, but rather just present its ligands (39). Interaction of ACKR1 and tumor cell suppressor markers such as CD82, inhibit tumor cell proliferation and induce senescence by upregulating p21 and downregulating TBX2 (41, 42). ACKR1 may be involved in the regulation of angiogenesis, sequestering CXCL1, CXCL5, and CXCL8 (35, 43). In transgenic mice engineered to overexpress ACKR1 in endothelial cells (via a preproendothelin promoter/enhancer), ACKR1 decreased the pro-angiogenic properties of ELR^+ CXC chemokines (44), whereas ACKR1 deficient mice showed increased levels of these chemokines as well as increased angiogenesis in a model of prostate adenocarcinoma (35).

ACKR3/CXCR7

Atypical chemokine receptor 3 (ACKR3), also known as CXCR7, has a modification in its DRYLAIV motif that does not allow it to bind heterotrimeric G-proteins after binding its ligands CXCL11 or CXCL12. Instead, it preferentially signals via the beta-arrestin pathway. ACKR3 is known to play a critical role in guiding progenitor cell migration during embryo- and organo-genesis (45). But following fetal development and birth, expression of ACKR3 protein is difficult to detect on the surface of cells or tissues, except in the context of cancer (46). ACKR3 is upregulated in many different cancer types including lung, cervical, pancreatic, myeloid, glial, and prostate cancer cells and brain cancer. ACKR3 may provide an advantage for tumor cells that favors their metastasis, driving cells through CXCL12 gradients by binding and degrading CXCL12–regulating bioavailability and gradient control, as it does during development. ACKR3 generally is not considered a chemotactic receptor, however, addition of CXCL12 enhances ACKR3+/CXCR4+ cancer cell migration across endothelial cells toward CCL19 and CXCL13, chemokines expressed by endothelial cells inside the lymph nodes (47, 48). Interestingly, this effect is abrogated when ACKR3 is inhibited with the small molecule CCX771, while not as diminished when CXCR4 is inhibited with AMD3100. Therefore, targeting ACKR3 could prevent lymph node entry and distant metastasis of CXCR4+/ACKR3+ positive tumor cells (48, 49). In addition, inhibiting ACKR3-dependent survival signals (50) may sensitize cells to chemotherapeutics or radiation (23). We and others have shown that ACKR3 is specifically up-regulated by activated (TNF-α treated) or inflamed endothelial cells while not in normal endothelial cells (29, 50–53). ACKR3 may also become upregulated in an unknown mechanism of tumor resistance to temozolomide (TMZ) where it could be identified as an independent factor for overall survival in the glioblastoma microenvironment (53), in line with mutations in isocitrate dehydrogenase mutations (IDH) and O6-methylguanine-DNA methyltransferase (MGMT) (54, 55). Moreover, inflammation augments ACKR3 expression on the abluminal surface of the brain microvessel endothelium, contributing to damage in the context of experimental autoimmune encephalomyelitis (EAE), and blocking ACKR3 decreases the damage to primary brain endothelial cells *in vitro* (56).

ACKR3 is upregulated in tumor endothelial cells, where it is induced under hypoxic and acidic conditions,

distinctive features of the tumor microenvironment. CXCL12 secreted by TECs promotes ACKR3-mediated angiogenesis via ERK1/2 phosphorylation but not normal endothelial cells (NECs), indicating an autocrine/paracrine loop affects TEC proangiogenic properties (52). Therefore, ligand blocking inhibition of ACKR3 such as in the form of specific monoclonal antibodies that inhibit CXCL12 binding and beta-arrestin2 activation could significantly reduce TEC angiogenesis and decrease tumor burden acting as true and specific inhibitors of CXCL12 (29, 53, 57). VEGF stimulation upregulates ACKR3 expression in NECs (52). VEGF is stimulated by hypoxia and ACKR3 is stimulated by hypoxia inducible factor (HIF1a) (58). Thus, once the tumorigenesis process creates a hypoxic tumor microenvironment, ACKR3 expression by TEC may be induced by both HIF1a and VEGF.

G-Protein-Coupled Chemokine Receptors

CXCR4

CXCR4 is the most common chemokine receptor overexpressed in human cancers and is implicated in over 25 different types of cancers (59, 60). CXCR4 is considered a novel marker in tumor endothelium, specifically on tip cells forming the sprouting tumor vessels within hepatocellular carcinoma (HCC). In the same study, high levels of TEC CXCR4 predicted poor prognosis for patients with HCC (61). Inflammatory cytokines derived from tumor conditioned monocytes/macrophages (Mo/Mϕ), especially TNF-α, upregulate CXCR4 expression on ECs (61). TNF-α induces activation of the Raf-ERK pathway and induces expression of CXCR4 on activated endothelial cells. CCR2 KO mice showed reduced infiltration of inflammatory Mo/Mϕ in tumor tissues and reduced vascular CXCR4 expression in HCC tumors (62). CXCR4 is among the genes enriched in tip cells vs. stalk endothelial cells, along with VEGFR2, platelet-derived growth factor (PDGF)- B, Dll4, and matrix metalloproteinase (MMP)14 (16, 28, 63).

CXCR4 is expressed by EPCs and is responsible for their maintenance in the bone marrow via CXCL12. VEGF/VEGF-R, which upregulates MMP9, and CXCL12/CXCR4 are considered the key pathways regulating bone marrow-EPC mobilization (64–66). On the other hand, inhibition of CXCR4 reduces VEGF secretion in tumor cells, which results in decreased neovascularization and tumor growth (67). CXCR4 is also expressed on the surface of LECs and is critical for lymphangiogenesis through CXCL12 stimulation (12, 68). Circulating levels of CXCL12 increase in patients who evade various anti-VEGF therapies, including rectal carcinoma with bevacizumab, GBM with cediranib, HCC with sunitinib, and soft tissue sarcoma with sorafenib (8, 69). The CXCL12/CXCR4 pathway is also involved in vessel co-option, vasculogenesis, fibrosis, lymphocyte trafficking, and cancer cell invasion, depending on the tumor and treatment. Clinical trials with AMD3100 (a CXCR4 small molecule antagonist) and the anti-VEGF mAb, bevacizumab are currently underway to treat recurrent GBM patients to address mechanisms of evasive resistance (8, 70).

CCR2

Although CCR2 is expressed in human endothelial cells, in the context of TEC, activation of CCR2 leads to phosphorylation of kinases JAK2 and p38MAPK and transcription factor Stat5, which enhances endothelial permeability and enables colon carcinoma extravasation and metastasis in preclinical mouse models (36). Pharmacological inhibition of CCR2 with a selective small molecule antagonist, CCX872, significantly suppressed tumor growth and enhanced survival in a spontaneous breast cancer model (*HER2/neu* transgenic mice) (71). A similar result was obtained using *HER2/neu* mice deficient in the single known CCR2 chemokine ligand, CCL2 (71). EPC express CCR2, migrate to CCL2 and contribute to tumor neovascularization (71). EPC development in the bone marrow and their mobilization to the blood was impaired in $CCL2^{-/-}$ *HER2/neu* mice compared with WT *HER2/neu* mice, providing a possible mechanism for the observed anti-tumor phenotype (71). ChemoCentryx, Inc., recently reported the improved overall survival of CCX872-treated patients in an ongoing Phase 1b clinical trial for locally advanced/metastatic pancreatic cancer targeting CCR2 in circulating immune cell and myeloid derived suppressor cells (MDSCs). In addition, monoclonal antibodies that bind either CCL2 or CCR2 to inhibit the CCL2/CCR2 interactions (37, 72) have been tested in several inflammation-related diseases with varying but promising results (37).

CXCR2—The Receptor for Angiogenic Chemokines

CXCR2 is expressed on microvascular EC and binds all the ELR^{+} CXC-chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) known to possess angiogenic activity and promote neovascularization (73, 74). In the gut, CXCR2 is expressed on human intestinal microvessels and on primary cultures of human intestinal microvascular endothelial cells (HIMEC) (75). CXCL8 secreted by malignant colon tumors had angiogenic effects on HIMEC (endothelial tube formation, EC chemotaxis), which was suppressed by CXCR2 antibody blockade or by ERK1/2 inhibition (75, 76). In lung cancer, CXCR2 is expressed on the vascular endothelium in human non-small cell lung carcinomas and in experimental mouse lung cancer models [orthotopic syngeneic Lewis lung carcinoma (LLC) tumors], and elevated levels of CXCL1, CXCL2/3 were detected in tumor vs. matched normal tissue controls (77). LLC tumor angiogenesis (as quantified by microvessel density) was inhibited in CXCR2-deficient mice or WT mice treated with anti-CXCR2 mAbs vs. controls, which correlated with increased necrotic area and reduced tumor growth *in vivo* (77). In a preclinical model of spontaneous prostate cancer, tumor growth was significantly suppressed in CXCR2-deficient mice compared with controls, with a corresponding reduction in tumor angiogenesis as measured by von Willebrand factor RNA expression (78). Similar results supporting a tumor-promoting angiogenic role for CXCR2 were also reported for pancreatic ductal adenocarcinoma (79) and ovarian cancer (80). Thus, targeting CXCR2, particularly in combination with immune checkpoint inhibitors, may offer new opportunities to slow or reverse cancer progression across multiple tumor types, as the approaches act via complementary anti-tumor pathways.

CXCR3—The Receptor for Angiogenesis Inhibiting Chemokines

CXCR3 interacts only with ELR$^-$ angiostatic chemokines CXCL9, CXCL10, and CXCL11. Interferon (IFN) gamma-induces CXCL9/10/11 expression in endothelial cells, where the chemokines play an important role in the vascular recruitment of IFN-gamma-producing T cells to atherosclerotic plaques (81). CXCR3 is significantly overexpressed in vessels from primary kidney tumors compared to matched normal tissue vessels (82). CXCR3 expression on primary cultures of human microvascular endothelial cells (HMVECs) is limited to the S/G2-M phase of the cell cycle vessels (82). CXCL9, CXCL10, and CXCL11 block HMVEC proliferation (either spontaneous or basic fibroblast growth factor-induced) *in vitro*, which can be reversed by treatment with an anti-CXCR3 antibody (82). CXCR3 is a particularly complicated chemokine receptor due to its alternative splicing of the human gene, which generates 3 different isoforms, CXCR3A, CXCR3B, CXCR3alt, with CXCR3B mediating angiostatic activity on ECs (74). Differential expression of the three CXCR3 splice variants was reported in ovarian tumors, with overexpression of CXCR3alt and CXCR3A vs. normal tissue, while CXCR3B was downregulated vs. normal tissue, suggesting that a specific pattern of CXCR3 RNA transcript processing and expression favors tumor progression and metastasis (83). In alignment with this hypothesis, in a preclinical model of spontaneous prostate cancer, tumor growth was significantly increased in CXCR3-deficient mice compared with controls, with a corresponding increase in tumor angiogenesis as measured by von Willebrand factor RNA expression (78). Given the complexity of the splice variants and expression of CXCR3 on activated T cells, additional research is needed to determine if and how TEC-expressed CXCR3 may be targeted for cancer immunotherapeutic purposes.

TEC-TARGETED CANCER THERAPY

Advantages and Considerations

There are several advantages to targeting TEC to slow tumor progression. First, TEC are more accessible to systemically delivered agents than tumor cells in solid organ cancers. Second, because TEC are not typically immortal, they are less likely to develop resistance to therapies than neoplastic cells (3, 4). Third, Aird suggests that inhibition of a single TEC can suppress the growth and survival of up to 100 tumor cells (4), an exciting concept. Thus, treatment aimed toward the endothelium may have an amplifying anti-tumor effect. State of the art treatments such as tubulin-targeted drugs are the most advanced vascular disrupting agents (84). Combining anti-angiogenic and vascular disrupting agents that target the tumor vasculature may realize the full potential of vascular targeted therapies (84). Chemokine receptors are excellent targets for vascular disruption therapies because they are typically not expressed on cells that comprise vital organs, so adverse events may be limited. Another advantage is the potential for multimodal effects. For example, CXCR4-targeting would inhibit angiogenic signals and prevent *de novo* TEC expansion. Since existing TEC in many cases express CXCR4, targeting CXCR4 with a cytotoxic monoclonal antibody (mAb) could lead to tumor vascular disruption and neoplastic necrosis. CXCR4 is also present on anti-tumoral leukocytes and synergistically attracts leukocytes with other chemoattractants. Thus, the outcome of CXCR4 inhibition might differ depending on the tumor type or even individual patient. For example, since CXCR4 is upregulated on immune suppressive regulatory CD4+ T cells (85), targeting CXCR4 could diminish these immune checkpoint cells and potentially unleash a potent anti-tumor immune response.

Recent Studies

Although most genes expressed in TEC are also upregulated in physiological angiogenic processes, there are important exceptions. The primary determinant of phenotypic heterogeneity in the context of the tumor is the surrounding physical microenvironment, which is typically hypoxic, acidic, and at a high interstitial fluid pressure (3, 86). Studies from high-throughput protein, gene arrays, and miRNA screens have identified unique molecular patterns in the tumor vasculature, but have failed to identify a consistency in TEC molecular signatures (3, 87). While the prevailing concept of the tumor endothelium was that of an admixture of cancer cells surrounded by normal endothelial cells, single cell expression profiling is currently demonstrating that tumor endothelium is distinct from the host's normal ECs.

A multicolor Cre-dependent marker system to trace clonality within the tumor endothelium showed that TECs and their vessels followed a pattern of dynamic clonal evolution. TECs were derived from a common precursor and evolved into a more invasive and immunologically silent phenotype. Gene expression profiling revealed selection for traits promoting upregulation of alternative angiogenic programs such as unregulated hepatocyte growth factor (HGF)/MET signaling and enhanced autocrine signaling through VEGF and platelet-derived growth factor (PDGF). As the TECs developed within the tumor, there was loss of normal EC function and markers including MAdCAM-1 that control lymphocyte homing. Changes in adhesive properties on tumor endothelial cells also showed decreased expression of lymphocyte-attracting chemokines CXCL16, CXCL13, CXCL12, CXCL9, CXCL10, and CCL19 (88). This study showed at high resolution how the tumor microenvironment co-opts endothelial cells and re-arranges their genetic program to drive tumor progression.

TECs and NECs isolated from human breast cancer tissues and reduction mammoplasty tissues were analyzed by single cell RNA sequencing (scRNA-seq) to characterize and compare their global gene expression profiles. Not surprisingly, chemokines and the GPCR pathways, which include the chemokine receptors, were some of the most differentially expressed genes and correlated with breast cancer (89). One limitation of the study is that the authors generate clusters from only 2 normal vs. 2 cancer endothelial cell sets, with a total of 280 viable ECs; thus, the conclusions are based on a relatively small data set.

In another study evaluating tumor endothelium by scRNA-seq analysis, the authors isolated TEC and NECs from xenografts of human colon carcinoma and successfully re-capitulated previously identified markers for tip and stalk cells. Most

interesting was the effect of anti-angiogenic therapy on the TEC populations. The authors used either aflibercept (VEGF inhibitor), anti-Dll4 (Notch inhibitor), or a combination to treat the colon carcinoma xenograft model. Blockade of VEGF rapidly inhibited cell cycle genes and reduced the proportion of endothelial tip cells in tumors. In contrast, blockade of Dll4 promoted endothelial proliferation as well as tip cell markers while blockade of both pathways inhibited endothelial proliferation but preserved some tip cells (90). While the results are potentially exciting, these conclusions were made using TECs derived from contrived xenografted NSG tumor model mice, which have a limited capacity to model tumorigenesis in human cancer patients. Furthermore, the authors compared TEC to cardiac endothelial cells, which would presumably be quite different from anatomically matched NEC. In this same study, the authors identified additional potential tip-like cell markers such as Ramp3, Ednrb and Cldn5 as well as stalk-like cell markers such as Ackr1 and Tmem252 (90). These types of studies, at single cell resolution, give us the opportunity to appreciate the heterogeneity of the TEC population, confirm known markers, and allow us to propose previously unidentified roles for induced genes such as chemokine receptors, which can be challenging to identify specifically at the protein level due to lack of effective anti-mouse receptor antibodies.

PERSPECTIVE FOR THE FUTURE

Current technologies are allowing us to decipher at the single cell resolution the heterogeneity and functionality of the tumor endothelium. This will provide profound insight into mechanisms governing tumor leukocyte infiltration and functional activation via the quantitative analysis of adhesion molecule, chemoattractant, and costimulatory molecule expression. Single cell analysis of the endothelium may also help identify escape mechanisms from current anti-angiogenic and vascular disrupting agents and lead the way to effective countermeasures (88).

Understanding which chemokine receptors are responsible for the early development of the tumor EC but change as the TECs progress along with the rest of the tumor, will help us understand the dynamics of this heterogeneous population and identify appropriate targets for treatment. In the near future, we should seek to identify the characteristics of TECs at the single cell level to understand the transcriptional controls that are altered in NECs, EPCs, and/or cancer-stem-like cells that give rise to TECs in the tumor microenvironment. Understanding how to control this developmental switch will allow us to do what we cannot do today: control the position, abundance, and physical properties of tumor blood vessels. For example, in poorly-vascularized tumors, we may seek to increase the abundance and permeability of tumor vascular endothelium to allow better irrigation of a tumor with a drug or cellular treatment in a patient. In other cases, we may seek to optimally disrupt existing tumor-associated vascular endothelium by targeting key chemokine receptors for therapeutic purposes. As we enter the era of personalized medicine, sequence and expression analysis of neoplastic cells, and supporting TEC will guide the selection of optimal treatments. Given the role of TEC-expressed chemokine receptors in tumor progression, it is likely that chemokine receptor-targeted drugs will play a prominent role in most combination treatment strategies to treat cancer.

AUTHOR CONTRIBUTIONS

NS organized the research topic, wrote, and edited the manuscript. BZ wrote and edited the manuscript.

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Chemokine Heterocomplexes and Cancer: A Novel Chapter to Be Written in Tumor Immunity

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Infiltrating immune cells are a key component of the tumor microenvironment and play central roles in dictating tumor fate, either promoting anti-tumor immune responses, or sustaining tumor growth, angiogenesis and metastasis. A distinctive microenvironment is often associated to different tumor types, with substantial differences in prognosis. The production of a variety of chemotactic factors by cancer and stromal cells orchestrates cell recruitment, local immune responses or cancer progression. In the last decades, different studies have highlighted how chemotactic cues, and in particular chemokines, can act as natural antagonists or induce synergistic effects on selective receptors by forming heterocomplexes, thus shaping migratory responses of immune cells. A variety of chemokines has been described to be able to form heterocomplexes both *in vitro* and *in vivo* under inflammatory conditions, but nowadays little is known on the presence and relevance of heterocomplexes in the tumor microenvironment. In recent years, the alarmin HMGB1, which can be massively released within the tumor microenvironment, has also been described to form a complex with the chemokine CXCL12 enhancing CXCR4-mediated signaling, thus providing an additional regulation of the activity of the chemokine system. In the present review, we will discuss the current knowledge on the synergy occurring between chemokines or inflammatory molecules, and describe the multiple functions exerted by the chemokines expressed in the tumor microenvironment, pointing our attention to the synergism as a possible modulator of tumor suppression or progression.

Keywords: chemokines, tumor microenvironment, heterocomplexes, CXCL12, HMGB1

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INTRODUCTION

The leukocyte infiltrate is a key component of the cancer stromal compartment. Within the tumor, the wide range of chemokines produced by both malignant and stromal cells can affect the composition and the phenotype of the cell infiltrate, and influence tumor growth, survival and metastasis (1–5). Chemokines that regulate leukocyte migration and play key roles in both physiology and pathological conditions (6–8), are small proteins of 8–12 KDa, which can be divided into 4 groups (CCL-, CXCL-, CX3CL1, and XCLs chemokines) according to the position of two conserved cysteine residues within their structure. The chemokine system is characterized by a set of almost 50 ligands, which engage in a promiscuous fashion a panel of more than 20 chemokine receptors, including conventional and atypical receptors, expressed by immune cells,

endothelial cells and cancer cells (9–12). The promiscuous pattern of interaction, together with the large number of ligands and receptors, enables the chemokine system to mediate a variety of cell functions. This is of particular relevance in tumors, since chemokines can influence angiogenesis, cell-adhesion, cell extravasation, and survival (7). Different chemokines can also interact together showing antagonistic or synergistic activity on specific chemokine receptors. They can trigger simultaneously different receptors, resulting either in the inhibition or in the enhancement of the intracellular cell signaling (13, 14), or a single receptor can be activated by a heterocomplex formed between two chemokines, resulting in a stronger cellular response (13, 15). Additionally, chemokines can also interact with inflammatory mediators released in the microenvironment, amplifying cellular responses induced by chemokine receptors (16, 17).

While the multiple roles of heterocomplexes in the early stage of inflammation and in regeneration have been clearly dissected (13, 18, 19), little is known about their functions in tumors (20), and further studies are necessary to define their significance. Von Hundelshausen and colleagues have performed a thorough study mapping the chemokine heterocomplexes by bidirectional immunoligand blotting (21). This study opens the debate on the *in vivo* relevance of the multitude of heterocomplexes found *in vitro*. In the present review, we discuss examples on how the concomitant expression of several chemokines with either anti- or pro-tumor functions could favor heterocomplexes formation in the tumor microenvironment (TME), thus adding an additional feature to be considered in tumor immunity.

TABLE 1 | Synergy-inducing chemokines heterocomplexes .

Receptor	Heterocomplexes	Synergistic Functions	References
CXCR1/2	CXCL8/Regakine-1 CXCL8/CXCL4 CXCL7/Regakine-1	Chemotaxis of neutrophils and of CXCR1-transfected Jurkat cells.	(24–27)
CCR7* CXCR5	CCL19/CXCL13 CCL21/CXCL13 CXCL13/CCL19 CXCL13/CCL21	Chemotaxis of CCR7+ transfected PreB cells and human leukocytes (DCs, B and T lymphocytes). Increased chemotaxis of CCR7+ Sezary Syndrome (SS) cells.	(23, 28)
CCR4**	CCL22/CXCL10 CCL22/CCL19 CCL17/CXCL10 and many others	Chemotaxis of human T lymphocytes (Th1-Th2).	(29)
CCR5	CCL5/CXCL4	Triggering of monocytes arrest on activated endothelium under flow conditions. Blockade of CCL5/CXCL4 heterocomplex inhibits atherosclerosis in hyperlipidemic mice.	(30, 31)
CCR2	CCL2/CCL19 CCL2/CCL21 CCL7/CCL19 CCL7/CCL21 CCL7/Regakine-1	Induction of chemotaxis and responses in monocytes and lymphocytes.	(24, 32)
CXCR4	CXCL12/CXCL9	Recruitment of tumor-infiltrating lymphocytes in primary central nervous system lymphoma.	(20)
CXCR4	CXCL12/HMGB1	Promotion of monocytes chemotaxis both *in vitro* and *in vivo*. Tissue regeneration (liver, muscle, bone).	(16, 18, 19)

** Additional CXC and CC chemokines have been shown in this study to act in synergy with the selective CCR7 agonists. Migration is enhanced in human mature dendritic cells, B cells, T cells, and CCR7-transfected cells.*

*** Additional CXC and CC chemokines have been shown in this study to act in synergy with CCL22. Migration is enhanced in CCR4-transfected cells.*

CHEMOKINE HETEROCOMPLEXES

Several studies in the last decade have described the heterodimerization between chemokines as a regulatory mechanism that governs their activity under inflammatory conditions. In the TME, chemokines play crucial roles either favoring immune responses against the tumor or promoting cancer progression and metastasis. Of note, similar chemokine expression profiles can result in a different tumor-specific leukocyte infiltrate. This phenomenon suggests that additional regulatory mechanisms might be involved, including the release of proteins able to modify chemokine activity. It is now well established that a chemokine receptor can be triggered by a low concentration of its selective agonist when a synergy-inducing chemokine, not selective for the receptor but able to form a complex with the agonist, is concomitantly present (17, 22, 23) (**Table 1**). The first evidence of the synergism induced by the presence of two chemokines was provided by Struyf and colleagues (24), who described the synergy between Regakine-1 and CXCL8, and between Regakine-1 and CCL7. Few years later, the same group has shown that CXCL8 can enhance CXCL12 responses and this enhancement is CXCR4 mediated (25). In 2005, three groups described the formation of heterocomplexes between chemokines, able to enhance the activity of CCR7, CCR4, CCR5, and CXCR2 (23, 26, 29, 30). In particular, CXCL13 forms a complex with CCL19 and CCL21, leading to CCR7 activation at lower agonist concentrations (23). The CXCR3- and CCR4-agonists, CXCL10 and CCL22, co-expressed in the inflamed skin, synergistically interact together, through the first β-strand of CCL22, enhancing CCR4-mediated chemotaxis of T cells, independently from CXCR3 or GAGs binding (29). Other studies showed that the heterocomplex between CCL5 and CXCL4, formed through heterophilic interactions, plays a crucial role in triggering monocyte arrest on the endothelium (30). In this case, the authors demonstrated the requirement of GAGs on the cell surface, and that the CCL5/CXCL4 complex showed paired N-terminus, resembling a CC-type heteromer that promoted a more efficient receptor activation (30, 31). NMR spectroscopy and molecular modeling, followed by *in vitro* analysis, have shown the structure of a heterocomplex between CXCL8 and CXCL4. This complex was shown to enhance the anti-proliferative effect of PF4 on endothelial cells, and the CXCL8-induced migration of CXCR2 transfected cells (26).

Later on, the CCR7-agonists, CCL19 and CCL21, were described as enhancer of monocytes recruitment by forming

heterocomplexes with CCL7 and CCL2, resulting in an augmented CCR2 response, and preventing CCL7 and CCL2 degradation by ACKR2 (32).

A study directly supporting the idea that the activity of heterocomplexes can be relevant also in cancer was performed in our laboratory, showing the role of the CXCL9/CXCL12 heterocomplex in primary central nervous system lymphoma (PCNSL). In this work, it was shown that CXCL9 and CXCL12 are co-expressed in the perivascular area of the tumor, and can form a complex enhancing CXCR4-mediated recruitment of tumor-infiltrating lymphocytes and malignant B cells. This synergism might serve as regulator of the recruitment of CD8+/CXCR4+/CXCR3+ T cells and CXCR4+/CXCR3- malignant B cells in the perivascular cuffs, forming the typical lesions of these tumors (20).

CXCL12/HMGB1 HETEROCOMPLEX

A synergism, mediated by the heterocomplex formed between CXCL12 and the DAMP protein HMGB1 has been shown to be relevant in monocyte recruitment (16, 33) and in tissue regeneration (19, 34). However, its involvement in modulating tumor progression and metastasis has never been assessed. Nonetheless, both CXCL12 and HMGB1 are key players in the TME, where they orchestrate a variety of functions that sustain cancer progression. Indeed, the CXCL12/CXCR4 axis is hyper-activated in lymphomas and in many solid tumors. Their activity is central in the promotion of tumor progression and metastasis to the lungs, brain and bone (35, 36). HMGB1 plays a variety of functions based on its cellular location: in the nucleus, is essential for nucleosomes dynamics and chromosomal stability; in the cytosol or mitochondria, modulates autophagy and apoptosis and regulates mitochondrial morphology and functions; on the cell surface of neurons, promotes axon sprouting and neurite outgrowth (37). Stressed and cancer cells release HMGB1 in the extracellular space, where it activates different receptors in a redox-sensitive manner. The disulphide-HMGB1 promotes TLR-4 activation and mediates production of inflammatory cytokines and chemokines, whereas the reduced-HMGB1 triggers RAGE to promote autophagy and CXCL12 secretion. The reduced form is also able to complex with CXCL12 mediating CXCR4-dependent chemotaxis (33, 38). The CXCL12/HMGB1 heterocomplex acts as an enhancer of CXCR4-mediated signaling, potentiating ERK activation, calcium rise and chemotaxis, both *in vitro* and *in vivo* (17). The effect can be blocked by glycyrrhizin and by anti-CXCL12 antibodies, which prevent the formation of the heterocomplex, or by AMD3100, proving the sole involvement of CXCR4 (16, 39). Moreover, the heterocomplex induces a rearrangement of the N-terminus of CXCL12 and conformational changes in the CXCR4-dimers (16) that might suggest a different mode of receptor triggering.

Recently an important role of the CXCL12/HMGB1 heterocomplex has been described in tissue regeneration. Fully reduced HMGB1 promotes liver and muscle regeneration through CXCR4, by acting on muscle stem cells, hepatocytes, and infiltrating cells (18). In a similar study, HMGB1 was detected after fracture both in humans and in animal models, and the heterocomplex acting via CXCR4 promotes *in vivo* skeletal, hematopoietic and muscle regeneration (19).

CHEMOKINE FUNCTIONS IN THE TUMOR MICROENVIRONMENT

During the different phases of cancer progression, many types of inflammatory cells that exhibit either anti- or pro-tumoral functions are recruited from the blood stream by specific chemotactic cues. The leukocyte infiltrate includes neutrophils with different phenotypes (40), macrophages (41), natural killer cells (NK) (42), myeloid-derived suppressor cells (MDSC) (43, 44), dendritic cells (DCs) (45), T and B lymphocytes (46, 47). Several chemokines have been shown to be expressed in tumors, guiding leukocyte recruitment and positioning, and to support tumor spread at distal organs (7). Below we provide some examples in which different cell types present in tumors can be recruited in the TME thanks to the activity of chemokines, and possibly to the presence of heterocomplexes.

Anti-tumoral Functions

Chemokines mediate anti-tumor activities through the recruitment of specific immune cell types (48). CXCL9 and CXCL10, agonists of the CXCR3, promote the recruitment of CD4+ Th1 lymphocytes, NK cells, and CD8+ cytotoxic T lymphocytes (CTL) to the TME, where they exert a potent anti-tumor activity (7, 49). Th17 cells further sustain the recruitment of CTL, NK cells (50), and DCs (51). In particular, CTL specific for tumor-associated antigens (TAA), together with Th1 and NK cells expressing IFNγ, guide immunity against the tumor promoting tumor cell apoptosis, and releasing effector cytokines and cytotoxic molecules (48). Indeed, evidence in patients with ovarian cancer demonstrated that the increased expression of CXCL9 and CXCL10 correlates with an increased number of tumor-infiltrating CTL and a high CD8+/regulatory T cells ratio that lead to a reduction in cancer metastasis and to a better prognosis (52). IFNγ produced within the TME induces CXCL9, CXCL10, and CXCL11 expression, which correlates with tumor infiltrating CTL and Th1-effector cells and with a positive survival rate in colorectal cancer (53). Moreover, the presence of CTL, CXCL9, and CXCL10 within the tumors is associated to a positive response to PD1/PD1L blocking therapies (54, 55). In recent years, Bronger and colleagues demonstrated that CXCL9 and CXCL10 expression can predict survival in high-grade serous ovarian cancer patients (56).

Tumor tissues from ovarian cancer patients show a dynamic T cell infiltration at different disease stages. Th17 and Th1 cells are present in the early stages, associated with an anti-tumor immune response and production of CXCL9 and CXCL10 (56). In the later stages Treg, expressing CCR4, correlate with CCL22 production, and are associated to pro-tumoral immunosuppressive functions (57). The role of the CCL22/CXCL10 heterocomplex (29) in the switch from an anti- to a pro-tumoral TME should be investigated.

CXCL9 and CXCL12 can form heterocomplexes, and in PCNSL are coexpressed on the tumor vasculature. CXCL12-induced migration is enhanced in CXCR4+/CXCR3+/CD8+ T lymphocytes and in CXCR4+/CXCR3− malignant B cells, indicating that chemotactic cues in the perivascular environment serve as regulators for the recruitment of tumor infiltrating lymphocytes (TILs) (20). Tumor associated macrophages (TAM) are also a source of CXCL9 and CXCL12. Interestingly, the expression of CXCL9 is restricted to macrophages present in the perivascular area, indicating heterogeneity among macrophages within the tumor, and suggesting this cell type as the most important player for the recruitment of CTL in the perivascular space (20).

TAM, recruited to the tumor in response to chemokines, polarize toward different subtypes (M1 or M2) accordingly to the presence of activating stimuli generated by the cytokines expressed in the microenvironment. M1 macrophages produce CXCL9 and CXCL10 and exert an anti-tumoral activity, while M2 macrophages sustain cancer growth (5, 41, 58). CXCR3 agonists are also important for the polarization toward a M1 phenotype, since CXCR3 deficiency of this receptor induces a M2 phenotype (59).

Tumor associated neutrophils (TAN) polarized toward a N1 phenotype exert an anti-tumoral activity. In particular, TGF-β blockade increased neutrophil attracting chemokines (CXCL2, CXCL5, CCL3) specific for CXCR1/2 and CCR2-5. This resulted in an influx of CD11b+/Ly6G+ TAN with enhanced tumor cytotoxic activities and higher levels of pro-inflammatory cytokines (60).

The expression of CCR5 on CD4+ and CD8+ T lymphocytes has been described to be essential for an efficient tumor rejection in mouse model of Lewis lung adenocarcinoma and pancreatic adenocarcinoma (61). The activity of CCL5, a selective CCR5 agonist, can be enhanced by CXCL4 (30), a chemokine expressed by a variety of tumor types (62). Interestingly, in both tumor types the expression of CXCL4 have been documented (62), and could represent an additional tool for enhancing CCR5 responses.

The recruitment of other cell types including DCs and B cells with antigen presenting functions is essential for the expansion and activation of leukocytes within the TME (48). High levels of B cell-infiltrates, recruited into the microenvironment through the CXCL12/CXCR4 axis, are positively associated with a good survival rate in breast cancer, high-grade serous ovarian cancer, and cervical cancer (63–65). B cells infiltrating the tumor can organize in tumor-associated tertiary lymphoid structures, where they act as antigen presenting cells enhancing T cell responses or producing tumor-specific antibodies (66). In breast cancer, a specific subset of T follicular helper cells, through the production of CXCL13, has been linked to tertiary lymphoid structures formation, generation of germinal centers and maturation of B cells, migrating into the TME via CXCR5 (67).

A recent study showed that NK cells, through the production of CCL5 and XCL1, recruit DCs into the TME promoting cancer immune control, which is associated with patient survival (68).

Pro-Tumoral Functions

Chemokines can also support tumor progression and metastasis, either acting as angiogenic factors (69), or through the recruitment of different immune cell types into the TME, which inhibit effector cell functions (7, 48).

Within the CXC-family of chemokines, an important role in inducing angiogenesis has been demonstrated for chemokines containing the ELR motif (glutamic acid-leucine-arginine). Neovascolarization is an essential process that sustains solid tumor growth and metastasis. In humans, CXCR2 is considered the receptor mainly involved in angiogenesis through the interaction with ELR+ chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8). CXCR2 activity has been directly correlated with the aggressiveness of a number of tumors, including melanoma (70), pancreatic cancer (71), gastrointestinal cancers (72), and renal cell carcinoma (73).

CCL22 and CCL28, expressed in many human tumors, are mediators for the recruitment of CCR4+/CCR10+ Treg cells, involved in the suppression of both spontaneous and therapy-induced local tumor immunity. The presence of these cells is associated to a poor prognosis (74–76). It has been demonstrated also that Treg directly support angiogenesis through the secretion of VEGF and promote metastasis via the induction of NK cells apoptosis (75, 77). Interestingly, the expression of CXCR3 by Treg resulted in an immunosuppressive effect mediated by the control of Th1-associated responses (78). In addition, Treg with a memory phenotype are frequently recruited through CXCR4/CXCL12 signaling to the bone marrow, a common target of metastasis in humans, further supporting the idea that this cell subset provides an anti-inflammatory environment that sustains cancer progression (79, 80).

Th22 cells, that under physiological conditions express CCR10, CCR6 and CCR4, and home to the skin (81), have been shown to be recruited to the tumor site, supporting tumorigenesis through the activation of STAT3 and the enhancement of the expression of the methyltransferases DOT1L (82) and of the Polycomb repressive complex 2 (PRC2) (83). B cells, as well, can exert a regulatory function by inhibiting T cells activity through the production of TGF-β and IL-10, or further support tumorigenesis via the production of TNF (84, 85). Their recruitment to the tumor sites is mediated by the CXCL12/CXCR4 axis, and might be enhanced by the chemokines known to form a complex with CXCL12.

Myeloid-derived suppressor cells (MDSCs) are deeply investigated in tumor models and in cancer patients, due to their relevant role in promoting cancer stemness (43, 44). Granulocytic MDSCs, mainly composed by different subsets of neutrophils, express CXCR1 and CXCR2, and are recruited to the tumor by CXCL8, produced by tumor cells or by Treg (86). In the TME, they release molecules that sustain angiogenesis, further supporting tumor progression and metastasis (44). Interestingly, CXCL8 has been shown to synergize with CXCL4, which is produced by a variety of tumors at different stages (62). Monocytic MDSCs, that include macrophages at different maturation stages, express CCR2, CXCR2 and CXCR4, and can

reach the tumor via their specific ligands CCL2, CXCL5 and CXCL12 respectively (87, 88). These cells are able to sustain tumor growth via the induction of arginase-I, iNOS, and TGF-β, and favor the recruitment of Treg at the tumor site through the production of CCR5-binding chemokines (89).

The M2 subset of TAM is negatively correlated with survival in cancer, and is associated with responses that sustain tumor growth and progression (41, 90).

Plasmacytoid DCs can reach the TME via the CXCR4/CXCL12 axis. Their recruitment sustains tumor growth by the induction of IL-10 producing Treg that in turn suppress the activation of tumor specific effector T cells (91, 92). As shown by Vanbervliet and colleagues, the sensitivity of this cell type to CXCL12 can be enhanced by the CXCR3 agonists (93). Nonetheless, this type of synergy was interpreted as the activity of both CXCR4 and CXCR3, and was not demonstrated if this effect was due to a heterocomplex formation, as shown later in the PCNSL (20).

CONCLUSIONS

Many chemokines are abundantly and concomitantly expressed in the TME and orchestrate a variety of functions that sustain cancer progression or suppression. While the activity of chemokine heterocomplexes has been deeply investigated in inflammatory conditions, and in models of tissue regeneration, a direct prove that a heterocomplex can enhance the responses of tumor cells to chemokines has been demonstrated only for the CXCL12/CXCL9 heterocomplex in PCNSL (20). The concepts covered in the present review suggest that the nature and function of tumor infiltrating immune cells might not be the simple result of the interaction occurring between a chemokine agonist and its specific receptor, but, could be mediated by chemokine heterocomplexes that can differently modulate the activation of a variety of chemokine receptors regulating cell recruitment, positioning, and the switch in the components of the cellular infiltrate in different tumor stages.

The mapping of the possible chemokine-chemokine interactions by bidirectional immunoligand blotting suggests that the synergism might preferentially be mediated by CC-type heterodimers, whereas the CXC-types might promote inhibitory effects (21). Additional studies are required to determine whether this distinction can be applied to the whole chemokine system, and in particular if the heterocomplexes identified are relevant in the TME.

As testified by the diverse expression of chemokine receptors in tumors and by the multiple activities of the heterocomplexes studied so far, we might expect different responses to the same heterocomplex according to the distinctive features of each TME. A deeper understanding of the modulation of the chemokine system in TME, will tell us the relevance of the heterocomplexes, and their possible involvement in shaping the activity of the microenvironment.

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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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REVIEW
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Chemokines Modulate Immune Surveillance in Tumorigenesis, Metastasis, and Response to Immunotherapy

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Chemokines are small secreted proteins that orchestrate migration and positioning of immune cells within the tissues. Chemokines are essential for the function of the immune system. Accumulating evidence suggest that chemokines play important roles in tumor microenvironment. In this review we discuss an association of chemokine expression and activity within the tumor microenvironment with cancer outcome. We summarize regulation of immune cell recruitment into the tumor by chemokine-chemokine receptor interactions and describe evidence implicating chemokines in promotion of the "inflamed" immune-cell enriched tumor microenvironment. We review both tumor-promoting function of chemokines, such as regulation of tumor metastasis, and beneficial chemokine roles, including stimulation of anti-tumor immunity and response to immunotherapy. Finally, we discuss the therapeutic strategies target tumor-promoting chemokines or induce/deliver beneficial chemokines within the tumor focusing on pre-clinical studies and clinical trials going forward. The goal of this review is to provide insight into comprehensive role of chemokines and their receptors in tumor pathobiology and treatment.

Keywords: chemokine, cancer, immune surveillance, immune therapy, metastasis, chemokine receptor

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INTRODUCTION

Migration of the immune cells to specific organs is controlled in part by small proteins called chemokines (i.e., chemotactic cytokines) (1, 2). Chemokines bind to seven transmembrane G protein-coupled receptors that trigger intracellular signaling that drives cell polarization, adhesion, and migration (3, 4). They are divided into four families based upon structure: CXC, CC, CX3C, and C chemokines. The receptors follow a similar nomenclature system, based upon the family of chemokines to which they bind. In addition there is a family of atypical chemokine receptors that do not directly couple to G proteins, but are reported to have a variety of roles in development, homeostasis, inflammatory disease, infection, and cancer (5). Chemokines are also classified as homeostatic or inflammatory (4, 6–8) and both subsets play important roles in cancer (9, 10).

CHEMOKINE/CHEMOKINE RECEPTORS IN THE REGULATION OF LEUKOCYTES

Since chemokines and their receptors are highly promiscuous, with most chemokines binding multiple receptors, and receptors binding multiple chemokine ligands. One must consider this complexity in reference to functional significance of each chemokine or receptor in reference to cancers **(Figure 1)**.

Of key importance in immune surveillance is the recruitment of dendritic cells (DCs), CD4+Th1 cells, and CD8+ T effector cells to the tumor microenvironment. Chemokine receptors CCR4, CCR5, CXCR3, CXCR4, CCR6, and CCR7 play a pivotal role in the regulation of T cell homing to inflammatory sites (13). T cells (αβ, γδ, T_{FM}, T_{FH}, Th22, Tregs, ILCs, NKT), NK cells, B cells and immature DCs (14–16) are recruited to the tumor by CCL20 interaction with CCR6. CCL19 and CCL21 recruit Tregs, CD4T helper, T_{CM}, T_{RCM}, activated T cells, monocyte-derived dendritic cells (mDC) and B cells to the TME through interaction with CCR7 (7, 17–20). Dendritic cells home to XCR1, CCL3, CCL4, CCL5, CCL20, and CCL25 in the TME or LN (21–23). When antigen-specific CD4 T cells interact with DC, CCL3, and CCL4 are released and this can guide CCR5-positive naïve CD8+T cells into tissues for activation (24). As such, secretion of ligands for these receptors (CCL4/5 for CCR5, and CXCL9/10/11 for CXCR3) at the site of inflammation is necessary for the initiation of a specific immune response (25).

In contrast, tumor-promoting leukocytes are comprised of macrophages expressing arginase, IL4, IL10, and IL13, as well as myeloid-derived suppressor cells (MDSCs), T regulatory cells (Tregs) and specific B cell subsets. Ligands for chemokine receptors CCR1, CCR2, CCR3, and CCR5, CCR8, CXCR1, CXCR2, and CXCR4 recruit macrophages to the TME (4, 26–39). Neutrophils and myeloid derived suppressor cells (MDSCs) are recruited to the tumor through ligands for CCR2, CCR3, CXCR1, CXCR2, and CXCR4. Tregs express the chemokine receptors CCR2, 3, 4, 6, 7, 8, 10, CXCR3, and CXCR4 (40–48). Because the same chemokines that recruit anti-tumor leukocytes can also recruit pro-tumor leukocytes (for example CCL19 and CCL21 recruit both Tregs, mDCs, and activated T cells), therapeutically targeting chemokines or chemokine receptors in cancer is complicated.

For naïve T cells to become activated, antigen presenting DCs migrate from the developing tumor to the lymph node where they present antigen to the T cells via the T cell receptor (TCR) and stimulate a process that leads to T cell activation. CD4 cells can be activated by antigen presenting cells (APCs) and mature into helper cells [T helper type I cells (Th1) or T helper type II cells (Th2)]. Th1 cells produce cytokines including interferon-γ (IFNγ), tumor necrosis factor-alpha (TNFα), while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13. The cytokines produced by the DCs influence the differentiation of naïve helper T cells into either Th1 or Th2 cells. For example, if DCs secrete IL-12, the naïve helper T cells differentiate into Th1 cells. Th1 cells express CD40L on their plasma membrane and this ligand binds to CD40 expressed by the DC or other APC. Engagement of CD40 on the DCs or other APC primes them to a higher activation level resulting in elevated expression of class I MHC, B7 and co-stimulating molecules such as 4-1BBL.

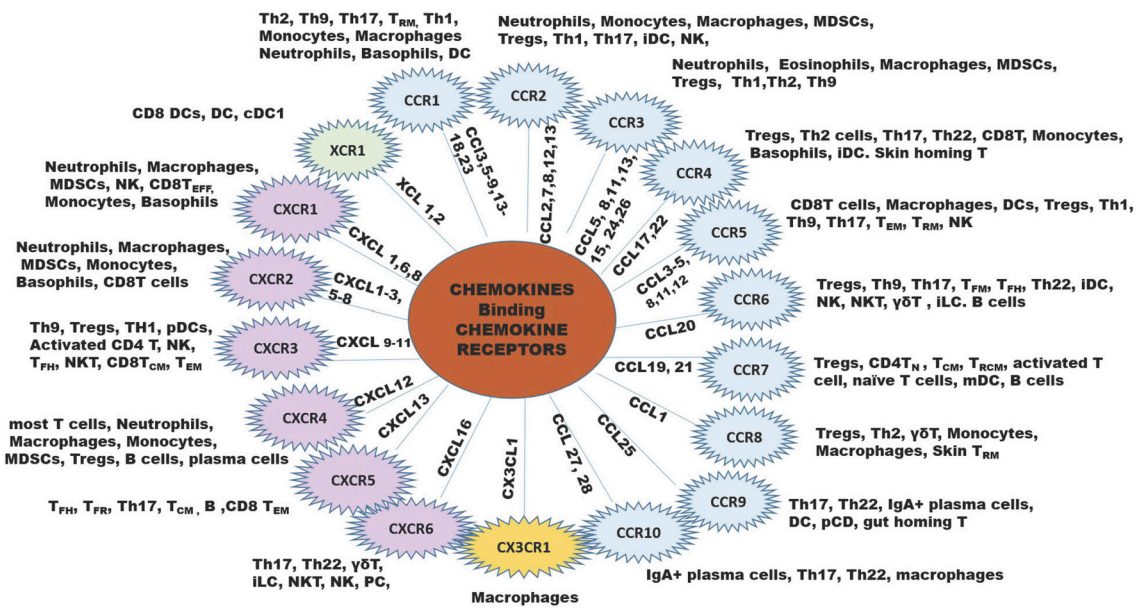

FIGURE 1 | The chemokine family of chemokine ligands and chemokine receptors. The XC chemokine receptor is in green, CC chemokine receptors are in blue B, CX3CR chemokine receptor is in yellow, and CXC chemokine receptors are in lilac. The inner lines leading to each chemokine receptor shows the ligands that bind to the receptor. Outside the chemokine receptor wheel shows the types of cells that express the receptor to respond to the ligands for each chemokine receptor. B, B cell; iDC, immature DC; NK, natural killer cell; NKT, natural killer T cell; MDSCs, myeloid-derived suppressor cells; pDC, plasmacytoid DC; Th, T helper cell; T_{CM}, central memory T cell; T_{EFF}, effector T cell; T_{FH}, follicular helper T cell; T_{FR}, follicular regulatory T cell; T_N, naïve T cells, T_{RCM}, recirculating memory T cell (11, 12).

When CD8+ T cells come into contact with one of these highly activated DCs, its TCRs recognizes the peptides presented by the MHC Class I molecule on the DC/APC. This, in turn, leads to the activation of CD8 T cell upon binding of its TCR to the MHC presented peptide(12).The clone subsequently expands in response to IL-2 induced stimulation of cell proliferation. CD4 T cells are important for the survival and expansion of activated CD8 T cell clones and for the survival of memory CD8 T cells during recall expansion, but there is some priming in the absence of Major Histocompatibility Complex, Class II (MHCII) activation (49). Different subsets of T cells migrate in response to a variety of chemokines (12). For example CCR7 is expressed on all naïve CD4 T cells and it's ligand CCL21 is expressed by the endothelial cells of the high endothelial venules (HEV) which are specialized vessels that facilitate lymphocyte recruitment. CCL21 is presented by heparin sulfate into the luminal surface (49). CCL19 can also bind to CCR7 on CD4 cells and is thought to mediate survival of naïve T cells as they move into the LN (50). Once in the LN, naïve CD4 T cells search for APCs using a random walk along a fibroblastic reticular cell network (51) which expresses adhesion molecules in addition to ligands for CCR7, CCL19, and 21, as well as CXCL12, which binds CXCR4. To escape the LN, CCR7 gradually becomes down-regulated and the CD4 cells bind the sphingosine-1-phosphate receptor 1 (S1PR1) (52) and follow S1P signals into the lymphatic vesicles, other LNs, or the circulation. FOXO1 is a key transcription factor in CD4 T cells, as is KLF2. FOXO1 regulates expression of CD62L and CCR7, while KLF2 represses CXCR3, CCR3 and CXCR5 expression (53).

When CD4 T cells are activated, there is upregulation of CXCR3 and CXCR5, both or which are associated with differentiation into T_{H1} cells (54) and can be linked to Bcl6 and cell division, though the order is controversial (55, 56). TCR engagement, IL12, IL21, and IFNγ expression along with induction of T-bet are associated with escape from a plastic state into a definitive Th1 phenotype (57). The cells migrate from the T zone to the B-T zone interface usingCXCR5 and EB12 (58) to escape areas with high IL-2. In contrast, contact with an environment high in IL-2 will suppress T_{FH} differentiation.

CD4+T cells undergo priming by DCs and upregulate CXCR3 expression, then CXCR3 mediates the migration of CD4+T cells between different DC populations in the LN. These CD8α+DCs are producing CXCL10 in response to IFNγ stimulation. CXCL9, CXCL10, and CXCL11 are produced by many cell types including fibroblasts, leukocytes, and keratinocytes and all bind CXCR3, though the most potent ligand in humans for CXCR3 is CXCL11 (59). CXCR3 is essential for T cell recruitment into tumors and through the thymus (60, 61) and Th1 cells also produce IFNγ that induces additional production of CXCL9 and 10 to enhance the recruitment of cytotoxic CD8+ T cells into the tumor (62).

Th2 cells express CCR4 and this receptor responds to ligands CCL17 and CCL22. CCR4 expression is induced in response to IL-4 and CCR4 expressing Th2 cells may also produce IL-4 (63–65). In contrast, those Th2 cells that express CCR8 produce IL-5 (66). Another key population of CD4 cells is the CD4+ memory T cells that express CCR7 and CD62L. These cells produced IL-2 when there is restimulation (67).

In the tumor microenvironment, chemokines are produced by tumor cells, endothelial cells, mesenchymal stem cells (MSC), cancer-associated fibroblasts, myeloid cells, and neutrophils, providing a very rich "soil" to facilitate the recruitment of immune cells into the tumor microenvironment (TME). For example, tumor cells, macrophages, and neutrophils produce CXCL1, CXCL2, CXCL5, and CXCL8 and these chemokines recruit MDSCs, both the PMN-MDSCs and the Monocytic-MDSCs (68, 69). The MDSCs suppress the activity of CD8+T effector cells to prevent tumor cell killing by these cells. Dendritic cells (DCs), Tregs, CD8+ T cells, Th1, Th9, Th17, T_{EM}, T_{RM}, and macrophages are recruited into the TME by CCL3-5, CCL8, CCL11-12, and CCL28 (70). Mature DCs release CXCL5, CXCL9-11 and these chemokines recruit CD4+Th cells, CD8+T cells, Tregs, pDCs, NK, and NKT cells into the TME (71) **(Figure 2)**. Additional interactions of chemokines and chemokine receptors that facilitate recruitment of diverse immune cells are shown in **Figure 1**.

TUMOR CHEMOKINES AND PATIENT PROGNOSIS

According to the analysis of the TCGA collection of human cancers using either The Human Protein Atlas (www.proteinatlas.org) (72, 73) or CBioPortal (74, 75), chemokine expression can be prognostic in many human cancers. However, same chemokines can be either favorable or unfavorable prognostic indicators depending on the type of malignancies. For instance, T cell-recruiting chemokines CXCL9, CXCL10, and CXCL11 are favorable prognostic indicators in ovarian cancer, but are unfavorable indicators for pancreatic and renal cancer. CXCL9 is also favorable in endometrial and breast cancer. Elevated expression of CXCL1 is unfavorable indicator in renal, liver and cervical cancers, but it is favorable in breast cancer. High CXCL5 is associated with poor survival in renal, liver, pancreatic and cervical cancer, while CXCL12 is not prognostic in any of the common TCGA malignancies. High expression of CCL4 and CCL5 are associated with better outcome in melanoma, endometrial, and colorectal cancer, but with worst outcome in renal cancer (**Figure 3**). Furthermore, a study of 14,492 distinct solid tumors (primaries and metastases) with at least 30 per tumor type revealed that a 12-chemokine expression signature (CCL2, CCL3, CCL4, CCL5, CCL8, CCL18, CCL19, CCL21, CXCL9, CXCL10, CXCL11, and CXCL13) correlated with the presence of tertiary lymph node-like structures and was also associated with better overall survival of the subset of melanoma patients (76). Moreover, loss of CCL5 expression was found to be associated with enhanced melanoma aggressiveness (77) and poor therapeutic response (78). Interestingly, tumor genomic instability can affect chemokine expression and patients' outcome. For instance, chromosomal instability in colorectal cancer can lead to deletion of the *CXCL13* gene which is associated with greater risk of tumor relapse (79). Of note, in human breast cancer CXCL13 is produced by follicular helper T cells which are linked with activation of adaptive antitumor humoral responses (80).

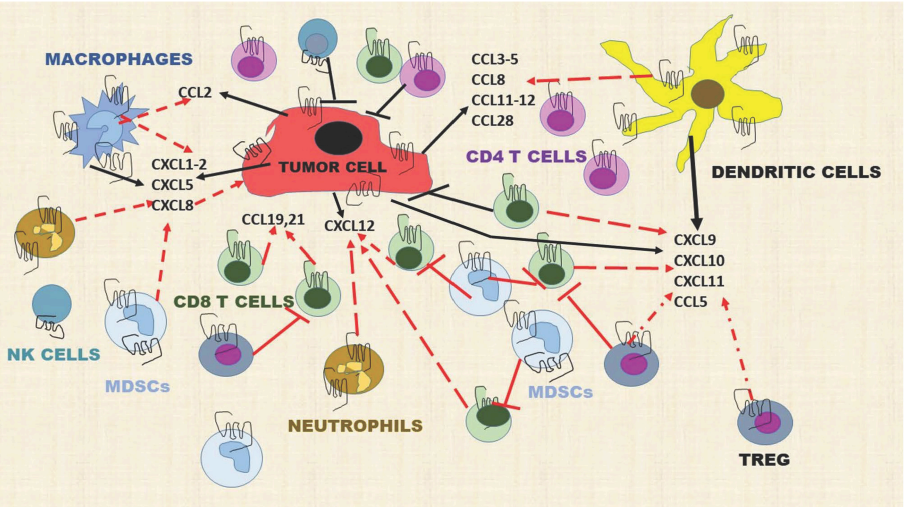

FIGURE 2 | Chemokines produced by stromal cells, tumor cells, and immune cells dynamically modulate the immune landscape of the tumor microenvironment. Dashed red lines indicate a cell moving toward a chemokine gradient. Solid T red lines indicate inhibition. Solid black lines indicate chemokines released by a cell type. Solid black T lines indicate immune cell killing of tumor cells. This diagram includes representative chemokines recruiting immune cells but does not include all possible interactions.

Thus, primary tumor data indicate that chemokines play an important role in tumor progression, which, in part, may relate to the direct effect of chemokines on cancer cell growth and metastasis (9). However, the main effect of chemokines is likely due to their ability to recruit specific subtypes of immune cells into the tumor that, in turn, can modulate tumor growth and metastasis. Indeed, immune cells within the tumor are among the key determinants of cancer outcome, based on the pan-cancer meta-analysis that correlated gene expression with overall survival outcomes in ∼18,000 human tumors across 39 malignancies. This study showed that genes associated with immune cells, especially T cells, are the most significant indicators of favorable patient outcome (81). Furthermore, the presence of T cells or T cell expression signature within the tumor is associated with greater likelihood of response to immune checkpoint inhibitors (22, 76, 82–85). Below we summarize recent studies demonstrating that chemokine-mediated recruitment plays a central role in the regulation of the levels of different immune subtypes within the tumor.

CHEMOKINES REGULATE TUMOR AGGRESSIVENESS AND METASTASIS

Pro-metastatic Chemokine Signaling in Tumor Cells

Tumor cells express a wide range of chemokine receptors, and there are extensive reports that tumor cells utilize both autocrine and paracrine pathways to respond to chemokines with altered migration, proliferation, and gene expression. Importantly, chemokine receptors have been reported to play a crucial role in maintenance of cancer stem cells. For example, a CXCR1 blockade has been shown to selectively target breast cancer stem cells (86) and its expression has been correlated with poor prognosis in breast cancer (87). CXCR1 and CXCR2 have been linked to melanoma tumor growth and metastasis (88–91).

Similarly, CCL2 expression by cancer-associated fibroblasts has been shown to support the growth of breast cancer stem cells (92), while CXCR4 was shown to be enriched in a subset of glioma cancer stem cells (93). Furthermore, CXCR2 is expressed in MSC and CXCR2 overexpressing MSCs can be used to accelerate mucosa wound healing (94). Both CXCR5 and CXCR4 are involved in metastasis of PCSLC prostate cancer stem-like cells (95), and inhibition of CXCR4 alters the homing of quiescent stem-like prostate cancer cells to bone (96). Furthermore, expression of the CXCR4 ligand, CXCL12, by tumor-associated fibroblasts has been shown to promote immune evasion in a murine model of pancreatic cancer, while targeting CXCR4 with specific antagonist AMD3100 facilitated immunotherapy response in these model (97). CCR5 has also been implicated in breast cancer growth and metastasis (98–100). These findings provide a rationale for targeting these chemokine receptors within the tumor microenvironment.

Pro-metastatic Chemokine Signaling in Metastatic Niche

Chemokines play a crucial role in establishing the make-up of the "pre-metastatic niche." Yang et al. reported that when CXCR2 and CXCR4 are inhibited, recruitment of MDSCs to the pre-metastatic niche of the lung is inhibited and, as a result, breast cancer metastasis to the lung is significantly reduced (37). Granot et al. reported that tumor-entrained neutrophils (TENs) inhibit metastatic seeding in the lungs by generating H_2O_2 and tumor-secreted CCL2 is a critical mediator of optimal anti-metastatic entrainment of G-CSF-stimulated neutrophils. Tumor entrained neutrophils inhibit seeding in the pre-metastatic lung (101).

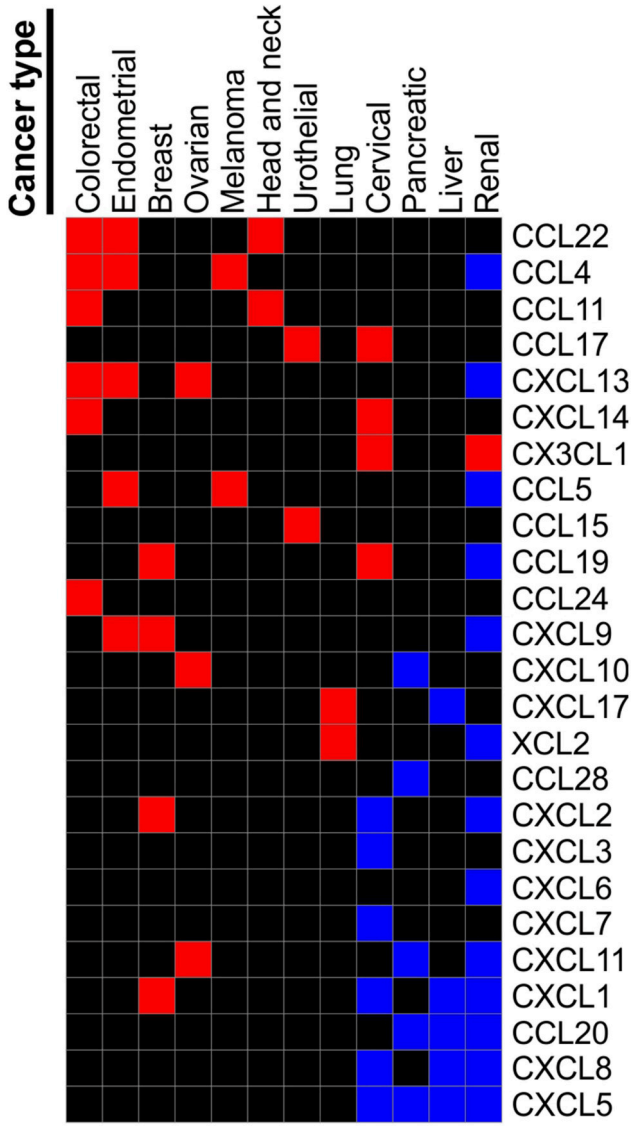

FIGURE 3 | Chemokines associated with patient survival in various malignancies. Prognostic data was obtained from The Human Protein Atlas. We reviewed Kaplan-Meier plots for all cancers where high expression of indicated chemokine genes has significant ($p < 0.001$) association with patient survival. Based on this review we constructed a table where chemokines associated with better survival in one of the reviewed malignancies were assigned the value of "1." Chemokines that were significantly associated with worse survival in a given malignancy were assigned the value of "−1." Chemokines not strongly associated with survival ($p > 0.001$) were assigned the value of "0." Chemokines that were not prognostic in any of the tested malignancies were excluded. Based on the resulting table the heat map was constructed using Morpheus online tool (https://software.broadinstitute.org/morpheus).

In contrast, Lavender reported that while *in vitro* delivery of CCL2 to 4T1 TENs enhanced the killing of the less aggressive 67NR variant of 4T1 tumor cells, intranasal delivery of CCL2 enhanced the seeding and outgrowth of tumor cells in the lung (102). However, it has been shown that patients with high CCL2 expressing basal-like, HER2+ and luminal B breast cancer exhibit a higher probability of longer survival in comparison to those patients with low expression of CCL2. These results are contradicted by findings showing that CCL2 and CCL3 are pro-tumor based upon their recruitment of pro-tumor macrophages into the TME (26). Presumably, the contribution of different chemokines to tumor growth and metastasis may be context dependent reflecting the overall complexity of cancer-associated chemokine-chemokine receptor network.

CHEMOKINES FACILITATE "T CELL-INFLAMED" TUMOR PHENOTYPE

Cytotoxic CD8 T cells are Th1-differentiated CD4 T cells are the main drivers of anti-tumor immunity, and there is a strong clinical and experimental evidence that chemokines are necessary to for the recruitment of these cells into the tumor. Analysis of patient samples indicates that chemokine expression is associated with T cell infiltrate. For example, in melanoma, the lack of CCR5 ligands (CCL3, CCL4, CCL5) and CXCR3 ligands (CXCL9 and CXCL10) has been associated with limited infiltration of antigen-specific T cells (103). The critical role of CXCR3 ligands in the recruitment of T cells into tumors of various origin has been well-documented (4). This critical role was further confirmed by the recent meta-analysis study which examined 5,953 cancer specimens from breast, colorectal, lung, ovary, melanoma, and head and neck carcinomas. This study demonstrated a positive correlation of CXCL9, CXCL10, and CXCL11 mRNA expression with the density of tumor-infiltrating T and NK cells (104). Interestingly, this study also uncovered a surprising negative correlation between the expression of CXCR3 ligands and neutrophil levels within tumors, indicating a possibility of a mutually exclusive pattern of T cell and neutrophil recruitment. Functional studies revealed that blockade of CXCL9 and CXCL10, or their receptor CXCR3, impairs recruitment of adoptively transferred T cells into melanoma tumors (61, 105). Furthermore, B16 melanoma tumors grow more rapidly in mice lacking CXCR3, and their tumors have lower levels of T cells as compared to wild-type mice. Notably, response to T cell-reactivating therapy, such as PD-1 blockade, is also impaired in CXCR3 knockout animals (105). These findings implicate CXCR3 ligands as major regulators of T cell tumor homing. Interestingly, there is evidence that tumors can find ways to neutralize anti-tumor chemokines within the tumor microenvironment. For example, a study Barreira da Silva et al. showed that dipeptidylpeptidase DPP4 produced by stromal cells within the tumor truncated and inactivated chemokine CXCL10 in transplanted murine melanoma tumors, resulting in reduced T cell infiltration and enhanced tumor growth and metastasis (106). These findings suggest that DPP4 inhibitors which are used as anti-diabetic drugs could potentially be used to stimulate

tumor immunity. Indeed, the prospective clinical study showed that DPP4 inhibition can preserve the bioactive form of CXCL10 in humans (107) and a clinical trial of DPP4 inhibitor linagliptin with PD-L1-antagonist is underway (NCT03281369).

Certain C-C chemokines can also contribute to T cell recruitment into the tumor. Clinical data indicate that CCR5 ligands, CCL4, and CCL5, can promote anti-melanoma immune response. This observation is based on our analysis of the TCGA set of 287 melanoma samples which identified a robust association of the CD8+ T cell marker CD8A with the expression of chemokine CCL5 (78). One of the receptors for CCL5, CCR5, is expressed on T cells, and it has been reported to direct CD8 trafficking to sites of inflammation (24). However, mouse studies showed that CCR5 is dispensable for homing of T cells into melanoma (61). Recent studies indicate the critical role of CCL4 and CCL5 within the tumor microenvironment is the recruitment of cells of myeloid lineage that support adaptive anti-tumor T cell responses, such as dendritic cells. For instance, NK cell-derived CCL5 in cooperation with XCL1 has been shown to drive DC1 recruitment into the tumor (108). Furthermore, tumor-derived CCL4 has also been linked with the recruitment of DC cells in a mouse model of melanoma. These DCs, in turn, recruited cytotoxic T cells into the tumor by producing CXCR3 ligands CXCL9 and CXCL10 (109). Similar data were obtained in urothelial bladder cancer (110).

Besides CXCR3 and CCR5 ligands, additional chemokines are now emerging as key players in the regulation of anti-tumor immunity. For example, CXCL16 has been implicated in driving immune response against liver cancer by recruiting anti-tumor NKT cells. Sinusoidal endothelial cells were the major source of CXCL16 which was induced by gut microbiome-mediated primary-to-secondary bile acid conversion (111). Cremonesi et al. demonstrated that recruitment of T cells into colorectal tumors is controlled by many chemokines, including CCL5, CXCL9 and CXCL10, CCL17, CCL22, CXCL12, CXCL13, CCL20, and CCL17 (112). Expression of these chemokines was induced upon exposure of patient-derived colorectal cancer cells to gut microbiota and thus was sensitive to antibiotic treatment. These chemokines predominantly induced recruitment of T cells with an anti-tumor activity which was associated with improved survival in an animal model and clinical samples (112).

These reports suggest that many different chemokines contribute to anti-tumoral T cell recruitment. However, experimental evidence suggests that not all of these chemokines directly regulate T cell chemotaxis. For instance, an *in vivo* analysis of anti-tumoral T cell chemotaxis using competitive homing assay showed that key tumor-derived chemotactic factors are CXCR3 ligands, while CCL5, which was also produced by melanoma tumors, is dispensable for direct homing of T cells into the tumor (61). Furthermore, as shown by Yagawa et al. who used a standardized chemokine assay to test immune cell recruitment by 48 recombinant chemokines, resting CD4+ and CD8+ T cells displayed concentration-dependent chemo-attraction toward CCL19, CCL21, CXCL10, and CXCL12 and, to a lesser extent, toward CCL13, CCL16, CXCL9, CXCL11, CXCL13, and/or CXCL16 (113). None of the other tested chemokine molecules, including CCL4 and CCL5, were chemotactic for T cells in this experimental setting. These data suggest that the observed correlation of T cell markers and CCL5 observed in human melanoma tumors could be a result of indirect promotion of T cell recruitment or proliferation by myeloid and antigen-presenting cells recruited by CCL4 and CCL5. Notably, some chemokines may even play a role in repelling T cells as shown by Li et al. who identified CXCL1 as a determinant of the non-T-cell-inflamed microenvironment (114). In summary, these data point out that complex chemokine profiles orchestrate diverse immune microenvironment of tumors, including "T cell-inflamed" phenotype.

CHEMOKINES AND TUMOR RESPONSE TO IMMUNOTHERAPY

Analysis of samples from melanoma patients undergoing various immunotherapeutic treatments, including cancer vaccines and immune checkpoint blockade with CTLA-4 and PD-1 antagonists, revealed that tumors responsive to immunotherapy tend to be infiltrated with T cells, which is described as "T cell-inflamed" tumor microenvironment (22, 82–84). It is not yet fully understood why immune cells are present in some but absent in other tumors. It has been hypothesized that tumors with high mutation burden are more immunogenic because peptides derived from mutated proteins can serve as neo-antigens when bound by MHC molecules for presentation to T cells and thus can trigger an immune response (115, 116). However, a study of a TCGA tumor sample collection found no correlation between the T cell gene expression signature and mutational burden in any cancer type (117). An explanation of this interesting data came from the recent study by Cristescu et al. which analyzed over 300 patient samples across 22 tumor types from four KEYNOTE clinical trials (85). This study found that tumor mutational burden and a T cell-inflamed gene expression profile were independently predictive of response to the PD-1 antibody pembrolizumab. Notably, these parameters demonstrated a low correlation between each other, suggesting that they reflect distinct features of tumors that independently promote immunotherapy response. Consistent with this conclusion, tumors that exhibited both high mutation burden and prominent T cell signature were most likely to respond to PD-1 blockade (27% response rates). Tumors exhibiting only one of these immunotherapy response-promoting phenotypes had an intermediate likelihood of response (11–12%), while response rates were low on "T cell cold" tumors with low mutation burden (0% response rate) (85). These data suggest that many tumors, including potentially immunogenic tumors with high mutation burden, find ways to exclude immune cells to escape immune-mediated destruction. Indeed, regardless of the mutational load and ability to produce neo-antigen peptides, if tumor antigen-specific T cells are not mobilized to infiltrate the tumor, the presence of mutations and neoantigens is not going to be sufficient to mount anti-tumor immunity.

Based on this logic, chemokines are likely to facilitate immunotherapy responses by bringing immune cells with anti-tumor activity into the tumor and, thus, counteracting

T cell exclusion. The data from patients' samples supports this hypothesis. For example, Ayers et al. published a gene expression signature that accurately predicts response to PD-1 therapy in patients with HNSCC and gastric cancer (23). Notably, several chemokine genes including *CCL5, CXCL9, CXCL10,* and *CXCL11* were in this signature. Furthermore, a Genentech-sponsored study of therapeutic anti-PD-L1 antibody showed a significant positive correlation between therapeutic response and baseline CXCL9 levels in melanoma. This correlation, however, did not reach statistical significance in NSCLC or renal carcinoma tumors (118). Interestingly, the same study found that fractalkine CX3CL1 negatively correlates with anti-PD-L1 response in all tested indications. This is an unexpected finding because this chemokine is generally associated with T-cell infiltration.

It is important to mention that chemokines are essential not only for the response to PD-1/PD-L1 therapeutic targeting, but they are also implicated in response to other immunotherapeutic agents. For instance, functional mouse studies revealed the requirement of CXCR3 ligands for response to anti-TIM-3 immune checkpoint inhibitor when administered in combination with chemotherapeutic drug paclitaxel (119). Of course, not all chemokines play a beneficial role in immunotherapy outcome. It has been shown that high levels of chemokines CCL3, CCL4, and CXCL8 in pre-treatment tumor specimens were associated with worse patient overall survival after anti-CTLA4 and Carboplatin/paclitaxel treatment in melanoma (120).

The key question that remains is how the expression of immunotherapy response-promoting chemokines is induced in tumors? An interesting hypothesis came from a study by Topalian's group which found that chemokines CCL5 and CXCL1 were upregulated in PD-L1-positive melanoma tumors along with IFNγ and several IFNγ-regulated genes based on the analysis of 49 archived melanoma specimens that were either PD-L1 positive or negative (121). Notably, Topalian's group also showed that CCL5 and CXCL1 had no direct effect on PD-L1 expression *in vitro*. The rationale for this study relates to the fact that PD-L1 positive tumors are more likely to respond to anti-PD-L1 immunotherapy, even though PD-L1 is not a definitive predictor of response (118, 122). The connection between chemokines and IFNγ was later confirmed in HNSCC and gastric cancer where CCL5 and CXCL9-11 along with a number of IFNγ-regulated genes comprised an expression signature associated with response to PD-1 blockade (23). However, it is not entirely clear from these correlative studies whether IFNγ stimulates chemokine expression in tumors or whether chemokines recruit immune cells that produce IFNγ. Perhaps both mechanisms take place *in vivo*. On the one hand, chemokines such as CXCL9-11 have been shown to be induced by IFNγ *in vivo* (www.interferome.org) (123). On the other hand, chemokines orchestrate tumor homing of cells that are the major producers of IFNγ, such as Th1-polarized CD4+ T, CD8+ T cells, and NK cells (124). IFNγ released by these cells activates JAK-STAT signaling in tumor and other cells of the tumor microenvironment which leads to increased PD-L1 surface display (125–128). This compensatory PD-L1 induction mediated by IFNγ inhibits the anti-tumor activity of T cells which is a key mechanism of adaptive immune resistance. Furthermore, Benci et al. showed that prolonged IFNγ signaling contributes to tumor growth as a result of expression of interferon-driven inhibitor ligands (IDILS) which, in addition to PD-L1, include TNF Receptor Superfamily Member 14/Herpes Virus Entry Mediator (TNFRSF14), galectin-9 (LGALS9), MHCII, CD28 Antigen Ligand 2/B7-2 (CD86), and the Interferon Stimulated genes (ISGs), such as Interferon-Induced Protein with Tetratricopeptide Repeats 1 (IFIT1) and MX Dynamin Like GTPase1 (MX1)(129). This same study showed that CRISPR ablation of multiple of these IDILS or ISGs enhances response to anti-CTLA4+anti-PD1 (129). This CRISPR ablation worked better than the addition of anti-LAG3 and or anti-TIM3. These data are complicated by reports of JAK1 mutation being associated with resistance to anti-PD1 (130).

In addition to driving adaptive immune resistance, IFNγ also promotes chemokine expression which, in turn, can recruit additional immune cells into the tumor (123). Based on these findings, a model can be proposed where IFNγ-producing immune cells increase tumor chemokines to recruit more immune cells that will further induce chemokine expression and so on. At the same time, tumor cells try to escape immune-mediated killing by inducing PD-L1 and other immune checkpoint proteins. The remaining question not explained by this model is how IFNγ-producing cells are recruited into the tumor in the first place. We and others have identified key molecular signals and pathways regulating basal chemokine expression in tumor cells that can be modulated therapeutically. We discuss these studies in the following chapter.

THERAPEUTIC IMPLICATIONS

Chemokines as Therapeutic Targets

Accumulating evidence suggests that CXCR2 and CXCR4 are promising therapeutic targets in multiple malignancies. There are now over 2,400 publications describing a role for CXCR4 in cancer and over 300 publications describing a role for CXCR2 in cancer progression. These receptors are expressed on tumor cells, endothelial cells, leukocytes, including MDSCs. These studies provide significant evidence that CXCR2 and CXCR4 promote tumor growth through a variety of mechanisms (30, 37, 68, 131, 132). For example, Yang et al. demonstrated that targeted deletion of CXCR4 in myeloid cells reduced melanoma and breast cancer tumor growth through a mechanism that involved enhanced recruitment and activation of NK cells in the tumor. Likewise, systemic treatment with a CXCR4 antagonist also significantly inhibited tumor growth (131). Moreover, in an organotypic tumor spheroid-immune cell co-culture model inhibition of CXCL12 enhanced T cell recruitment and the anti-PD-1 immunotherapy response in a colon carcinoma cell model (133). Other reports show that ablation of CXCR2 signaling inhibited metastasis of in pancreatic adenocarcinoma in mouse models (114, 134–139) and improved response to anti-PD1 (114, 135, 140, 141). CXCR2 antagonism also inhibits metastasis of breast cancer, lung, ovarian, melanoma cells in mouse models (32, 33, 89–91, 142–148). A meta-analysis study of 2,461 patients revealed that CXCR2 predicts poor overall and relapse-free survival in laryngeal SCC, lung cancer, pancreatic ductal

carcinoma, clear-cell renal cell carcinoma, and hepatocellular carcinoma, but not for digestive tract cancer (149).

Currently, clinical trials are ongoing with both CXCR2 and CXCR4 antagonist (150–153).

Therapeutic Induction of Chemokines

Chemokines control infiltration of diverse immune cells into the tumors. The immune cell infiltrate, in turn, is essential for mounting an effective anti-tumor immune response with immunotherapy. Thus, therapies that induce chemokine secretion in tumors and restore immune cell entry into non-inflamed tumors are likely to facilitate immunotherapy response. One of the previously explored approaches to induce infiltration of T cell into the tumors was to inject them directly with interferons. In a mouse model interferon injection into melanoma tumor-induced chemokine production and improved response to anti-PD-L1 therapy (154). One drawback of this approach is that since not all melanoma lesions are injectable, this strategy may miss potential micrometastases and therapeutic effects are likely to be transient. Indeed, a recent clinical study in melanoma patients did not find increased T cell infiltration after a single intra-tumoral injection of IFNγ (34). Other studies reported that chemo-and radio-sensitivity could increase chemokine expression (155, 156). However, melanoma tumors are notoriously resistant to chemotherapy and radiation.

We have discovered that senescent-inducing drugs increase chemokine secretion by melanoma cells (78). Senescence is a cell state of irreversible (or stable) cell cycle arrest accompanied by an induction of a complex secretory program known as senescence-associated secretory phenotype (SASP) (157). Using small molecules targeting cell cycle kinases, such as alisertib that inhibits mitotic kinase Aurora A, or palbociclib that inhibits CDK4/6, to induce senescence we demonstrated that the melanoma SASP includes a number of chemokines implicated in T cell trafficking (78, 158, 159). These chemokines included CCL5 and CXCR3 ligands which are up-regulated in tumors responsive to PD-1-targeting immune checkpoint therapy (23). Taken together, these data suggest that senescence-inducing therapy promotes chemokine secretion in melanoma cells which facilitates an inflamed tumor microenvironment.

Another approach to re-activate chemokine expression in immunologically cold tumors is by targeting the epigenetic blocks that impede chemokine expression in tumor cells. For instance, treatment of ovarian cancer cells with epigenetic modifiers reversed the EZH2 and DNMT1 suppression of expression of the CXCR3 ligands, CXCL9, and CXCL10, resulting in T cell influx into the tumor and improved response to T cell transfer and anti-PD-L1 blockade therapy (160). Interestingly, another study showed that DNMT1 inhibitor treatment induced expression of CXCL12 in osteosarcoma tumors. Activation of CXCR4 by CXCL12 has been reported to have pro-tumor activity. In contrast, in the context of DNMT1 inhibition in osteosarcoma, activation of the CXCL12-CXCR4 axis reduced metastasis and promoted T cell recruitment (161). Expression of CCL5 can also be epigenetically regulated as shown by the study in non-small cell lung cancer showing that a combination of DNA-demethylating agents with histone deacetylase inhibitors reversed tumor immune evasion and modulated the T cell phenotype away from a T cell exhaustion state toward memory and effector T cell phenotypes (162). These experiments indicate that epigenetic modifiers can be utilized for cancer treatment to rescue expression of key chemokines important for the recruitment of T cells and DCs to the tumor.

Also, viral delivery of chemokines can be used to increase T cell homing into the tumor and promote immunotherapy response. For instance, intra-tumoral injection of vaccinia virus delivering CXCL11 promoted response to adoptive T cell therapy and vaccines (163). In addition, it has been shown that oncolytic viruses can enhance secretion of CXCL2 and CXCL10 chemokines by tumors (164). Another promising approach to elevate chemokine levels within the tumor is nanoparticle delivery as demonstrated by CXCL10-loaded folate-modified chitosan nanoparticles that showed anti-tumor activity (165). Another study showed that resistance to PD-L1 blockade could be overcome by targeting tumors with tumor necrosis factor superfamily member, LIGHT. Administration of antibody-guided LIGHT activated lymphotoxin-beta receptor signaling which, in turn, facilitated production of chemokines CCL21 and CXCL13 that recruited T cells into the tumor (166). Finally, immune adjuvants, including double-stranded (ds) RNAs of Sendai Virus (SeV), poly-I:C, and rintatolimod (poly-I:C12U), has been shown to promote the production of CXCR3 ligand within the tumor (167). In glioblastoma poly(I:C) stimulated expression of chemokines CXCL9, CXCL10, CCL4, and CCL5 (167). Similarly, an engineered RIG-I agonist-induced expression of lymphocyte-recruiting chemokines in breast cancer cells (168). Altogether, these approaches of delivering agents that elevate levels of T cell-recruiting chemokines within the tumor can be used to stimulate anti-tumor immunity when tumors are in an injectable location.

CONCLUDING REMARKS

In the last 30 years, we have made extensive progress in identifying chemokines and chemokine receptors, characterizing their roles in the development of the immune system, in angiogenesis, wound healing, inflammation, tumorigenesis, and host defense. Extensive effort was put into developing antagonists of chemokine receptors and some of these were investigated in various clinical trials. CCR5 antagonists, like maraviroc, have been developed and used in AIDs patients with some success (169). CXCR2 antagonists are currently in clinical trials to block MDSC recruitment to tumors and the pre-metastatic niche (NCT03177187 in metastatic castration-resistant prostate cancer (not yet recruiting). CXCR2 antagonists are also being evaluated in combination with immune checkpoint inhibitor pembrolizumab in advanced solid tumors (NCT03473925) and in metastatic melanoma (NCT03161431, not yet recruiting) (ClinicalTrials.org). CXCR4 antagonists have been and are in clinical trials: NCT02179970–to assess safety of continuous IV administration of plerixafor in patients with advanced pancreatic, ovarian and colorectal cancers (recruiting); NCT03277209–continuous IV administration of plerixafor to assess impact on

immune microenvironment in patients with pancreatic, ovarian and colorectal adenocarcinomas (active but not recruiting); NCT02605460-chemo-sensitization before hematopoietic stem cell transplantation in patients with acute leukemia in complete remission–recruiting; NCT02737072–LY2510924 combined with durvalumab for solid tumors (terminated and results not posted); NCT01068301—a Phase I study plerixafor in combination with fludarabine, thiotepa, and melphalan for a second allogeneic stem cell transplantation has been completed but results are not posted; NCT01010880—safety study of CXCR4 antagonist in multiple myeloma patients-study was completed but no results are posted. Additional trials are ongoing for the CXCR4 antagonist BL-040 in NSCLC (NCT03337698), in AML in combination with atezolizumab (NCT03154827), in metastatic pancreatic cancer (NCT02907099), and in aplastic anemias or hypoplastic myelodysplastic Syndrome (NCT02462252) and several others. In addition, the Polyphor CXCR4 antagonist, balixafortide, combined with eribulin has completed Phase I trials in HER2-negative metastatic breast cancer patients and demonstrated an objective response in 16/54 evaluable patients (30%) with an additional 25 patients exhibiting stable disease (46%) (153). Xue et al. have recently reviewed additional reports showing CXCR4 is a potential target for cancer (170). Similarly, therapeutic approaches to increase chemokine expression in tumors to facilitate anti-tumor immune response are also explored in clinical studies. This includes trials of combined epigenetic and immunotherapy agents, such as DNA demethylating drug azacitidine with anti-PD-1 immunotherapeutic pembrolizumab (NCT03264404) or with anti-PD-L1 antibody avelumab (NCT03699384), as well as HDAC inhibitor entinostat and anti-PD-1 agent pembrolizumab (NCT02437136) and similar approaches (171). It will be interesting to follow the results from these ongoing clinical trials to learn what works and what revisions are needed to successfully modulate chemokines and chemokine receptors in combination with other key targets for treatment of cancers.

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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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Chemokines and Chemokine Receptors: New Targets for Cancer Immunotherapy

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Immunotherapy is a clinically validated treatment for many cancers to boost the immune system against tumor growth and dissemination. Several strategies are used to harness immune cells: monoclonal antibodies against tumor antigens, immune checkpoint inhibitors, vaccination, adoptive cell therapies (e.g., CAR-T cells) and cytokine administration. In the last decades, it is emerging that the chemokine system represents a potential target for immunotherapy. Chemokines, a large family of cytokines with chemotactic activity, and their cognate receptors are expressed by both cancer and stromal cells. Their altered expression in malignancies dictates leukocyte recruitment and activation, angiogenesis, cancer cell proliferation, and metastasis in all the stages of the disease. Here, we review first attempts to inhibit the chemokine system in cancer as a monotherapy or in combination with canonical or immuno-mediated therapies. We also provide recent findings about the role in cancer of atypical chemokine receptors that could become future targets for immunotherapy.

Keywords: immunotherapy, cancer related inflammation, atypical chemokine receptor, chemokine receptor, chemokine

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ROLE OF CHEMOKINES IN TUMORS

Inflammation is an essential component of the tumor microenvironment and one of the hallmarks of cancer (1, 2). Chemokines, are a family of small, secreted, and structurally related cytokines with a crucial role in inflammation and immunity (3). They are also key mediators of cancer related inflammation being present at tumor site for pre-existing chronic inflammatory conditions but also being target of oncogenic pathways (4). Initially identified with a prominent role in determining the composition of tumor stroma, they were found able to directly affect cancer cell proliferation and metastasis (5, 6).

Leukocyte Recruitment

The proper movement of immune cells is orchestrated by the spatial and temporal expression of chemokines. Inflammatory CC (CCL2, CCL3, CCL5) and CXC (CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8) chemokines recruit at the tumor site $CCR2^+$ monocytes and $CXCR2^+$ neutrophils that differentiate into tumor associated macrophages (TAMs) and tumor associated neutrophils (TANs), exerting pro- or anti-tumoral role (7–10). Some chemokines present at tumor site can modify leukocyte activation, for instance CXCL16 acting on CXCR6 induces macrophage polarization toward a pro-tumoral phenotype in solid tumors (11, 12). CXCL9 and CXCL10 are strongly associated with Th1 immune response by recruiting NK cells, $CD4^+$ Th1 and $CD8^+$ cytotoxic lymphocytes, which can elicit antitumoral responses (13, 14). Moreover, potent attractant of dendritic cells (DC) are CCL20, CCL5, and CXCL12 (15); CCL21 and CCL19 recruit $CCR7^+$ DC

but also regulatory T cells (T_{regs}) (16, 17). CCL17 and CCL22 acting on CCR4 can directly recruit T_{regs} and Th2 lymphocytes, that promote tumor growth and proliferation (18).

Angiogenesis

Both CC and CXC chemokines play a critical role in tumor angiogenesis, essential for tumor growth and metastatic spreading (19, 20). CXC chemokines, based on the presence of glutamic-leucine-arginine (ELR) motif at the N-terminal, can be divided in ELR^+ chemokines with angiogenic and ELR^- chemokines with angiostatic effects. CCL2, CCL11, CCL16, CCL18, and CXCL8 promote tumor angiogenesis and endothelial cell survival (21, 22). Moreover, CXCL16 interacting with CXCR6, acts as a potent angiogenic mediator (23). CXCL12 and CCL2 can promote angiogenesis and inhibit apoptosis of endothelial cells by directly binding their receptor (CXCR4 and CCR2, respectively) expressed on tumor vessels or indirectly promoting the recruitment of leukocytes (24, 25). On the contrary, chemokines, such as CCL21 and ELR^- chemokines (CXCL4, CXCL9, CXCL10, and CXCL11) inhibit angiogenesis and endothelial cell proliferation (26).

Tumor Growth and Proliferation

Chemokines produced by tumor itself, cancer-associated fibroblasts and infiltrating leukocytes (27, 28), through the binding of chemokine receptors expressed by tumor cells, directly promote cancer cell proliferation activating different signaling pathways, such as PI3K/AKT/NF-κB and MAPK/ERK pathway (29–31). Additionally, they can promote tumor cell survival by preventing their apoptosis and regulating the balance between pro- and anti-apoptotic molecules (e.g., downregulation of Bcl-2 expression or inhibition of caspase-3 and caspase-9 activation) (32, 33).

Metastasis

Chemokine receptors expressed by cancer cells promote their migration to metastatic sites (34). Chemokines and chemokine receptors involved in this phenomenon are several: CCR7 mediates the migration of tumor cells to lymph nodes where their ligands, CCL19 and CCL21, are produced (34, 35). The CCR10/CCL27 axis facilitates the adhesion and survival of melanoma cells during metastatic spreading (36). CCL28 promotes breast cancer growth and metastasis spreading through MAPK/ERK pathway (37). Finally the chemokine receptor CXCR5 and its ligand CXCL13 support bone metastases in prostate cancer (38). However, the main player of this process is the CXCL12/CXCR4 axis. In several tumors, CXCR4 expression endows cancer cells with the ability to migrate and metastasize into organs secreting high levels of CXCL12 (6, 39).

CHEMOKINES IN CANCER THERAPY

Targeting the immune system represents a concrete approach against cancer (40–42). Starting from Coley's toxin development in 1893, many strategies have been set to enhance the antitumor activity of leukocytes (42, 43). Given that chemokines and their receptors have been found involved in several aspects of cancer biology, their possible targeting was evaluated in many preclinical studies and clinical trials (**Table 1** and **Figure 1**). Actually, a monoclonal antibody (anti-CCR4 mAb, Mogamulizumab) and a chemokine receptor inhibitor (CXCR4 antagonist AMD3100) are already in the clinical practice for hematological malignancies (see below).

CCR1

Inhibition of CCR1 reduces cancer growth and metastatization mainly by targeting myeloid cells. In mouse models of Multiple Myeloma (MM) the CCR1 antagonist CCX721 reduced tumor growth and osteolysis targeting osteoclasts and their precursors (44, 45). The same effect was also given by blocking the CCR1 ligand CCL3 that is highly produced by MM cells (95). In a murine model of colon cancer liver metastasis, the CCR1 antagonist BL5923 inhibited metastasis by limiting the recruitment of immature myeloid cells (46). The CCR1 receptor antagonist CCX9588 was recently used in combination with anti-PD-L1 in a murine model of breast cancer showing a synergistic antitumoral effect by reducing the myeloid infiltrate (47). Due to the fact that CCR1 antagonists did not show adverse effects when used in autoimmune disease patients (96), they are ideal candidates to modulate the myeloid infiltrate in combination treatments.

CCR2 and CCL2

Interference with the CCL2/CCR2 axis exerts antitumoral activity in many cancers for the reduced recruitment of monocytes with pro-tumorigenic and pro-metastatic activities.

Many data are available in the context of pancreatic tumors. In a preclinical model, the oral CCR2 inhibitor PF-04136309 reduced the number of TAMs and exerted a modest effect on tumor growth when used alone, while it acted synergistically with the chemotherapeutic drug Gemcitabine (GEM) (48). Encouraging results of a Phase Ib/II trial with pancreatic cancer patients, in which PF-04136309 is used in combination with nab-Paclitaxel [(PTX), a nanoparticle albumin-bound formulation of PTX able to induce TAM activation toward an M1 like phenotype] (97), and GEM, were recently published (NCT02732938) (49). The same inhibitor was used in another clinical trial (NCT01413022) performed on borderline resectable or locally advanced pancreatic ductal adenocarcinoma patients in combination with the standard chemotherapy FOLFIRINOX (FX). Preliminary results demonstrated that the combination therapy increased the percentage of objective responses (51). Another CCR2 inhibitor, CCX872, is really promising in the context of pancreatic tumors. In a preclinical setting, it improved the efficacy of the anti-PD-1 treatment (50) and positive results were also obtained in a clinical trial (NCT02345408) when used in combination with FX (53). In murine models of hepatocellular carcinoma (HCC), CCR2 targeting with the antagonists RDC018 or 747 in combination with Sorafenib, reduced tumor growth and metastasis with a corresponding decrease in macrophage infiltration (52, 54). In prostate and breast cancer, CCR2 was found expressed by tumor cells and to promote cancer growth and migration (98, 99). However, targeting CCL2 with the humanized monoclonal

TABLE 1 | Chemokine and chemokine receptor inhibitors in preclinical models and clinical trials.

	Preclinical models			Clinical trials		
Target	**Inhibitor**	**Tumor model**	**References**	**Inhibitor**	**Tumor type**	**References**
CCR1	CCX721	Multiple myeloma	(44, 45)			
	BL5923	Colon cancer liver metastasis	(46)			
	CCX9588 + anti-PD-L1	Breast cancer	(47)			
CCR2	PF-04136309 + GEM	Pancreatic cancer	(48)	PF-04136309+nab-PTX+GEM	Pancreatic cancer	NCT02732938; (49)
	CCX872 + anti-PD-1	Pancreatic cancer	(50)	PF-04136309 + FX	Pancreatic ductal adenocarcinoma	NCT01413022; (51)
	RDC018	Hepatocellular carcinoma	(52)	CCX872 +FX	Pancreatic cancer	NCT02345408; (53)
	747 + Sorafenib	Hepatocellular carcinoma	(54)			
	iCCR2	Ovarian cancer	(55)			
CCL2	CNTO 888 + radiotherapy	Breast cancer	(56)	CNTO 888	Solid tumors	NCT00537368
				CNTO 888	Metastatic prostate cancer	NCT00992186; (57, 58)
CCR4	Anti-CCR4 CAR-T cells	T cell malignancies	(59)	Mogamulizumab	Relapsed/refractory	
	Affi 5	Renal tumor	(61)		ATL	(60)
	AF399/420/1802	Melanoma, lung tumor and CRC	(62)	Mogamulizumab	CTL	NCT01728805; (63)
CCR5	Maraviroc	CRC	(64)	Maraviroc + chemotherapy	CRC	NCT01736813; (64)
CCR7	siRNA	Metastatic CRC and prostate cancer	(65, 66)			
	MSM R707	Metastatic T- ALL	(67)			
CXCR2	$Cxcr2^{-/-}$ + PTX	Breast cancer	(68)	AZD5069	Pancreatic cancer	NCT02583477
	Navarixin + anti-MEK	Melanoma	(69)	Reparixin + PTX	Breast cancer	NCT02370238; (70)
	SB225002 + Sorafenib	Ovarian cancer	(71)			
	Reparixin + 5-fluorouracil	Human gastric cancer	(72)			
	$Cxcr2^{-/-}$	Pancreatic cancer	(73)			
	$Cxcr2^{-/-}$+ anti-PD-1	Pancreatic cancer	(74)			
	SB225002+RS504393+FX	Pancreatic cancer	(75)			
	SB265610 + Docetaxel	Prostate cancer	(76)			
CXCR4	AMD3100 + Ara-C	AML	(77)	AMD3100	Relapsed AML	NCT00512252; (78)
	LY2510924	AML	(79, 80)	LY2510924	CRC, lung, breast, prostate cancer	NCT02737072; (81)
	BKT140 + Rituximab	NHL	(82)	BMS-936564	AML	NCT01120457; (83)
	AMD3465	GBM and Medulloblastoma	(84)	PF-06747143	Hematologic malignancies	NCT02954653
	POL5551 + anti-VEGF	GBM	(85, 86)	USL311 + Lomustine	Solid tumors and GBM	NCT02765165
	AMD3100	Ovarian cancer	(87)	Balixafortide + Eribulin	$HER2^{-}$ metastatic breast cancer	NCT01837095; (88)
	AMD3100 + anti-PD-L1	Pancreatic cancer	(89)	AMD3100	Recurrent GBM	NCI2012-00149;
	AMD3100 + VIC-008	Mesothelioma	(90)			NCI2013-02012
	PRX177561+Bevacizumab+ Sunitinib	GBM	(91)			
ACKR2	$Ackr2^{-/-}$	Metastatic breast cancer and melanoma	(92, 93)			
ACKR3	X7Ab + Temozolomide	GBM	(94)			

CCL2 neutralizing antibody CNTO 888 in a phase I trial (NCT00537368) in solid tumors and in a phase II trial (NCT00992186) in metastatic prostate cancer, was unsuccessful due to ineffectiveness of CNTO 888 in reducing CCL2 serum level (57, 58). More recent preclinical data indicated that in breast cancer models inhibition of CCL2 improved the response to radiotherapy (100) and was effective in preventing metastasis (56), but its discontinuation caused a rebound in

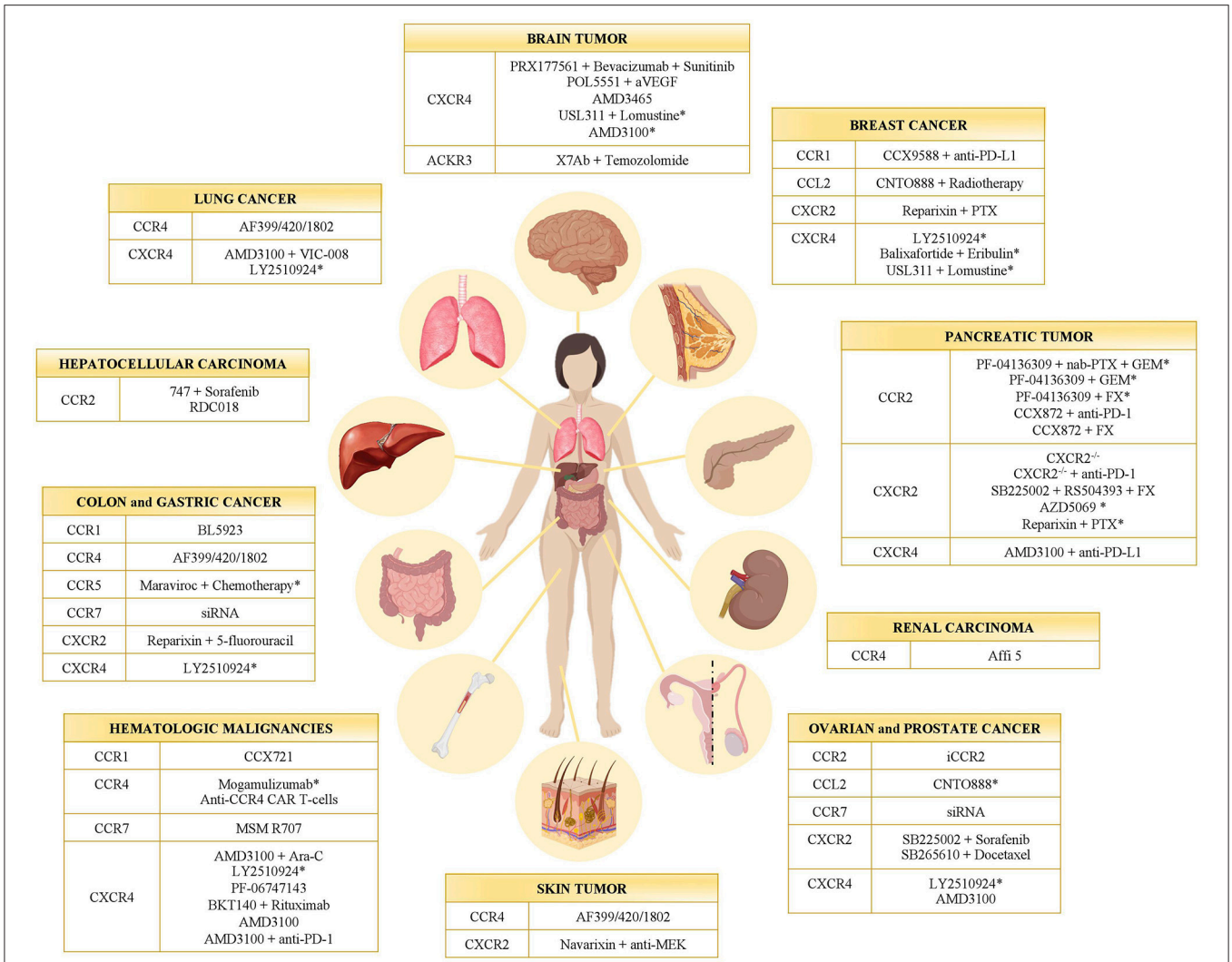

FIGURE 1 | Chemokine receptor inhibitors in cancer. Inhibitors of CC- and CXC-chemokine receptors tested in different tumor types in preclinical models and clinical trials (*). GEM, Gemcitabine; PTX, Paclitaxel; FX, FOLFIRINOX.

the number of circulating monocytes increasing metastatic spreading. Finally, in ovarian cancer, a CCR2 inhibitor enhanced peptide vaccination (55). All these data suggest that targeting the CCL2-CCR2 axis could be effective especially in combination therapies but attention has to be given to fluctuations in the number of circulating monocytes that can produce controversial effects (56).

CCR4

CCR4 is overexpressed in many hematologic malignancies such as Adult T-cell leukemia (ATL) and Cutaneous T-cell lymphoma (CTL). The human anti-CCR4 antibody Mogamulizumab eliminates tumor cells via antibody-dependent cellular cytotoxicity (ADCC) and is actually in use in Japan for the treatment of relapsed/refractory ATL (60). It is also considered the best therapy for previously treated CTL patients according to an international phase III trial (63). In addition, in preclinical studies, CAR-T cells generated against CCR4, were found effective in the treatment of a wide spectrum of T cell malignancies (59).

CCR4 is also considered a promising target for solid tumors for its activity in modulating leukocyte infiltrate, in particular for depleting T_{regs}. In a preclinical model of renal cancer, Affi 5, a CCR4 blocking mAb, reduced tumor growth affecting the phenotype of myeloid cells and increasing the number of infiltrating NK cells (61). CCR4 is now considered a target for renal carcinoma patients (101). However, there are major concerns about the safety of the use of mAbs against CCR4 especially in patients previously subjected to allogenic bone marrow (BM) transplant. Anti-CCR4 mAbs are also depleting T_{regs} for few months, increasing the risk of graft-vs-host disease (102). For this reason, small molecule antagonists of CCR4 with less harmful side effects are in development and one of them, AF399/420/1802, considerably improved the efficacy of cancer vaccines in different preclinical tumor models (melanoma, lung, and colon cancer) by preventing T_{regs} induction (62).

CCR5

The role of CCR5 in cancer remains still controversial; depending on the cell type on which it is expressed it can have a pro- or anti-tumoral role. When expressed by tumor cells it drives their growth and metastatization, while when expressed by T cells potentiates anti-tumoral responses (103). For instance in breast cancer, a dual role of the receptor has been reported in promoting antitumor immune responses, but being also associated with cancer progression and metastasis (104). More recent data indicate that CCR5 induces the mobilization of myeloid cells with pro-tumoral activity (105) and results obtained with preclinical and clinical models of colorectal cancer (CRC) indicate that targeting CCR5 with the negative allosteric inhibitor Maraviroc promoted the polarization of macrophages toward an antitumoral state. Very interestingly, objective partial response was reported in three out of five patients who received a combination of Maraviroc (NCT01736813) and chemotherapy (64). These data suggest that targeting CCR5 could have a major antitumoral effect on tumors that are CCR5 positive and have a prevalent myeloid infiltrate with immunosuppressive activity, while in other tumors CCR5 activity on T cells needs to be preserved for the correct development of the immune response.

CCR7

The therapeutic application of CCR7 inhibitors is also extremely promising. CCR7 is overexpressed by many tumors driving both tumor growth and metastatization. By the use of siRNA technology, CCR7 inhibition resulted in decreased number of metastasis in a model of colon carcinoma (65) and inhibited the growth of prostate cancer (66). Moreover, reduction of CCR7 expression in breast cancer inhibited metastasis (106) and single-chain antibodies blocking CCR7 (MSM R707) were found able to inhibit brain metastasis of T-cell acute lymphoblastic leukemia (107).

CXCR2

CXCR2 is expressed by many tumor cells and is involved in the chemotherapy resistance in different preclinical models of cancer. In breast cancer cells, CXCR2 deletion resulted in better response to Paclitaxel (68). In a melanoma model, the CXCR2 inhibitor Navarixin synergized with MEK inhibition (69) whereas, in an ovarian tumor model, the CXCR2 inhibitor SB225002 improved the antiangiogenic therapy Sorafenib (71). Finally, in human gastric cancer, Reparixin, a CXCR1 and CXCR2 inhibitor, enhanced the efficacy of 5-fluorouracil (72).

CXCR2 targeting inhibits tumor growth also because it affects myeloid cell infiltration. In pancreatic tumors, CXCR2 inhibition prevented the accumulation of neutrophils unleashing the T cell response (73), resulting in inhibition of metastatic spreading and improved response to anti-PD-1 (74). Interestingly, the combined treatment of CXCR2 and CCR2 inhibitors limited the compensatory response of TAMs, increased antitumor immunity and improved response to FX (75). Finally, in a prostate cancer model, CXCR2 inhibition by SB265610, decreased recruitment of myeloid cells and enhanced Docetaxel-induced senescence, limiting tumor growth (76).

Following these promising preclinical results, a phase II clinical trial with the CXCR2 inhibitor AZD5069 is ongoing in pancreatic cancer patients (NCT02583477). In addition, the safety of using Reparixin in combination with Paclitaxel was assessed (70) and a double-blind study with these drugs for metastatic triple-negative breast cancer is in progress (NCT02370238).

CXCR4

The CXCR4 antagonist AMD3100 (Plerixafor) is clinically approved for the mobilization of hematopoietic stem cells (HSCs) for transplantation in patients with Non-Hodgkin's lymphoma (NHL) or MM (67). Beside the HSCs mobilization effect, many preclinical data and clinical trials with AMD3100 or other CXCR4 inhibitors are now suggesting their effectiveness in tumors.

Referring to hematological malignancies, some CXCR4 antagonists, like AMD3100 and the derivative AMD3465, enhanced the efficacy of conventional therapies inducing the mobilization of cancer cells from the protective environment of the BM. In murine models of AML, AMD3100 improved the efficacy of chemotherapy with Ara-C (77). Similar results were obtained in a phase I/II study in patients with relapsed AML (78). The CXCR4 antagonists LY2510924 was also able to suppress the proliferation and progression of AML used as monotherapy (79). Another CXCR4 antagonist, BKT140 had an anti-leukemic effect in a murine model of NHL and its action was synergic with Rituximab (82). Phase I trials are ongoing to evaluate the safety and tolerability of the anti-CXCR4 mAbs BMS-936564 in AML patients (NCT01120457) and PF-06747143 in hematological malignancies (NCT02954653) (83).

CXCR4 inhibitors have strong antitumor and anti-metastatic effects also in solid tumors. In glioblastoma (GBM), CXCR4 expression is higher in more aggressive tumors and is further upregulated by anti-angiogenic therapies (85). AMD3465 reduced the growth of xenografts of glioblastoma multiforme and medulloblastoma cell lines (108) and the CXCR4 antagonist PRX177561, increased the antitumor effects of Bevacizumab and Sunitinib in subcutaneous or orthotopic xenografts of glioblastoma models (91). The CXCR4 antagonist POL5551 inhibited GBM growth and dissemination after anti-VEGF therapy (86). Current clinical trials with AMD3100 in newly diagnosed or recurrent GBM patients are evaluating the safety and efficacy of daily subcutaneous injection (NCI2012-00149) or 2 weeks continuous intravenous infusion (NCI2013-02012). A phase I/II study of the CXCR4 antagonist USL311 alone and in combination with Lomustine is ongoing in patients with advanced solid tumors and relapsed/recurrent glioblastoma multiforme (NCT02765165).

In addition to brain tumors, AMD3465 and LY2510924 have been found to inhibit tumor growth and metastatization in many preclinical models (80, 84). LY2510924, tested in a phase I trial (NCT02737072), was found clinically safe and well-tolerated in advanced solid cancers (colorectum, lung, breast, and prostate) (81). A phase I trial (NCT01837095) of the CXCR4 antagonist Balixafortide plus Eribulin in HER2-negative metastatic breast cancer has given promising results (88).

Notably, CXCR4 inhibition is not only acting on tumor cells but is also promoting antitumoral T cell responses. In a pancreas tumor model, AMD3100, blocking the interaction

of CXCR4 positive tumor cells with CXCL12 producing fibroblasts, unleashed a rapid accumulation of T cells and acted synergistically with anti-PD-L1 (89). In a mesothelioma model, AMD3100 increased the efficiency of the vaccine against mesothelin (VIC-008) by inhibiting PD-1 expression on CD8 T cells and by converting T_{regs} in T helper like cells (90). The inhibition of T_{regs} infiltration and the promotion of antitumoral T cell response by AMD3100 were also demonstrated in a mouse model of ovarian cancer (87).

THE ATYPICALS IN THE IMMUNOTHERAPY LANDSCAPE

Atypical chemokine receptors (ACKRs) are emerging as crucial regulatory components of the chemokine network in a wide range of homeostatic and pathological conditions (109, 110). In this section, we reported preclinical observations and clinical data that provide evidences on their importance in cancer biology suggesting the possibility to validate them as new targets for innovative immunotherapies.

ACKR1 is mainly expressed on post-capillary and small collecting venular endothelial cells (ECs) and red blood cells (111), but also in many tumors such as GBM, hemangiosarcoma, erythroleukemia, breast, and colorectal cancers (112). It is able to bind a broad panel of both CC and CXC inflammatory chemokines acting as chemokine transporter. However, its role remains unclear in cancer because when expressed by ECs promotes tumor growth generating a chemokine gradient that sustains leukocyte infiltration (113). On the contrary, ACKR1 was reducing tumor growth in a model of prostate cancer (114) through the binding of angiogenic ELR^+ CXC-chemokines that decreased angiogenesis and in a melanoma lung metastasis model, interacting with the tetraspanin CD82/KAI that induced tumor cells senescence (115). Finally, in breast carcinoma, ACKR1 expression correlated with a more favorable prognosis with less lymph nodes metastasis and better survival (116, 117).

ACKR2 plays a non-redundant role in the control of inflammatory response by scavenging and degrading most inflammatory CC chemokines, acting as agonists for receptors from CCR1 to CCR5 (118). It is expressed by trophoblast cells in placenta, lymphatic endothelial cells and at low levels by subsets of leukocytes (92, 119, 120). ACKR2 acts as a tumor extrinsic suppressor gene. Indeed, by dampening inflammation, it has a protective role in different inflammation-driven tumor models (121, 122). ACKR2 prevents tumor growth also when it is expressed by Kaposi's sarcoma cells where it is down-regulated by the oncogenic pathway KRAS/BRAF/MEK/MAPK (123), while in anaplastic thyroid carcinomas ACKR2 expression is downregulated by miR-146a (124). In both tumors ACKR2 downregulation unleashes pro-tumoral leukocyte infiltration.

On the contrary, ACKR2 has a tumor promoting role in the Apc-Min model of CRC limiting mast cells infiltration and activation of $CD8^+$ T cells (125) and it has a pro-metastatic function in breast and melanoma cancer models, by limiting neutrophil and NK activity (92, 93).

ACKR3, is a high affinity receptor for CXCL12 and CXCL11 expressed by hematopoietic cells, mesenchymal cells, activated ECs, and neurons. ACKR3 negatively regulates CXCL11 and CXCL12 bioavailability and modulates CXCR4 expression and function (126, 127). In cancer, ACKR3 was found expressed on many tumor cells (such as renal carcinoma, breast cancer, and glioblastoma) and by tumoral ECs. It promotes tumor cell growth and metastasis (128, 129) acting on mTOR pathway (130). In lung adenocarcinoma, ACKR3 mediates TGF-ß1 promoted epithelial to mesenchymal transition (EMT) and tumor growth (131). ACKR3 is also expressed by aggressive prostate carcinoma cells (132) and in renal carcinoma patients with decreased survival and poor prognosis. In renal cell carcinoma, ACKR3 expressed by endothelial progenitor cells and tumoral ECs exerts a proangiogenic role inducing their migration and survival (133). In a glioblastoma murine model, mice treated with X7Ab against ACKR3 in combination with Temozolomide (TMZ) showed significant tumor reduction and longer survival, enhancing M1 macrophage activation (94).

The last member of the family, ACKR4 is a scavenger receptor for CCL19, CCL21, CCL25, and CXCL13. It is expressed by keratinocytes, thymic epithelium and bronchial cells (134). Some papers indicated a protective role of ACKR4 in tumors. In HCC tumors, it impaired chemotactic events associated with CCR7, limiting tumor progression and metastasis (135). ACKR4 down-regulation in human breast and colon cancer correlated with a worse outcome (136, 137). However, in breast carcinoma ACKR4 had a pro-metastatic role regulating EMT (138).

CONCLUDING REMARKS

Being chemokines and chemokine receptors expressed by both tumor cells and leukocyte infiltrate they represent an ideal target for immunotherapy. However, better understanding of their roles in different malignancies is still necessary to avoid potential side effects. In hematological malignancies targeting of overexpressed chemokine receptors directly kill tumor cells but can potentially induce unwanted immune reactions (e.g., CCR4).

In the context of solid tumors, chemokine receptor inhibitors are giving encouraging results when used in combination with chemotherapy or with antibodies against immune checkpoints. For this reason, it is possible to envisage that chemokine receptor inhibitors will be used in the future to modulate the stromal component, to overcome chemotherapy resistance and to optimize the immune response of the patients.

AUTHOR CONTRIBUTIONS

VMP wrote the initial draft. AC, MM, and RB made substantial contributions and discussed the content. All authors reviewed and/or edited the manuscript prior submission.

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