

The Role of CXCR3/Ligand Axis in Cancer

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Abstract— CXC chemokines and their receptors (CXCR) influence the tumor microenvironment (TME) by regulating angiogenesis, recruiting activated immune cells and effecting tumor cell proliferation/metastases. CXCR3/ligand expression in tumors has divergent roles, either promoting or inhibiting tumor progression. These effects can be explained by the tumor CXCR3 receptor isoform expression with CXCR3-A isoform promoting; whereas, CXCR3-B isoform inhibiting tumor growth. CXCR3/ligand axis recruits immune cells into the TME. The types of leukocytes infiltrating tumors modulate tumor progression with activated T and NK effectors inhibiting while M2 macrophages and T regulatory cells supporting tumor growth. Macrophages that lack CXCR3 expression are M2 polarized and promote cancer growth. Antibody-blockade of programmed cell death protein 1 (PD-1) leads to a CXCR3 ligand-mediated recruitment of T cells following adoptive T cell transfer therapy in melanoma. In lung cancer models enhancing the CXCR3 ligands in the TME following treatment with cytokines recruit activated NK and T cells with potent anti-tumor activity. This review discusses the tumor supportive and inhibitory role of CXCR3/ligand axis in several cancer types to show the significance of the axis in the modulation of tumor growth. A full comprehension of these mechanisms is critical for the development of CXCR3 targeted strategies against cancer.

Keywords —CXCR3, chemokines, CXCL9, CXCL10, CXCL11, metastases, immune activation, cancer.

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I. INTRODUCTION

Chemokines, a group of homologous yet functionally divergent proteins, mediate leukocyte migration and activation; regulate angiogenesis, impact immune homeostasis and secondary lymphoid organ architecture. Chemokines are classified into four groups (designated CC, CXC, C, and CXXC), depending on the spacing or presence of four conserved cysteine residues near their amino-terminus. In the CC subgroup, the first two cysteine residues are adjacent, whereas in the CXC subgroup the first 2 cysteine residues are separated by a non-conserved amino acid residue (hence the CXC designation). The CXC chemokine ligands are further classified on the basis of the presence or absence of three amino acid residues (Glu-Leu-Arg; “ELR” motif), preceding the first conserved cysteine amino acid residue in the primary structure of these proteins [1].

The IFN γ inducible CXCR3 ligands (CXCL9, CXCL10 and CXCL11) are produced by endothelial cells, fibroblasts, mononuclear cells and tumors. CXCL4, another CXCR3 ligand is produced by activated platelets [1]. These chemokines exert their biological effects by binding to the 7 transmembrane domain G-protein coupled CXCR3 receptor [2]. Binding of CXCL9, CXCL10, or CXCL11 to CXCR3 increases intracellular Ca²⁺ levels and activates phosphoinositide 3-kinase and mitogen-activated protein kinase pathways in target cells [3]. CXCR3 expression by tumors has pro or anti-tumor role based on the isoforms expressed. In breast cancer [4] and glioma [5] CXCR3-A expression is associated with increased metastases and poor prognoses. Tumor expression of the CXCR3-A receptor in patient breast cancer samples has been suggested to enhance invasion and metastases [6]. The findings from this study suggest that the co-expression of the CXCR3/ligand in breast cancer is associated with poor prognosis. On the other hand, CXCR3-B isoform expression by prostate [7] and breast cancer [8] has been reported to be associated with reduced invasion and growth.

Anti-tumor reactivity is dependent on the types of leukocytes infiltrating the tumors. The numbers and types of leukocytes in the tumor infiltrate are related to the chemokines produced in the TME. CXCL9 and CXCL10 elaboration in the TME recruit activated CXCR3 expressing T lymphocyte effectors with anti-tumor reactivity [9, 10]. Several studies have shown that the anti-tumor activities in the TME are enhanced by CXCR3 expressing T and NK infiltrates [9, 11, 12]. Contributing to the

anti-tumor role, the CXCR3-ligand interaction attracts T helper type 1 lymphocytes and promotes their maturation [13]. CXCR3 ligands that attract lymphocyte effectors into the tumor can serve as therapeutic agents. In addition to inducing chemotactic migration, CXCR3 ligands cause expansion of CD4+ and CD8+ T cells and induce Th1 polarization [14, 15]. The function of CXCR3 ligands to attract T cells, co-stimulate their proliferation, differentiation and activation suggest that the ligands are important for priming T cell responses that have therapeutic implications following local delivery. Activation of this receptor also leads to angiogenesis inhibition and the promotion of CD4 Th1 cell-mediated cellular immunity [14]. Th1 cells produce interferon- γ and enhance anti-tumor immunity by activating macrophages and CD8 cytotoxic T lymphocytes, which are crucial effectors for anti-tumor immunity. The anti-tumor effectors, NK and NKT cell subsets that express CXCR3 are responsive to the ligands. The recruitment of NK and NKT cells is advantageous because these effectors can recognize tumor targets in the absence of MHC expression [16, 17]. Thus, the use of CXCR3 ligands that attract lymphocyte, NK and NKT effectors into tumors can serve as an anti-tumor strategy.

Tumor associated macrophages (TAMs) play an important modulatory role in the generation of anti-tumor responses. The TAMs are heterogeneous, with diverse and opposing biological properties. Recent findings suggest that the CXCR3/ligand axis regulates macrophage polarization in the TME that affects tumor growth and progression. The production of chemotactic factors such as CCL2, VEGF, and macrophage colony stimulating factor [18, 19] in the TME recruits macrophages. The type of macrophages in the tumor correlates with favorable or unfavorable prognoses [20]. The M1 (classically activated) have tumoricidal activities, and M2 (alternatively activated) macrophages contribute to tumor progression and poor prognosis in cancer patients. The M1 macrophages have potent antigen presentation function and stimulate Type1 immune responses that lead to tumor rejection, tissue destruction, and host defense. M1 macrophage density in the tumor islets is positively associated with extended survival of non-small cell lung cancer patients [21]. The M1 macrophages produce high levels of IL-12, CXCL10, and iNOS [22].

In contrast, M2 macrophages are thought to promote tumor formation by enhancing wound healing and tissue remodeling via inhibition of Type1 immune responses by IL-10 and TGF β secretion. The M2 macrophages express high levels of IL-10 and arginase that suppress anti-tumor immune responses [22-25]. Until recently, little was known on the mechanisms of macrophage polarization in the TME. A recent study showed that CXCR3 expression was important for macrophage polarization in a murine breast cancer model. The study demonstrated that the absence of host CXCR3 expression led to increased tumor growth and progression with enhanced levels of TAMs with M2 polarization [26]. The absence of macrophage CXCR3 expression led to M2 polarization and regulated innate and immune cell-mediated anti-tumor

responses that present important therapeutic implications for breast cancer.

Apart from macrophages, another important immune evasion pathway in cancer is the up-regulation of immune regulatory checkpoint molecules. CXCR3 ligand expression in the tumor is influenced by the programmed cell death protein 1 (PD-1) receptor blockade through increased IFN γ production. PD-1 is an immune regulatory checkpoint molecule. Immune checkpoint molecules are coupled with inhibitory pathways in the immune system and crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues in order to minimize tissue damage. Cancer cells usurp this pathway to evade the host immune system. Thus, activation of immune regulatory checkpoint molecules on T cells and their ligands on tumor cells is important for immune evasion of tumors. For example, cancer cells often express the PD-L1 protein that helps tumors evade the immune system by interacting with PD-1 receptor on T cells. An immune strategy that targets this pathway and leads to activation of T cells has demonstrated promise in early phase trials against cancer. This therapy works by releasing the brakes on T cell immune activation by blockade of co-inhibitory checkpoint PD-1 and its ligand (PD-L1, B7-H1) to disable mechanisms of tumor immune escape and improve anti-tumor immune activity [27]. The PD-1/PDL-1 pathway modulates CXCR3 ligand expression in the TME and influences adoptive T cell transfer (ACT) therapy.

Although ACT therapy is a promising modality for cancer treatment, many patients do not experience clinical benefits. The TME limits the recruitment of T cells in ACT therapy. In a recent study, the coordinate over-expression of CXCL9, CXCL10, CXCL11, and CCL5 in pretreatment tumors was associated with responsiveness to ACT therapy in metastatic melanoma patients [28]. In another study, [29] the induction of CXCR3 ligand CXCL10 by antibody-mediated blockade of immune regulatory checkpoint molecule programmed cell death protein 1 (PD-1) resulted in improved ACT therapy. Blocking the PD-1 pathway increased IFN- γ in the tumor, thereby increasing chemokine-dependent trafficking of T cells into malignant disease site with enhanced tumor regressions [29]. Thus, mechanisms that increase the levels of CXCL9 and CXCL10 in the TME have shown to promote effective cell-mediated anti-tumor activity through the CXCR3 expressing effector NK and/or T lymphocytes. While these studies demonstrate the favorable anti-tumor activity mediated by the CXCR3/ligand system, T regulatory cells also express the CXCR3 receptor and recruitment of these suppressor cells in the tumor could lead to pro-tumor effects.

II. CXCR3 RECEPTOR BIOLOGY

CXCR3 is a chemokine G protein coupled receptor for the interferon inducible chemokine ligands CXCL9 (monokine induced by interferon- or MIG), CXCL10 (interferon-inducible

10 kDa or IP-10) and CXCL11 (interferon-inducible T-cell chemo attractant or I-TAC). In humans, the receptor is widely expressed on fibroblasts, endothelial cells, T cells, NK cells, dendritic cells, airway epithelial and smooth muscle cells, type two pneumocytes [30] and a variety of cancers [e.g. glioma [5], prostate [31], renal [32], breast [33], ovarian [34], colon [35], osteosarcoma [36], melanoma [37] and multiple myeloma cells [38]]. In comparison to high levels of CXCR3 expression on hematopoietic and tumor cells, fibroblast and endothelial cells express lower levels of the receptor.

There are three functional CXCR3 isoforms: CXCR3-A and CXCR3-B formed by alternative splicing of the CXCR3 gene and CXCR3-alt formed by translation of a shorter CXCR3 transcript. CXCR3-A and CXCR3-B bind and respond to CXCL9, CXCL10 and CXCL11 whereas CXCR3-alt mediates CXCL11 function. CXCL4 chemokine binds to CXCR3-B [39, 40].

The expression of CXCR3 isoforms and ligand binding interactions on the various cell types determine chemotactic or angiostatic responses. CXCR3-A mediates proliferation, chemotaxis, cell migration and invasion, while CXCR3-B mediates the anti-proliferative, angiostatic and pro-apoptotic effect of the CXCR3 ligands. CXCR3-B has been suggested to mediate the inhibitory activities of CXCL9, CXCL10 and CXCL11 on the growth of several cell types such as human microvascular endothelial cells. In tumor cells, CXCR3-A plays a key role in cell survival, proliferation and migration [41]. Studies have suggested that the biological function of CXCR3 receptor is further impacted by hetero-dimerization with other chemokine receptors such as CXCR4 and CCR5 that influence tumor cell migration and T cell activities and present novel therapeutic opportunities [42, 43].

The effects of CXCL9, CXCL10 and CXCL11 on CXCR3-A are well established whereas the effects on CXCR3-B and CXCR3-alt and the effects of CXCL4 on CXCR3 remain to be characterized. For example CXCL4 binds with weak affinity to CXCR3-A and CXCR3-B in transfected murine pre-B cell L1.2 [44]. CXCL4 exerts its effects on T cells in a CXCR3-independent manner [45]. Campanella et al demonstrated that CXCL10 exerts its effects on endothelial cells in a CXCR3-independent manner by displacing heparan sulfate proteoglycan binding growth factors [46]. In a study by Wu et al, ectopic over-expression of CXCR3-B isoform in prostate cancer cells reduced motility and invasion [7]. Balan et al [8] demonstrated the growth-inhibitory signal mediated by CXCR3-B induced cell death in MCF-7 and T47D breast cancer cells. Further studies are required to differentiate the effects of endogenous and exogenous CXCR3-B expression on tumor cell proliferation, motility and invasion. CXCR3-alt isoform was cloned as a PCR product generated from post transcriptional excision from a portion of CXCR3-A full length mRNA [47]. This putative mRNA codes for a truncated protein with only 4 transmembrane domains and raises concern whether it is a functional receptor. In the subsequent sections of this review, we will focus the discussion on the

well-established CXCR3-A isoform referred to as CXCR3 from here on.

III. CXCR3 EXPRESSION IN CANCERS

Current therapeutic options benefit cancer patient survival slightly when the tumor invades and disseminates to surrounding tissues or metastasizes to distal sites. An understanding of the molecular underpinnings of this transition from the localized to the metastatic site can provide patients with the benefits of rational approaches to ablate these processes. Several studies have demonstrated that endogenous tumor CXCR3 expression enhanced tumor cell invasion and migration with poor prognosis in cancer patients. The mechanism of CXCR3/ligand system support of metastases is facilitating the migration of CXCR3 expressing tumor cells to ligand rich metastatic sites. The results from several studies on the role of CXCR3 expression on tumor growth/metastases are discussed below.

Kawada et al demonstrated that knockdown of endogenous CXCR3 expression in melanoma cells by anti-sense RNA reduced the metastatic frequency to lymph nodes (LNs) in a murine model [37]. Studies by the same group [35] demonstrated that ectopic over-expression of CXCR3 in colon cancer cells increased the frequency of macroscopic metastatic foci in the draining LNs, probably due to increased migration and or survival/expansion of tumor cells at the metastatic site. This group reported CXCR3 expression in clinical colon cancer samples cases (34%), most of which had LN metastasis. Patients with CXCR3-positive cancer had poor prognosis compared to those without CXCR3 expression.

In related studies, endogenous CXCR3 knockdown in breast cancer cells inhibited lung colonization and spontaneous lung metastasis from mammary gland-implanted tumors [4]. In the same study, NK depletion in mice transplanted with the CXCR3 knockdown cells abrogated the reduced metastatic frequency to the lung. This suggests that tumor cells with CXCR3 knockdown bind negligible amounts of CXCR3 ligand, with sufficient ligand remaining in the tumor for a gradient-ligand mediated recruitment of effector NK infiltrate. Furthermore, Ma, et al. [4] demonstrated that a high CXCR3 expression correlates with poor overall survival in early breast cancer patients (node negative).

In murine models, pharmacological antagonism of CXCR3 reduced lung metastases of breast [48] and colon carcinoma cells [49]. In addition, CXCR3 antagonism prolonged median survival times with anti-tumor progression effects in mice bearing glioblastoma multiforme [5]. Consistent with these findings, Pradelli et al. [36] demonstrated in osteosarcoma that CXCR3 antagonism inhibited lung metastasis, decreased migration, matrix metalloproteinase activity and proliferation/survival, but increased caspase-independent death. Although CXCR3 antagonism presents a therapeutic opportunity, it poses the challenge of impacting CXCR3

mediated immune cell infiltration. However, considerable redundancy exists in chemokine/chemokine receptor mediated recruitment of immune cells that present opportunities for non-CXCR3 chemokine receptor/ligand mechanism for immune effector recruitment. This approach would achieve pharmacological targeting of CXCR3 on tumors, yet allow non-CXCR3 mechanism such as CCR1, CCR2, CCR5, or CXCR5 mediated immune effector cell recruitment into the tumor.

IV. CXCR3 MEDIATED RECRUITMENT OF IMMUNE INFILTRATES INTO THE TME

CXCR3/ligand axis plays a key role in mediating the recruitment of leukocytes to inflammatory sites. This axis recruits anti-tumor effectors into the TME. CXCR3, highly expressed in activated but not resting T cells, mediates T-cell chemotaxis in response to CXCL9 or CXCL10. The results of the effects of CXCR3 ligand mediated recruitment of anti-tumor effectors are discussed below.

In experimental murine cancer models, the elaboration of CXCR3 ligands caused migration of CXCR3+NK and CD8+T cells that resulted in reduction in tumor growth and metastases. CXCL9 production by tumor cells was critical for T cell-mediated suppression of cutaneous tumors [50]. Expression of CXCL10 in breast cancer cells enhanced tumor-specific T cell infiltration and extended the survival of treated mice [51]. In another study [52], mice challenged with EL4 T-cell lymphoma cells genetically modified to produce murine CXCL11 showed a CD8 T cell dependent tumor rejection and induction of immunological memory that rejected a secondary tumor challenge. In a mouse model of glioma, recombinant IP10-EGFR fusion protein (IP10-scFv) and CTL administration inhibited tumor growth, increased CXCR3+CD8+T cell recruitment and extended survival [53].

In contrast to these findings, in patient breast cancer samples, CXCL10 expression was associated with increased CXCR3 expressing FOXP3+ regulatory T cell [6]. This suggests that CXCL10/CXCR3 axis has a role in tumor invasiveness and progression. Based on these findings, further investigations are needed to determine the relationship between expression of the CXCR3 ligands in cancer patient samples and prognoses.

To exploit the anti-tumor benefit of CXCR3 ligands, modes of delivery in the tumor, combination with therapeutic vaccination and the impact on immune regulatory checkpoint molecule blockade need to be adequately addressed in several tumor models prior to clinical application. For example, CXCR3 ligand combined with therapeutic vaccination can enhance the recruitment of antigen specific T cells in the tumor. A combination of CXCR3 ligand with immune regulatory checkpoint blockade can enhance the recruitment of effector T cells into the tumor with sustained anti-tumor activities. CXCR3 ligands can be delivered intra-tumorally or by chemokine/antibody chimeras to facilitate uptake by tumor

specific antigen expression. However, further work is needed in this area to validate the applicability of the approach as tumor cells expressing CXCR3 could exhibit a proliferative response to the administered CXCR3 ligand thereby limiting the approach to non- CXCR3 expressing tumors.

V. THERAPEUTIC IMPLICATIONS OF CXCR3/LIGAND AXIS IN CANCER

Agents that reduce tumor CXCR3 or augment tumor paracrine CXCR3 ligands have shown anti-tumor activity in several tumor model systems. This adds to the rationale for further investigation of the therapeutic potential of CXCR3/ligand axis in cancer. Studies in murine lung cancer models have shown that cytokine therapy with CCL21 [54], IL-7 [9] and IL-7/IL-7R α -Fc [10] promote T cell dependent anti-tumor immunity that require CXCL9 and CXCL10.

In a murine RENCA tumor model, systemic IL-2 and intra-tumor CXCL9 administration suppressed tumor growth, enhanced tumor necrosis, reduced tumor-associated angiogenesis, and increased tumor infiltration of CXCR3+ mononuclear cells [11]. Similarly in the RENCA tumor model, treatment with IL-2 and agonistic CD40 antibody increased CXCL9/CXCL10 and inhibited tumor growth [55].

The significance of CXCR3/ligand axis in cancer is further strengthened by the observations that COX-inhibitors increased CXCL9/CXCL10 expression [56] and promoted anti-tumor effects in breast cancer [57]. Bronger et al [56] demonstrated that suppressing endogenous PGE2 synthesis by cyclooxygenase inhibition increased CXCL9 and CXCL10 release from breast cancer cells and enhanced intra-tumoral immune infiltration. In this study, the unselective COX inhibitors aspirin and indomethacin were preferable in increasing CXCL9/CXCL10 in comparison to celecoxib that at higher concentrations reduced ligand release from breast cancer cells. The decrease in ligand release from breast cancer cells at high concentrations of celecoxib was attributed to COX independent mechanisms [56].

Blockade of T cell immune regulatory molecules sustain anti-tumor T cell activity. In murine models of melanoma and colon adenocarcinoma, PD-1 blockade enhanced T cell migration to tumors by elevating tumor CXCL10 [29]. In another study, inhibition of miRNA-21 enhanced RANTES and CXCL10 release from breast cancer cells that has therapeutic implications [58]. Taken together these studies demonstrate that increasing CXCR3 ligands in the tumor facilitated an enhanced anti-tumor T cell response to control tumor growth.

VI. FUTURE PROSPECTS

The CXCR3/ligand axis and the intra cellular signaling pathways that stimulate cell survival and motility signify probable targets in cancer, but important questions remain to be addressed. CXCR3-A and CXCR3-B isoforms mediate

opposing effects of CXCR3 ligands. CXCR3-A mediates proliferation, chemotaxis, cell migration and invasion, while CXCR3-B mediates the anti-proliferative, angiostatic and pro-apoptotic effect of the CXCR3 ligands. Although CXCR3-B has been reported to mediate the inhibitory effect of the CXCR3 ligands, further studies are required to differentiate the mechanisms of endogenous and exogenous CXCR3-B expression on tumor cell survival and motility in different tumor systems. Investigations are also required to determine the CXCR3 isoforms/ligand status in tumor stem cells and their contribution to tumor growth and metastases. These studies would provide key information and allow for the benefits of rational personalized approaches to inhibit tumor cell proliferation and metastatic dissemination.

Studies are needed to evaluate cellular and non-cellular vehicles for CXCR3 ligand delivery to facilitate immune effector recruitment into the tumor. This approach may prove effective for non-CXCR3-A expressing tumors. However, for CXCR3-A expressing tumor cells that secrete CXCR3 ligands and promote their growth and survival, pharmacological inhibition of CXCR3-A ligation or signaling combined with CXCR3 independent mechanism for immune cell recruitment may be needed. For example CCR1, CCR2, CCR5, and CXCR5 ligands that recruit T cell effectors may serve this purpose. Some candidate drugs that reduce tumor CXCR3-A are COX inhibitors, agents that target enzymes downstream of the COX pathway and CXCR3 small molecule antagonists. Further work is needed to understand the role of miRNAs in the modulation of CXCR3/ligand axis in cancer. This could lay important groundwork for therapeutic options as well as provide important biomarkers of response and/or patient selection. There is an urgent need for prognostic biomarkers to predict patient responses to specific therapies for cancer in order to provide safe and effective treatment options. Moreover, since tumor CXCR3-A expression enhances metastases, studies are needed to fully determine the relationship between CXCR3/ligand expression in patient cancer samples and prognoses.

Although cell-based delivery systems (fibroblasts, dendritic and mesenchymal stem cells) are efficient modes of cytokine delivery, an efficacious off-the-shelf reagent would facilitate the widespread therapeutic applicability of CXCR3 ligands in cancer. For this purpose, nanoparticles such as vault nanocapsules could prove useful for ligand delivery. The vault nanocapsules have been an efficient mode for CCL21 chemokine delivery to induce systemic anti-tumor immune responses against lung cancer [59]. The vault nanocapsule ubiquitously expressed and highly conserved throughout eukaryotes is a unique therapeutic delivery system. The vaults are highly stable structures and non-immunogenic. Once packaged, the particles are stable; they protect their protein contents, yet act as time-release capsules to deliver their payloads. As a naturally-occurring nanocapsule, the vault particle is an ideal structure to engineer for targeting CXCR3 ligands to recruit immune effectors into the tumors.

Although CXCR3 ligands are effective at promoting T cell activation/recruitment, little is known about their impact on T cell immune regulatory checkpoint molecules that inhibit sustained anti-tumor T cell activity. Immune inhibitory molecules are up-regulated on T cells in tumors with an overall effect of down-regulation of anti-tumor activity. Inhibitory receptors that regulate immune responses include cytotoxic T-lymphocyte-associated antigen 4 (CTLA4, also known as CD152); programmed cell death protein 1 (PD1, also known as CD279); T cell membrane protein 3 (TIM3) and Lymphocyte activation gene-3 (LAG-3). Antibody-mediated blockade of PD-1 or PDL-1 has shown benefit in patients with lung cancer [27, 60]. Patients do not benefit from PD-1 or PDL-1 blockade therapy if they lack lymphocytic infiltration of the tumors. Recent findings indicate that tumor regression after therapeutic PD-1 blockade requires pre-existing CD8+ T cells that are negatively regulated by PD-1/PD-L1 [61]. Perhaps the blockade of immune regulatory checkpoint molecules will be more efficacious when used in conjunction with chemokines that augment activated T cell infiltration into the tumors. Such investigations will bring the potential CXCR3/ligand axis closer to clinical fruition.

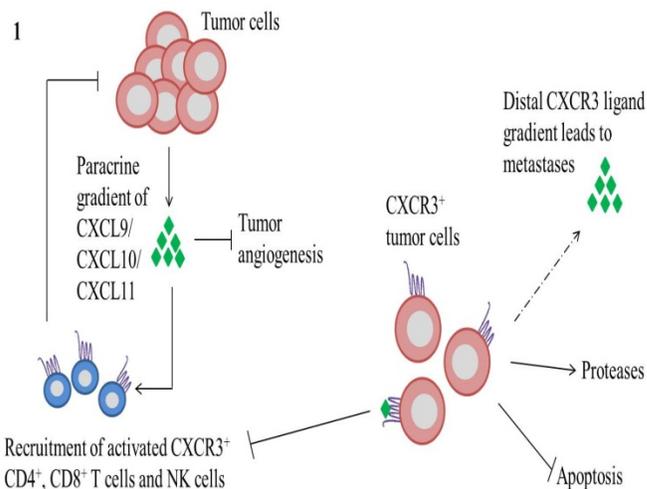


Fig. 1. Differential response of tumor and immune cells to CXCR3/ligand expression

Paracrine expression of tumor CXCR3 ligands recruits activated CXCR3+ T lymphocytes and NK cells that inhibit tumor growth and induce immune angiostasis. Tumor CXCR3 expression enhances tumor cell migration to distal ligand abundant sites. Ligand binding to tumor CXCR3 localizes tumor growth, increases tumor cell survival/proliferation, induces proteases and inhibits immune cell recruitment.

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