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At the Bench: Pre-Clinical Evidence for Multiple Functions of CXCR4 in Cancer

Summary:

We review CXCR4-dependent mechanisms in hematologic and solid malignancies, the current status of radioimaging methods for CXCR4, and the landscape of CXCR4 modulators.

Keywords:

tumor · chemokine · CXCL12 · radioimaging · CXCR4 inhibitor · CXCR4 antagonist

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Abbreviations

Abbreviation	Definition
ACKR3	Atypical chemokine receptor 3
ADT	Androgen deprivation treatment
AKT	RAC-alpha serine/threonine-protein kinase 1/PKB
APC	Antigen-presenting cell
BBB	Blood brain barrier
BC	Breast cancer
BM	Bone marrow
BMDC	BM-derived dendritic cells
CAF	Carcinoma-associated fibroblasts
CCR	C-C chemokine receptor
CSC	Cancer stem cell
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
Cu	Copper
CXCL	C-X-C motif chemokine
CXCR	C-X-C chemokine receptor type
DC	Dendritic cell
ECM	Extracellular Matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
Ga	Gallium

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Abbreviation	Definition
GAG	Glycosaminoglycan
GBM	Glioblastoma multiforme
GSC	Glioma stem(-like) cells
ERG	Erythroblast transformation specific-related gene
h	human
HPV	Human papilloma virus
HSC	Hematopoietic stem cells
HSPC	Hematopoietic stem and progenitor cells
Ig	Immunoglobulin
IL	Interleukin
LC	Langerhans cells
LN	Lymph node
m	murine
MAPK	Mitogen activated protein kinase
MDSC	Myeloid derived suppressor cell
MIF	Macrophage migration inhibitory factor
MM	Multiple myeloma
mTOR	Mammalian target of rapamycin
NK	Natural killer cells
PCa	Prostate cancer
PD-1	Programmed death-1
PET	Positron emission tomography
PI3K	Phosphatidylinositol-3-kinase
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid

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Abbreviation	Definition
S1P	Sphingosine-1-phosphate
Tc	Technetium
Th17	T helper 17 cell
TIME	Tumor immune microenvironment
TME	Tumor microenvironment
Treg	T-regulatory cells
USA	United States of America
VEGF	Vascular endothelial growth factor
WHIM	Warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis
WM	Waldenström's macroglobulinemia

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Abstract:

Signaling through chemokine receptor, C-X-C chemokine receptor type 4 (CXCR4) regulates essential processes in normal physiology, including embryogenesis, tissue repair, angiogenesis, and trafficking of immune cells. Tumors co-opt many of these fundamental processes to directly stimulate proliferation, invasion, and metastasis of cancer cells. CXCR4 signaling contributes to critical functions of stromal cells in cancer, including angiogenesis and multiple cell types in the tumor immune environment. Studies in animal models of several different types of cancers consistently demonstrate essential functions of CXCR4 in tumor initiation, local invasion, and metastasis to lymph nodes and distant organs. Data from animal models support clinical observations showing that integrated effects of CXCR4 on cancer and stromal cells correlate with metastasis and overall poor prognosis in more than 20 different human malignancies. Small molecules, antibodies, and peptidic agents have shown anti-cancer efficacy in animal models, sparking ongoing efforts at clinical translation for cancer therapy. Investigators also are developing companion CXCR4-targeted imaging agents with potential to stratify patients for CXCR4-targeted therapy and monitor treatment efficacy. Here we review pre-clinical studies demonstrating functions of CXCR4 in cancer.

Clinical aspects of patient selection and results of initial clinical trials are covered in the related review: “At the Bedside: Profiling and treating patients with CXCR4-expressing cancers. J. Leukoc. Biol. XX: XX–XX; 2020”.

I. Introduction: CXCR4 and Cancer

C-X-C chemokine receptor type 4 (CXCR4), also known as CD184, is a member of the seven-transmembrane (G-protein coupled) receptor family, the largest class of cell surface receptors and the targets of ~35% of all approved drugs (1). CXCR4 signaling critically regulates essential processes in normal physiology, including embryogenesis, tissue repair, and hematopoiesis. Homozygous deletion of CXCR4 in mice causes embryonic lethality due to widespread defects, affecting vascularization of the gastrointestinal tract, generation of B lymphocytes and myeloid cells, formation of the cerebellum, and ventriculoseptal defects in the heart (2). Conditional knockout of CXCR4 in mice reveals even more functions in development, such as myogenesis, innervation of limbs, and formation of renal vasculature (3, 4). In adults, high expression of CXCR4 occurs in bone marrow (BM) and multiple cell types in the immune system, with modest levels in most other tissues and organs. CXCR4 on hematopoietic stem cells (HSC) controls homing and retention of these cells in the BM, a function that has been targeted therapeutically to mobilize these cells into the circulation for recovery and transplantation (5). Expression of CXCR4 on more differentiated immune cells controls homeostatic trafficking for immune surveillance and host defense. CXCR4 on T lymphocytes also serves as a co-receptor for some strains of human immunodeficiency virus. Essential functions of CXCR4 in immunity contribute to pathogenesis of multiple common diseases, including cancer, autoimmunity, atherosclerosis, and neurodegeneration (6-9).

Beyond regulation of immunity, CXCR4 on cancer cells directly enhances multiple steps in tumor initiation, growth, and metastasis. Müller et al. first discovered that CXCR4 promotes organ-specific patterns of breast cancer (BC) metastasis to lung, liver, bone, and brain. These sites all express high

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levels of C-X-C motif chemokine 12 (CXCL12, also known as stromal cell-derived factor 1), the chemokine ligand for CXCR4 (10). Blocking CXCR4 with neutralizing antibodies markedly reduced metastatic BC in mice, highlighting CXCR4 as a therapeutic target to block or prevent metastasis. Subsequent studies expanded functions of CXCR4 on cancer cells to local growth of tumors in orthotopic mouse models of BC and established high levels of CXCR4 as a marker of poor prognosis in patients with BC (11-13). CXCR4 is reported to enhance local tumor growth, invasion, and metastasis in more than 20 different cancers (6), pointing to CXCR4 as a general driver of human malignancies. Functions of CXCR4 in cancer typically result from upregulation of a normal, non-mutated receptor. However, congenital or de novo mutations that truncate the intracellular C-terminus of CXCR4, resulting in defective internalization and amplified signaling, occur in warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (abnormal retention of neutrophils in bone marrow) (WHIM) syndrome (14). Truncation of the CXCR4 C-terminus also occurs as a somatic mutation in the hematologic malignancy, Waldenström's macroglobulinemia (WM) as described in Section II (15). Discoveries about CXCR4 also sparked a new area of research about chemokine receptors in cancer. Ongoing studies continue to link multiple chemokine receptors to hallmark features of cancer, including proliferation, survival, and metabolism (16).

Environments in primary tumors and metastases highlight interconnections between cancer and stromal cells that activate CXCR4 with multiple direct and indirect effects on tumor progression. Using BC as an example, carcinoma-associated fibroblasts (CAFs) in a primary breast tumor secrete chemokine CXCL12 (17-19), driven in part by cytokine signals from cancer cells in a feed-forward loop that directly enhance survival, proliferation, and invasion of malignant cells (20). CXCL12 secreted by CAFs also selects BC cells primed to metastasize to bone, another environment rich in CXCL12 (21). Hypoxia, a feature common to both primary and metastatic tumors, drives transcription of both CXCL12 and CXCR4 to further stimulate tumor growth and metastasis (22-24). CXCR4 is expressed on both immunosuppressive and effector populations of immune cells, so signaling through this receptor regulates the balance of pro- and anti-tumor leukocytes recruited to a tumor. CXCR4 promotes tumor angiogenesis by recruiting endothelial progenitor cells to a tumor and/or amplifying pro-angiogenic effects of molecules such as vascular endothelial growth factor (VEGF) (25, 26). In response to chemotactic gradients of CXCL12, CXCR4 promotes local invasion, intravasation and extravasation of cancer cells from the vasculature into environments with high levels of CXCL12. Within the BM, CXCR4 enables disseminated cancer cells to displace HSC from CXCL12-rich vascular niches, an environment that establishes dormancy and protects malignant cells from chemotherapy (27, 28). These findings highlight integrated, multi-faceted functions of CXCR4 in tumor environments and emphasize both challenges and opportunities to improve cancer therapy by targeting CXCR4.

II. Activating mutations in CXCR4: WHIM syndrome and Waldenström's macroglobulinemia

Both WHIM syndrome and WM share mutations in CXCR4 that truncate the intracellular C-terminus of the receptor. Such mutations prevent ligand-induced internalization of CXCR4, resulting in higher levels of cell surface receptor and amplified signaling. Mutant CXCR4 enhances and prolongs activation of AKT and extracellular signal-regulated kinases (ERK), as well as reducing apoptosis (29).

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Truncated CXCR4, or amino acid mutations that mimic effects of truncation, impairs stability of the immunologic synapse between T cells and antigen presenting cells (30). In mouse and zebrafish models, expressing truncated CXCR4 in HSCs increases bone marrow engraftment while impairing release of leukocytes (lymphocytes and neutrophils) into the peripheral circulation (31-33). These phenotypes reproduce clinical abnormalities in patients with WHIM syndrome. Immune defects caused by truncated CXCR4 account for increased frequencies of bacterial and viral infections in patients with WHIM syndrome. Infection with human papilloma virus (HPV) leads not only to warts but also to squamous cell carcinomas, such as cervical and head and neck cancers (34). Similar truncating mutations in CXCR4 occur in ~30% of patients with WM, an indolent lymphoma marked by expansion of immunoglobulin (Ig) M-producing lymphoplasmacytic cells in BM (35-37). Mutations in CXCR4 confer resistance to ibrutinib, the only drug approved for WM (38). Effects of amplified CXCR4 in WHIM syndrome and WM point to key signaling pathways and processes that contribute to tumor-promoting effects of CXCR4 in other malignancies (38).

III. CXCR4 Signaling and Cancer Stem Cells

Regulation of CXCR4 signaling occurs at multiple levels (Fig 1). Chemokine CXCL12 was the first ligand identified for CXCR4 (39). For many years, investigators regarded CXCL12 as the only ligand for CXCR4, based in large part on the nearly identical phenotypes of mice with genetic knockout of either CXCL12 or CXCR4 (40). However, recent studies have revealed unexpected complexity and diversity of ligands for CXCR4. Alternative splicing generates six biochemically distinct isoforms of CXCL12 (α, β, γ, δ, ε, and ϕ). CXCL12- γ has an extended C-terminus with a high number of positively-charged amino acids that confer nanomolar binding affinity to glycosaminoglycans (41-44). CXCL12-γ normally is expressed predominantly in heart and brain (43), but transcripts for all isoforms have been identified in human BCs (44). Various isoforms of CXCL12 vary in kinetics, amplitude, preferential activation of β-arrestin versus G protein signaling outputs, and outputs such as chemotaxis (41, 43, 45, 46). Notably, CXCL12-γ increases metastasis in BC and castration-resistant prostate cancer (PCa) in part by increasing frequencies of cancer stem cells (41, 47).

CXCL12 exists as monomers and dimers that also produce divergent CXCR4 signaling responses, likely dependent upon cell type and environmental context (48, 49). The nuclear protein high mobility group box 1 released from necrotic cells forms a heterocomplex with CXCL12 that induces conformational changes in CXCR4 distinct from those produced by CXCL12 alone (50). In addition, extracellular ubiquitin, CXCL14, and macrophage migration inhibitory factor (MIF) activate aspects of CXCR4 signaling in at least some cell types, likely through allosteric effects on the receptor (51-54). The extent to which different single ligands or combinations of agonists determine activation of specific CXCR4 signaling pathways and/or functions requires further investigation under physiologic and pathophysiologic conditions.

Formation of CXCR4 dimers, higher order homo-oligomers, and heterocomplexes with other receptors provide additional regulation of CXCR4 receptor dynamics and signaling. Although potentially dependent upon cell type, multiple techniques generally show that CXCR4 exists as a homodimer even in the absence of ligand (55-58). Homodimers of CXCR4 may occur due to confinement of the receptor within lipid rafts (59). Ligand binding increases oligomers of CXCR4 that internalize more efficiently than monomers, regulating intracellular signaling and subsequent

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desensitization (60-62). CXCR4 forms heterocomplexes with other chemokine receptors (CXCR7, now designated as atypical chemokine receptor 3 [ACKR3], C-C chemokine receptor type 2 [CCR2], and CCR5), and the T cell receptor [TCR]) (56, 63-69). ACKR3 binds CXCL12 with higher affinity than CXCR4, so cells co-expressing both receptors exhibit modified signaling outputs and functions including migration (70-73). Heterocomplexes with other chemokine receptors also cross-sensitize CXCR4 to inhibitors of the binding partner, making CXCR4 signaling and functions vulnerable to cross-inhibition (74). Similarly, CXCR4-TCR heterodimers cross-regulate cytokine responses and chemotaxis of T lymphocytes by engaging distinct downstream signaling molecules (75). Particularly in tumor environments where cells encounter multiple ligands and inhibitors, homodimers and heterodimers of CXCR4 potentially increase plasticity of signaling and open potential opportunities to target multiple receptors and signaling pathways with a single drug.

As a seven-transmembrane receptor, CXCR4 signals through G proteins. CXCR4 and other chemokine receptors predominantly activate Gαi, resulting in release of intracellular calcium, activation of pathways including mitogen activated protein kinases (MAPK)/ERK and phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), and inhibition of adenyl cyclase and cyclic adenosine monophosphate (76). However, CXCR4 may signal through other Gα proteins, such as Gα13 (77). CXCL12 binding to CXCR4 causes phosphorylation of the receptor and recruitment of adapter protein β-arrestin 2, causing receptor internalization and activation of arrestin-dependent signaling to ERK. A recent study suggests biased antagonists that selectively block CXCR4-dependent signaling to G proteins but not β-arrestin 2 can overcome tolerance to chronic inhibition of this receptor (78). Activation of ERK, AKT, and mTOR promotes proliferation, survival, and protein synthesis, all of which enhance tumor initiation and metastasis. CXCR4 activates small guanosine triphosphatases such as Rac1 and Rho to remodel the actin cytoskeleton, an essential step in cell motility and chemotaxis. CXCR4/CXCL12 signaling activates integrins necessary for cell adhesion to the extracellular matrix (ECM) and endothelial cells, although CXCL12 also may allosterically activate integrins independent of CXCR4 (79). Functions of these downstream effectors could collectively or independently promote CXCR4-mediated tumor progression and metastasis.

CXCR4 marks cancer stem cells (CSCs) in malignancies including breast, lung, esophagus, stomach, prostate and glioblastoma (80-84), and CXCL12-CXCR4 signaling through pathways such as ERK leads to invasion and metastasis. CSCs, also referred to as tumor-initiating cells, define a dynamic sub-population of malignant cells with enhanced capabilities for self-renewal, tumor formation, metastasis, and differentiation into the full range of cancer cell types present in a primary or metastatic tumor. A variety of resistance mechanisms enable CSCs to survive standard chemotherapy drugs and radiation, making these cells a key cause of treatment failure and recurrent cancer. Expression of CXCR4 on CSCs is consistent with clinical data showing that upregulation of CXCR4 typically correlates with poor prognosis across malignancies. Upregulation of CXCR4 through mechanisms including PI3K/AKT/mTOR signaling and transcription activated by ΔNP63α, the predominant isoform of TP63 in epithelium, increases abundance and phenotypes of CSCs (80). Activation of CXCR4 stimulates key functions of CSCs, including self-renewal, local invasion, and dissemination to secondary organs and tissues (85-87). In pancreatic cancer, CXCR4 demarks a highly metastatic subset of CSCs (88). Key functions of CXCR4 in CSCs suggest that inhibiting CXCR4 signaling may be an effective strategy to reduce or eliminate CSCs in multiple malignancies.

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IV. CXCR4 in the tumor microenvironment

The tumor microenvironment (TME) describes all components of a tumor mass, including malignant cells, ECM, immune cells, mesenchymal stem/stromal cells, fibroblasts, vasculature, metabolic products, and signaling molecules (89, 90). The TME shapes different phases of cancer, from initiation to metastasis, with reciprocal interactions between cancer cells and surrounding cellular and biophysical components that establish conditions permissive or restrictive to tumor progression (90). The tumor immune microenvironment (TIME) defines the immunologic environment composed of infiltrating immune cells and their secreted products (cytokines, chemokines, growth factors, etc). Chemokines expressed in the TIME regulate polarization of immune cells and the overall balance of immunosuppressive T-regulatory cells (Tregs), some subgroups of T helper 17 cells (Th17), myeloid derived suppressor cells (MDSCs) and M2 macrophages versus effector natural killer (NK) and $CD8^+$ T cells (91). Recruited leukocytes, along with endothelial cells and fibroblasts, make up the stromal cell compartment, and these stromal cells interact with tumor cells to modulate the TME, where both chemokines and chemokine receptors participate in this process (92). Emerging evidence identifies chemokine CXCL12 as one of the most important factors controlling recruitment of immunosuppressive cells to the TIME (91, 93-95). CXCL12, particularly CXCL12-γ, binds to glycosaminoglycans (GAGs) on the surface of endothelial cells, forming chemotactic gradients that promote migration of leukocytes and cancer cells (96-98). In the following sections, we describe functions of CXCR4 in various components of tumor environments, with a particular emphasis on tumor immunity.

CXCR4 in endothelial cells

Endothelial cells express both CXCR4 and CXCL12, and this receptor-ligand interaction facilitates intravasation and extravasation of cancer cells as well as tumor angiogenesis through a process that involves integrin activation, resulting in enhanced adhesion of CXCR4 expressing cells to the endothelium during extravasation or intravasation (99-101). Tumor growth and metastasis require angiogenesis and increased vascularization; otherwise, tumor cells may replicate rapidly, but their growth and metastasis are restricted by inadequate blood supply (102). Cancer cells stimulate angiogenesis through the CXCR4/CXCL12 signaling pathway, leading to tumor neovascularization, growth, and progression to metastasis. Owing to potential clinical benefits of therapeutically manipulating tumor angiogenesis, the mechanisms controlling this process attracted scientists to focus on vascular research over the last two decades. Blood vessel formation by angiogenesis is a complex multistep process that critically requires tight control and coordination of endothelial cells. Angiogenesis is a process including ECM dissolution, endothelial cell mitoses, and new branches sprouting into lumens to form a complex vessel network (103, 104). During the process, endothelial cells interact together in a process termed “tip-stalk cell selection” (103). The endothelial tip senses attractive and repulsive signals and extends filopodia, leading sprouts toward the angiogenic chemokines, whereas the stalk cells, trailing behind the tip cells, facilitate lumen formation and mitosis to support sprout elongation (105). Angiogenesis is orchestrated not only by VEGF, but also by CXCR4/CXCL12. CXCR4 facilitates angiogenesis by recruitment of endothelial progenitor cells or BM-derived accessory cells. VEGF promotes sprouting angiogenesis by inducing tip cell filopodia and serving as an attraction cue (106), whereas the CXCR4/CXCL12 axis stimulates tip cells and migration in neovascular sprouting (107). Thus, sprouting endothelial cells are hierarchically organized into leading tip cells and trailing stalk cells that exhibit very distinct and specialized cell behaviors. These

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studies indicate critical roles for CXCR4 in endothelial tip cell behavior *in vitro* (108-110), *ex vivo,* and *in vivo* (111).

CXCR4 in bone marrow myeloid cells

CXCR4/CXCL12 signaling regulates trafficking and tissue localization of human HSC to hematopoietic organs (10, 101). CXCR4 expressed on myeloid cells binds CXCL12 that is presented to the receptor by GAGs in the BM microenvironment (10, 101). Once bound to ligand, CXCR4-expressing BM myeloid cells remain tethered to GAG-bound CXCL12 in the BM. CXCR4 antagonists break this tethered interaction and release CXCR4-expressing cells into the vascular system (112, 113). Recently in an *in vivo* model of CXCR4 genetic knockout in myeloid cells, Yang et al. demonstrated that disruption of the CXCR4/CXCL12 axis in these cells led to increased neutrophil but not monocyte release from BM, and enhanced NK-mediated antitumor immune response, significantly reducing melanoma tumor growth. The observation from the GEM model that targeted deletion of CXCR4 in myeloid cells results in increased circulating neutrophils is in agreement with prior studies by Devi et al. who demonstrated that CXCR4 antagonism with the Food and Drug Administration (FDA)-approved small molecule plerixafor results in a statistically significant increase in neutrophils in peripheral blood due to the mobilization of neutrophils from a marginated pool in the lung and prevention of the return of neutrophils to bone marrow (114). Yang et al. did not observe pan leukocyte mobilization in the $CXCR4^{myeΔ/Δ}$ mice or with treatment with systemic treatment with the LY2510924 CXCR4 antagonist but did observe an increase in the total percentage of CD45+ cells in peripheral blood that were neutrophils or NK cells. Either genetic deletion of CXCR4 in myeloid cells or systemic inhibition of CXCR4 with LY2510924 increased the population of circulating neutrophils that release interleukin (IL)-18 to activate NK cells, resulting in enhanced antitumor immunity (115). Jaeger et al. suggested neutrophils may play a role as non-redundant regulatory cells ensuring terminal maturation of NK cells, which occurs not only in the BM but also in the periphery, where neutrophils can interact with NK cells (116). IL-18 can activate NK cells to release interferon γ (117). Hence, neutrophils offer a significant source of IL-18, which increases the NK cell population and their activation in myeloid CXCR4 knockout mice (115). In contrast, plerixafor treatment of healthy control or patients with severe leukopenia in myelokathexis or WHIM syndrome resulted in ~3-fold increase in neutrophils, monocytes, and lymphocytes in peripheral blood (118). In another study, treatment of three WHIM syndrome patients with plerixafor reduced myelofibrosis, panleukopenia, anemia, thrombocytopenia, and wart burden (112, 113). Moreover delivery of the CXCR4 antagonist plerixafor after HSC transplant mobilized residual recipient cells into the circulation and enhanced the proliferation and engraftment of HSCs (119, 120). Differences observed in response to either treatment with LY2510924 or myeloid CXCR4 deletion and treatment with plerixafor may be due to mechanisms by which these 3 ways of inhibiting CXCR4 take place.

CXCR4 in dendritic cells

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) in the immune system, especially for maturation and activation of T cells (121, 122). Maturation of DCs is induced by cytokines such as tumor necrosis factor-α, lipopolysaccharide, IL-1β, and CD40 ligand. These cytokines induce expression of CXCR4 in murine BM-derived DCs (BMDCs) and skin Langerhans cells (LCs) (123, 124). BMDCs produce CXCL12 that interacts with DCs in an autocrine manner to promote DC maturation and survival (123, 125). CXCR4 antagonist treatment results in reduction of mature, but not immature, BMDCs and LCs. CXCL12 enhances the proliferative response of BMDCs, which is

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suppressed by a CXCR4-antagonist. Thus, the CXCR4/CXCL12 signaling axis controls survival and maturation of DCs (126). High numbers of plasmacytoid DCs have been observed in human ovarian carcinoma, BC metastases, and lymphoma due to high levels of CXCL12 that attract DCs into the TME (127-129).

CXCR4 in T-regulatory cells

Immune cell progenitors develop and differentiate in primary lymphoid organs such as BM, thymus, and fetal liver. CXCR4/CXCL12 signaling regulates thymocyte trafficking inside the thymus and leads to migration of T cells to lymph nodes (LNs) (130, 131). In a TME, the anti-tumor immune response changes drastically during tumor progression. Cancer progression is often accompanied by escape from the host immune system. $CD4^+CD25^+$ Tregs are increased and linked to compromised immune responses in patients with multiple solid cancers. Tregs are identified through expression of CD4, CD25, FOXP3, CD127, and CD45RA. Also, members of the CD28/cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and CD39/ENTPD1 families define a highly suppressive Treg population (132). CTLA-4 and programmed death-1 (PD-1) have been proposed as critical molecules in the generation of suppressive function of Tregs (133). Tregs suppress a range of immune cells (134) and are associated with poor prognosis in solid cancers such as renal (135), ovarian (136), pancreatic (137) and liver (138). Tregs derived from cancer patients usually express a distinct profile of chemokine receptors such as CXCR4 and can migrate toward a gradient of CXCL12 produced in a TME (139, 140). Antagonists of CXCR4 reduce infiltration of Foxp3 cells into a tumor and enhance responses to anti-PD-1 therapy (141).

CXCR4 in $CD8^+$ T cells

Adaptive immunity with a capacity to develop long-lived memory T cells counters cancers and infection. Memory $CD8^+$ T cells are classified into two main subsets: central memory (CD44hi CD62Lhi) cells and effector memory (CD44hi CD62Llo) cells. Central memory cells, which preferentially home to secondary lymphoid organs, have longer life spans and greater capacity for homeostatic proliferation than do effector memory cells (142). CXCR4 promotes homeostatic self-renewal of central memory $CD8^+$ T cells and maintains the $CD8^+$ T cell memory pool (143). CXCR4/CXCL12 signaling directs leukocyte migration among blood, lymph, and tissues. In addition to its chemotactic functions, CXCR4 fine-tunes immune responses. During T cell activation by APCs, CXCR4 is recruited into the immunological synapse, where it delivers costimulatory signals (63).

CXCL12 controls recruitment of immunosuppressive cells (91, 93-95). The first evidence that CXCR4/CXCL12 regulates T-mediated immune response was more than a decade ago when Nomura et al. demonstrated that transducing CXCL12 into two murine immunogenic tumor cells (fibrosarcoma and ovarian cancer, Meth A and HM-1) increased infiltration of $CD4^+$ and $CD8^+$ T cells and anti-tumor immune responses (144). Dunussi-Joannopoulos then demonstrated that CXCL12 secreted at the tumor site by genetically modified murine tumor cells regulated the *in vivo* priming and effector phase of immune responses required for successful rejection of tumors, and supported development of long-lived tumor-specific CTL responses (145). The most updated model of melanoma demonstrated that CXCL12 has a bimodal effect on CXCR4-expressing T effector cell migration. Low CXCL12 serves as T cell chemoattractant, while high CXCL12 concentrations can repel T cells via a mechanism termed chemorepulsion or fugetaxis. This chemoattraction mechanism contributes to the physiological process of T cell migration from the thymus. Moreover, repelling

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T effector cells inside the TME may represent a mechanism by which high CXCL12-expressing tumors evade the immune system (146-148). CXCR4 activation by a recombinant CXCL12 agonist significantly reduced IL-23 and IL-12, cytokines that polarize to Th17 and Th1 states, respectively (149). These results show that CXCL12 is not only involved in attracting APCs and T cells but also in directing their anti-inflammatory properties, potentially explaining how tumors that produce high levels of CXCL12 evade the immune system.

Reflecting on the concept of fugetaxis leads one to ask how it is that recirculating T cells manage to migrate into BM or LN where there are fairly high concentrations of CXCL12. Indeed, CXCR4 expressing T cells recirculate back to the BM, and extravasate from the blood back into BM where they are retained. Here, the concentration of CXCL12 in the blood is lower than in the bone marrow, so T cells are able to migrate into the BM or LN tissue following the CXCL12 gradient. Then, a CXCL12/CXCR4 and integrin interaction keeps the T cells in the BM or LN. Egress from LN or BM does not involve a CXCL12 gradient but involves sphingosine-1-phosphate (S1P) interaction with its receptor S1PR1 (150). Since the levels of S1P are lower in the BM and LN than in the blood, CD4 and CD8+ T cells can respond to a S1P concentration gradient and be recruited into the blood. However, this whole process is quite complicated and can be offset by CD69 which mediates retention of memory CD4+T cells in the BM (150-153).

CXCR4 in B cells

During infection, B cells respond by maturing and differentiating into plasma cells. CXCR4 signaling is involved in late B cell lymphopoiesis, whereby CXCR4 activation of MAPK signaling leads to development of small pre- and immature B cells that then stop dividing and initiate Igk recombination as well as expression of the B cell receptor (154). Plasma cells secrete antibodies (Igs) that help T lymphocytes attack and kill targets. Plasma cells express CXCR4 and reside mainly in the BM and lamina propria of epithelia. As with myeloid cells, CXCR4/CXCL12 signaling holds B cells in the BM, and antagonism of this ligand-receptor interaction releases B cells into the peripheral blood. Intestinal plasma cells are IgA-producing cells and contribute to the maintenance of gut homeostasis. Plasma cells producing IgG antibodies are highly abundant, and their numbers increase in response to infection, chronic inflammation, and autoimmune diseases (155). CXCR4 directs infiltration of IgG plasma cells and accumulation into inflamed mucosa. This process mediates pathogenesis by exacerbating mucosal inflammation (156). When plasma cells become cancerous, multiple myeloma (MM) develops. CXCR4/CXCL12 is a critical regulator of MM cell migration and homing through activation of PI3K and ERK/MAPK pathways, but not p38 MAPK (157). These data imply that CXCR4 might be a target for the abrogation of metastasis by MM cells. Moreover, CXCR4, MYD88, and ARID1A somatic mutations are often found in patients with WM as outlined in chapter II of this review. CXCR4 mutations led to diminished B cell differentiation and a reduction in the mutant MYD88-mediated expression of tumor suppressors (158). Interestingly, CXCR4 and CCR7 are expressed at higher levels in stage IV than in stage 0 B cell chronic lymphocytic leukemia (B-CLL) patients (159). CXCR4 receptors play a key role in B-CLL and B cell acute lymphoblastic leukemia where they are involved in homing and in generating signals that yield these cancers resistant to ibrutinib (160-162). Recruitment of B cells into developing tumors promotes tumor progression, thus indirectly implicating a role for CXCL12 and CXCR4 due to expression of CXCR4 on B cells. Both recruitment of B-regulatory cells and antibodies produced by B cells have been associated with enhanced tumor growth and tumor progression (163-165).

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CXCR4/CXCL12 in TIME

The ability to evade immune responses constitutes a hallmark feature of cancer (166-168). In fact, persons with genetic immunodeficiency or chronic immunosuppression exhibit a higher incidence of cancer (169). For example, acquired immune deficiency syndrome patients are immune deficient and frequently develop lymphoma (170), while patients with prolonged immune suppression frequently develop cancers such as Merkel cell carcinoma where immune suppression is associated with poorer overall survival (171). The incidence of cancer is elevated in patients who are immune suppressed and in those who undergo organ transplant (169, 172, 173).

Primary resistance to immunity principally occurs due to the lack of recognition by T cells because of absence of tumor antigens or defects in antigen presentation (174, 175). The lack of immune effector cells, presence of immune suppressive cells, and polarization of immune cells in the TME play a fundamental role in shifting the balance from an immune active "hot" to immune suppressive "cold" TIME (176). In progressing cancers, tumors and associated TIME are not static, but interactions between cancer and immune/stromal cells evolve with tumor progression. As a result of this process, tumor heterogeneity is not just referred to as the heterogeneity of cancer cells/clones, but also includes heterogeneity of TME and TIME (166). CXCL12 is mainly secreted into the TME from CAFs that favor a tumor permissive microenvironment as shown in Fig 2. In BC metastases, tumor-infiltrating lymphocyte counts and PD-L1 protein expression (5 fold lower, p=0.0004 based on quantitation from full slides) were substantially lower than in primary tumors, and this was accompanied by significant downregulation of messenger ribonucleic acid (mRNA) expression of chemotactic and immune activating cytokines along with reduced antigen presentation and upregulation of immune suppressive mechanisms (recruitment of M2 macrophages) demonstrating that metastases' TIME were immunologically inert and thus "colder" than primary tumors (177).

In the BM, CXCL12 plays a critical role in the regulation of hematopoietic stem and progenitor cells (HSPC) self-renewal, retention and differentiation, creating the so-called "HSPC niche". CXCR4 overexpression in engineered therapeutic $CD8^+$ T cells improves their functions and antitumor efficacy by redirecting and favoring their engraftment to the "memory niches" in BM. The BM microenvironment drives homeostatic expansion of IL-15–dependent engineered therapeutic $CD8^+$ T cells and promotes differentiation of memory precursor-like cells with low expression of PD-1, resistance to apoptosis, and amplified capacity to generate polyfunctional cytokine-producing effector cells. Thus, following transfer to lymphoma-bearing mice, CXCR4-overexpressing engineered therapeutic $CD8^+$ T cells showed a greater capacity for effector expansion, a memory precursor-like gene signature, increased responsiveness to IL-15, a nearly 2-fold increase in CD62Lhi effector T cells, and better anti-tumor capability, with a median survival of >42 days vs 31 days for controls (178).

(Insert Figure 1)

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V. CXCR4 in cancer development and progression: tumor examples

The role of the CXCR4/CXCL12 axis is described for glioblastoma, PCa and BC, where these pathways may modulate growth, epithelial to mesenchymal transition (EMT), CSC maintenance, metastasis, and reduced survival.

Gliomas and glioma stem cells

World Health Organization grade IV glioma, defined as glioblastoma multiforme (GBM), represents the most frequent and malignant primary astrocytoma, accounting for more than 60% of all brain tumors in adults (179, 180). GBM continues to have a dismal prognosis with median survival of 14.6 months (181). GBM tumor cells commonly infiltrate healthy brain tissue (182, 183), contributing to failures of current therapies. GBMs commonly have areas of necrosis and hypoxia, which increases expression of CXCL12, MIF and CXCR4 to drive EMT through a MIF-CXCR4-AKT pathway in GBM cells (184, 185). This same signaling pathway also causes GBM cells to form blood vascular channels, a process known as vascular mimicry.

Recently, the Cancer Genome Atlas categorized GBM into four subtypes based on gene expression patterns and clinical characteristics. CXCR4 is preferentially expressed in the mesenchymal subgroup, which contains frequent mutations in the NF1, phosphatase and tensin homolog (PTEN) and TP53 tumor suppressor genes. Patients in this subgroup have significant increases in survival after aggressive treatment. CXCR4/CXCL12 signaling regulates many other aspects of brain tumor biology, including resistance to radio- and chemotherapy, migration of cancer cells through the brain, angiogenesis, and recruitment of vascular progenitor cells (186, 187). These properties suggest that CXCR4 antagonists may help control this disease. CXCR4 expression varies significantly among different subtypes of GBM and in glioblastoma stem-like cells (GSCs) (188-192). Low expression of CXCR4 due to processes such as promoter hypermethylation might predict favorable overall survival.

GBM cells tend to migrate along blood vessels and white matter structures, both of which express high levels of CXCL12 in concert with other growth factors such as epidermal growth factor and platelet-derived growth factor. Migrating GBM cells show a reduced propensity to undergo apoptosis and proliferation and exhibit upregulation of CXCR4 (193, 194). Since the highest levels of CXCR4 expression are associated with stemness, the poor prognosis of GBM may reflect increased CXCR4-positive GSCs. In addition, expression of CXCR4 increases in recurrent tumors compared with matched primary GBMs (182).

Pre-clinical studies support addition of CXCR4 inhibitors to therapy in GBM. Knockdown or pharmacological inhibition of CXCR4 reduced GBM cell migration toward brain-derived endothelial cell monolayers (195), and knockdown of CXCR4 in GBM cells prior to intracranial injection in mice reduced tumor growth and perivascular invasion with increased survival. CXCR4 knockdown resulted in reduced local invasiveness (193). Similarly, these strategies increased apoptosis of cells treated with radiotherapy, prolonging median survival of mice with established GBM (196). Inhibition of CXCR4/CXCL12 signaling reduced the viability and number of GSCs (197) and recruitment of glioma-associated macrophage/microglial cells (198). In addition, the brain-penetrating CXCR4 antagonist PRX77561 inhibited growth of established tumors, local cell recruitment and inflammation in GBM. PRX77561 also induced differentiation of glioma cells (199). CXCR4 inhibitors may improve efficacy of anti-angiogenic therapy since CXCL12-CXCR4 signaling contributes to resistance to the anti-VEGF

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agent, bevacizumab (199). Combining PRX77561 with anti-angiogenic agents further improved control of tumor growth and overall survival (199). Similarly, the selective CXCR4 antagonist POL5551 VEGF reduced glioma growth and dissemination by inhibiting tumor cell migration and local invasion (193). POL5551, similarly to PRX177561 or plerixafor (200) dose-dependently reduced hypoxia- and CXCL12-mediated migration. Combining POL5551 with a VEGF-blocking antibody significantly reduced invasion of GBM cells and vascular density to greater extent than single agents (193). Analysis of GSC and more malignant GBM cell content suggested that inhibition of CXCR4-regulated cell recruitment (tumoral stem and immune cells) accounted for improved efficacy of antiangiogenic therapy. CXCR4 antagonists also may improve treatment of brain metastases, particularly for CXCR4-positive primary tumors.

CXCR4/CXCL12 signaling and prostate cancer

PCa is the most common cancer in men and the second leading cause of cancer-related death among men in developed countries (201). The most common cause of mortality is not the primary tumor growth but rather its spread to other organs, predominantly bone (202). Understanding molecular mechanisms of metastasis is crucial in developing new therapy strategies (203). Recent advances in cancer biology have indicated the critical role that CXCR4/CXCL12 play in CSC renewal and metastasis of various cancers, including PCa (6, 204).

Some data report that PCa expresses 35x higher levels of CXCR4 than non-malignant prostate tissue, suggesting that CXCR4 expression could be a diagnostic biomarker for PCa (205, 206). CXCR4 expression correlates significantly with LN or bone metastasis (207). Bone metastatic PCa cells express higher levels of CXCR4 than primary tumors, suggesting that CXCR4 expression may be a useful prognostic marker for metastasis in PCa (208, 209). However, the clinical relevance of CXCR4 in PCa remains controversial, and its association with clinicopathological features remains inconclusive due to relatively small sample sizes in studies. A meta-analysis by Lee et al. (210) reported that increased CXCR4 expression in PCa correlates with presence of metastasis but not tumor stage (of the TNM classification of malignant tumors, UICC TNM Project) of PCa (211). A meta-analysis by Chen, on the other hand, found significantly higher amounts of CXCR4 protein in T3-4 than in T1-2 stages of PCa, as well as being significantly associated with the presence of LN and bone metastasis (207). Darash-Yahana et al. reported that CXCR4 inhibitory antibodies blocked CXCR4-dependent vascularization and growth of previously established tumors (212). Moreover, anti-CXCR4 antibodies and peptide analogs disrupt tumor-stromal interactions and reduce intraosseous growth of established prostate cancer cells in mouse xenograft models of PCa (213-217). Taken together, inhibition of CXCR4/CXCL12 signaling could be a potentially beneficial addition to treatment in PCa.

Androgen deprivation treatment (ADT) is the most effective intervention for advanced and metastatic PCa. Although approximately 80% patients initially respond to ADT, incurable castration-resistant PCa develops almost invariably (207, 218, 219). Importantly, androgen receptors continue to be critical for PCa growth and progression after ADT. It has been demonstrated that several putative consensus-binding sites for the oncogenic erythroblast transformation specific-related gene

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(ERG) transcription factor are present in the promoter region of CXCR4; thus androgen-dependent regulation of ERG could induce CXCR4 expression in PCa cells. Findings also link TMPRSS2-ERG translocations and enhanced metastasis of tumor cells through CXCR4 function in PCa cells (220). Androgen-independent PCa cells commonly lose the PTEN tumor suppressor, resulting in metastasis (221, 222). The pathways of CXCR4 and PTEN converge, leading to promotion and regulation of tumorigenesis, respectively. Loss of PTEN may permit CXCR4 to shift PCa to advanced disease (213). Reconstitution of PTEN in PTEN-negative PC3 cells reversed EMT and inhibited CXCR4-mediated migration and proliferation in PC3 cells, suggesting that loss of PTEN permits CXCR4-mediated functions in PCa cells (213). These data may indicate that targeting CXCR4 may improve treatment for PCa patients with resistance to androgen deprivation.

CXCR4/CXCL12 signaling in breast cancer

BC is the most common malignancy among females, diagnosed in nearly 1.7 million women and causing more than 40,000 deaths in the United States of America (USA) alone in 2019 (201). CXCR4 is significantly overexpressed in BC, not only increasing metastasis but also promoting survival and proliferation of BC cells. The first evidence for this was the much smaller tumor mass resulting from subcutaneous inoculation in immunodeficient mice of low CXCR4-expressing MCF-7 BC cells than high CXCR4-expressing MDA-MB-231 cells. In syngeneic, immunocompetent mice, implanted tumor cells with RNAi-downregulated expression of CXCR4 grew more slowly than cells with upregulated or normal levels of CXCR4 (12, 223). CXCR4 facilitates growth of primary BC through mechanisms including angiogenesis as discussed, activation of numerous downstream signaling pathways (including PI3K/AKT, Src/ERK1-2, NF-κB, STAT3, Notch, Wnt, and sonic hedgehog) (224), and recruitment of immunosuppressive immune cells (225).

Estrogen regulates tumorigenesis, metastasis, and drug resistance in estrogen receptor (ER)-positive BC, the most common subtype. Several studies have reported the relationship between hormone-dependent BC cell proliferation and CXCR4 signaling. CXCL12 was shown to be a target of ER action and to mediate the mitogenic effects of estradiol in ovarian and BC cells (226). Co-overexpression of ER coactivators and Her2/neu indicates poor prognosis: coactivators increase CXCL12 expression while Her2/neu stabilizes CXCR4 (227). 17beta-estradiol promotes CXCL12-mediated transactivation of the epidermal growth factor receptor (EGFR) transactivation, increasing proliferation of BC cells (228). CXCR4 activation transduces proliferative signals from ER to EGFR, whose inhibition reverts BC

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cell proliferation induced by activation of multiple receptors (228). CXCR4/CXCL12 and ERalpha/ERbeta form an autocrine signaling loop that dictates ER-dependent gene expression and growth of BC cells (229). Expression of CXCL12 in invasive BC correlates with ER status and patient prognosis (230). CXCR4 also mediates estrogen-independent tumorigenesis, metastasis, and resistance to endocrine therapy in human BC, although the study did not address overall survival (231). Thus, CXCR4 is a rational therapeutic target for ER-positive, estrogen-independent BC. More recently, it was demonstrated that estrogen stimulated CXCR4 transcription via ER-binding sites at the CXCR4 promoter, thus enhancing mediated cellular growth (232).

Estrogen may also promote progression of ER-negative BC, by stimulating CAFs to secrete CXCL12, which can recruit MDSCs to the TME to exert tumor-promoting effects. ER-negative BC cells xenografted into ovariectomized mice, followed by continuous injection of estrogen, demonstrated high levels of CXCL12 and tumor-infiltrating MDSCs. Blocking CXCR4 with plerixafor prevented recruitment of MDSCs (233).

CXCR4 promotes BC metastasis to organs, bone, liver, and lung, with high levels of CXCL12 (217). The interaction between CXCR4 and CXCL12 makes BC cells move out of the circulation and into organs with high amounts of chemokines, thus forming metastases. CXCR4-high, but not CXCR4-low, MCF-7 human BC cells spontaneously metastasized to lung in severe combined immunodeficient mice (234). Bone metastatic BC cells in patients have increased CXCR4 (235). CXCL12-producing adventitial reticular cells and other stromal cell types in bone and BM produce CXCL12, facilitating bone metastasis (236). Similarly, stromal cells in liver and lungs produce large amounts of CXCL12 to recruit BC cells and support metastases (237). Giving a peptide CXCR4 antagonist (CTCE-9908) to athymic mice prior to inoculation of GFP-MDA-MB-231 cells, while not reducing the incidence of metastasis, did decrease the metastatic burden (size of metastases) in all organs examined (lungs, bone, heart, liver, kidneys, pancreas and spleen) (235, 238).

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A recent report found an association between tumoral CXCR4 expression in patients with locally advanced BC and increased metastases and rapid tumor progression. Moreover, high CXCR4 expression identified a group of patients with BM disseminated tumor cells at high risk for metastasis and death (239). POL5551, another peptide CXCR4 antagonist, *in vitro* had no direct effect on tumor cell viability, but reduced migration of BC cells. In two orthotopic models of triple-negative BC, POL5551 administered after formation of tumor xenografts had little inhibitory effect on primary tumor growth but significantly reduced distant metastasis. In mice after resection of the primary tumor, combination of POL5551 with the microtubule inhibitor eribulin additively reduced metastasis, and overall survival was longer than with single-agent eribulin (239).

About 20–30% of BC patients with metastatic disease develop brain metastases (240-242). Disruption of the blood–brain barrier (BBB) is critical to the development of metastases (243, 244). An *in vitro* study demonstrated that CXCL12 introduces BC cells into the brain by increasing vascular permeability. Several CXCR4 cofactors are involved in BC cell invasion. The CXCR4/CXCL12-mediated activation of PI3K/AKT and phosphorylation of focal adhesion kinase and Forkhead transcription factor FKHR-L1 were necessary for BC cell migration through the BBB (245).

BC cells that metastasized to the lungs exhibited high CXCR4 expression compared with their parental cells, supporting the role of CXCR4 in organ directed-metastasis and invasion of BC. Neutralizing antibodies to CXCR4 markedly inhibited metastases of BC to LNs and lung (246). Local LN involvement in BC patients has been widely investigated. Kato et al. analysed CXCR4 expression in 79 surgically resected invasive ductal carcinomas and found that patients with high CXCR4 levels had more extensive metastasis to LNs than those with low levels (247). A recent meta-analysis (13) of 13 studies (3,865 participants) across USA, Canada, China, Japan, Korea, and Taiwan showed CXCR4 expression to be significantly associated with both LN status and distant metastasis and indicated poor overall and disease-free survival. Xu et al. analyzed 15 studies (3,104 patients) from USA, Canada, Germany, France, China, and Japan, and found results consistent with those of Zhang, with both overall and disease-free survival significantly lower in BC patients with high CXCR4 expression

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than in those with low expression (248). CXCR4 thus appears to be an efficient prognostic factor for BC and promising target for therapy.

VI. Imaging CXCR4 – bridge to pharmacodynamics *in vivo*

Given the aforementioned pivotal involvement of CXCR4/CXCL12 in various aspects of cancer, development of molecular imaging tools for CXCR4 offers the potential to identify patients with CXCR4-positive tumors for therapy and quantify pharmacodynamics of compounds. Excellent reviews summarizing the emergence of diverse CXCR4-targeted radiolabeled, fluorescently labeled and bimodal ("hybrid") probes for *in vivo* imaging have been published (6, 249-251). We present only the most promising classes of CXCR4-targeted imaging probes with potential clinical applications: small-molecule ligands based on plerixafor; CXCL12-derived disulfide-bridged 14 amino acid peptides based on T140; and FC-131 based cyclic pentapeptides. All three classes of imaging agents were adapted from compounds initially designed as potent inhibitors of human immunodeficiency virus (HIV) infection and subsequently investigated as therapeutic agents in oncology.

Bicylam tracers and small molecules

Plerixafor and the structurally related AMD3465 allow direct complexation of some metal ions, a feature exploited for radiolabeling with ^{64}Cu (252, 253), ^{68}Ga, or ^{99m}Tc (254-256). Both mouse and first-in-human studies with [^{64}Cu]-plerixafor showed high levels of radiotracer accumulation in liver and BM due to endogenous expression of CXCR4 in these organs. Normal accumulation in these organs limit detection of primary and metastatic tumors in these sites and produces unwanted radiation doses.

T-140 based tracers

As described in "Targeting CXCR4 therapeutically" below, research in the 1990s yielded polyphemusin II analogs with anti-HIV activity and CXCR4 binding affinity: T22, T140, and then more stable analogs with improved CXCR4 affinity and anti-HIV potency e.g. FB-TN14003 and Ac-TZ14011 (257). These second generation T140 analogs represent the targeting agent for CXCR4-targeted imaging probes (nuclear and/or fluorescent) (255, 258-265). Despite substantial differences in binding affinity (half maximal inhibitory concentration values in the range of 2-200 nM), preclinical evaluation of diverse T140-based imaging probes invariably showed reasonably specific uptake in CXCR4-expressing cancer cells *in vitro* and *in vivo* in mouse xenograft models. One feature, however, that is common to all T140-based molecular imaging agents developed so far, is their high accumulation in non-target tissues; this includes strong binding to blood cells and high uptake in liver, kidney, intestines and spleen. These results suggest a contribution of CXCR4-mediated tracer uptake to background activity accumulation, particularly in the liver, and is in line with results with the different radiolabeled AMD analogs. Thus, although having undisputed potential as tools for *in vitro* characterization and quantification of CXCR4 expression, the *in vivo* characteristics of T140-based molecular imaging agents generally limit clinical translation. The only compound of this class

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that has been evaluated in a clinical setting so far is [^{68}Ga]-1,4,7-triazacyclononane-N,N',N''-triacetic acid-N-terminal 4-fluoro-benzoyl in glioma (266) (see matching Bedside review for more details).

FC-131 based cyclic pentapeptides

Based on the insights and experience gained from development and optimization of T140 ligands, further downsizing the T140 pharmacophore to the essential amino acids led to discovery of the small cyclic pentapeptide FC-131 as a fully stable and highly potent CXCR4 antagonist (267). A proof-of-concept study using radio-iodinated FC-131 in a human small cell lung cancer mouse xenograft model demonstrated the general applicability of this class of CXCR4 antagonists for sensitive and specific *in vivo* detection of receptor expression (268). Further research to improve biodistribution of this class of peptide imaging agents for CXCR4 produced [^{68}Ga]-Pentixafor. [^{68}Ga]-Pentixafor (269) shows high affinity and selectivity for human CXCR4, rapid renal rather than hepatobiliary excretion, and very low non-specific background accumulation, thus allowing sensitive and high-contrast imaging of CXCR4-expressing tissues *in vivo* using positron emission tomography (PET). After its first successful application in patients with lymphoma (270), [^{68}Ga]-Pentixafor-PET has now found widespread clinical applications in various hematological and solid cancers (271-273). Data from PET imaging studies in patients with adrenocortical carcinoma showed good correlations with mRNA and protein levels of CXCR4 in tumors and revealed that imaging with [^{68}Ga]-Pentixafor could detect heterogeneous expression of CXCR4 among different metastases. Derivatives of Pentixafor also have been developed for targeted radiotherapy of CXCR4-positive tumors (274, 275). In two patient-derived xenograft models of leukemia with different levels of CXCR4 expression, CXCR4-directed radiotherapy with [^{177}Lu]-Pentixather efficiently reduced leukemic burden in spleen and bone marrow. This radiotherapy approach is to date being further explored in the clinic (see Bedside review).

VII. Targeting CXCR4 therapeutically

Multiple companies have and continue to develop approaches to target CXCR4, and hundreds of clinical trials have investigated safety profiles and efficacy in hematologic and solid cancers and other diseases. However, plerixafor remains the first and so far only approved CXCR4 antagonist. In 2008, the FDA approved plerixafor in combination with granulocyte-colony stimulating factor to mobilize HSCs to the peripheral blood for collection and subsequent autologous transplantation in adult patients with non-Hodgkin's lymphoma and MM (276).

Preclinical antitumor therapies using either plerixafor (277) or structurally related small molecule CXCR4 antagonists such as the plerixafor analog AMD3465 (278, 279) reduced tumor burden by ~50% in mouse models of a variety of human hematological as well as solid cancers, primarily by effectively preventing distant organ metastasis (280). Plerixafor sensitized BC cells to radiotherapy *in vitro* and *in vivo* by increasing cell-cycle arrest and apoptosis (281) Low-dose plerixafor also sensitized cells to low-dose taxol, substantially reducing colony formation and proliferation (282).

While plerixafor remains the most studied CXCR4 antagonist, the drug lacks selectivity and also has a narrow safety profile. Plerixafor acts as allosteric agonist of ACKR3/CXCR7, the second receptor for CXCL12 (283). In a Phase 1/2 trial in 2004, 8 of 40 enrolled HIV patients who received plerixafor from 2.5 to 160 µg/kg/h for 10 days by intravenous infusion discontinued treatment due to adverse

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events including 2 cases of ventricular ectopy, thrombocytopenia and paresthesia (284). However, low-dose (4-8% of the dose used for mobilization of HSCs) plerixafor administered twice daily by self-administered injection has been well-tolerated in clinical trials for WHIM syndrome. Results from trials for patients with WHIM syndrome show declines in infections, stabilization of HPV-associated oropharyngeal squamous-cell carcinoma, and improved quality of life. Adverse events were mainly infections attributable to the underlying immunodeficiency (285, 286).

Several more clinically viable non-cyclam small molecule antagonists with high potency, improved selectivity, and better tolerability profiles were identified over the past two decades. When known, we highlight the current status of development for each agent in preclinical and clinical studies. Currently, the most advanced compound is the tetrahydroquinoline compound mavorixafor (X4P-001, X4 Pharmaceuticals, formerly AMD11070/AMD070 Genzyme Corp) (287). A recent Phase 2 clinical trial with mavorixafor in patients with WHIM syndrome showed increases in total white blood cell counts, neutrophils, lymphocytes (288). Mavorixafor also reduced frequencies of infections and warts. The company website for X4 Pharmaceuticals lists ongoing studies with additional CXCR4 antagonists X4P-002 and X4P-003, in preclinical models for brain cancers and primary immunodeficiencies, respectively. X4 Pharmaceuticals also previously studied X4-136 in ovarian cancer and melanoma models (289, 290). The structures of these compounds have not been published. More recently, Emory University and Bristol-Myers Squibb disclosed newer generations of tetrahydroisoquinoline-containing CXCR4 antagonists with improved *in vitro* absorption, distribution, metabolism, excretion, and toxicity properties, such as TIQ-15 and analogues (291, 292). The compounds were also optimized for higher selectivity against a panel of proteins known to interact with many nitrogen-based chemotypes, such as phenethyl amines, pyridines, piperazines, and piperidines similar to those found in the tetrahydroisoquinoline class (293). GMI-1359 (GlycoMimetics) is a rationally-designed small molecule that simultaneously targets both E-selectin and CXCR4, discovered by applying a platform that produces mimetics of naturally-occurring carbohydrates . The structure of GMI-1359 is undisclosed. Kureha Chemical Industries identified another promising candidate through a large screening campaign (294). The bis-imidazole lead compound KRH-1639 was further optimized; however, the Phase 1 candidate KRH-3955 did not enter clinical trials (295). There have been other promising small molecule CXCR4 antagonists that have not advanced beyond late preclinical or early clinical stages. A detailed review about small molecule CXCR4 antagonists was published by Peng et al. in 2018 (296).

In parallel with these small molecule approaches, other companies and research groups worked on peptide-based CXCR4 antagonists. Polyphemusin II is a self-defense β-sheet-like peptide isolated from the hemocytes of horseshoe crab species *Tachypleus tridentatus* and *Limulus polyphemus*. In 1992, Masuda et al. synthesized the first polyphemusin II analogs and examined antiviral activity against HIV type 1 *in vitro* (297). By binding to CXCR4, low concentrations of the 18-residue peptide T22 inhibited infection of human T cells with CXCR4-tropic strains of HIV type 1. Further optimization led to BL-8040 (BKT-140, 4F-benzoyl-TN14003, or T-140 as described in the section for imaging agents), a 14-mer peptide that binds CXCR4 with greater affinity than T22 and better biostability (298). BL-8040 is currently in clinical trials for acute myeloid leukemia and pancreatic cancer. The maximal tolerated dose of BL-8040 in man is 1.5 mg/kg subcutaneously daily.

Protein epitope mimetic technology applied to the β-sheet-like structure of polyphemusin II yielded POL3026 (Polyphor) (299), followed by POL5551 and the clinical candidate POL6326 (balixafortide)

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(300). In a mouse model of triple-negative BC, POL5551 nearly completely prevented distant metastases while having minimal effect of growth of orthotopic mouse 4T1 tumors (239). A Phase 1 trial combining balixafortide with eribulin in patients with metastatic BC showed objective responses in 38% and clinical benefit in 67% of heavily pre-treated patients (301). Based on these data, the FDA granted fast track designation for balixafortide plus eribulin for patients with metastatic Her-negative BC and prior treatment with at least 2 chemotherapy drugs in the metastatic setting. A Phase 3 trial now is underway. Pharmacologic properties of balixafortide that enabled it to progress to a Phase 3 trial remain uncertain but may relate to modest binding affinity and half-life combined with greater selectivity for CXCR4 (302). Both balixafortide and POL5551 also induce efficient stem cell mobilization (300, 303).

Other peptide inhibitors of CXCR4 include FC131, a cyclic pentapeptide derived from T22 and LY2510924 (Eli Lilly) (304-306). LY2510924 has very high *in vitro* potency but a low maximum tolerated dose (~20 mg subcutaneously daily) in people (307). This compound remains in clinical trials for solid tumors such as pancreatic cancer (308). E5, a synthetic peptide (Chinese Academy of Medical Sciences) inhibits migration and adhesion of leukemia cells *in vitro*, modestly extended survival when given as monotherapy in mice with leukemia, and significantly inhibited tumor growth when combined with paclitaxel or cyclophosphamide in a murine BC model (309, 310).

The design of other peptidic antagonists has been inspired by natural ligands of CXCR4, such as a three-residue reverse order segment identified in CXCL12 that was similar to the inhibitory chemokine secreted by human herpes virus 8 vMIP-II. Further optimization delivered three peptides (R, S, and I) that nearly completed eliminated experimental lung metastases in an immunocompetent mouse model of melanoma (311). TCM Biotech is exploring PTX-9908 (CTCE-9908), a CXCL12 N-terminus–derived peptide that is a dimerized sequence of CXCL12 amino acids 1–8 (312). A Phase 1/2 study in hepatocellular carcinoma is starting.

Therapeutic antibodies represent a third large pool of CXCR4 antagonists. Several candidates reached clinical trials, e.g. PF-06747143 (Pfizer) (313), ulocuplumab (BMS-936564/MDX1338 from BMS/Medarex) (314), LY2624587 (Eli Lilly) (315), or the nanobody ALX-0651 (Ablynx) (316). However, the Phase 2 trial of ulocuplumab in WM (NCT03225716) is the only active trial with a CXCR4-targeted antibody. Most of these antibodies have been described as exhibiting antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, possible advantages for elimination of tumor cells. Others, such as ulocuplumab induce apoptosis through a reactive oxygen species-dependent pathway. However, blocking CXCR4 on various subsets of leukocytes may limit anti-tumor immune responses. Moreover, permanent CXCR4 blockade by high affinity/slow off-rate antibodies may impact normal, regular functions of the organism such as stem cell mobilization or tissue regeneration. For example, LY2624587 induces receptor internalization and down-regulation of CXCR4 density on the cell surface, which may impair trafficking of CXCR4-positive immune cells. While generally safe, anti-CXCR4 antibodies may produce short-term myelosuppression or abnormally elevated leukocyte counts (317, 318). More recent work shows that AD-114, a human single domain antibody (so-called i-body) binds CXCR4 without mobilizing hematopoietic stem cells (319). Based on pre-clinical work showing reductions in collagen synthesis by fibroblasts from patients with idiopathic pulmonary fibrosis and fibrosis in a mouse model, AD-114 received FDA Orphan Drug Designation for idiopathic pulmonary fibrosis (319).

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In summary, several CXCR4 antagonists have progressed to clinic trials, raising hope that a second CXCR4 antagonist may become available to treat patients over a decade after FDA approval of plerixafor. These agents are described in more detail in the linked Bedside (clinical) review in this issue.

Conclusion

In the past 10 years, numerous investigations have been conducted on the role of the CXCR4/CXCL12 signaling pathway in solid tumors and hematologic malignancies. CXCR4 antagonists could be promising agents for treatment of local and metastatic disease because of direct effects against cancer cells and broader effects in TMEs. However, CXCR4-targeted therapy also may disrupt essential functions of this receptor in development and normal physiology. Since CXCR4 regulates trafficking and functions of both immunosuppressive and effector immune cells, CXCR4-targeted therapy has the potential to dampen host immune responses and decrease anticancer effects. Balancing these effects will require ongoing investigations in immunocompetent animal models of cancer, informed by live-animal imaging studies of pharmacodynamics of CXCR4-targeted therapy. Reciprocal feedback from clinical trials and animal models will help fulfill the promise of CXCR4-targeted therapy to improve outcomes for patients with cancer.

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Figure Legends

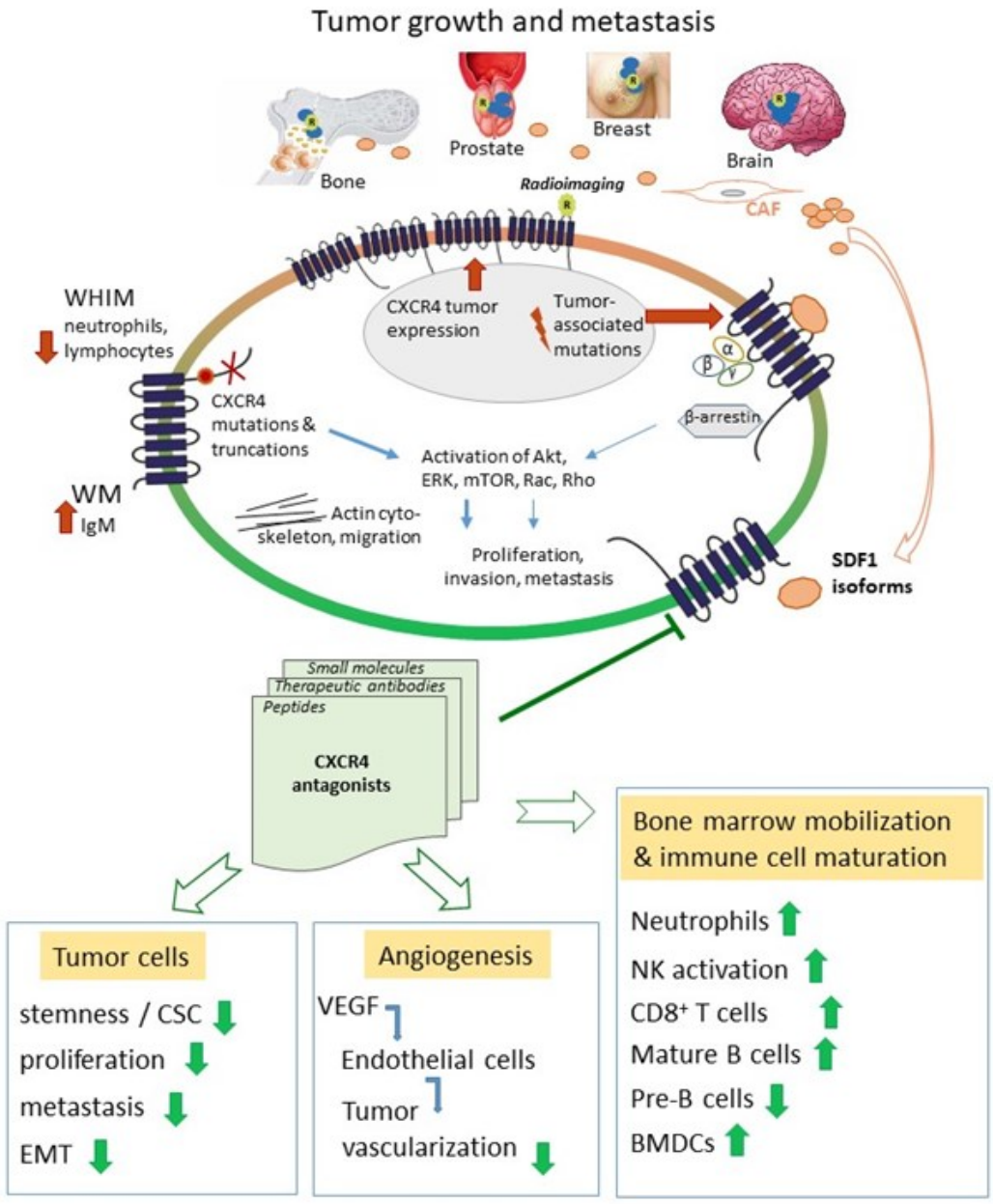

Figure 1. CXCR4 signaling promotes tumor growth and organ-specific metastasis. CXCR4 signaling promotes tumor growth in primary tumors from sites including prostate, breast, and brain. Various isoforms of CXCL12 produced by carcinoma-associated fibroblasts in a primary tumor environment

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activates CXCr4 signaling through G protein and β-arrestin-dependent pathways. CXCR4 activates Akt, ERK, mTOR, Rac, Rho, and other effector molecules to drive survival, proliferation, and actin assesmbly for migration and invasion. Truncating mutations in CXCR4 as present in WHIM syndrome increase signaling through the receptor and reduced circulating neutrophils and lymphocytes. CXCL12-CXCR4 signaling promotes metastasis to sites including lung, liver, brain, and bone. Inhibitors of CXCR4 reverse aggressive features of cancer cells, angiogenesis, and abnormalities in circulating leukocytes. Targeted imaging agents can detect expression of CXCR4, potentially informing selection of patients and measurements of treatment efficacy.

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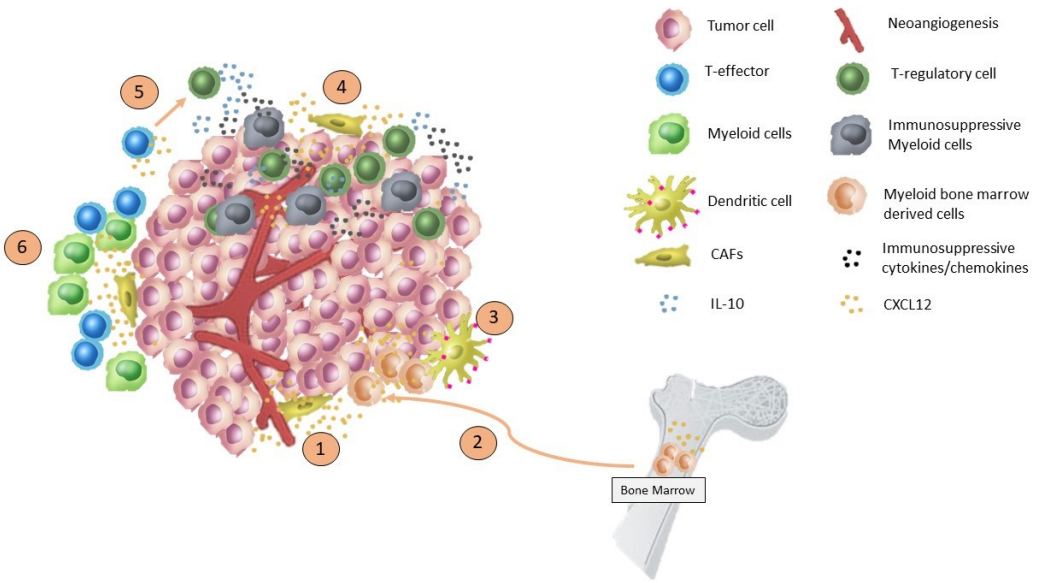

Figure 2. CXCR4 in the tumor microenvironment. CAF-produced CXCL12 acts on different TME cells and regulates the recruitment of immune cells. 1. CXCR4/CXCL12 axis stimulates endothelial cells promoting neovascularization, tumor growth and metastatic progression. 2. CXCL12/CXCR4 axis regulates trafficking and tissue localization of human HSC in the BM. 3. CXCR4/CXCL12 axis recruits BM derived myeloid cells promoting DC maturation and survival. 4-6. CXCL12 recruits immunosuppressive cells and excludes effector cells designing a “colder” TME that impair immunotherapy response. CXCL12 also redirects the polarization of effector Th1 cells into $CD4^{+}CD25^{-}Foxp3^{-}IL\ 10^{high}$ regulatory T cells.

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Authorship

All authors read and approved the final manuscript.

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Conflict of Interest Disclosure

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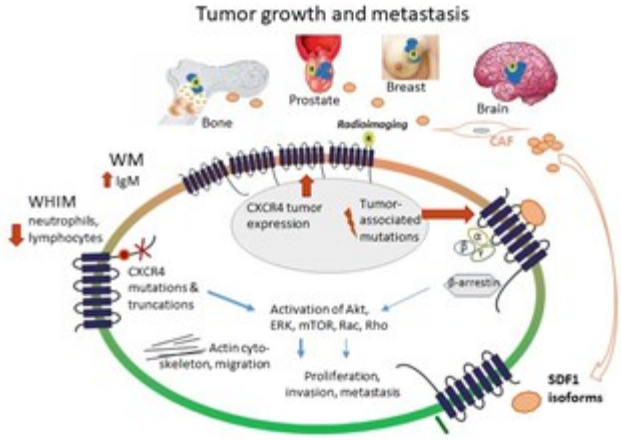

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