CXCL12 Signaling in the Tumor Microenvironment

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Abstract

Tumor microenvironment (TME) is the local environment of tumor, composed of tumor cells and blood vessels, extracellular matrix (ECM), immune cells, and metabolic and signaling molecules. Chemokines and their receptors play a fundamental role in the crosstalk between tumor cells and TME, regulating tumor-related angiogenesis, specific leukocyte infiltration, and activation of the immune response and directly influencing tumor cell growth, invasion, and cancer progression. The chemokine CXCL12 is a homeostatic chemokine that regulates physiological and pathological process such as inflammation, cell proliferation, and specific migration. CXCL12 activates CXCR4 and CXCR7 chemokine receptors, and the entire axis has been shown to be dysregulated in more than 20 different tumors. CXCL12 binding to CXCR4 triggers multiple signal transduction pathways that regulate intracellular calcium flux, chemotaxis, transcription, and cell survival. CXCR7 binds with high-affinity CXCL12 and with lower-affinity CXCL11, which binds also CXCR3. Although CXCR7 acts as a CXCL12 scavenger through ligand internalization and degradation, it transduces the signal mainly through β-arrestin with a pivotal role in endothelial and neural cells. Recent studies demonstrate that TME rich in CXCL12 leads to resistance to immune checkpoint inhibitors (ICI) therapy and that CXCL12 axis inhibitors sensitize resistant tumors to ICI effect. Thus targeting the CXCL12-mediated axis may control tumor and tumor microenvironment exerting an antitumor dual action. Herein CXCL12 physiology, role in cancer biology and in composite TME, prognostic role, and the relative inhibitors are addressed.

Keywords

Cancer · Chemokines · Tumor microenvironment · Chemokine receptors · CXCL12 · CXCR4 · CXCR7 · CXCR4-CXCL12-CXCR7 axis · Metastasis · Tumor progression · Angiogenesis · Immunotherapy · Checkpoint inhibitors · CXCL12 antagonist · Antitumor immune response

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5.1 Introduction

Tumor microenvironment (TME) is the local environment of tumorigenesis and tumor growth, a dynamic space that determines cancer fate. It is composed of tumor and surrounding cells such as bone marrow-derived dendritic cells, mesenchymal stem/stromal cells, fibroblasts, pericytes, and immune cells [1, 2]. TME components support tumors, angiogenesis, and growth and represent the site of critical interactions mostly promoting tumor initiation, resistance, metastasis, and recurrence [3]. Chemokines, or chemotactic cytokines, are small chemoattractant secreted molecules regulating directed cell migration, proliferation, and survival with a role in physiological and pathological processes including cancer [4]. Structurally chemokines are classified on the basis of a specific cysteine motif at the N-terminal into CC, CXC, XC, and CX3C subfamilies: the CC chemokines have two adjacent conserved cysteine residues, XC chemokines have only one N-terminal cysteine residue, whereas CXC and CX_3C chemokines have one or three other amino acids in between their conserved N-terminal cysteine residue; they accordingly bind to their respective chemokine receptor subfamilies CCR, CXCR, XCR, and CX3CR [5] (Fig. 5.1). Chemokines can be divided into inflammatory and homeostatic based on their prominent functions: mainly inflammatory chemokines, that are expressed during inflammatory processes, are CXCL1, CXCL2, CXCL3, CXCL5, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, and CXCL14. On the other hand, homeostatic chemokines such as CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12, and CXCL13 are constitutively expressed and regulate homeostatic leukocyte trafficking [6]. The chemokine system is complex with several chemokines that bind the same receptor with similar affinities and receptors that bind the same ligand. Nevertheless, the system is not redundant as cells dynamically express receptors at the cell membrane simultaneously or during different stages of their life [7]. Chemokines are expressed by neurons, glia, and neural progenitor cells, the major cell types of the nervous system, and are induced by neuroinflammatory responses [8]. Combined with the normal and/or pathological nervous system expression of chemokine receptors, chemokines potentially initiate a cascade of events leading to neuroinflammation [9]. The chemokines generated in association with neuroinflammation are crucial for the migration of leukocytes into inflamed neural tissue, just as in other parts of the body [10]. Chemokines also regulate the migration of mesenchymal stem cells (MSCs) to tumor sites where they promote tumor development and differentiate to tumor-promoting cancer-associated fibroblasts (CAFs). Moreover, chemokines expressed in metastatic sites are key players in attracting tumor cells that express the corresponding receptors [11].

Twenty-three human chemokine receptors and about 50 chemokines [12] have been identified. Chemokine receptors are G protein-coupled/seven-transmembrane domain receptors. A new family of chemokine receptors named atypical chemokine receptors (ACKR) has recently emerged as important regulators of chemokine functions. The ACKRs are unable to trigger the canonical G protein-mediated signaling. Four chemokine receptors belong to ACKR: 1. ACKR1, previously called Duffy antigen receptor for chemokines (DARC); 2. ACKR2, also known as D6; 3. ACKR3, also called CXC-chemokine receptor 7 (CXCR7); and 4. ACKR4, previously called CC chemokine receptor-like 1 (CCRL1) [13]. In the TME chemokines can be expressed by tumor, immune, and stromal cells such as leukocytes, fibroblasts, pericytes, and endothelial cells crucial for tumor vascularization and metastatic spread [14].

CXCL12 is a homeostatic chemokine that binds CXCR4 and CXCR7 receptors and physiologically functions in hematopoiesis, leucocyte trafficking, cardiogenesis, and neurogenesis. CXCR4 (352 amino acids, 48 kDa) [15–17] is a G protein-coupled chemokine receptor encoded on chromosome 2.1 in human. CXCL12 binding to CXCR4 triggers multiple signal transduction pathways that regulate intracellular calcium flux, chemotaxis, transcription, and cell survival [18]. CXCR7 binds with high-affinity CXCL12 and with lower-

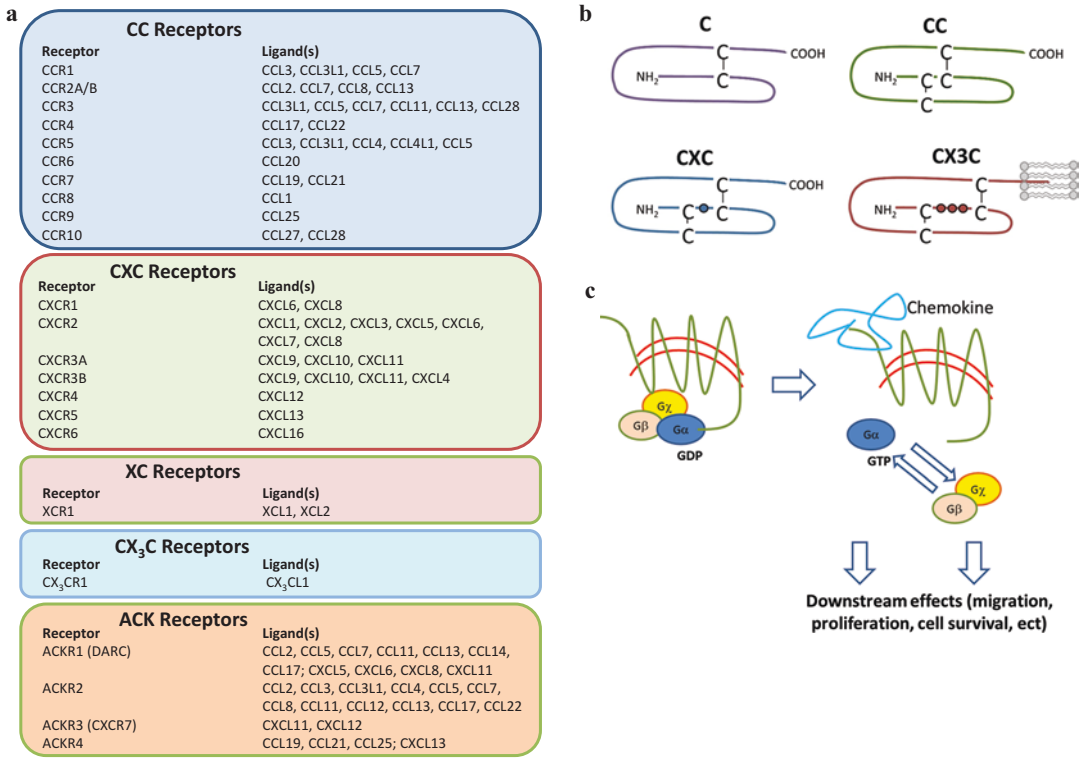

Fig. 5.1 The chemokine and chemokine receptor superfamily. (**a**) The chemokine receptors' subfamilies and ligands. (**b**) Schematic representation of chemokine structure according to cysteine residue position. (**c**) Schematic representation of chemokine and chemokine receptor interactions at cell surface. Heterotrimeric G proteins associate with the intracellular domains of chemokine receptors. Specific ligand-receptor interactions lead to triggering of the receptor and dissociation of the heterotrimeric G protein complex into the Gα and Gβγ subunits. These second messengers then play a critical role in activation of the various signal transduction cascades, leading to migration and other responses driven by chemokines. (Panel (b) adapted from *de Munnik S.* et al. *Modulation of cellular signaling by herpesvirus-encoded G protein-coupled receptors Front. Pharmacol., 2015*)

affinity CXCL11, which binds also CXCR3 [19]. Although CXCR7 acts as a CXCL12 scavenger through ligand internalization and degradation [20], it transduces the signal mainly through β-arrestin with a pivotal role in endothelial and neural cells.

5.2 The CXCR4-CXCL12-CXCR7 Axis

5.2.1 CXCL12

Initially known as stromal cell-derived factor-1α (SDF-1 α) or pre B-cell growth-stimulating factor (PBSF), CXCL12 is the most studied member of the chemokine family [21, 22]. CXCL12 gene in human is located on chromosome 10 (10q11.21) and recognizes seven isoforms deriving from alternative gene splicing (α, β, γ, δ, ε, θ) with α and β being the most studied [23] and three (CXCL12α to γ) in mice [24, 25] (Fig. 5.2). CXCL12α (89aa, 10 kDa) is the most common isoform produced in lymph nodes (LNs), brain, liver, colon, kidney, testis, lung, pancreas, skin, and placenta and by different cell types including stromal cells, osteoblasts, fibroblasts, dendritic cells, and monocytes, among others. CXCL12 is the major chemokine produced in the bone marrow (BM), where it regulates quiescence, retention, and differentiation of hematopoietic stem cells (HSC) [26]. CXCL12

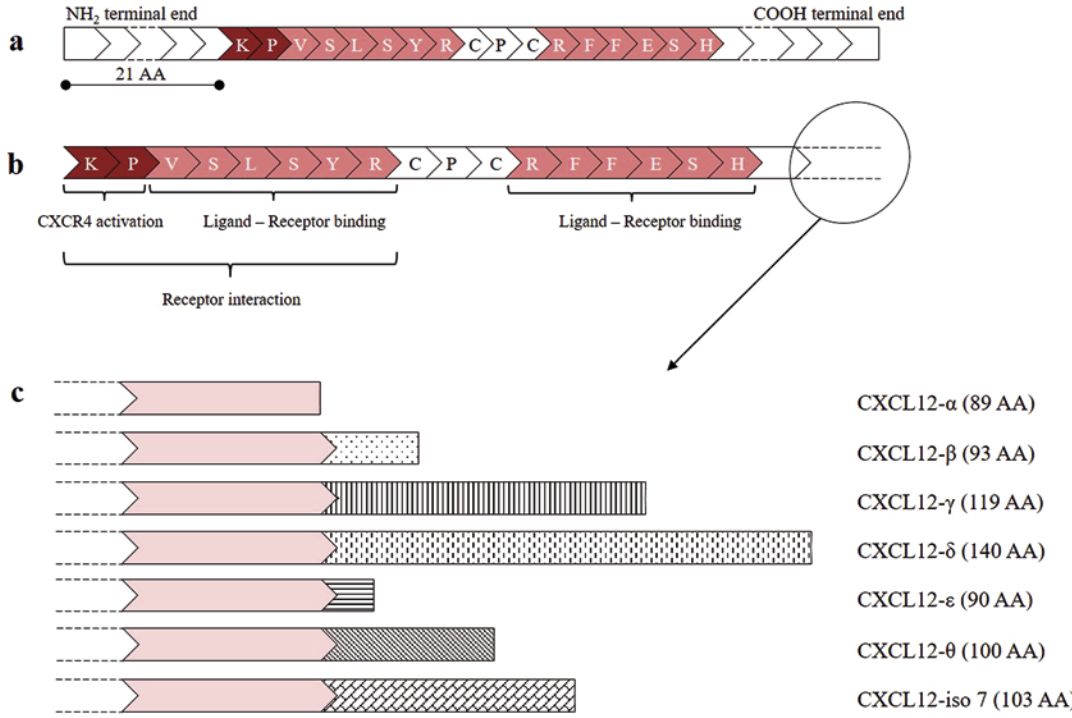

Fig. 5.2 CXCL12 isoforms. (**a**) The CXCL12 immature form, the propeptide, which includes the 21 amino acids at the N-terminal end that will be removed. (**b**) The mature CXCL12 form has undergone a proteolytic cut of 21 amino acids at the N-terminal end. The first eight amino acids of the mature CXCL12 allow the receptor interaction; in particular, the first two, lysine and proline, activate the CXCR4 receptor, while the other six are used for the receptor binding. In addition, the "RFFESH" sequence allows the ligand-receptor binding. (**c**) Representations of all CXCL12 isoforms are shown. They all have the same starting sequence, but each one differs from the others in the terminal region length. (Adapted from *Righetti A. et al CXCL12 and Its Isoforms: Different Roles in Pancreatic Cancer? Journal of Oncology* [8]*:1–13. (2019)*)

half life is approximately 26 minutes. CXCL12 is then degraded at the N-terminal by enzymes matrix metalloproteinase-2/9 (MMP-2/9) crucial to extracellular matrix (ECM) remodeling. CXCL12 is described classically as a homing chemokine as it exhibits chemoattraction of tumoral cells toward the target tissues [27]. CXCL12, although being homeostatic in classification, also takes inflammatory activities [28]. CXCL12 binds to glycosaminoglycans (GAGs) exposed on the surface of endothelial cells through a cluster of basic residues—the BBXB motif (B for basic amino acid and X any amino acid) generating its chemotactic gradients and promoting leukocyte/cancer cell migration [29–31]. CXCL12-α is not present in blood due to enzymatic degradation [23], but it is expressed in adult BM, where it accounts for progenitor cell retention and chemotaxis of leukemia cells [32]. Unlike CXCL12α, CXCL12β (93aa, 10.6 kDa) promotes angiogenesis [33], while CXCL12γ (119aa, 13.6 kDa) is highly expressed in less vascularized organs, such as the heart and brain [34]. CXCL12γ is poor chemotactic in vitro but the most active in vivo [35] and, due to a stable binding interaction, produces a powerful inflammatory reaction in in vivo mouse models [23]. Recently CXCL12γ has been found to interact with CXCR4 inducing cancer stem cell (CSCs), with neuroendocrine phenotypes and development of metastatic castration-resistant prostate cancer (mCRPC) [36].

5.2.2 CXCL12 Signaling

CXCL12 binds the seven α-helical transmembrane domains (7TM) G protein-coupled (GPCR) conventional receptor CXCR4, and the atypical receptor CXCR7 (also known as ACKR3) and plays a key role in physiological and pathological processes, including embryogenesis, hematopoiesis, angiogenesis, and inflammation, regulating the migration of hematopoietic progenitor and stem cells, endothelial cells, and leukocytes [7, 37, 38] (Fig. 5.3). CXCL12 main receptor, CXCR4, also binds macrophage migration inhibitory factor (MIF), a cytokine involved in the regulation of innate immunity [39]. MIF binds to the N-terminal tail of CXCR4 and to the exterior side of TM helices, but not inside the TM pocket [39, 40]. MIF also binds to other receptors, including CXCR2, CD74/CD44, and ACKR3 [41]. CXCL12 can also activate CXCR4 through heterodimers like HMGB1-CXCL12. High-mobility group box 1 protein (HMGB1) is the archetypal damage-associated molecular pattern (DAMP) released from dead or severely stressed cells to alert their microenvironment and the innate immune system. However, the conformational rearrangements of CXCR4 differ when triggered by CXCL12 alone or by HMGB1•CXCL12, and the complex is extremely more potent than CXCL12 alone in inducing cell migration [42]. Extracellular ubiquitin (eUb), also considered a DAMP, is a CXCL12 antagonist [41] that binds to CXCR4 inside the cavity delimited by TMs [43], but makes contact to CXCR4 residues that are not contributing to CXCL12 binding [44]. Beta-defensin-3 (HBD3) also competes with CXCL12 for CXCR4 binding and promotes CXCR4 internalization without inducing calcium flux, ERK phosphorylation, or chemotaxis. CXCL12-CXCR4 forms a complex with the $G\alpha_i$ subunit G protein, inhibiting the adenylyl cyclase-mediated cyclic adenosine monophosphate (cAMP) production and promoting mobilization of intracellular calcium. Dissociation of the $G\alpha_i$ subunit from Gβγ leads to activation of multiple downstream targets, including protein kinase B (Akt), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MEK), and extracellular signal-regulated kinase-1 (ERK1/2) effectors [45]. In addition, Gα subunit activates Ras and Rac/Rho pathways, leading to the phosphorylation of ERK and P38 proteins, respectively [46]. CXCR4 homodimerization results in G protein-independent activation of the JAK/STAT pathway that, in conjunction with other signaling pathways, promotes polarization and chemotactic responses [47]. Recent evidence has shown CXCR4-dependent mTOR signaling in pancreatic, renal, and gastric cancer and T cell leukemia cells [18, 48]. CXCR7 homo- and heterodimerizes with CXCR4, and overall, the signaling properties of CXCR7 seem to be multifaceted and may be cell context-dependent. CXCR4-CXCR7 heterodimers regulate the subcellular distribution of CXCR4, recruit β-arrestins, and modify CXCL12-driven responses through CXCR4 [49]. Emerging evidence suggests that CXCR7 internalizes its ligands and is activated by CXCL12 to induce intracellular signaling and in particular Akt, MAPkinase (MAPK), and Janus kinase-signal transducer and activator of transcription (JAK/STAT3) through β-arrestin [19] or in heterodimers with CXCR4 [50, 51]. β-arrestin recruitment to the CXCR4/CXCR7 complex enhances downstream cell signaling (ERK1/2, p38, SAPK/JNK), which induces cell migration in response to CXCL12 [19]. CXCR4 is expressed by migrating cells, and CXCR7 acts by sequestrating CXCL12 from non-target areas, allowing the correct cell migration [20]. In the absence of CXCR7, migrating cells still respond to CXCL12, but their movement ends in undesirable sites due to the lack of a CXCL12 gradient required for a directional migration [67]. Continuous CXCL12 stimulation desensitizes CXCR4-expressing cells promoting CXCR4 endocytosis, as uncoupling from G proteins by GPCR kinase (GRK)-dependent phosphorylation and subsequent interaction of CXCR4 with β-arrestin, which mediates internalization of the receptor that is ubiquitinated and degraded in lysosomes [52, 53]. Activated YY1, a transcription factor, can inhibit CXCR4 expression favoring C-terminal phosphorylation of Src kinase in breast cancer cells [54, 55]. The histone deacetylase CREB3 and the

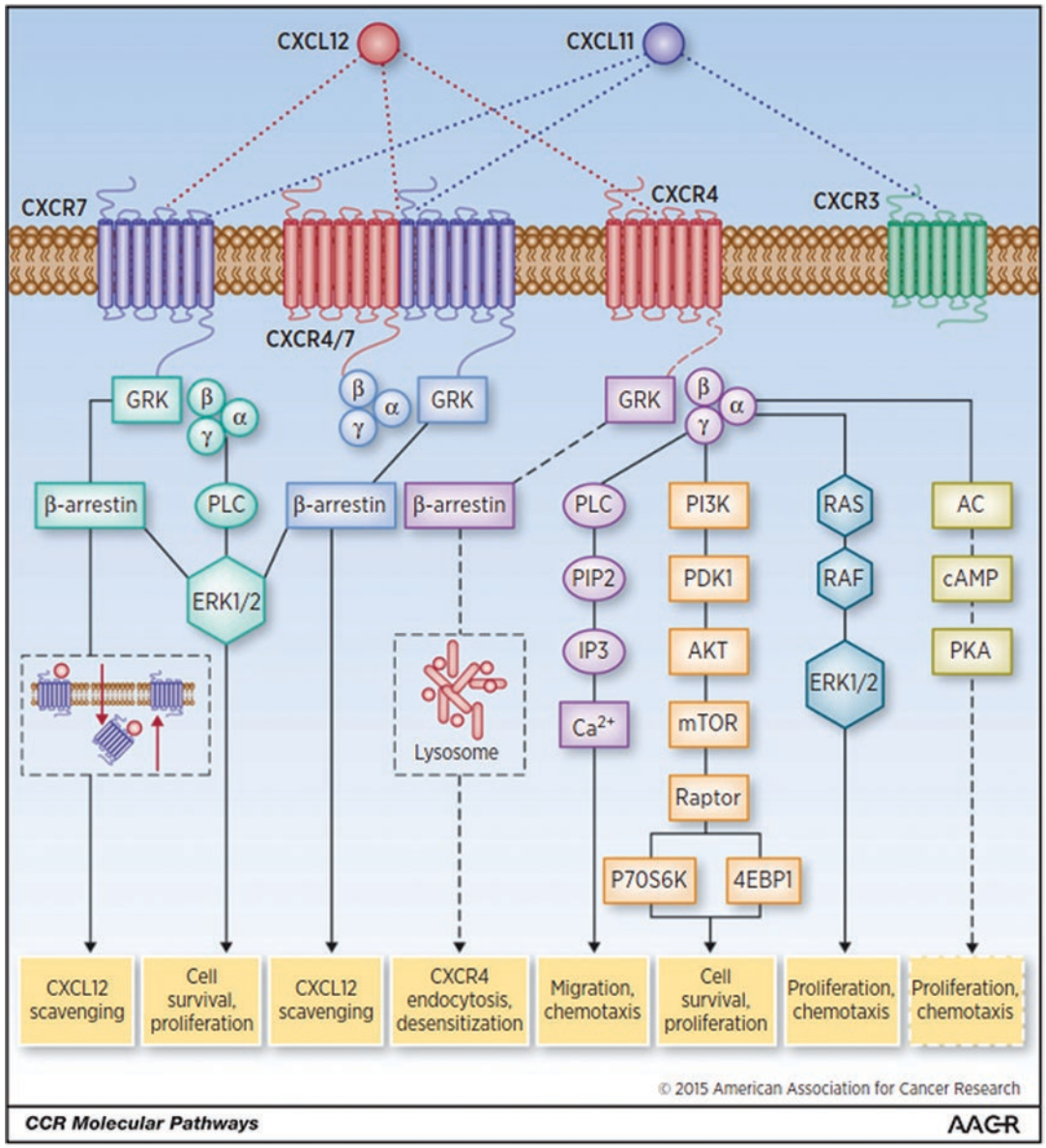

Fig. 5.3 The CXCR4–CXCL12–CXCR7 transduction pathway. CXCL12 acts on two distinct receptors, CXCR4 and CXCR7, which are seven-membrane GPCR receptors. CXCR4 and CXCR7 can form homodimers or heterodimers. CXCL12 shares CXCR7 binding with another chemokine, CXCL11, that is also a ligand for CXCR3. CXCR4 triggers preferentially G protein-coupled signaling, whereas activation of CXCR7 or the CXCR4-CXCR7 complex induces b-arrestin-mediated signaling. The Gai monomer inhibits the adenylyl cyclase activity regulating cell survival, proliferation, and chemotaxis. Although Gai triggers PI3K/AKT/mTOR and ERK1/2, the Gbg dimer triggers intracellular calcium mobilization through PLC. When CXCL12 binds CXCR7, the receptor signals through b-arrestin, inhibits G protein-coupled signaling, and activates the MAPK pathway. CXCR7 can also signal through PLC/MAPK to increase cell survival. The CXCR4-CXCR7 heterodimers-b-arrestin pathway can be activated through GRK-dependent phosphorylation to internalize CXCR4, scavenging CXCL12, and/or control cell survival through ERK1/2. CXCL12 also causes CXCR4 desensitization, uncoupling from G protein by GRK-dependent phosphorylation, and b-arrestin-dependent endocytosis. In contrast with CXCR4, when CXCL12 binds CXCR7, the interaction between b-arrestin and CXCR7 internalizes the receptor and subsequently recycles it to the cell membrane. Upon binding to CXCR4 or CXCR7, CXCL12 is internalized and subjected to lysosomal degradation. Activator signals are represented by straight lines. Inhibitory symbols are represented by dashed lines. (From *Scala S., Molecular Pathways: Targeting the CXCR4-CXCL12 Axis--Untapped Potential in the Tumor Microenvironment. Clin Cancer Res;21* [19]*:4278-85 (2015)*)

Kruppel-like factor 2 can inactivate CXCR4 [56, 57]. The oncogene Her2 can block CXCR4 ubiquitination and degradation after CXCL12 binding in breast cancer cells [58, 59]. CXCL12 is considered a key molecule for normal development as 50% of the CXCL12$^{-/-}$ knockout mice die before birth around day 18.5 of embryogenesis and neonates die within an hour. CXCL12$^{-/-}$ mice show severely reduced B cell progenitors in fetal liver and bone marrow and myeloid progenitors virtually absent in the bone marrow. In addition, the mutants have a cardiac ventricular septal defect [60]. Similar defect was reported for CXCR4$^{-/-}$ mice suggesting a strong relationship between CXCR4 and CXCL12 [61]; moreover, in the CXCR4$^{-/-}$ mice, nervous system defects were also observed with the cerebellum characterized by an irregular external granule cell layer, ectopically located Purkinje cells, and numerous chromophilic cell clumps of abnormally migrated granule cells within the cerebellar anlage [61]. CXCR4$^{-/+}$ knockout mice presents few mature B and T cells within the peripheral lymphoid organs, impaired vascularization in various organs such as the intestines, stomach, and heart, and ventricular septal defect that occurs during embryogenesis [62]. CXCR7 has a role in the development of the central nervous system [50], angiogenesis [63], neurogenesis [64], and cardiogenesis [65], while CXCR4 is involved in vascularization, homing of immune cells in the bone marrow [62], and neurogenesis [66]. CXCR7$^{-/-}$ knockout mice die perinatally due to semilunar heart valve malformation and ventricular septal defects and show furthermore disrupted lymph angiogenesis and cardiomyocyte hyperplasia, while their hematopoiesis remains normal.

5.3 The CXCL12-CXCR4/CXCR7 Axis in Cancer

The expression of CXCL12-CXCR4/CXCR7 axis is mostly reported in aggressive tumors [68] and correlates with tumor recurrence [69, 70], poor prognosis and patient survival [16, 71, 72]. CXCR4 is overexpressed in a wide range of tumors comprising prostate, brain, breast, lung, liver, gastric, colon, ovary, and pancreas [73–76]. CXCL12 confers several advantage to CXCR4-expressing cancer cells including a chemo-resistant phenotype via crosstalk with several pathways of survival, proliferation, tumorigenesis, epithelial to mesenchymal transition (EMT), and acquisition/maintenance of stem-like proprieties [36, 77–82]. In addition, CXCL12 hypermethylation was reported in gastric cancer [83], breast cancer [84, 85], colon cancer [86], lung cancer [87], as well as prostate cancer [88]. Within the TME, CXCL12/CXCR4 regulates trafficking of immune and tumor cells promoting tumor-related inflammation and metastasis [47, 89, 90]. In addition, endothelial cells express both CXCR4/CXCR7 and CXCL12 that facilitates intravasation and extravasation of cancer and immune cells as well as tumor angiogenesis [91]. CXCR4 facilitates angiogenesis by recruitment of endothelial progenitor cells or BM-derived accessory cells, while VEGF promotes sprouting angiogenesis by inducing tip cell filopodia and serving as an attraction cue [92]; the CXCL12/CXCR4 axis stimulates tip cells and migration in neovascular sprouting [93]. CXCR7 is highly expressed by most tumor-associated blood vessels of human breast and lung cancers as well as melanoma, but not by normal vasculature [49]. Overexpression of CXCR7 and its activation in the vascular endothelium enhance invasive and migratory ability toward breast, prostate, and lung cancer [94]. In ovarian cancer, estrogen induces CXCR7 expression that favors tumor cell migration and invasion through CXCL11 [95], while CXCL12-stimulated EMT depends on CXCR4 [70]. Although CXCR7 is not considered a chemotactic receptor, addition of CXCL12 enhances CXCR4+/CXCR7+ cancer cell trans-endothelial migration toward CCL19 and CXCL13, chemokines expressed by endothelial cells inside the lymph nodes [96]. Moreover, in ovarian cancer CXCL12 stimulation reduced the expression of ARH-GAP10, a member of Rho GTPase-activating proteins considered a tumor suppressor gene [97].

MicroRNAs have been reported to play critical roles in regulating tumor progression through

CXCL12/CXCR4 axis [98]. MiR-302a decreased the invasion and metastasis of breast cancer cells by reducing CXCR4 production [99]. MiR-9 reduced the proliferation of oral squamous cell carcinoma cells by the inhibition of CXCR4 via the Wnt/β-catenin signaling pathway [100]. MiR-146a down-modulated CXCR4 production in target cells [101]. CXCR4 was inhibited upon miR-451 treatment in lung cancer cells [102]. MiR-204-5p may function as an inhibitory RNA molecule in oral squamous cell carcinoma by targeting CXCR4 [103]. Artificial microRNA was demonstrated to effectively block invasion and metastasis of breast cancer cells by targeting CXCR4 [104]. MiR-126 may also act as a tumor suppressor by inactivating RhoA signaling via CXCR4 in colon cancer [105]. In addition, miR-101 was recently discovered to directly target CXCL12 in lung cancer cells [106].

CXCL12-CXCR4 axis mediates also chemotherapy-induced transition toward a mesenchymal/stem cell phenotype. Cisplatin induces upregulation of both CXCR4 and CXCL12 expression in NSCLC cells. In colon cancer, chemotherapy or chemoradiotherapy induces CXCR4 expression as part of a mesenchymal transition [107]. Chemotherapy- induced cytokines also have a direct effect on tumor cells. In vivo CXCL12 or S1p/S1PR1 inhibition prevented chemotherapy-enhanced metastasis in tumor-bearing mice. In glioblastoma (GMB) CXCR4 inhibition through Pep R impairs the metabolic activity and tumor growth of GMB cells in vitro and reduced tumor cellularity, promoted M1 features of TAMs and astrogliosis, and hindered intratumor vasculature in orthotopic GMB model [108].

5.4 CXCL12 as Prognostic Factor in Cancer

In a meta-analysis of 38 studies involving 5807 patients, high CXCL12 expression was associated with reduced overall survival in patients with esophageal, colorectal, gastric, pancreatic, ovarian, and lung cancer, while in breast cancer patients, high CXCL12 expression conferred an overall survival advantage [109]. In esophageal cancer patients' meta-analysis, high expression of CXCL12 and its receptors (CXCR4 and CXCR7), CXCL8 and its receptor (CXCR2), CCL21 and its receptor (CCR7), or CCL20 was associated with worse prognosis [110]. CXCL12γ was detected only in breast cancers from patients with advanced disease suggesting CXCL12γ as a prognostic marker for breast cancer [34]. CXCL12γ, mostly expressed by carcinoma-associated fibroblasts, confers to CXCR4-positive breast cancer cells the ability to metastasize to the bone marrow through the expression of the receptor activator of NFκB ligand (RANKL) [33]. High levels of CXCL12 and CXCR4 were reported in sinusoidal endothelial cells in hepatocellular carcinoma (HCC) specimens, breast tumors, metastatic lung cancer, bladder cancer, head and neck squamous cell carcinoma, glioblastoma, and pancreatic tumors [17, 100, 108, 111–115]. In metastatic germ cell tumors, CXCL12 is almost exclusively expressed in non-seminoma [116]. CXCL12/CXCR4 axis is centrally involved in ovarian cancer progression since CXCL12 induces ovarian cancer cell migration and invasion and was reported as prognostic factor in ovarian cancer [117]. In lung cancer patients, CXCL12 protein and mRNA expression levels were significantly higher in metastatic lymph nodes than in primary site. CXCL12 high expression in metastatic lymph nodes was associated with poor overall survival [118]. In another study on 63 patients undergoing surgical resection for lung adenocarcinoma, CXCL12 overexpression was a significant poor prognostic factor in patients with surgical resected lung adenocarcinoma. In a study including 596 patients, CXCL12 and relative CXCL12-CXCR4 expression was independent prognostic factors for 5-year DFS in TNM stage III colon cancer [119]. In renal cancer (RCC) patients with high expression of CXCR4, CXCR7, and CXCL12 had shorter overall survival and recurrence-free survival than those with low expression [120]. Moreover, CXCR4 and CXCR7 expression, alone and in combination, was prognostic in RCC [121]. Even if CXCL12 was not prognostic, CXCR4 and CXCR7

correlate with poor prognosis in melanoma, hepatocellular carcinoma (HCC), and colorectal cancer (CRC) [122–125].

5.5 The CXCL12-CXCR4/CXCR7 Axis in the Tumor Microenvironment

Tumor microenvironment is the tumor local environment composed of immune cells, fibroblasts, epithelial cells, extracellular matrix (ECM) proteins, blood and lymphatic vessels, metabolites, chemokines, and cytokines [1, 126]. TME is defined "hot" when it is highly infiltrated by T cell lymphocyte generating "inflamed" TME; in contrast, lack of T cells infiltrating the tumor characterizes "non-inflamed" or "cold tumors" in which other immune populations, like myeloid cells (mostly immunosuppressive), prevail. Tregs (regulatory T cells) and MDSC (myeloid-derived suppressor cells) prevail. Tregs and MDSCs (myeloid-derived suppressor cells) prevail in a COLD TME; moreover, there are few TH1, NK, and CD8+ T cells and few functional antigen-presenting cells (APCs) and it is enriched in immunosuppressive cytokines. Hot TME displays high PDL1 expression and is enriched in TH1-type chemokines, effector immune cells (TH1 cells, NK cells, and CD8+ T cells), and functional APCs [1, 126, 127]. Recent evidence showed that the CXCL12/CXCR4 axis can regulate the recruitment of specific immune cell populations within the TME (facilitating the access of immune cells with suppressive function and repelling immune effector cells) and drive the polarization of CXCR4-expressing immune cells toward an immunosuppressive phenotype (Fig. 5.4). CXCL12-CXCR4 signaling mediates plasmacytoid DC trafficking into tumors and Treg cells homing to the bone marrow microenvironment [128]; moreover it has an anti-inflammatory role by mediating T cell polarization toward Tregs [129, 130] and generating poorly functional DCs to stimulate antigen-specific T lymphocyte and macrophages expressing proangiogenic factors [131]. Cytokines produced by stromal cells such as TGF-β elicit epithelial CXCR4 expression that, activated by stromal CXCL12, mediates the activation of the Akt pathway in the epithelial cells promoting malignant progression [132]. CXCL12/CXCR4 signaling is central to the retention of neutrophils in the BM, mobilization from the bone marrow, and the homing back of senescent neutrophils [133]. Mice carrying a myeloid-specific deletion of CXCR4 (myeloid-specific knockout (MKO) mice) display a marked redistribution of neutrophils from the bone marrow to the blood and spleen [134]. In a melanoma mice model, disruption of CXCL12/CXCR4 signaling in myeloid cells via genetic knockout of CXCR4 inhibits the outgrowth of circulating B16 melanoma cells in the lung and inhibits tumor growth in an inducible BrafV600E/PTEN null melanoma mouse model as IL18 overexpression activates NK cells and enhances antitumor immunity [135]. BMDCs attracted at the tumor site produce CXCL12 that interacts with DC cells in an autocrine manner to promote DC maturation and survival [136, 137]. High numbers of plasmacytoid DCs have been observed in human ovarian carcinoma due to CXCL12 in malignant ascites that attracts DCs into the TME [138]. Intratumoral DC plays an important role in stimulating cytotoxic T cells and driving antitumor immunity. In metastatic ovarian tumor model, locoregional delivery of the CXCR4-antagonist-armed virus reduced the tumor load and the immunosuppressive network in the TME, leading to infiltration of CD103+ DC capable of phagocytic clearance of cellular material from virally infected cancer cells [139]. As natural and synthetic amines inhibit DC activation, CXCR4 has been identified as receptor used by amines to inhibit DCs [140]. Thus, CXCR4 was described as a potential "on-off" switch of DC activity with therapeutic potential [140]. Tregs are recruited at the tumor site by chemokines such as CXCL12 [141] and CXCR4 overexpression by Tregs is reported in advanced cervical cancers [142], malignant pleural mesothelioma [143], ovarian [144] and renal cell carcinoma [145]. The CXCR4 antagonist AMD3100 reduced intratumoral Treg by conversion of Treg cells into T-helper-like cells conferring survival

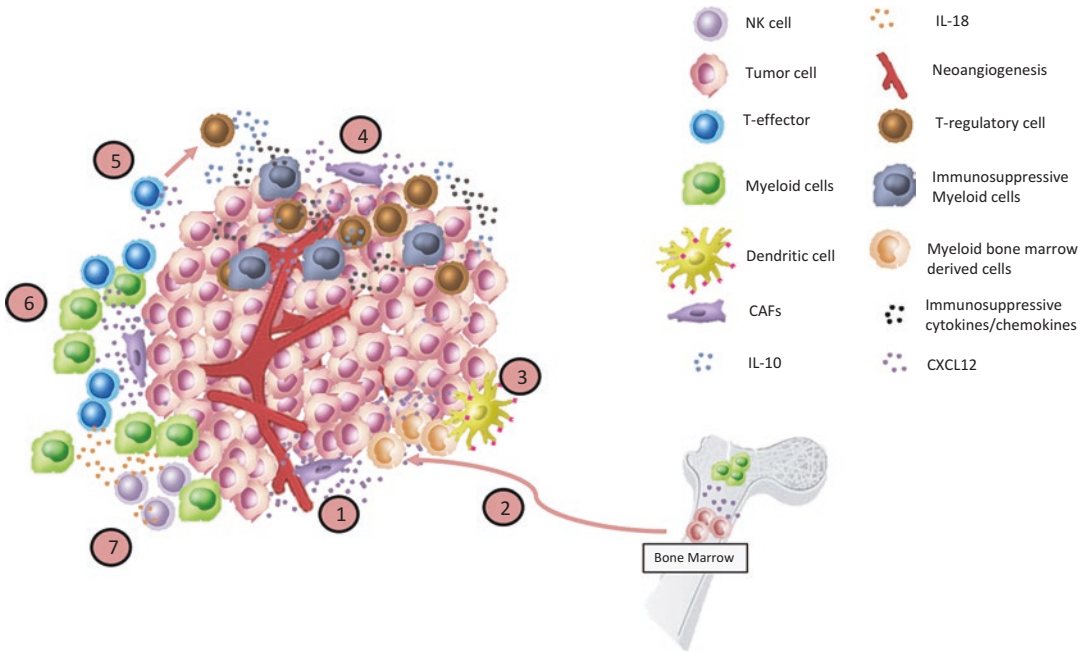

Fig. 5.4 CXCL12 in the tumor microenvironment. CAF-produced CXCL12 acts on different TME cells and regulates the recruitment of immune cells. 1. CXCL12/CXCR4 axis stimulates endothelial cells promoting neovascularization, tumor growth, and metastatic progression. 2. CXCL12/CXCR4 axis regulates trafficking and tissue localization of human HSC in the bone marrow. 3. CXCL12/CXCR4 axis recruits bone marrow-derived myeloid cells promoting DC maturation and survival. 4. CXCL12 recruits immunosuppressive cells and 5. excludes effector cells designing a "colder" TME that impairs immunotherapy response. 6. CXCL12 also redirects the polarization of effector Th1 cells into $CD4^{+}CD25^{-}Foxp3^{-}$interleukin (IL) 10^{high} regulatory T cells. 7. Neutrophils produce IL-18 which increases the NK cell population and their antitumor activity. Adapted from Luker et al: At the Bench: Pre-clinical evidence for multiple functions of CXCR4 in cancer (2021) J Leukoc Biol. 2021;109:969–989

advantage to the ovarian tumor [144]. In ovarian cancer CXCR4 antagonism potentiates the PD-1 blocking antibody activity impairing the recruitment of immunosuppressive cell components and increasing tumor-specific cell-mediated immune responses [146]. AMD3100 alone and in combination with a mesothelin-targeted, immune-activating fusion protein VIC-008 modulated immunosuppression in tumors inhibiting PD-1 expression on CD8+ T cells and promoting the conversion of Tregs into CD4+CD25–Foxp3+IL2+CD40L+ helper-like cells [147]. Ex vivo treatment with the new developed CXCR4 antagonist R29 suppresses Treg function and restores T effector cell proliferation without affecting Treg viability in RCC patients [145]. 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 antitumor immune responses [148, 149]. However, CXCL12-overexpressing melanoma mice model (B16/OVA cells engineered to overexpress CXCL12) demonstrated that CXCL12 has a bimodal effect on CXCR4-expressing T effector cell migration attracting them at low concentrations and repelling them at higher concentrations of the chemokine [150]. This mechanism is termed chemorepulsion or fugetaxis [150–152] and contributes to the physiological process of T cell migration from the thymus, while repelling T effector cells inside the TME may represent a mechanism by which high CXCL12-expressing tumors evade the immune system [150–152].

Interestingly, recent studies are investigating the role of a novel component of TME: nerves. Nerves are gaining attention for their role in

cancer as their presence in the TME correlates to cancer metastasis and poor prognosis [153]. Cancer-nerve crosstalk is based on perineural invasion (PNI, cancer cells invading and migrating along the perineurium) and tumor innervation (nerve fibers recruited by neoplastic tissues); both mechanisms cause pain, poor prognosis, and higher risk for recurrence and depend on the chemokine network [153]. In a PNI-associated pancreatic cancer model, in vitro and in vivo analyses revealed that the CXCL12/CXCR4 signaling axis can promote PNI, which can be inhibited by AMD3100 or CXCR4 short hairpin RNA [154].

5.6 Targeting the CXCL12-CXCR4/CXCR7 Axis in Combination Therapy

Different CXCL12/CXCR4 antagonists have been developed and validated (Table 5.1), showing promising anti-cancer activities in several tumor types and can be divided in five major classes: (1) small modified peptides, including BKT140 [155], FC131 [156], T140 [157], and POL6326 [158]; (2) small molecules, including the bicyclam AMD070 [159], AMD3100 [160], AMD11070 [161], MSX-122 [162], GSK812397 [163], and KRH-3955 [164]; (3) antibodies, such as MDX-1338/BMS 93656 [165]; (4) modified agonists and antagonists for CXCL12 such as CTCE-9908 [166]; and (5) microRNAs, such as miR-302a [98], miR-9 [100], miR-204-5p [103], and miR-126 [105].

CTCE-9908 is a CXCL12 analogue which actually received the orphan drug status for osteogenic sarcoma treatment [166]; CTCE-9908 showed in vivo and in vitro antitumor activity in different tumor models such as osteosarcoma, prostate, esophageal, and breast cancer [167–171]. NOX-A12 (OLA-PEG) is a high-affinity anti-CXCL12 Spiegelmer which binds CXCL12 blocking its interaction with both CXCR4 and CXCR7. OLA-PEG antitumor activity has been evaluated in multiple myeloma, leukemia, colorectal cancer, and glioma and in association with immune checkpoint inhibitors in pancreatic cancer and colorectal cancer (NCT03168139, NCT01521533, NCT01486797, NCT04121455) [172–175]. Combinations of OLA-PEG with other chemotherapeutic agents were investigated [176]. Peptide R and Peptide R54 are CXCR4 inhibitors generated by rational design. Peptide R inhibits CXCL12-induced cell migration and lung metastasis development [177–180] and potentiates standard/immune therapy in colorectal cancer models in vivo [107, 181].

Several evidence showed that combinatorial blockade of CXCR4 and PD-1 greatly reduces specific cellular and functional elements within the immunosuppressive tumor microenvironment and augments tumor-specific cell-mediated immune responses. Combined treatment with the Pep R and anti-PD-1 reduced tumor progression in two syngeneic murine models, anti-PD-1 sensitive and resistant, increasing Granzyme+ and inhibiting Foxp3+ cell tumor infiltration. In addition Pep R54, a Peptide R derivative [182], synergizes with nivolumab in inhibiting the growth of the PD-1 expressing human PES43 melanoma xenograft [181]. Fibroblast activation protein-α (FAP)-positive CAF are the major source of CXCL12 in TME that regulates TME exclusion of T cells. The conditional depletion of the FAP+ CAF permits immune control effects of both anti-PD-L1and anti-CTLA-4; administering AMD3100 induced rapid T cell accumulation in this autochthonous model of pancreatic ductal adenocarcinoma (PDA) [183, 184]. Moreover, CXCL12 upregulation in HCC models increased hypoxia and the recruitment of immunosuppressive cells, PD-L1, regulatory T cells, and M2-type macrophages after treatment of sorafenib. PD-1 blockade combined with CXCR4 inhibition and sorafenib decreased HCC growth [185]. NOX-A12 in colon cancer spheroid increased numbers of T and NK infiltrating cells and reduces tumor growth in combination with anti-PD-1 compared with anti-PD-1 alone [172].

5.7 Future Trends or Directions

CXCL12 has two main interaction partners, specifically CXCR4 and CXCR7. CXCL12 is a crossroad molecule that modulates crucial

Table 5.1 CXCL12/CXCR4 axis antagonists in cancer clinical development

Drug name	Phase	Active indication	Combination therapy	Trial number
Small molecules				
Plerixafor (AMD3100)	Phase 2	Metastatic pancreatic cancer	Cemiplimab	NCT04177810
	Phase 2	Acute myeloid leukemia, acute lymphoid leukemia	Busulfan, cyclophosphamide	NCT02605460
X4P-001	Phase 1	Melanoma	Pembrolizumab	NCT02823405
	Phase 1/2	Renal cancer	Axitinib	NCT02667886
CX-01	Phase 1	Myelodysplastic syndromes, acute myeloid leukemia	Azacitidine	NCT02995655
	Phase 2	Acute myeloid leukemia	Idarubicin, cytarabine	NCT02873338
CXCL12 Spiegelmer				
NOX-A12	Phase 1/2	Glioblastoma	Radiotherapy	NCT04121455
	Phase 1/2	Metastatic colorectal cancer Metastatic pancreatic cancer	Pembrolizumab	NCT03168139
CXCR4 peptide antagonists				
Balixafortide	Phase 3	Metastatic breast cancer	Eribulin	NCT03786094
LY2510924	Phase 1	Leukemia	Idarubicin, cytarabine	NCT02652871
BL-8040	Phase 2	Metastatic pancreatic adenocarcinoma	Pembrolizumab	NCT02826486
	Phase 2	Malignant neoplasms of digestive organs, metastatic pancreatic cancer	Pembrolizumab	NCT02907099
	Phase 1/2	Pancreatic adenocarcinoma	PEGPH20, cobimetinib, atezolizumab, gemcitabine, Nab-paclitaxel, oxaliplatin, leucovorin, fluorouracil	NCT03193190
	Phase 1/2	Gastric adenocarcinoma or gastroesophageal junction adenocarcinoma	PEGPH20, linagliptin, paclitaxel, ramucirumab, 5-FU, leucovorin, oxaliplatin, atezolizumab, cobimetinib	NCT03281369
	Phase 1/2	Carcinoma, non-small cell lung	Gemcitabine, carboplatin, pemetrexed, CPI-444, tazemetostat, atezolizumab, cobimetinib, RO6958688, docetaxel	NCT03337698
Anti-CXCR4 antibodies				
Ulocuplumab (MDX-1338)	Phase 1/2	Waldenstrom's macroglobulinemia	Ibrutinib	NCT03225716

mechanisms such as proliferation and migration of tumor and tumor microenvironment cells. CXCL12 targeting affects tumor primary growth, mesenchymal transition, and migration but also shapes the TME toward immunoresponsive TME, potentiates the efficacy of checkpoint inhibitors targeting drugs, and interferes in building distant pre-metastatic niches. CXCL12-CXCR4 antagonists, although suboptimal, need deeper evaluation in terms of patient's selection, schedule, and combination therapies.

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