Cytokine and Growth Factor Reviews xxx (xxxx) xxx

Contents lists available at ScienceDirect

Cytokine and Growth Factor Reviews

journal homepage: www.elsevier.com/locate/cytogfr

An emerging paradigm of CXCL12 involvement in the metastatic cascade

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ARTICLE INFO

Keywords:
CXCL12
CXCR4
CXCR7
Tumor microenvironment
Epithelial-to-mesenchymal transition
Cancer cell stemness
Invasion and migration
Metastatic colonization
Angiogenesis
Immunosuppression

ABSTRACT

The chemokine CXCL12, also known as stromal cell-derived factor 1 (SDF1), has emerged as a pivotal regulator in the intricate molecular networks driving cancer progression. As an influential factor in the tumor microenvironment, CXCL12 plays a multifaceted role that spans beyond its traditional role as a chemokine inducing invasion and metastasis. Indeed, CXCL12 has been assigned functions related to epithelial-to-mesenchymal transition, cancer cell stemness, angiogenesis, and immunosuppression, all of which are currently viewed as specialized biological programs contributing to the "metastatic cascade" among other cancer hallmarks. Its interaction with its cognate receptor, CXCR4, initiates a cascade of events that not only shapes the metastatic potential of tumor cells but also defines the niches within the secondary organs that support metastatic colonization. Given the profound implications of CXCL12 in the metastatic cascade, understanding its mechanistic underpinnings is of paramount importance for the targeted elimination of rate-limiting steps in the metastatic process. This review aims to provide a comprehensive overview of the current knowledge surrounding the role of CXCL12 in cancer metastasis, especially its molecular interactions rationalizing its potential as a therapeutic target.

1. Introduction

The chemokine CXCL12, also known as stromal cell-derived factor 1 (SDF1), has emerged as a pivotal regulator in the intricate molecular networks underpinning cancer progression. By interacting with its receptor, CXCR4, CXCL12 orchestrates a multitude of biological processes within the tumor microenvironment, such as tumor growth, invasion, angiogenesis, and metastasis. While traditionally known for its role in chemotaxis and migration, our understanding of CXCL12 simply as a "homing" chemokine has expanded. Today, metastasis is seen as a complex interplay of biological programs, which collectively induce metastatic phenotypes. Consequently, our grasp on the role of the CXCL12/CXCR4 pathway in the "metastatic cascade" has deepened and been reevaluated. In this article, we will explore the CXCL12 signaling pathway in the context of cancer metastasis, reexamining both traditional views and emerging insights into its regulatory functions in metastasis-specific programming. This renewed understanding will inform strategic considerations when targeting this pathway therapeutically.

2. CXCL12 gene/transcript organization and regulation

CXCL12 has been originally described as an essential growth factor for B cell lineage development, but was later demonstrated to be indispensable for various developmental processes, including but not limited to the colonization of gonads by primordial germ cells, and heart and cerebellar development. Given that CXCL12 was found to be highly produced by stromal cells in the bone marrow, it was named "stromal-

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https://doi.org/10.1016/j.cytogfr.2023.10.003
Received 15 October 2023; Accepted 20 October 2023
Available online 31 October 2023

derived growth factor" (SDF1) [1,2]. CXCL12 is the only member of the chemokine family that is essential for life, as its genetic ablation leads to embryonic lethality, characterized by a partial failure of nervous system development, among other lesions [3]. This section will thus focus on basic gene- and transcript-organization principles, as a prelude to understanding CXCL12-dependent signaling, dysregulation at disease state, and implication in cancer development and progression.

2.1. Gene organization

The human *CXCL12* gene is located in chromosome 10q11.1 and is comprised of a total of six exons. Within the promoter region of *CXCL12*, there are binding sites for the transcription factors SP1 and CTF with three GC boxes (the first, from position −451 to −446; the second, from position −87 to −82; and the third, from position −63 to −58), and one CAAT box (from position −423 to −419) [4–6]. A natural insertion mutation (GA+) has been identified in the GC-rich region of the 3'UTR regulatory domain of the CXCL12 isoform-alpha, which most likely affects the regulation of its expression by modifying the secondary structure of the RNA [7]. An association between CXCL12 gene polymorphisms and resistance to human immunodeficiency virus type 1 infections has been proposed [8]. The mature CXCL12 protein is an 8 kDa chemokine of the intercrine family, and represents the only known endogenous ligand for the G-protein coupled receptor, chemokine (C-X-C motif) receptor 4 (CXCR4) [5,9]. Human and mouse CXCL12 show an exceptional homology on both genome and protein level, making the latter an appropriate model organism for investigating its role in pathology [10].

2.2. Transcript organization

CXCL12 is the only known CXC chemokine undergoing differential mRNA splicing among the chemokine family [3]. In particular, six splice variants have been identified for the human CXCL12 (CXCL12α to φ) and three for the mouse ortholog (CXCL12α to γ), all resulting from alternative CXCL12 gene splicing. Of those, CXCL12α and CXCL12β are the most well-studied to-date, with the former being expressed in nearly all organs and representing the predominant isoform in bone-marrow stromal and endothelial cells. While both isoforms are derived from a single gene, the β-isoform differs only by four additional amino acids (RLKM) at the C-terminal end, as a result of alternative splicing [11]. Despite this small difference, the two isoforms have surprisingly diverse biochemical and even functional properties. For example, although the α-isoform is abundantly increased in tissue damage, it undergoes rapid proteolysis in the blood, thus only contributing as an acute responder to the injury. On the other side, the β-isoform is more resistant to blood-dependent degradation, it can still stimulate angiogenesis equally effectively with the α-isoform, but it builds up more progressively and slowly over time [12]. The remaining splice isoforms of CXCL12 are all derived from alternative splicing events sharing the same first three exons, but using a different fourth exon each time, and have been designated as CXCL12γ, CXCL12δ, CXCL12ε, and CXCL12φ [13]. Among the six human CXCL12 isoforms, only the CXCL12α, -β and -γ isoforms are conserved in mice. As such, it is rather challenging to decipher isoform-specific differences (if any) in CXCL12-driven chemotaxis, mainly because of the inability to develop relevant animal models. However, a few insights have been drawn in the case of CXCL12γ, which is quite spatially and functionally diverse from CXCL12α/β. For example, the C-terminus of CXCL12γ contains a positively charged glycosaminoglycan (GAG) binding site, which allows CXCL12γ to generate more stable chemokine gradients, when compared to other isoforms [3]. Interestingly, CXCL12γ has a nucleolus localization signal, which originates in the unique fourth exon of the splice variant, allowing CXCL12 to translocate into differentiated mouse cardiac cells, whereby it exerts tissue-specific functions, unrelated to cell-to-cell communication [14].

2.3. Regulation of gene expression and alternative splicing

As opposed to the "inflammatory" chemokines that are upregulated in response to various proinflammatory stimuli, CXCL12 belongs to the group of "homeostatic" chemokines, and thus it is constitutively expressed in certain tissues, such as bone marrow, spleen, and lung. Under circumstances of hypoxia and growth arrest however, CXCL12 production can be significantly upregulated [15]. During wound repair and cancer progression for instance, CXCL12 expression can be induced in endothelial cells via the hypoxia-inducible factor-1 (HIF-1) transcription factor [16,17]. Nevertheless, there is a plethora of signals in the tumor microenvironment that can instead suppress CXCL12 expression, such as transforming growth factor-β (TGF-β). This mechanism is thought to abolish CXCL12 chemokine gradients from the primary tumor sites, thus enhancing chemotactic migration of prometastatic $CXCR4^+$ tumor cells toward the distant sites, where CXCL12 is more abundant and unaffected [18].

As mentioned, CXCL12 exists in multiple splice variants in humans and mice, all encoded by the same gene and possessing the same first three exons, but differing in the nature of the fourth exon, which gives them exclusive physico-/biochemical properties and biological activity [1,4,13]. In general, CXCL12α, and to a lesser extent CXCL12β, are the most abundant variants in adult tissues, while the remaining ones exert a narrower distribution pattern, suggesting tightly regulated mRNA splicing and/or spliced mRNA stability [13]. In a prominent example, it has been shown that the microRNA, miR-141 controls mRNA stability of CXCL12β, but not of other CXCL12 splice variants [19]. In certain disease states, such as in Chron's disease, miR-141 is downregulated in inflamed colonic tissue, which consequently promotes increased CXCL12β mRNA and protein expression [19]. Moreover, pharmacologic intervention using pre-miR-141 can sufficiently reverse the CXCL12β isoform switch, and ameliorate the severity of colonic inflammation [19]. Together, these observations suggest that different CXCL12 splice variants have distinct patterns of expression and regulation, which may be perturbed in the context of disease, including during neoplastic transformation.

3. The CXCL12-CXCR4/7 signal transduction pathway

In recent years, the intricacies of the CXCL12/CXCR4/CXCR7 signaling circuitry have profoundly emerged. It has been shown that CXCL12 signaling stimulates a diversified repertoire of downstream signaling relays, which may together regulate cancer cell survival, proliferation, invasion/migration, chemotaxis, and cancer stem cell phenotype, among other hallmarks of the disease [20]. The downstream signaling events are particularly intricate, and are further complicated by signaling pathway cross-talk, especially in the context of neoplastic disease [20]. Outlining the full spectrum of downstream signaling activation is beyond the scope of the current review. Rather, we will provide a concise overview of the key organizational principles behind CXCL12/CXCR4/CXCR7 signaling axis, to appreciate its contextual regulation of the metastatic cascade in the following sections.

3.1. The canonical CXCL12-CXCR4 pathway

The canonical/cognate receptor for CXCL12 is considered to be the C-X-C motif chemokine receptor-4 (CXCR4), also known as fusin or cluster of differentiation 184 (CD184). To-date, CXCL12 is considered as the exclusive *endogenous* ligand of CXCR4, although CXCR4 has been identified as one of several chemokine co-receptors for HIV entry into $CD4^+$ T cells [21]. The human CXCR4 gene was first reported by Bleul *et al.* (1996) [22], but various groups have cloned alternatively spliced variants ever since [23–25]. The *CXCR4* gene is located in chromosome 2q21, and is comprised of two exons with 103 nucleotides and 1564 nucleotides, respectively. As of today, five splice variants have been confirmed in humans, all likely to be translated into mature and

functional proteins. However, only two murine CXCR4 transcripts have been found, both of which are similar to human CXCR4 variant 2, and are almost identical to each other, except for a two-amino acid addition in the splicing region between exons 1 and 2 [26]. The mature CXCR4 receptor is a 48 kDa protein with 352 amino acids, and is primarily expressed by cells of hematopoietic origin [27]. *CXCR4* gene locus amplification has been suggested as an early event in many cancer types [28,29], while CXCR4 gene mutations have been reported in several cancer types and cell lines [27,30].

CXCR4 is a Class-A (rhodopsin-like) seven-pass transmembrane domain G Protein Coupled Receptor (GPCR). One of the intracellular loops of the CXCR4 chemokine receptor is coupled with heterotrimeric G proteins, composed of Gα, Gβ, and Gγ subunits, with the former bound to GDP at resting state. Upon binding of CXCL12 to CXCR4, a series of conformational changes allow CXCR4 to function as a "guanine nucleotide exchange factor", thus facilitating the exchange of the GDP for a GTP. This exchange triggers further conformational changes within the Gα subunit, eventually causing its physical dissociation from the Gβ/Gγ dimer, and the complete release of all subunits from CXCR4. The dissociated Gα and Gβ/Gγ components can then become available to interact with various intracellular mediators and second messengers, to eventually induce transcriptional changes at the target cell [31–35].

The specific downstream effectors of the CXCR4 signaling axis depend on the coupled Gα subunits, which are generally categorized into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$. For instance, $G\alpha_s$ signals through the adenyl cyclase-cAMP system, which in turn, triggers cAMP-dependent protein kinase and the MAPK pathway. On the contrary, $G\alpha_i$ inhibits this pathway and instead activates the Src tyrosine kinase. CXCR4 mostly functions through the $G\alpha_i$ family, in particular the $G\alpha_{i1}$ and $G\alpha_{i2}$, in response to CXCL12 stimulation [32,36–39]. The $G\alpha_q$ subunit is also associated with CXCR4 signaling in certain cellular contexts, such as in dendritic cells and granulocytes [20,40], and activates phospholipase C (PLC)-β, which catalyzes hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP2) into two secondary messengers, inositol (1,4,5)- trisphosphate (IP3) and diacylglycerol (DAG). Upon binding with IP3, IP3 receptor (IP3R) triggers release of Ca^{++} from intracellular stores into the cytoplasm [41,42]. DAG further promotes the activation of protein kinase-C (PKC) and the mitogen-activated protein kinase (MAPK), which are main contributors of chemotaxis [42]. Finally, the $G\alpha_{12/13}$ subunit acts primarily through Rho-GEF, which in turn activates the small G protein RhoA, and is thus involved in the regulation of cell movement, cell polarity, and chemotactic migration [43–47]. The Gβ/Gγ dimer on the other side regulates an intracellular cascade, which initially involves activation of phosphoinositide-3 kinase (PI3K), followed by the activation of serine-threonine kinase AKT, and then followed by activation of downstream targets, such as mammalian target of rapamycin (mTOR), forehead family transcription factors (e.g., FOXO), and nuclear factor-κB (NF-κB) among others. The above are not only known to regulate cancer development and metastasis, but also cross-talk with other oncogenic signaling mechanisms [48–58].

Following activation of CXCR4 by CXCL12, various G protein-coupled receptor kinases (GRKs) gain access to the now G protein-uncoupled receptor, and can phosphorylate critical residues in the CXCR4 intracellular loops, especially those positioned in the third loop (e.g., Ser^{324}, Ser^{325}, Ser^{330}, and Ser^{339}), to promote the recruitment of β-arrestins-1/2 [36,59–61]. The binding of CXCR4 to β-arrestins causes internalization of the receptor via clathrin-coated pits, and represents the major regulatory mechanism for G protein signaling attenuation and desensitization [59,61–64]. Indeed, knockout mice for either β-arrestin-2, or specific GRKs, exhibit decreased desensitization of CXCL12/CXCR4 signaling and enhanced G protein coupling to CXCR4, which translates into defective lymphocyte chemotaxis [65]. The internalized CXCR4 receptors are eventually led to lysosomal degradation, although there is increasing evidence that GPCRs may also signal from intracellular sites, such as endoplasmic reticulum, Golgi apparatus, and nucleus, after internalization and ligand binding [66–70].

3.2. The "Atypical" CXCL12-CXCR7 pathway

Although for years it has been believed that CXCR4 was the exclusive, cognate receptor for CXCL12, subsequent work demonstrated that CXCL12 also binds to and activates another G-protein-coupled receptor, CXCR7 [71,72]. Also known as atypical chemokine receptor 3 (ACKR3), CXCR7 was initially described as an "orphan" receptor, but was later demonstrated to bind to CXCL11 with lower, and to CXCL12 with higher affinity [73–75]. The mature CXCR7 protein is widely expressed in adult tissues, and plays an important role in the development of both cardiovascular and central nervous systems [76–79]. Similar to CXCR4, CXCR7 is also overexpressed in many cancers, and plays key roles in tumor development, inflammation, and progression [50,80–83].

The CXCR7 signal transduction pathway is less well-understood compared to CXCR4. It was originally thought that CXCR7 is simply a "decoy" receptor that is not bound to G proteins, and as such, it was proposed that CXCR7 exerts a "quenching" function, thus internalizing and scavenging the CXCL11/CXCL12 ligands upon receptor binding. Thus, a definitive role of CXCR7 in regulating chemotaxis has been long debated [73,84–88]. This hypothesis was further supported mainly due to the opposing CXCR4/CXCR7 functions in certain contexts, such as in breast cancer metastasis [89]. It was eventually discovered that CXCR7 propagates intracellular signaling via at least two molecular mechanisms. In the first mechanism, a narrow set of cells, for example astrocytes, was found to utilize a CXCR7/$G\alpha_i$-dependent mechanism, to elicit ERK/Akt phosphorylation in response to CXCL12 and CXCL11. This mechanism could not be reproduced in a broader context however, thus suggesting that astrocytes and glioma cells may be the only candidate cells, capable of hijacking the pathway via G proteins [90–93]. In the second mechanism, CXCR7 was found to activate downstream pathways, like ERK/Akt, MAPK and JAX/STAT in a β-arrestin-dependent manner, thus functioning in a G protein-independent fashion [94–97]. These data collectively suggest that although in principle, CXCR7 acts antagonistically to CXCR4, its functions extend beyond just restricting CXCR4 signaling.

3.3. Contextual regulation of the CXCL12-CXCR4/7 pathway

As mentioned above, a key regulatory mechanism of CXCL12-CXCR4 signaling pathway is the antagonistic effects of CXCR7, since both receptors compete for the bioavailable CXCL12 ligand. Although a co-expression of CXCR4 and CXCR7 has been observed in many cell types, including tumor cells [72,89,98–102], their precise interactions and cross-talk has been rather underexplored. At least two distinct modes of CXCR4-CXCR7 interaction/cross-talk have been proposed. The first mode suggests that CXCR7 can regulate CXCR4 signaling by scavenging CXCL12 from the surrounding environment, thus finetuning the CXCL12 gradient [103]. For example, primordial germ cell migration in zebrafish is highly dependent on CXCR7-mediated scavenging of CXCL12, because excess CXCL12 production at the destination site can eliminate the gradient's capacity to chemotactically attract these cells [86]. The second mode suggests a direct physical interaction between the two receptors with potential of altering the signaling propagation. In a prominent example, it was demonstrated that the hetero-oligomerization of CXCR4/CXCR7 enforces the negative modulation of CXCR4 by CXCR7, thus impacting the ability of T lymphocytes to adhere to integrins and to traffic into and out of tissues [104]. However, another study suggested that the knockdown of CXCR7 in T cells potentiates their sensitivity to CXCL12 gradients, due to CXCL12 scavenging, and not due to physical dissociation of the CXCR4/CXCR7 hetero-oligomer [92].

At the transcriptional level, hypermethylation of the CXCL12 promoter and hypomethylation of the CXCR4 promoter have been reported as frequent events during neoplastic transformation, and lead to CXCL12

down- and CXCR4 upregulation [105,106]. The combination of these epigenetic events results in the relatively lower expression of CXCL12 in the primary tumor site, when compared to other sites, such as bone marrow, liver, and lungs, which allows $CXCR4^{+}$ tumor cells to become more sensitive to seeding at these distant sites [29]. Therefore, dissemination-competent tumor cells hijack a typical behavior of immune cells, which uses homing chemokine signaling to traffic into and out of lymphoid organs and peripheral tissues [29]. In addition, various microRNAs have been found to regulate the CXCL12/CXCR4 pathway at the post-transcriptional level, thus affecting cancer development and progression [5]. Perhaps the most prominent regulatory mechanism is the regulation of mRNA stability of different CXCL12 splice isoforms by specific mRNA, as is the instance of mir-141/CXCL12β [19]. Dysregulation of such microRNAs observed during neoplastic transformation could account for selective CXCL12 splice isoform biases, which may in turn give different signaling properties in the tumor microenvironment.

Homodimerization and higher-order oligomerization of CXCR4 receptors has been reported in certain cellular contexts, especially when CXCR4 is highly expressed [107–109], as frequently seen during neoplastic transformation [110,111]. This observation could explain for example the high dependency of tumor cells on the CXCL12/CXCR4 pathway, to achieve their metastatic potential. CXCR4 oligomerization is a key regulatory mechanism of the CXCL12/CXCR4 pathway because the conformational landscape of CXCR4 dimers/oligomers along with their bound ligand(s) produces different downstream signaling properties, propensity for receptor internalization, and signaling outcome strength [112–114]. CXCR7 can also form homodimers, although the functional consequences of this phenomenon are not well-understood [92,115]. To make matters even more complicated, both CXCR4 and CXCR7 have been shown to form heterodimers with other receptors, again with functional consequences of altering downstream signaling events. For example, heterodimerization of CXCR4 and CCR2 yields higher Ca^{++} responses compared to either monomer alone, but the heterodimer loses the ability to bind to and signal through the $G\alpha_{13}$ subunit [107,116,117]. In another paradigm, CCR7 was demonstrated to be unable to properly localize in the plasma membrane of T cells, unless it formed a heterodimer with CXCR4 [118]. Similarly, CXCR7 has been shown to form heterodimers with the CXCR4 receptor, as well as with receptors of different GPCR classes, such as α1-adrenoreceptor (α1-AR) in vascular smooth muscle cells, exerting a regulatory-inhibiting role on the latter [119].

Altogether, the functions of both CXCR4 and CXCR7 seem to be strongly influenced by context-dependent transcriptional and post-transcriptional mechanisms, a highly complicated intracellular signaling cross-talk, and multiple interacting partners found in the cells. As a consequence, CXCR4/CXCR7 functions may vary significantly in different cell types. More profound complexity is anticipated in various pathologies, and especially during neoplastic transformation, due to the extreme heterogeneity of the transcriptional, post-transcriptional, and translational landscape.

4. Biochemical properties of CXCL12

Because CXCL12 primarily functions as a homing chemokine for a variety of immune cells within normal lymphoid as well as within neoplastic tissues, its ability to form stable gradients by binding to extracellular matrix glycosaminoglycans (GAGs), and the biochemical balance between production/degradation are pivotal to ensure proper CXCL12 biological activity. This chapter will explore key biochemical features of CXCL12, which are critical for its chemokine function, with relevance in the context of neoplastic progression.

4.1. Glycosaminoglycan binding and chemokine gradient formation

GAGs are unbranched polymers that harbor negatively charged sulfated disaccharides, allowing them to participate in various interactions with positively charged protein structures, and as such, their interactions with chemokines are paramount for the formation of chemokine gradients [120]. The interaction of CXCL12 with GAGs, such as heparin and heparan sulfate, is especially critical for the proper tethering and "presentation" of CXCL12 in tissues and blood vessel endothelia [121–123]. Moreover, CXCL12-GAG interactions promote CXCL12 homodimerization, which further strengthens the chemotactic cues elicited as a result [123]. Indeed, when GAG-binding motifs are specifically eliminated from CXCL12α, CXCL12β, and CXCL12γ isoforms using genetically engineered mouse models, there is critical failure in the induction of the CXCL12 chemokine gradient, and as a consequence, a failure in the CXCL12/CXCR4-dependent homing mechanism of hematopoietic stem cells (HSC) in the bone marrow [124].

Detailed investigations of the GAG-binding region of CXCL12 revealed the β-strand cluster $Lys^{24}His^{25}Leu^{26}Lys^{27}$, which follows the BBXB motif, as critical for CXCR4-dependent leukocyte diapedesis in response to CXCL12 gradients in tissue endothelia [122,125]. Besides this dedicated motif, it has been postulated that CXCL12 retains other positively-charged regions, which could be necessary for GAG interactions. For example, the selective truncation of the first two N-terminal amino acids, which includes a positively charge Lys residue, results in reduced heparin-binding capability [123,126,127]. It has also been proposed that the tertiary/quaternary structure of CXCL12 is another critical factor bringing together individual and isolated amino acid residues, thus creating positively-charged "pockets", capable of GAG-binding with high affinity [122]. Finally, certain spliced isoforms, such as the CXCL12γ, have been revealed to exert higher GAG-binding activity when compared to the others, mainly because the alternatively spliced variants possess exons with higher content of positively charged amino acids. In this case, CXCL12γ exhibits a C-terminal 20-amino acid long extension, which is composed of ~60% positively charged amino acids that follow the BBXB motif [128–130]. It could therefore be assumed that changes/shifts in the splice isoform ratio during neoplastic progression could significantly alter the capacity of CXCL12 in forming chemokine gradients, even in the absence of a change in CXCL12 expression [131].

4.2. Proteolytic truncation and inactivation

Following controlled transcription and translation, CXCL12 activity is regulated extensively through enzymatic and chemical modifications at the post-translational level. Among those, the selective truncation/inactivation of CXCL12 through a plethora of extracellular proteases, is perhaps the most important regulatory mechanism altering its biological activity, e.g., the GAG-binding and receptor-binding activities. The proteolytic truncation of CXCL12 is mediated via multiple extracellular proteases. which selectively target either the N- or the C- terminus of the mature chemokine, thus the CXCL12 degradation products are reflected by spatiotemporal expression of these proteases in normal tissues, or by dysregulated expression in disease states, such as cancer [1].

Cleavage sites at the CXCL12 N-terminus have been identified for serine protease dipeptidyl peptidase IV (DPP4) [132–138] and VIII (DPP8) [139], neutrophil elastase [140], matrix metalloproteinases (MMP)− 1, − 2, − 3, − 9, − 13, and − 14 [141], and cathepsin-G [142], and even undetermined proteases [126]. Due to cleavage of the first 2–6 amino acids of the N- terminus, these enzymatic truncations primarily interfere with the CXCR4-binding domain, which partially or completely eliminate CXCL12-CXCR4 interactions. Nevertheless, a

reduced GAG-binding activity has also been demonstrated for CXCL12 variants truncated at their terminus, possibly because the misfolding of truncated CXCL12 alters the tertiary structure and exposure of its GAG-binding domains [1]. Interestingly, proteolytic truncation by DPP4 does not only diminish the ability of CXCL12 to bind to CXCR4, but it causes an increased and biased affinity towards binding to the atypical CXCL12 receptor, CXCR7 [127], thus providing an additional layer of complexity in the spatiotemporal regulation of CXCL12 activity. C-terminal truncation has also been reported, especially for the CXCL12α and CXCL12φ isoforms, which possess C-terminal Lysine (Lys) and Arginine (Arg), respectively [135,143,144]. These amino acids can be recognized and cleaved by two different carboxypeptidases, the membrane-bound -M (CPM), and the soluble -N (CPN), both leading to moderate decrease in the ability of CXCL12 to induce chemotaxis and GAG-binding [143,144]. Because C-terminal truncation is significantly less potent than N-terminal truncation, it has been proposed that the former represents the first step of a proteolytic cascade that makes CXCL12 more vulnerable and available to the N-terminal proteases [135]. Finally, a different mode of C-terminal truncation is mediated by another member of the cathepsin family, cathepsin-X, which sequentially removes one amino acid at a time from the C-terminus of CXCL12, until a Proline (Pro) amino acid is encountered [145]. Via this modification, cathepsin-X can sequentially cleave up to 15 amino acids from the C-terminus, thus significantly reducing CXCL12 activity.

4.3. Nitration and citrullination

Besides proteolytic inactivation, CXCL12 citrullination and nitration have been reported as two additional forms of post-translational modification. Citrullination occurs after hydrolysis of Arg into citrulline, under regulated catalysis of any of the five peptidylarginine deiminase (PAD) isozymes that are found in mammals [146]. Under in vitro conditions, PAD2 has been shown to induce conversion of Arg residues into citrulline, leading to weakened CXCL12 chemotactic strength and receptor-binding [147]. In particular, CXCL12 possesses multiple Arg residues in its N-terminal domain, and the degree of Arg citrullination is proportional to the loss of CXCL12 activity [147]. PAD2 and other PAD enzymes are widely expressed in many cancers, both in tumor cells and in host immune cells, whereby they induce widespread citrullination of many proteins, including histones, with often underappreciated functional consequences [148].

The nitration of aromatic Tyrosine (Tyr) and Tryptophan (Trp) residues is mediated by peroxynitrite, a product originating from the chemical reaction between superoxide anion and radical nitric oxide [149]. Key enzymes regulating production of nitric oxide are known as nitric oxide synthases (NOS), which are constitutively expressed in many cell types [e.g., neuronal (nNOS), and endothelial (eNOS)], but they can also be induced via proinflammatory stimuli (iNOS) in immune cells, such as macrophages and neutrophils [149]. CXCL12 nitration under peroxynitrite incubation in vitro, or under inflammatory conditions with iNOS induction in vivo, both impair its ability to function as chemoattractant for lymphocytes and monocytes, and suppress leukocyte diapedesis in intra-articular injection animal models [150,151]. In cancer, the expression of NOS, and especially iNOS, is very high, and it has been established that nitric oxide modulates various cellular activities and cancer hallmarks, including angiogenesis and invasion/migration [152]. Whether Tyr/Trp nitration in certain proteins, such as CXCL12, plays a fundamental role in the process remains to be elucidated. Together, observations presented in this section suggest that various elements, factors, enzymes, and pathways within the tumor microenvironment, which may be perturbed in the course of neoplastic progression may have indirect impact on CXCL12 activity, without necessarily changing its expression levels.

5. Emerging roles of the CXCL12/CXCR4/CXCR7 pathway in cancer metastasis

Although the CXCL12/CXCR4 pathway is hijacked by tumor cells to achieve many cancer hallmarks, such as survival and proliferation, the chemotaxis-regulating nature of the pathway has placed it at the frontier of the invasion and metastasis hallmark. After the premise that "cancer metastasis is a multistep process" was proposed [153], the CXCL12/CXCR4 pathway has been viewed as a master regulator of the metastatic cascade in its entirety, in cancers of different embryological origins, including breast [154–156], lung [157], gastric [158–161], colorectal [162–165], ovarian [166–168], prostate [169], melanoma, [162,170] esophageal [171–173], bladder [174], osteosarcoma [175], neuroblastoma [176], glioblastoma [177] and acute lymphoblastic leukemia [178], among others. In this section, we will critically review available literature that demarcates molecular and cellular mechanisms on the involvement of CXCL12 and CXCR4/7 in the metastatic cascade, focusing on both the traditional steps of metastasis (e.g., epithelial-to-mesenchymal transition, intravasation/extravasation, cancer cell seeding, and colonization), and emerging biological programs that support the metastatic cascade (e.g., cancer stem cell induction, immune cell invasion, and angiogenesis).

5.1. Epithelial-to-mesenchymal transition

The epithelial-to-mesenchymal transition (EMT) program recapitulates a developmental process, through which epithelial cells acquire mesenchymal properties, and when enabled in the course of cancer progression, it helps tumor cells acquire the essential properties to enter the invasion and metastasis cascade [179–186]. Although proinvasive/promigratory properties associated with the metastatic cascade may be acquired in the absence of an EMT, this is a rather rare, and context-dependent phenomenon [187–195], suggesting that the loss of epithelial morphology in carcinoma cells is a major prerequisite of the invasion/metastasis hallmark. As paralleled through developmental studies, EMT in the course of cancer progression is mainly characterized by loss of cell-cell adhesion and cell-matrix adhesion ability, as well as by morphological changes of cell shape and membrane polarity, and the subsequent development of invasive protrusions [179−181,196−198]. There is now ample experimental evidence that the CXCL12/CXCR4/CXCR7 axis regulates EMT in cancer cells [199–201], although the underlying mechanisms are not completely understood.

Although it has been established for a long time that cancer-associated fibroblasts (CAFs), the main producers of CXCL12 in the tumor microenvironment, are capable of promoting EMT in tumor cells, it has not been deciphered whether CXCL12 is directly involved in EMT, given that other CAF-secreted factors, such as TGF-β and IL6, are also known to be highly-transformative [9,202–207]. In one study, an overexpressing CXCL12 derivative of the parental MCF7 human breast cancer cell line was developed, and exhibited dramatically increased proliferative, migratory, and invasive capabilities [208]. Importantly, these phenotypic augmentations were associated with the down-regulation of E-cadherin and the simultaneous upregulation of vimentin, N-cadherin, and α-smooth muscle actin [208], which represent the most archetypal indicators of an active EMT program. Interestingly, this study also showcased that the overexpression of CXCL12 in MCF7 cells promoted the increase of OCT4, Nanog, and SOX2 [208], which all are well-known factors of pluripotency and stem cell reprogramming, confirming that EMT and the stem cell program are closely related in the context of cancer [183,209−211]. In this model system however, CXCL12-driven EMT induction was shown to be dependent on the Wnt/β-catenin pathway [208], and the role of the CXCR4/CXCR7 was not clarified, thus offering the possibility that CXCL12 regulates EMT in a non-canonical fashion. In another study however, in which neuroendocrine tumor (NET) cell lines were investigated, CXCL12 treatment was demonstrated to induce EMT with expected changes in cell morphology

and the reported cadherin switch, but the EMT phenotype in NETs was abrogated upon genetic CXCR4 ablation [212], indicating that the CXCL12/CXCR4 pathway may indeed directly convey EMT-promoting signals in cancer cells.

It is now well-established that certain pleiotropic transcriptional factors, such as Snail, Slug, Twist, and Zeb1/2, are master orchestrators of EMT during embryogenesis, and as an extension, during cancer progression. Overall, these transcriptional regulators are spatiotemporarily expressed in various combinations and in a wide variety of malignant cells, and can modulate the key transcriptional and morphological events associated with EMT [213–216], as outlined above. There is now available evidence that the CXCL12/CXCR4 pathway may signal through, or better, affect the expression of several of these transcriptional EMT regulators. In a few studies, for instance, the overexpression of CXCL12, or even a basal activity of CXCR4, was capable of inducing downregulation of E-cadherin along with upregulation of mesenchymal markers, such as vimentin/fibronectin and matrix metalloproteinase-2, as well as an increase in certain transcriptional regulators, such as Slug and/or ZEB1 [217,218]. In salivary adenoid cystic carcinomas, which represent a unique tumor microenvironment whose metastatic cascade is directly associated with perineural invasion at the tumor-nerve interface, it was demonstrated that CXCL12/CXCR4 could induce EMT exerting both the archetypal EMT markers and Schwann cell hallmarks [219]. Subsequent investigations revealed that genetic silencing of the transcriptional EMT regulator Twist could entirely impair CXCL12/CXCR4-dependent EMT and perineurial invasion [219]. These data suggest that the CXCL12/CXCR4 axis may induce EMT in various malignant cancers, by utilizing EMT programing as elicited by the traditional transcriptional EMT master regulators.

As mentioned earlier, signaling of CXCL12 via the G-protein coupled receptor CXCR4 may yield a plethora of intracellular signaling pathways, including, but not limited to MAPK, PI3K/AKT, PKC, and JAK/STAT pathways. Therefore, pursuing the precise cellular signaling relays and second messengers leading up to CXCL12-dependent induction of the master transcriptional EMT regulators is a challenging task. In this context, the nuclear factor kappa-B (NF-κB) pathway has been recently identified as an attractive intracellular mediator of CXCL12-dependent EMT [217,220]. In one study, it was demonstrated that the CXCL12/CXCR4 axis activates NF-κB to induce EMT, as evidenced by E-cadherin decrease and N-cadherin increase in cancer cells [221]. Along the same lines, it was also shown that a pharmacological IκB phosphorylation inhibitor could significantly attenuate the CXCL12/CXCR4-dependent EMT, and invasive/migratory phenotype [221]. In general, the pleiotropic NF-κB pathway has been linked to a plethora of cancer hallmarks, including regulation of EMT. In particular, the nuclear translocation of NF-κB p65 has been shown to directly regulate the cadherin switch, but also to induce the transcription of a second wave of EMT regulators, such as transcription factors ZEB1/2, and Slug, as well as other EMT-promoting growth factors, such as TGF-β [222]. Although these observations may explain why CXCL12/CXCR4 does not seem to signal directly through the traditional transcriptional EMT regulators, it can still induce their upregulation, thus triggering a cascade of EMT-promoting events.

Although the aforementioned studies demonstrate that the CXCL12/CXCR4 axis upregulates and may thus function upstream of the master transcriptional EMT regulators, the possibility of CXCL12/CXCR4 contributing to EMT as a downstream event has also been proposed. For instance, retroviral-enforced expression of Slug in prostate cancer cells has been shown to upregulate CXCR4 expression, and as a consequence, prostate cancer cell sensitivity to CXCL12-mediated cell invasion and migration [223]. However, the same study demonstrated that CXCL12 was necessary in Slug-induced MMP9 overexpression in prostate cancer cells, because the ablation of CXCL12 using shRNA in this model system failed to induce a complete EMT through Slug transcription factor [223]. These data collectively suggest that regardless of whether CXCL12/CXCR4 functions as upstream orchestrator of EMT or a downstream/later event, it is an indispensable pathway to the full completion of the EMT program.

Although both CXCR4 and CXCR7 have been identified as equal EMT-triggering receptors in certain cancer models, such as in obesity-associated EMT in prostate cancer [224], and in JAK/STAT and manganese superoxide dismutase (MnSOD)-dependent EMT [225], many studies have also dictated receptor-specific bias towards EMT induction. In esophageal cancer for example, CXCL12/CXCR7 has been reported as a potent EMT-promoting pathway, while CXCR4-dependent signaling fails to induce EMT [171]. When investigating in more detail the downstream events there is sufficient evidence that the CXCL12/CXCR7 axis induces EMT through similar transcriptional EMT regulators (e.g., Snail), and intracellular signaling cascades (e.g., JAK/STAT and PI3K/AKT) as CXCR4. As such, the nature of the observed CXCR4-CXCR7 bias in EMT regulation in certain scenarios is poorly understood. In one notable example however, CXCL12/CXCR7 was demonstrated to induce EMT in prostate cancer cells through nuclear translocation of YAP1 and the subsequent targeting of vimentin and doublecortin-like kinase 1, a sequence of events that could not be reproduced via CXCR4-dependent signaling [226]. It was eventually demonstrated that the biased CXCR7 signaling through β-arrestin1 was the upstream trigger for the YAP1-dependent EMT-promoting signaling relay [226]. The propensity of CXCR7 to induce stronger recruitment of β-arrestin1 could be one possible explanation of CXCR7-specific EMT.

To summarize (Fig. 1), the involvement of the CXCL12/CXCR4 pathway in regulating EMT, can be viewed from a dual standpoint. On one side, CXCL12/CXCR4 can signal through its canonical signaling pathways such as PI3K/AKT, JAK/STAT, NF-κB and MAPK, to promote EMT through transcriptional EMT regulators (Snail, Slug, Twist, Zeb1/2). On the other side, the induction of these master transcriptional regulators from tertiary EMT-promoting pathways could lead to CXCR4 overexpression. Regardless, the CXCL12/CXCR4 axis is capable of orchestrating the induction of conventional EMT pathways and hallmarks, although certain biases between CXCR4 and CXCR7 have been proposed with regards to the downstream EMT effector molecules.

5.2. Cancer stem cell induction and maintenance

The cancer stem cell (CSC) hypothesis proposes that a small subpopulation of cells within a tumor, called “cancer stem cells”, are responsible for tumor initiation, progression, metastasis and therapeutic resistance. CSCs have the ability to self-renew and differentiate into multiple cell types within the tumor, including non-stem cancer cells, and allow tumor cells to adapt to hostile conditions and cellular/molecular barriers that are encountered along the metastatic cascade. The existence of CSCs has been demonstrated in a wide range of solid and hematological cancers, and their presence is associated with poor prognosis and increased risk of disease recurrence. CSCs are surrounded by various stromal and immune cells, blood and lymphatic vessels, and the extracellular matrix (ECM), creating a CSC “niche”, which promotes tumor cell survival, immune evasion, migratory and invasive potential, therapeutic resistance, plasticity, and self-renewal, most of which are paralleled with biological programming of normal adult stem cells [227]. In general, the CSC niche components include a constellation of cytokines, chemokines, growth factors, the ECM itself, and extracellular vesicles, such as exosomes, which together exert a paracrine effect on CSC homeostasis and behavior. As will be discussed in the current section, accumulating evidence suggests that CXCL12, which is mainly produced by mesenchymal stem cells (MSCs), cancer-associated fibroblasts (CAFs), and CSCs themselves within the tumor microenvironment, may represent a crucial component of the CSC niche, capable of partially orchestrating the aforementioned CSC phenotypes.

A number of studies have spatially positioned CSCs at perivascular regions, where the CXCL12/CXCR4 chemokine pathway could serve as a major orchestrator for the contextual positioning of the CSC niche. As described above, hypoxic conditions often lead to aberrant CXCL12

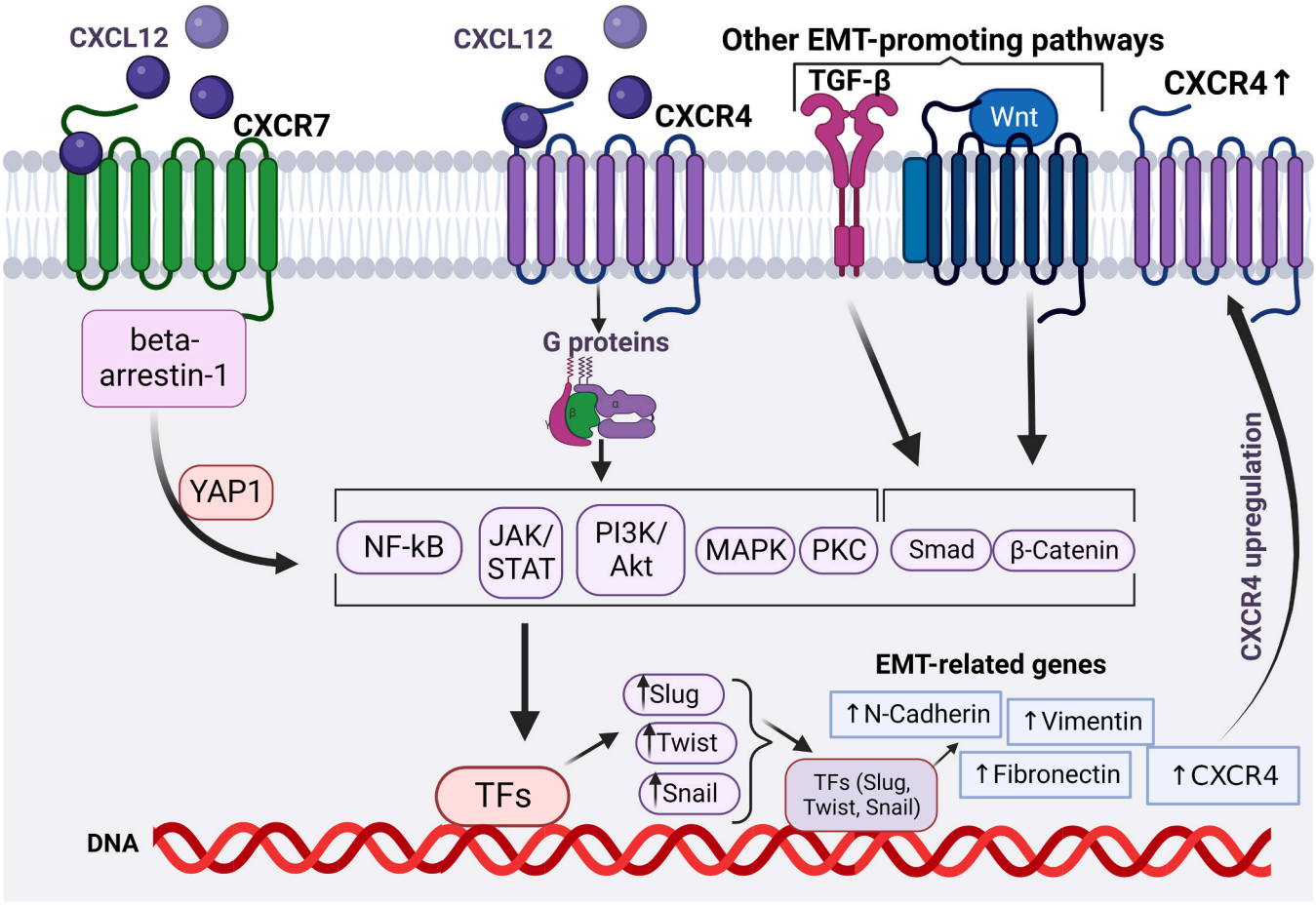

Fig. 1. Emerging Roles of the CXCL12 Axis in the Regulation of Epithelial-to-Mesenchymal Transition (EMT). The conceptual model suggests that the CXCL12/CXCR4/CXCR7 chemokine axis may represent either the cause or the consequence of EMT, depending on the context. As a putative causative factor, CXCL12 signals through CXCR4 or CXCR7 via disparate downstream effectors, to transactivate established pathways, functioning as EMT inducers (e.g., PI3K/Akt, MAPK, PKC etc.). These pathways eventually promote the transcription of other EMT-promoting transcription factors (e.g., Slug, Twist, Snail), which in turn activate the EMT program. However, under specific circumstances, i.e., when EMT transcriptional programs are activated by factors other than CXCL12 (e.g., TGF-beta, Wnt), it has been shown that CXCR4 could be one of the downstream target genes associated with EMT induction. This transcriptional relationship results in high CXCR4 expression at the cell surface, which in turn, amplifies the (pro)metastatic program, including the EMT program itself. Illustration created by BioRender (biorender.com).

production, generating intratumoral CXCL12 chemokine gradients that originate in the vasculature and diffuse into the tumor parenchyma. In response to such CXCL12 gradients, $CXCR4^+$ stromal cells, which include MSCs, EPCs, and $CXCR4^+$ myeloid cells (e.g., macrophages) are chemotactically attracted and home in the perivascular niche. Of note, most of these cells, in particular the $CXCR4^+$ myeloid suppressors and macrophages often exhibit strong immunosuppressive properties, particularly due to production of mismatching cytokines for T cell trafficking and expression of inhibitory checkpoint receptor ligands, such as PDL1. Moreover, these cells, in particular EPCs and other stromal cells may exert angiogenic and vasculogenic properties, either directly by affecting the adjacent endothelium, or indirectly by remodeling the perivascular ECM. Finally, these cells, especially the perivascular $CXCR4^+$ macrophages co-expressing the receptor tyrosine kinase Tie2, may locally remodel the tumor microenvironment and pave the pathway in a manner that favors metastatic dissemination and transendothelial migration of CSCs [228–231]. Interestingly, in a glioma cancer model, it has been determined that the CSC themselves attract $CXCR4^+$ EPCs from the bone marrow into the CSC niche, by secreting CXCL12 in the perivascular space [232]. In breast cancer, which is relatively refractory to most common immunotherapies, it has been shown that CXCL12 not only sustains breast CSC self-renewal and the CSC niche, but it also promotes tumor progression and immune escape programming [233]. Together, these observations suggest that the CXCL12/CXR4 pathway is crucial for the construction of a proangiogenic, prometastatic, and immunosuppressive CSC niche.

Besides assembling the cellular/molecular infrastructure of the CSC niche as described above, the CXCL12/CXCR4 pathway is also responsible for the induction and maintenance of CSCs through stimulation and reinforcement of critical CSC properties, such as environment-mediated resistance to chemotherapy/radiotherapy and self-renewal of CSC compartment. Of note, CXCR4 is the most commonly expressed receptor and is used as a surface marker to identify CSCs of many different human tumor types [230,231,234,235]. Specifically, CXCR4 defines CSC subpopulations in pancreatic [235], breast [236], renal [237], gastric [238], colorectal [239], glioma [240–242], liver [243], and lung [239,244–246] cancers. In pancreatic ductal adenocarcinoma (PDAC), $CXCR4^+$ CSCs were identified as an exclusive metastatic subpopulation of cells, and metastasis was completely abrogated after depletion of this CSC subset [235]. Inhibition of CXCL12/CXCR4 signaling in another study looking at PDAC led to decreased tumorigenicity in vivo [247]. In a study looking at Lewis lung carcinoma cells, the $CXCR4^+$ CSC subset was found to be more proangiogenic and metastatic [244]. In non-small cell lung carcinoma, $CXCR4^+$ CSCs were shown to confer chemoresistance and increased metastatic abilities. Inhibition of CXCR4 decreased the migratory and metastatic abilities of the lung CSCs [245]. In glioma and glioblastoma, inhibition of CXCR4 decreased tumor cell survival and tumor progression [241,242]. Treatment of glioblastoma

stem cells with radiation therapy increased invasion of a subset of cells, which were found to be radioresistant and characterized by increased CXCL12 and CXCR4 expression [248]. Together these data suggest that CXCR4^{+} CSCs may hijack the CXCL12/CXCR4 pathway in two ways, first for homing into the CSC niche similar to other supportive CXCR4^{+} niche cells, and second, for exerting prominent CSC features.

Besides hijacking the CXCL12/CXCR4 axis to maintain the CSC properties [249], less is known about whether CXCL12 can directly induce the CSC phenotype. In general, the CSC program has been investigated in close conjunction with EMT, mainly due to profuse parallels with regards to transcription factors and signaling pathways that serve as common denominators for both. Evidence on the role of CXCL12 on promoting EMT in cancer cells was provided in the prior section. Importantly, during CXCL12-mediated EMT, upregulation of transcription factors that elicit CSC programing, such as SOX2, OCT4, and NANOG, has been observed [208]. Moreover, CXCR4 overexpression in tumor cells is mediated by the microenvironment, which primarily includes hypoxia (e.g., HIF1a) and conditions of chronic inflammation that are often encountered during cancer progression (e.g., NF-κB), both of which are well-known to promote EMT, and to constitute critical elements of the CSC niche [217,250,251]. Through the development of the CSC niche, CSCs could come in physical contact with niche cells, and a recent study has determined that juxtracrine contact with macrophages is essential to induce a CSC program [252]. In the future, more studies are necessary to determine if the CXCL12/CXCR4 pathway is directly and causatively involved in CSC induction, or if it simply serves as a chemotactic determinant that brings in close proximity all the cell and molecular elements that are necessary for the *de novo* induction of prometastatic CSCs.

To summarize (Fig. 2), the involvement of the CXCL12/CXCR4 pathway in CSC programing in the tumor microenvironment includes primarily the generation of the CSC niche, mediated by the chemotactic recruitment of key niche cells, such as mesenchymal cells, macrophages and fibroblasts. However, CSCs also express CXCR4 with direct implications on their intrinsic properties, such as ability to self-renew and metastasize. Overall, the CXCL12/CXCR4 axis orchestrates an immunosuppressive sanctuary at the perivascular niche, from where highly aggressive CSCs may undergo their metastatic journey unscathed.

5.3. *Cancer cell migration, intravasation and metastatic dissemination*

Cell migration is a critical component of the metastatic dissemination of tumor cells from the primary tumor to either local or distant sites, and is typically driven by tumor cells that have already undergone an EMT program [253–261], as discussed in the previous section. Although tumor cells generally can exert a hyper-motile phenotype, characterized by random migratory behavior, it is only *directional migration* that gives them a profound competitive advantage to disseminate distally, overcome physical/chemical barriers, and survive within a hostile tissue microenvironment [87,262–266]. Both invasion within the primary tumor and dissemination to distant sites require chemotaxis, among other forms of cell migration, such as haptotaxis [262]. The CXCL12/CXCR4 pathway in particular is a central chemokine pathway, which is hijacked by tumor cells to achieve proinvasive/promigratory behavior [29]. Indeed, *de novo* expression of CXCR4 has been detected in at least 23 different types of cancer, while expression of other chemokine

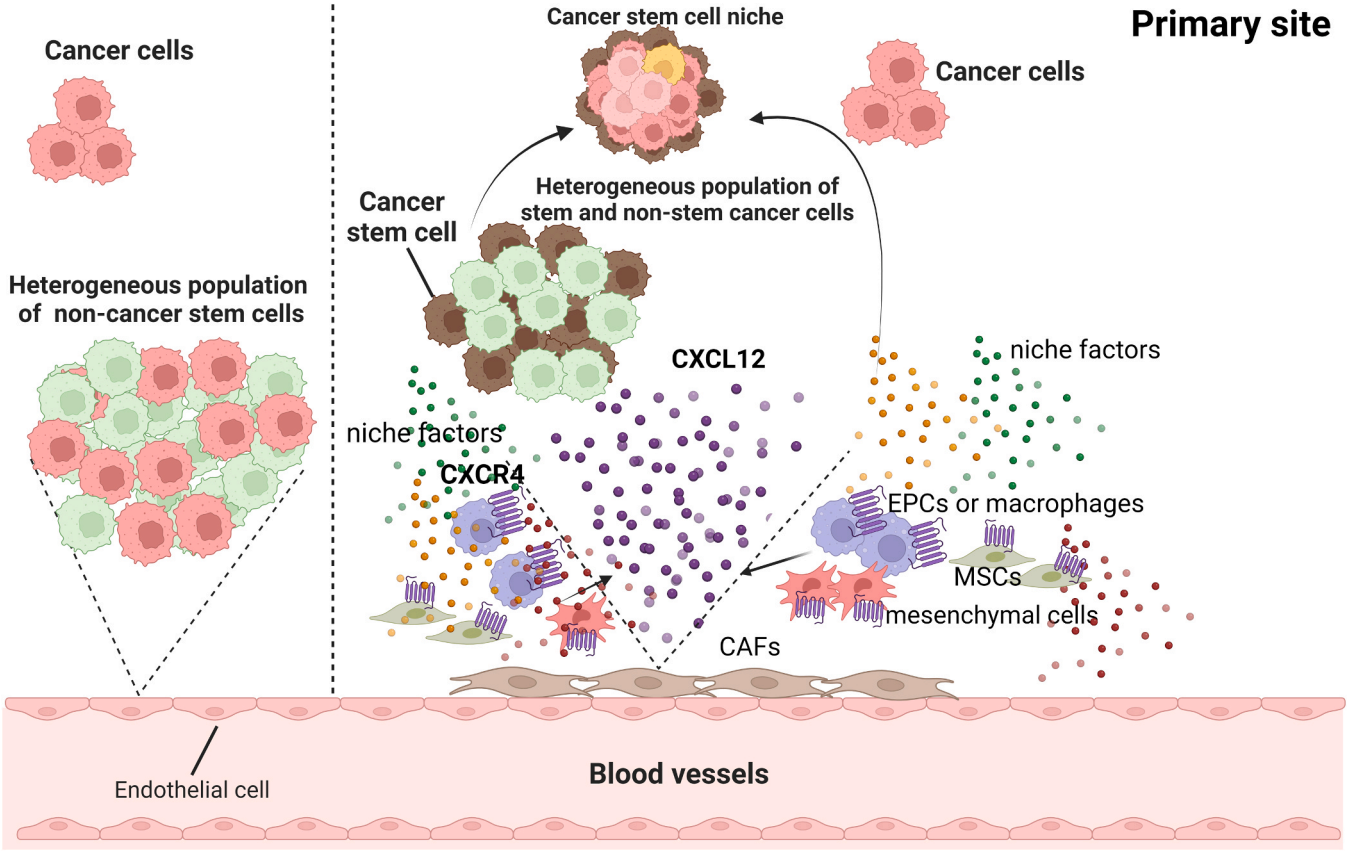

Fig. 2. Emerging Roles of the CXCL12 Axis in the Regulation of the Cancer Stem Cell Niche. The conceptual model suggests that intratumoral CXCL12^{High} niches that are supported by CXCL12-producing cells, such as for example the perivascular cancer-associated fibroblasts (CAFs), can attract various CXCR4^{+} cells from the local tumor microenvironment, such as wandering macrophages and/or mesenchymal stem cells. In turn, these cells may "enrich" the CXCL12^{+} niche by secreting a plethora of cancer stem cell (CSC) factors, collectively termed "niche factors", which nurture and maintain CSCs. On the left side of this conceptual model, a hypothetical CXCL12^{low} niche is visualized in a CAF-deficient tumor microenvironment, which does not contain the essential "niche factors" to maintain CSCs. Illustration created by BioRender (biorender.com).

receptors, such as CXCR7, has also been documented [267–269]. Together, these observations indicate that prometastatic tumor cells mimic the behavior of immune cells by overexpressing chemokine receptors, thus utilizing chemokine gradients to traffic across the different tissues and organs [29,267]. This section will focus on emerging concepts that link CXCL12/CXCR4-dependent chemotactic invasion/migration of tumor cells in primary tumors.

When single cell imaging was pioneered with the use of multiphoton intravital microscopy in live mice, the process of directed cell migration was adequately resolved, and the simplistic paradigm of tumor cells going through the metastatic cascade as single entities, was radically reformed [270]. Tumors possess complex microenvironments, which entail multiple migratory behaviors by prometastatic tumor cells, which include distinct patterns of single cell migration, such as amoeboid [192−194,271−274] or mesenchymal [258,275−279], and patterns of multicellular migration, such as collective [191,280−286] or streaming [287–291]. Chemokine signaling has been extensively studied in this context, for multiple cancers, although their precise impact on the migration pattern is not always assessed. Regardless, the CXCL12/CXCR4 axis has been implicated in both single cell and multicellular directed migration during metastatic dissemination. In an early study by Koshiba et al. (2000), it was shown that single cell migration of pancreatic cells, highly expressing the CXCR4 receptors, was highly dependent on CXCR4 signaling, using standard in vitro migration assays [292]. In another study by Scotton et al. (2002), it was shown that various ovarian cancer cell lines could invade through Matrigel toward a CXCL12 gradient, in a CXCR4-dependent manner, and the phenotype was abrogated upon broad-spectrum matrix metalloproteinase and TNFα converting enzyme inhibitors. Interestingly, single CXCL12/CXCR4-dependent invasion was noted at the single cell level, but also in highly metastatic ovarian cancer cell lines developed from the ascitic fluid [293], which are known to form multicellular spheroids to invade the peritoneum [294–296]. In another study by McCutcheon et al., the authors developed a microfluidic device to study the response of medulloblastoma, medulloblastoma-derived glial progenitors, and retinal progenitor cells, to CXCL12 gradients, either at single cell level, or after formation of neuroclusters. The study revealed increased CXCR4 expression upon spontaneous neurocluster formation, but variable propensity of collective migration towards CXCL12 concentration gradients [297]. These data suggest that the CXCL12/CXCR4 pathway represents a non-discriminatory chemotactic force in the tumor microenvironment that attracts $CXCR4^{+}$ tumor cells, regardless of whether these are spatially located as single or collective cell entities.

Revolutionary intravital imaging of live tumors at single cell resolution revealed a type of collective migratory pattern with a unique phenotype, later described as “multicellular streaming” migration [289,290,298]. It has been specifically demonstrated that invasive/migratory tumor cells form pairs with intratumoral macrophages, and migrate in a unidirectional fashion, toward the adjacent blood vessels for transendothelial migration and dissemination. Underlying this capacity of developing the “streaming sequences”, tumor cells were found to secrete colony stimulating factor-1 (CSF1), the ligand for CSF1 receptor at the surface of macrophages, while macrophages, could instead secrete epidermal growth factor (EGF), the ligand for the tumor-cell-specific EGF receptor [299]. This highly-contextual paracrine EGF/CSF1 loop not only ensures the chemotactic attraction of the two cells with each other, but also facilitates the exchange of factors or juxtracrine signals that promote cancer cell survival, immunoevasion, induction of stem-like capabilities, and increased invasive capability. Indeed, genetic suppression of any of the aforementioned elements of the paracrine loop is sufficient in disturbing streaming migration, and as a consequence, metastatic dissemination [252,289,298,300−304]. Although this mechanism can explain the nature of these multicellular streams, it cannot efficiently explain their propensity to migrate in a unidirectional and biased manner toward the blood vessel. It was thus speculated that such multicellular streams could respond to chemokines originating in the blood vessel itself, and such chemokines represent another key prerequisite for metastatic dissemination. For instance, Leung et al. (2017) demonstrated that hepatocyte growth factor (HGF), which is abundantly expressed in endothelial cells and the plasma, could attract tumor cell/macrophage pairs in a Met-dependent manner, by forming HGF gradients from the blood vessels [305]. Moreover, Boimel et al. (2012) suggested that the EGF/CSF1 paracrine loop requires CXCR4 signaling to promote “streaming” migration [306]. Specifically, the authors utilized an in vivo migration assay in live mice, a technique that uses passive collection of tumor cells from a needle tethered in a tumor, and coated in a chemokine gradient. When such needles were coated with EGF, the multicellular streams could efficiently migrate to the interior of the needle. However, when animals were treated with AMD3100, a CXCR4 antagonist, tumor cells could no longer migrate into EGF-coated needles [306]. Although the relative contributions of HGF and CXCL12 chemokine gradients remain to be resolved intratumorally, these data collectively suggest that the CXCL12/CXCR4 pathway has a key role in directing tumor cells towards blood vessels.

To summarize (Fig. 3), the involvement of the CXCL12/CXCR4 pathway in tumor cell invasion and migration within the tumor microenvironment is based on the traditional rules of chemotaxis. It is not clear how CXCL12/CXCR4 achieves such distinct and unique migratory patterns in the prometastatic tumor cell population. To what extent the acquisition of a migratory pattern, such as multicellular or collective streaming, is regulated via disparate downstream effectors, or through chemokine cooperativity in the tumor microenvironment, remains to be resolved.

5.4. Metastatic seeding and colonization

Once the disseminated tumor cells (DTCs) seed a new metastatic site, a constellation of microenvironmental factors will determine their fate. The vast majority of DTCs are eliminated, either because they do not find appropriate nutrients, growth factors, and cytokines that are essential for their growth in this hostile new environment, or because they will be targeted and cleared by means of immune surveillance. A few DTCs, however, will be able to enter a state of dormancy that is achieved via growth arrest, and may eventually enter a dynamic state of population equilibrium, again strongly dependent on the organ microenvironment. During this period, the expansion of metastatic diseases is paused, sometimes in decade-long periods of time, but DTCs survive through this biological program, and may relapse into clinically overt macrometastasis [307–313]. As will be discussed in the current section, there exists both old and emerging literature that CXCL12 is a critical constituent of the (pre)metastatic niche, which regulates the aforementioned phenomena.

As mentioned in the previous section, circulating tumor cells (CTCs) often highly express chemokine receptors, including CXCR4, thus representing a highly invasive/migratory tumor cell subset from the primary tumor site [314–316], and represents a key molecular determinant that dictates organ-specific metastasis. When non-small cell lung cancer cells are grown into SCID mice for example, the expression of CXCR4 in tumor cells is identified in only ~30–35% of the population when measured at the primary tumor site, but in almost all the cells that form metastases [157]. As such, CXCL12 is a critical constituent and homing factor for DTCs at the premetastatic niche [29]. One study has suggested that CXCR4 signaling promotes the ability of prostate tumor cells to adhere to the bone marrow endothelium, thus facilitating extravasation and transendothelial migration for seeding into the premetastatic niche [317]. Although it is not clear how this phenotype is regulated, it has been proposed that a CXCL12/CXCR4-Akt-MMP9 axis, could contextually lead to elevated levels of MMP9, to facilitate breaching of these extracellular matrix obstacles [317]. Indeed, CXCL12 is typically produced by the microvasculature in these organ sites, which ensures that the CXCL12/CXCR4 signaling pathway will be activated as soon as any tumor cell begins the seeding process [29].

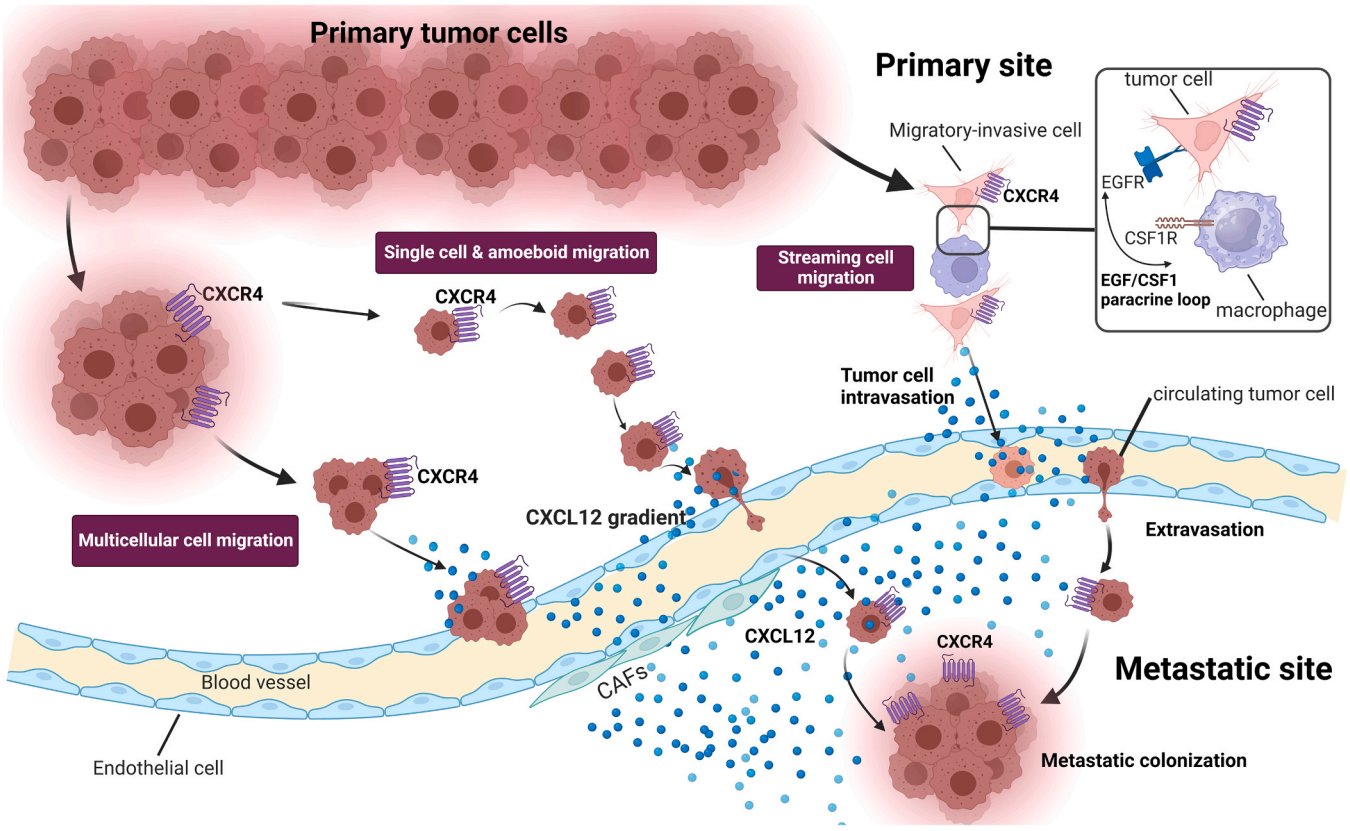

Fig. 3. Emerging Roles of the CXCL12 Axis in the Regulation of Metastatic Cancer Cell Dissemination. The conceptual model reveals the traditional and well-established role of CXCL12 in regulating chemotactic movement of CXCR4$^+$ cancer cell subpopulations from the tumor microenvironments, and selectively guiding them towards and through the cancer-associated vasculature. This model of CXCL12-dependent metastatic dissemination was originally proposed as a "hijacking" mechanism, exploited by tumor cells to mimic immune cell trafficking. Recent evidence in this context has revealed two emerging concepts: First, CXCL12-dependent chemotactic responses between the tumor parenchyma and the blood vessels are orchestrated by elaborate micro-gradients originating from spatially organized CXCL12-producing cells. Second, a wide range of cell migration patterns has been identified in promigratory tumor cells, such as unicellular migration, multicellular migration, amoeboid migration, and streaming migration, all of which seem to respond to the intratumoral CXCL12 gradients, albeit with different cellular and molecular features. Illustration created by BioRender (biorender.com).

Once the CXCR4$^+$ tumor cells reach the bone marrow, liver, or the lungs, by responding chemotactically to the high CXCL12 levels present in these organs, one could erroneously assume that the role of the CXCL12/CXCR4 pathway becomes redundant. However, CXCL12 is a critical component of the metastatic niche, and its functions extend beyond simply facilitating the dissemination step of metastasis. It has been shown that CXCL12 stimulates proliferation and survival of glioma, ovarian, small cell lung, and other cancer cells, especially under conditions of serum starvation [54,293,318], which could pinpoint CXCL12 as a critical adaptation mechanism for cancer cell survival at distant sites, where the microenvironment is less favorable. Indeed, when colon cancer cell-specific expression of CXCR4 is abrogated after metastatic colonization, the CXCR4-deficient tumor cells cannot proliferate in the distant location [319]. In another example, the antibody- or peptide-based inhibition of CXCR4 decreases the growth of pre-established prostate cancer lesions in the bone, indicating that CXCL12/CXCR4 may be necessary during the colonization step of metastasis [320]. In the case of prostate cancer, specifically, prostate cancer cells secrete CXCL12 themselves, which ensures constitutive autocrine signaling for survival in the bone marrow [320]. Interestingly, stimulation of ovarian cancer cells isolated from ascitic fluid with CXCL12, induces TNFα overexpression, which can further trigger a cascade of growth factors and cytokines promoting tumor growth [293, 321]. Finally, as will be discussed in the following two sections, there is plentiful data suggesting that CXCL12 is an inducer of angiogenesis and immunosuppressive factor. As such, the CXCL12/CXCR4 axis may not only sustain metastatic colonization directly, by positively affecting growth of metastatic cells, but also indirectly, by eliciting immunoevasive signaling and neoangiogenic signaling within the growing metastases.

Because CXCL12/CXCR4 signaling is important for growth and proliferation of DTCs and micrometastatic foci into clinically overt colonies, as described above, it has been postulated that CXCR4 may be responsible for shutting down the dormancy program in DTCs. In general, cellular dormancy in DTCs is regulated by various extrinsic factors that are locally produced by the metastatic microenvironment, such as bone morphogenetic protein-4 and-7 (BMP4/7), thrombospondin-1 (TSP1), and transforming factor beta-2 (TGFβ2) [322–326]. A recent study has revealed that CXCR4 is indeed downregulated in dormant cancer cells at the metastatic site, although the ligand CXCL12 is abundantly expressed [327]. CXCR4 downregulation was proven critical for the induction of dormancy because the treatment with CXCR4 antagonist, AMD3100, allowed the tumor cells to exit the dormant state and to start proliferating [327]. In a supportive study of prostate cancer colonization in the bone marrow, the treatment with a neutralizing CXCR4 antibody also suppressed total metastatic burden and forced

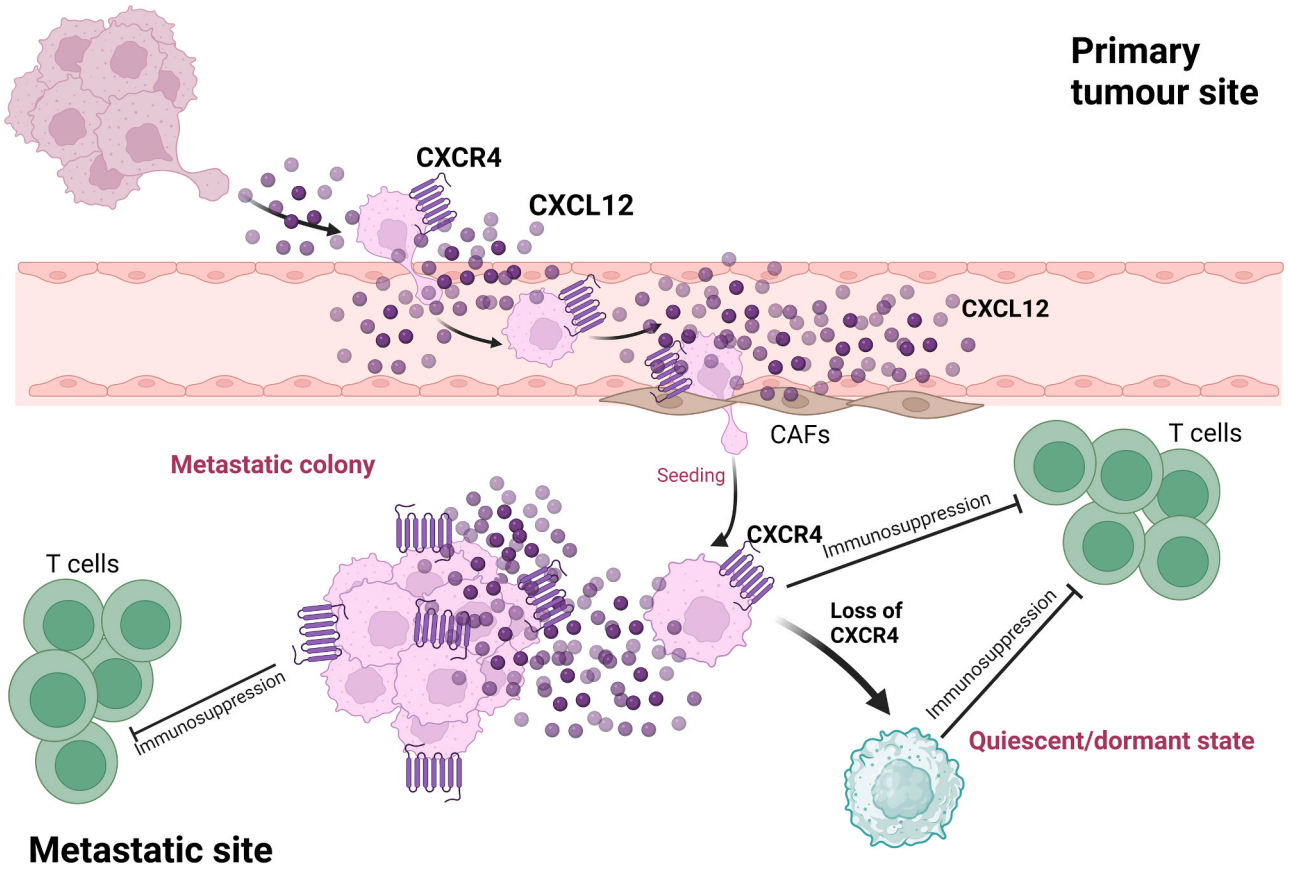

Fig. 4. Emerging Roles of the CXCL12 Axis in the Regulation of Metastatic Seeding and Colonization. The conceptual model reveals the traditional role of CXCL12 as organotropic factor in cancer metastasis. Specifically, when CXCR4$^+$ tumor cell clones are opted from the primary tumor to intravasate and disseminate under the control of CXCL12, these tumor cells follow CXCL12 gradients across the entire body of the host. Prominent metastatic sites that physiologically produce CXCL12, such as lungs, liver, and bone marrow, are preferentially selected by CXCR4$^+$ cells through chemotaxis, a phenomenon generally known as "organotropism". Besides seeding, the CXCL12$^+$ niches at secondary sides dictate additional fates for these disseminated tumor cells (DTCs). Certain DTCs epigenetically lose CXCR4 expression and are subsequently led into quiescence and cellular dormancy, exerting an immunosuppressive program against peripheral immune surveillance to thrive. However, DTCs that retain their expression of CXCR4 may undergo proliferation and generate micro- and macro-metastases. In this scenario, mechanisms of immunosuppression are also elicited to ensure proper metastatic colonization, although they may differ from dormancy-induced ones at the molecular level. Illustration created by BioRender (biorender.com).

DTCs into a quiescent/dormant state [328].

To summarize (Fig. 4), the contributions of the CXCL12/CXCR4 pathway in the final stages of the metastatic cascade have only recently begun to emerge. The available data are all supportive of a model that suggests that CXCL12/CXCR4 is critical for both the homing of CXCR4$^+$ tumor cells in the CXCL12-rich premetastatic niche, and the evolution of micro-metastases into overt metastatic colonies. As such, the pharmacological suppression of the CXCL12/CXCR4 axis could manifest as a targeted anti-metastatic therapy that locks DTCs into a perpetual state of cellular dormancy.

5.5. *Neoangiogenesis and neovascularization*

Angiogenesis, the process via which new blood vessel formation is sprouted from "parental" vasculature, is a well-established hallmark of cancer [153]. It is now widely accepted that tumor angiogenesis is contextually regulated by a complex interplay among cancer cells, endothelial cells, macrophages and other cells within the tumor microenvironment, and is not only critical for tumor growth as originally highlighted, but also for supporting tumor progression and metastasis [329]. Initial models of tumor (neo)angiogenesis described a paracrine relay, in which tumor cells secrete pro-angiogenic factors [e.g., vascular endothelial growth factor (VEGF-A)] in response to hypoxic stress, which in turn, promotes proliferation and migration of endothelial cells, which eventually leads to the formation of neoangiogenic vessels. Nowadays however, an enormous complexity of the signaling circuitries involved in the process has been appreciated, and the model of the "angiogenic switch" has been proposed. According to this model, a wide plethora of angiogenesis-promoting and angiogenesis-suppressing factors are concurrently dispersed in an evolving tumor microenvironment, and an imbalance of either or both arms may result in either a favorable or an unfavorable shift towards angiogenesis [330–335]. As will be discussed in the current section, accumulating data collectively suggest that CXCL12 is a putative pro-angiogenic chemokine, whose dominance in the tumor microenvironment can shift this "switch" towards new blood vessel formation.

In a non-neoplastic context, the CXCL12/CXCR4 pathway plays a key role in regulating angiogenesis, for instance in response to tissue ischemia and/or injury [336–339]. Within an injured/ischemic tissue, CXCR4 is upregulated in endothelial cells following hypoxia and VEGF-A production, thus increasing their sensitivity to CXCL12 expression gradients [338,340−342]. At the same time, CXCL12 is overproduced by the ischemic/injured tissues assuming the role of a damage-associated cytokine/alarmin, eventually forming an expression gradient between injured and uninjured tissue components, which may in turn chemotactically mobilize CXCR4$^+$ endothelial cells to the ischemia/injury site. An ensuing induction of CXCL12/CXCR4 signaling pathway additionally promotes survival and proliferation/expansion of the responder

endothelial cells, to support the development of neoangiogenic vessels at the sites of injury. Besides these local/paracrine interactions, CXCL12 also exhibits critical endocrine functions during physiological angiogenesis, by directly stimulating the mobilization of endothelial progenitor cells (EPCs) [17]. EPCs represent a bone marrow-derived population of non-hematopoietic stem cells with the capability of differentiating into endothelial cells and participating in vascular repair in peripheral tissues [343]. Similar to hematopoietic stem cell trafficking, CXCL8 and CXCL12 represent two key homeostatic chemokines, which may either individually or synergistically trigger the mobilization of EPCs at the sites of injury to elicit vascular remodeling [344].

Given the aforementioned roles of the CXCR4/CXCL12 axis in physiological angiogenesis, it is unsurprising that the pathway is hijacked in an almost identical way in cancer, given that most tumors elicit "wound-healing"-type of responses [345]. In several types of cancer for instance, such as in breast, prostate, and glioblastoma, it has been demonstrated that hypoxic conditions in the tumor microenvironment cause a significant increase of CXCL12 expression levels [346–350]. CXCL12 upregulation promotes the chemotactic recruitment of bone marrow-derived $CXCR4^+$ EPCs and monocytes. These newly-arriving stromal cells may in turn promote tumor growth and angiogenesis, mainly via secreting proangiogenic growth factors, chemokines, and cytokines, such as VEGF-A and angiopoietin [346]. Moreover, $CXCR4^+$ tumor cells may significantly increase the expression of VEGF-A in response to intratumoral CXCL12 increase, thus creating a positive feedback loop that can further trigger angiogenesis [351]. On another note, the role of tumor-associated macrophages (TAMs) in cancer angiogenesis and progression has been firmly established [333,352−358]. Genetic screenings suggest that TAMs secrete a wide variety of proangiogenic factors, including but not limited to VEGF, TNF, CXCL8, and PDGF, among others [359]. As such, tumors that are highly infiltrated by TAMs can exert significant proangiogenic pressure on parental endothelia. Besides the well-established CCL2/CCR2 pathway, it has been also shown that TAMs are recruited in primary tumors and subsequently transition into perivascular $TIE2^+VEGFA^+$ macrophages under the control of CXCL12/CXCR4 axis [291]. Pharmacological suppression of CXCR4 significantly reduces $TIE2^+VEGFA^+$ TAMs, leading to impaired angiogenesis, especially during chemotherapy-induced revascularization [360]. Together, these observations provide substantial evidence that the CXCL12/CXCR4 axis is hijacked via diverse mechanisms, to shift the balance of the angiogenic switch towards the promoting side.

As described above, there are multiple studies supporting induction of tumor angiogenesis by CXCL12/CXCR4 under context-specific in vitro and in vivo conditions [349,361,362]. However, a putative role of CXCR7 (if any) in the process has only been sparsely investigated. A study by Zhang et al. (2017) has recently shown that CXCL12-mediated directional polarization of endothelial cells is dependent on both CXCR7 and CXCR4 [76]. In the same study, it was revealed that the specific suppression of CXCR7 leads to downregulation of the AKT signaling pathway, and partially demoted CXCL12-dependent neovascularization [76]. The pharmacological suppression of CXCR7 using the CCX771 inhibitor, which is known to suppress CXCR7 but not CXCR4, reduced both invasiveness/metastatic potential and the angiogenic potential of breast carcinomas [82]. Moreover, CXCR7 has been implicated in the mobilization and survival of EPCs, although a clear association with expansion and proliferation of EPCs has not been firmly established [363,364]. Overall, although CXCR7 is considered a scavenger receptor for CXCR4 and thus assumed to exert opposing functions to CXCR4, growing evidence supports that it is also angiogenesis- and metastasis-promoting in cancer models. It is yet to be determined whether CXCR4 and CXCR7 functions are additive/synergistic, or even redundant to each other, in the regulation of these cancer hallmarks.

Ensuing characterization of the nature of neoangiogenic vessels defines them as structurally and functionally deficient, and bearing microanatomical impairments, such as low pericyte coverage, irregularly-shaped walls, and defective paracellular permeability. Pericyte recruitment in tumor neovasculature is primarily mediated by $PDGFRB^+$ perivascular progenitor cells, under the concerted contributions of PDGFRB/PDGFB signaling pathway and the extracellular matrix [365–367]. In certain non-neoplastic conditions such as chronic allergic inflammation, it has been shown that pericytes can upregulate CXCR4 expression, thus migrating more readily to the cognate chemokine, CXCL12, causing increased pericyte coupling to pulmonary vessels and respiratory distress [368]. On the contrary, intratumoral vessels are mainly devoid of pericyte coverage, despite CXCL12 production by cancer endothelial cells and the perivascular niche. To our knowledge, there are no studies that have determined whether intratumoral pericytes express CXCR4 during cancer development and progression. However, a study by Hamdan *et al.* (2014) determined that the CXCL12/PDGFB axis mediates pericyte differentiation of bone marrow cells, leading to the expansion of tumor vasculature in Ewing sarcomas [369]. In general, the neovasculature deficits resulting from poor pericyte coverage do not only impair normal blood flow, tissue oxygenation, and immune cell trafficking, but also promote the metastatic potential within the tumor microenvironment, thus forming the basis for the development of novel anti-angiogenic and/or vasculature-normalization therapies [370,371]. A more intricate role of the CXCL12/CXCR4/CXCR7 pathway in this context remains to be firmly established in the future.

Because CXCL12 expression is generally increased upon tissue injury and/or hypoxia, it has been proposed that CXCL12 may be an "alarmin"-type chemokine that triggers compensatory angiogenesis and tumor relapse after standard-of-care cancer treatments (e.g., chemotherapy, irradiation), which are known to act as cytotoxic insults [372–374]. For instance, treatment of mice modeling melanoma and lung cancer with paclitaxel/docetaxel causes the systemic induction of CXCL12, which in turn promotes mobilization and homing of EPCs to the primary tumor with angiogenesis-promoting effects [375]. As expected, treatment with a combination of chemotherapy and CXCL12 blocking antibodies significantly abrogates this effect, by neutralizing high CXCL12 serum levels and blocking EPC mobilization. Interestingly, cancer patients receiving paclitaxel-based chemotherapy exhibit a similar effect with increased CXCL12 serum levels and circulating EPCs, when compared to patients receiving other types of cancer therapies [375], indicating that the type of treatment may also play important role in the generation of intratumoral and systemic CXCL12 expression gradients. Besides chemotherapy, it has been shown that local irradiation in mouse models of glioblastoma enhances hypoxia and leads to increased CXCL12/CXCR4 levels, and enhanced tumor vascularization [376]. This study further revealed that pharmacological treatment with CXCR4 antagonist, AMD3100, could reverse irradiation-induced angiogenic and vasculogenic effects in murine glioblastomas [376]. Along the same lines, targeted inhibition of CXCR4 in a breast cancer mouse model decreases VEGF-A expression [377]. These observations collectively suggest that CXCL12 may not only serve as proangiogenic chemokine in the natural progression of cancer, but also as critical mediator of revascularization and cancer relapse following therapeutic interventions with cytotoxic anticancer agents. For instance, colorectal cancer (CRC) patients treated with bevacizumab, an approved anti-VEGF-A antibody-treatment strategy, typically present with increased CXCL12 and CXCR4 mRNA expression in tumor cells [378,379], thus the co-targeting of the CXCL12/CXCR4 pathway could potentially yield more beneficial outcomes if combined with bevacizumab in this context.

To summarize (Fig. 5), the contributions of the CXCL12/CXCR4 pathway in angiogenesis and metastatic progression have been firmly established in the past, but have only recently started to get dissected at molecular detail. CXCL12 promotes neoangiogenesis, both locally by eliciting direct vascular remodeling, and systemically by attracting endothelial and mural cell progenitors at the primary tumor site.

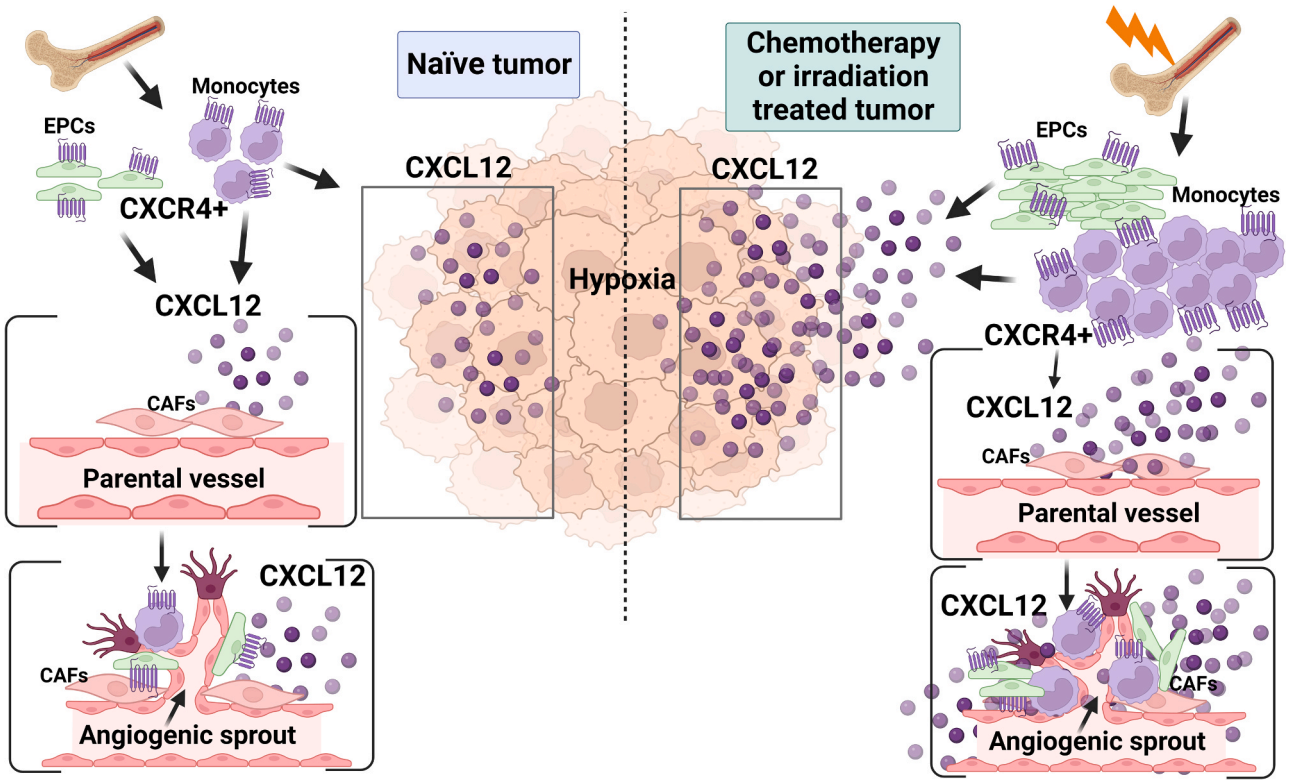

Fig. 5. Emerging Roles of CXCL12 Axis in the Regulation of Neovascularization and Neoangiogenesis. The conceptual model summarizes key mechanisms and cells involved in CXCL12-driven angiogenesis in primary tumors. In treatment-naïve tumors (left side of the illustration), hypoxic conditions may promote release of CXCL12 by the perivascular cancer-associated fibroblasts (CAFs). This increase of CXCL12 expression in the primary tumor microenvironment generates a strong chemotactic force, which attracts wandering bone marrow-derived progenitors, including monocytes and endothelial progenitor cells (EPCs) into the perivascular space. These cells will collectively function as effectors of angiogenesis and vascularization, by locally producing angiogenesis-promoting factors (e.g., VEGF), and by simultaneously antagonizing angiogenesis-suppressors (e.g., thrombospondins). Together these CXCL12-orchestrated perivascular niches shift the "angiogenic switch" towards eliciting neoangiogenesis/neovascularization. This model implies that CXCL12 mainly functions as a "master regulator" of angiogenesis by tethering an essential constellation of cellular and molecular factors at the perivascular space. Interestingly, following cancer treatment with chemotherapy and/or radiotherapy (right side of the illustration), an identical mechanism of angiogenesis is triggered albeit with severe amplification, mainly due to increased hypoxic stress after treatment. Illustration created by BioRender (biorender.com).

5.6. Establishment of immunosuppressive microenvironment

The chemokine CXCL12 serves major homeostatic functions in the immune system. In primary lymphoid organs, CXCL12 contributes to the maintenance of the hematopoietic stem cell niche in the bone marrow, serving as the main homing factor for HSC retention in their niche [380,381]. In the thymus, CXCL12 is expressed by endothelial cells at the corticomedullary junction and by cortical thymic epithelial cells, again serving as key homing factor for thymocyte progenitors entering the thymus to complete their development [382–386]. Outside the primary lymphoid organs, the CXCL12/CXCR4 pathway supports immune surveillance, via recruitment of innate immune cells, for instance neutrophils at peripheral tissues [387]. Finally, although not widely recognized, the CXCL12/CXCR4 pathway may also play a role during immunologic responses, as CXCR4 has been identified as part of the complex between antigen-presenting cells (MHC class receptors) and T cells (CD3 receptor) during T cell activation that leads to cytokine secretion from the activated T cells [388]. Therefore, CXCL12 serves as a pleiotropic chemokine for proper development and function of the immune system, and the CXCL12/CXCR4 pathway is contextually related to a plethora of leukocyte populations, belonging to both the innate and the adaptive immunity arm.

One of the emerging cancer hallmarks that has been widely appreciated in the past 20 years is the acquired ability of tumor cells to evade immunological destruction [153]. In this context, both intrinsic and extrinsic factors have been proposed. For instance, a paramount mechanism of intrinsic resistance to anticancer immunity is the downregulation of the Major Histocompatibility Complex Class-I (MHC-I) molecules from certain cancer cell variants, which makes them immunologically "invisible" to adaptive immune cells, because these variants no longer present any neoantigens in their surface [389–392]. However, it is not within the scope of the current review to define such mechanisms in great detail, because most immunosuppressive mechanisms associated with the CXCL12/CXCR4 pathway comprise extrinsic modulatory interventions of the tumor microenvironment, which eventually render it refractory to efficient cytotoxic $CD8^+$ T cell trafficking and function. Although cancer immunosuppression is not a cancer hallmark that is exclusive to the metastatic cascade, the community's viewpoint is that the molecular and cellular mechanisms promoting immunosuppression can efficiently shadow the prometastatic tumor cells, while in the act of metastatic dissemination to secondary sites, thus allowing them to seed new metastases and develop clinically overt metastatic colonies [393]. Of note, we have previously proposed a model of contextual immunosuppression, in which the specialized microenvironments associated with the regulation of metastatic dissemination express unique immunosuppressive signatures, thus justifying why prometastatic tumor cells are immune-privileged [393]. In this section, we will advocate that the pharmacologically-vulnerable CXCL12/CXCR4/CXCR7 pathway represents one such crucial orchestrator of local immunosuppression at the perivascular niche [229].

The key orchestrators of the immunosuppressive tumor microenvironment are myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), T regulatory cells (Tregs), and immature dendritic cells, which have all been reported to express CXCR4

[199,394]. The most prominent source of CXCL12 in the tumor microenvironment, capable of recruiting these cells, has been traditionally considered to be the CAFs, or at least certain mesenchymal cell subsets associated with the tumor vasculature [158,349,395]. In colorectal cancer, CAFs are known to promote M2 macrophage polarization in a CXCL12-dependent manner, thus promoting immunosuppression and tumor progression [396]. Inhibition of CXCL12 in colorectal cancer via ketogenic diet eliminates CXCL12 production and overcomes the immunosuppressive tumor microenvironment [397]. In general, CAFs have been identified as key sources of CXCL12 in the microenvironment of breast, endometrial, and pancreatic cancers [398–400], possibly among other cancer types, indicating a common underlying mechanism for CXCL12-mediated immunosuppression in the desmoplastic interface. In pancreatic cancer in particular, CXCL12 depletion has been shown to overcome resistance to immune checkpoint inhibitors, thus allowing activation of T cell anti-cancer responses [399]. Together, these data support a paracrine interplay among cells that form the desmoplastic response in primary tumors, such as CXCL12-producing CAFs, and a wide variety of $CXCR4^+$ immunosuppressive cells that obfuscate T cell trafficking and function.

As such, the combination of anti-CXCR4 with newly-engineered immune checkpoint blockade treatment or other immunotherapies has shown promising synergistic results in preclinical models. In a murine model of metastatic breast cancer for example, the pharmacological targeting of CXCR4 leads to increased infiltration of cytotoxic T lymphocytes, and significantly improves the outcome of anti-PD1/PDL1 immune checkpoint blockade [401]. This combination has also been shown to restore anticancer immunity in triple-negative breast cancers [402]. In addition, the use of another CXCR4 antagonist, AMD3465, not only suppresses breast cancer metastasis to the bone, but also eliminates Treg and MDSC infiltrates. A combined inhibition of indoleamine 2, 3-dioxygenase 1, an enzyme that inhibits T cell activation, further restores anti-cancer immunity [403]. In hematopoietic cancers such as B cell lymphomas, the CXCR4 antagonist AMD3100 has been shown to block Treg recruitment, thus alleviating Treg-mediated suppression of adaptive anticancer immunity [404]. In murine glioblastoma, CXCR4 suppression leads to decreased infiltration of immunosuppressive cells, including $CXCR4^+$ monocytic MDSCs, a favorable $CD8^+/CD4^+$ T cell ratio, and enhanced immunogenic cell death of glioblastoma cells [405] [406]. In ovarian cancer, the use of an oncolytic virus expressing a CXCR4 antagonist leads to increased levels of INF-γ, tumor-infiltrating T cells, and a favorable antibody response against the tumor [407,408]. From a mechanistic perspective, pharmacological CXCR4 ablation does not inhibit CXCL12 production by the CAFs per se, but antagonizes and eliminates the chemotactic response of the immunosuppressive $CXCR4^+$ cells within the tumor microenvironment. In the absence of an

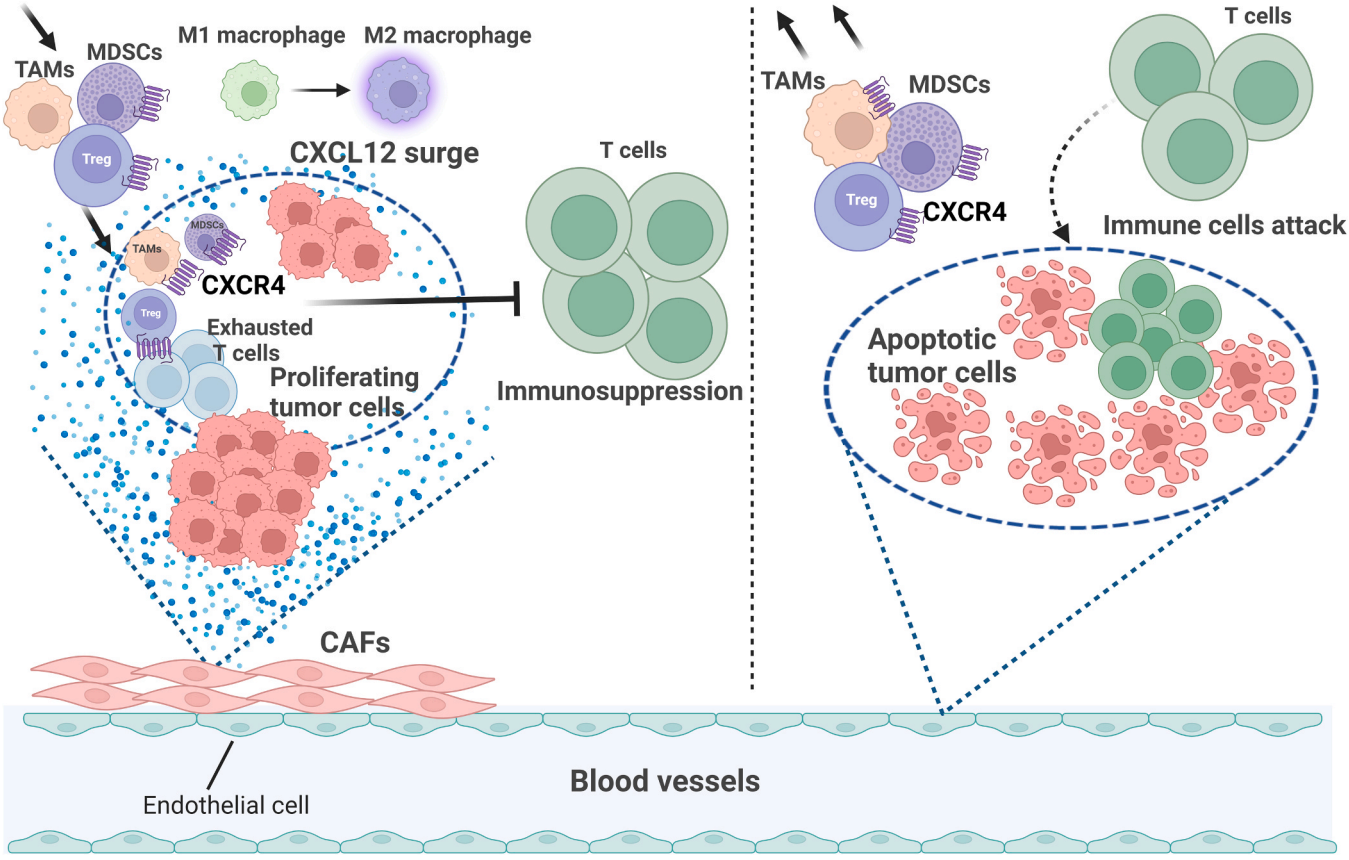

Fig. 6. Emerging Roles of CXCL12 Axis in the Regulation of Immunosuppression. The conceptual model summarizes the key mechanisms and cells involved in CXCL12-driven immunosuppression in primary tumors. As in prior biological programs examined (e.g., angiogenesis), CXCL12 plays mostly an indirect role in establishing an immunosuppressive niche in the perivascular space. In the presence of cancer-associated fibroblasts (left side of the illustration), the CAF-induced CXCL12 chemotactic force recruits a significant number of myeloid cells with immunosuppressive capabilities in a CXCR4-dependent manner, including T regulatory cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs). When assembled at the perivascular niche, these immunosuppressive myeloid cells create an immune-privileged sanctuary for the proliferating and prometastatic cancer cell subset, by secreting chemo-repulsive cytokines, or expressing ligands for immune checkpoint receptors. Together, the CXCL12-orchestrated immunosuppressive niche decreases trafficking and function of cytotoxic $CD8^+$ T cells mediating cytotoxic anti-cancer immunity. Under certain circumstances, CXCL12 has also been demonstrated to directly function as a repulsive chemokine for $CD8^+$ T cells although the underlying mechanisms are relatively underexplored. Indeed, in the absence of the $CXCL12^+$ niche described above (as surmised in the hypothetical scenario in the right side of the illustration), the immunosuppressive myeloid cells divert from the niche, and T cells can find their way to immunologically attack the tumor cells. Illustration created by BioRender (biorender.com).

immunosuppressive niche, other immunotherapeutic regimens, such as the selective blocking of checkpoint inhibitor pathways in T cells (e.g., anti-PD1/PDL1 immunotherapy), may then target inhibitory signaling in T cells, decrease T cell exhaustion, and promote the optimal and integrative T cell responses [409].

Because CXCL12 expression is elicited in both primary and secondary lymphoid organs as part of a homeostatic mechanism regulating immune cell development/trafficking, a role of the CXCL12/CXCR4 pathway in mediating immunosuppression at such metastatic sites has also been proposed. For instance, CXCL12 can attract Tregs in the bone marrow, facilitating immune evasion of disseminated prostate cancer cells [410]. CXCL12 is also physiologically expressed by mesenchymal cells in non-lymphoid organs, such as liver and lungs, not only explaining the propensity of $CXCR4^+$ tumor cells to metastasize in these sites, but also suggesting similar CXCL12-driven immunosuppressive mechanisms at secondary sites. For instance, hepatic stellate cells, which function as equivalents to CAFs, can amply produce CXCL12 in the liver microenvironment, which in turn induces $CXCR4^+$ MDSC infiltration [411].

To summarize (Fig. 6), the contributions of CXCL12/CXCR4 pathway in the establishment of an immunosuppressive tumor microenvironment are mostly attributed to reciprocal interactions between the CXCL12-secreting tumor mesenchyme and a plethora of $CXCR4^+$ immune (mostly myeloid) cells that target the $CXCL12^+$ niches. Once the immunosuppressive myeloid cells home into the intratumoral/perivascular $CXCL12^+$ niches, they elicit inhibitory responses that shut down the anticancer adaptive immunity.

6. Conclusion

In the intricate landscape of cancer biology, CXCL12 has emerged as a cornerstone modulator of metastatic progression. The traditional concepts related to its implication as a chemokine simply driving cancer cell dissemination and angiogenesis have been revisited and supplemented with novel context-dependent functions related to the development of highly-specialized niches for stemness, immunosuppression, and other key biological programs in the tumor microenvironment. As our understanding delves deeper into these multifarious roles, it is increasingly evident that targeting the CXCL12/CXCR4 axis could provide a promising avenue for therapeutic interventions. Indeed, there is a wide plethora of preclinical and clinical studies that investigate the pharmacological targeting of CXCL12/CXCR4 with/without chemotherapy or immunotherapy, but it was beyond the scope of the current review to discuss those. Instead, the current review provided new context and evidence, which could be used to re-direct our thinking on how to exploit the CXCL12/CXCR4 pathway as an optimal anti-metastatic therapy.

CRediT authorship contribution statement

All authors participated in writing different parts of the manuscript, combining these parts into a single narrative, and proof-reading the composition of the final manuscript. Panagiota S. Filippou and George S. Karagiannis additionally conceptualized the illustrations, and designed them using biorender. Panagiota S. Filippou has obtained the license from biorender to include all illustrations in the final manuscript. All the authors approved the final version of the manuscript before submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

G.S.K. receives funding support from the following: the NIH-NCI K99/R00 Transition to Independence Award (K99CA237851 and R00CA237851), the Integrated Imaging Program for Cancer Research (IIPCR), the Evelyn-Lipper Charitable Foundation, and the Montefiore-Einstein Cancer Center (MECC) start-up fund; C.L.D. receives funding support from the following: the Integrated Imaging Program for Cancer Research (IIPCR), the Evelyn Gruss-Lipper Charitable Foundation, and the Helen & Irving Spatz Foundation. P.S.F. receives funding support from the following: the Biochemical Society Eric Reid Fund for Methodology Grant, and the Teesside University seed corn fund.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cytogfr.2023.10.003.

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Due to reaching the reference number limitation, additional references are shown as Supplementary material, associated with this manuscript.

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