



Proteolytic chemokine cleavage as a regulator of lymphocytic infiltration in solid tumors

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Abstract

In the past decade, immune-based therapies such as monoclonal antibodies against tumor epitopes or immune checkpoint inhibitors have become an integral part of contemporary cancer treatment in many entities. However, a fundamental prerequisite for the success of such therapies is a sufficient trafficking of tumor-infiltrating lymphocytes into the tumor microenvironment. This infiltration is facilitated by chemokines, a group of about 50 small proteins capable of chemotactically guiding leukocytes. Proteolytic inactivation of chemokines leading to an impaired infiltration of immune effector cells appears to be an efficient immune escape mechanism of solid cancers.

The CXCR3 and CX3CR1 chemokine receptor ligands CXCL9-11 and CX3CL1, respectively, are mainly responsible for the tumor-suppressive lymphocytic infiltration into the tumor microenvironment. Their structure explains the biochemical basis of their proteolytic cleavage, while *in vivo* data from mouse models and patient samples shed light on the corresponding processes in cancer. The emerging roles of proteases, e.g., matrix metalloproteinases, cathepsins, and dipeptidyl peptidase 4, in chemokine inactivation define new resistance mechanisms against immunotherapies and identify attractive new targets to enhance immune intervention in cancer.

Keywords Chemokines · Proteases · Tumor-infiltrating lymphocytes · CXCR3 · CX3CR1 · Cathepsins · Matrix metalloproteinases · Dipeptidyl peptidase 4

1 Introduction

Although it was postulated more than 100 years ago that the immune system can fight established solid tumors, only in the past two decades substantial progress has been made towards a better understanding of the interaction between the different subtypes of immune cells with tumor cells and their environment [1, 2]. Starting point for many of these studies was the clinical observation that the number of tumor-infiltrating lymphocytes (TILs) is a strong and robust prognostic marker across various tumor entities, such as breast, colon, or ovarian cancer [3–5]. Further studies have dissected the different subpopulations of TILs delineating either their tumor-suppressive

(e.g., cytotoxic T cells, natural killer cells) or tumor-promoting functions (e.g., regulatory T cells) [6, 7]. Deciphering the immune cell-tumor cell interactions has prompted the development of new cancer immunotherapies, such as monoclonal antibody therapy against tumor antigens, immune checkpoint inhibition, adoptive T cell transfer, or various vaccination strategies [8]. Especially in highly immunogenic tumors, these immunotherapies have led to unprecedented improvements in survival and quality of life of cancer patients. Examples include the HER2-directed therapies in breast cancer or the immune checkpoint inhibitors in melanoma or non-small cell lung cancer [9, 10]. Moreover, there is ample evidence that classic chemotherapies also work *via* stimulation of an anti-tumor immune response in addition to their cytotoxic effects on the individual cancer cells [11].

However, a fundamental prerequisite for the success of all of these approaches is sufficient trafficking of the respective immune effector cells into the tumor microenvironment [12, 13]. This renders TILs or TIL subpopulations not only prognostic, but also feasible predictive biomarkers for the response to these therapies [7, 14]. In addition, it raises one of the most urgent questions in contemporary cancer immuno-oncology:

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how can these immune cells be efficiently recruited into the tumor? The ultimate goal is to transform a “cold” into a “hot” tumor [15].

The recruitment of immune cells into solid tumors is mediated by chemokines, a family of about 50 small proteins capable of chemotactically facilitating leukocyte migration [16]. Different chemokines can either attract tumor-suppressive or tumor-promoting leukocytes, and, thus, the intratumoral chemokine milieu is a strong determinant of the intratumoral immune milieu [17]. Besides their chemotactic function, chemokines also participate in activation or inactivation of immune cells.

Many chemokines can be posttranslationally modified by proteolytic cleavage which either activates or destroys their chemotactic function [18]. Expression of the corresponding proteases may thus significantly influence the modulation of the immune milieu and the anti-tumor immune response. Therefore, proteolytic inactivation of tumor-suppressive chemokines represents a potent immune escape mechanism of solid tumors [19]. Conversely, inhibition of these proteases might be an attractive adjuvant to immunotherapies, such as immune checkpoint inhibitors, whose function depends on the activity and presence of specialized chemokines [20].

In the following, the concept of chemokine cleavage as a modulator of the anti-tumor response will be discussed. The focus will be on the chemokines that are most notably known for their ability to recruit tumor-suppressive lymphocytes such as cytotoxic T cells (CTLs) or natural killer (NK) cells into solid tumors (Fig. 1). These chemokines are the CXCR3

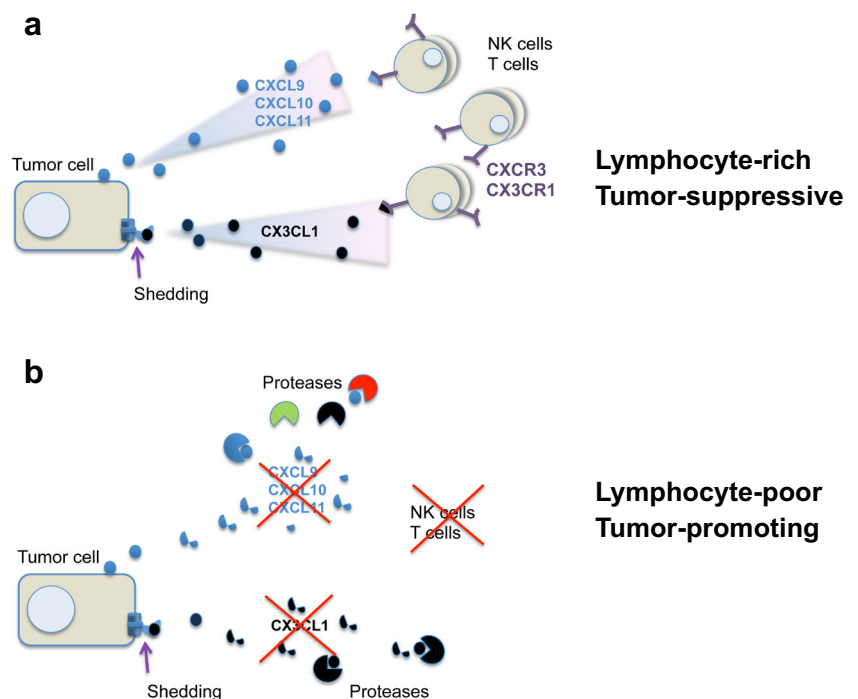
receptor ligands CXCL9, CXCL10, and CXCL11 as well as the CX3CR1 ligand CX3CL1, also named fractalkine [17, 21].

2 Chemokines

Chemokines or chemotactic cytokines make up the largest family of cytokines. With 48 members expressed in human tissue, they represent a structurally and functionally related group of low molecular weight proteins, which controls chemotactic recruitment of specific lymphocyte subtypes in a tissue- and time-dependent manner [22]. The distribution of immune cells not only plays a crucial role in the innate and adaptive immune response in case of inflammation or associated disease [23–27], but also supports tissue homeostasis [28] and angiogenesis [29, 30]. According to their major function chemokines are classified into two groups [25]: Homeostatic chemokines are constitutively expressed in order to initiate a proper immune response and to define the composition and organization of tissue-resident, blood-derived cells such as macrophages and dendritic cells [16]. On the contrary, inflammatory chemokines are mainly inducible and are strongly up-regulated upon demand [31]. It is of note that this classification is not strict as there are chemokines that can contribute to both groups.

The conventional chemokine signaling is thought to be transduced through a complementary receptor network consisting of transmembrane G protein-coupled receptors

Fig. 1 Inactivation of chemokine-mediated lymphocyte infiltration by proteolytic cleavage. **A** Secretion of the chemokines CXCL9, CXCL10, and CXCL11 as well as shedding of CX3CL1/fractalkine leads to chemotactic recruitment of tumor-suppressive lymphocytes to the tumor site. **B** Chemokine-targeted cleavage by proteases, such as MMPs, cathepsins, and DPPs, impairs lymphocytic infiltration, leading to reduced amounts of immune cells in the tumor microenvironment



[32]. However, also receptor-independent modulation of signaling has been proposed. Factors that can influence chemokine signaling are non-canonical receptors such as the atypical chemokine receptors 1–4, mRNA instability, alternative splicing, or glycosaminoglycan (GAG) binding [33–35]. Furthermore, regulation is even more complex due to considerable promiscuity between chemokines and their receptors, i.e., several ligands may bind to the same receptor and *vice versa* a single ligand can activate different receptors [16]. Moreover, chemokine receptors are involved in several signaling cascades which can be differentially activated by either distinct ligands or *via* oligomerization of the same ligand [36, 37].

The structure of chemokines is conserved across sub-families, in most cases built around four conserved cysteine residues, except the XC chemokines which lack two of these four cysteines [38]. The organization of the first cysteine residues in the N-terminal part of the chemokines divides them into four different groups: CXC, CC, XC, and CX3C, with X representing any amino acid [39]. The cysteines form one or two disulfide bridges, which stabilize a central three-stranded anti-parallel β -sheet with a rigid loop structure (Fig. 2). This central core is preceded by a flexible unstructured N-terminus. Both the flexible N-terminus and the following rigid N-loop, which often is C-terminally limited by a 3_{10} -helix, within the globular core are functional domains of the chemokine in terms of receptor binding and activation. Originally, a two-site/two-step model had been proposed, in which firstly the N-terminus of the receptor interacts with the N-loop of the chemokine and, subsequently, the unstructured N-terminus of the chemokine can enter the binding pocket of the receptor resulting in receptor activation and transmembrane signaling [40]. Recent results indicate that this model may have to be extended to a three step model, in which step 1 of the original model, binding of the chemokine to the receptor, is divided into an initial low-affinity, rather unspecific binding which is then followed by the specific high-affinity binding of the chemokine to the receptor [41]. C-terminal of the chemokine core, an α -helix is present which packs onto the β -sheet structure. The glycosaminoglycan (GAG) binding site of the chemokines is located within the β -sheet and C-terminus [42].

3 The CXCR3 chemokine system

The CXC chemokines can be further divided according to the presence or absence of a highly conserved Glu-Leu-Arg (ELR) motif, which is shared by CXCL1–3 and CXCL5–8. Among the chemokines that lack the ELR motif (CXCL4, CXCL4L1, and CXCL9–14), most of them interact with the CXCR3 receptor [43]. The CXCR3 receptor is a 368 amino

acid (aa) seven-transmembrane G protein coupled receptor with three isoforms. The originally identified, canonical CXCR3 receptor, named CXCR3-A, is bound by the ligands CXCL9, CXCL10 and CXCL11 and, upon activation, promotes chemotaxis, invasion, proliferation, and cell survival [44]. The first identified splice variant of CXCR3 was called CXCR3-B and interacts with CXCL4 and CXCL4L1 in addition to the classical ligands. This receptor isoform does not trigger chemotaxis but instead growth suppression, angiostasis, and apoptosis. The most recently discovered CXCR3 isoform, CXCR3-alt, only accepts CXCL11 as ligand [44, 45].

CXCR3 receptor expressing cells include, among others, regulatory T cells, CD4⁺ and CD8⁺ T cells, dendritic cells, NK cells, and NKT cells [21, 46]. CXCR3 ligand expression can be detected in endothelial cells, keratinocytes, and fibroblasts. Also, immune cells like T cells and monocytes are capable of secreting those chemokines. Especially CXCL9 and CXCL11 are secreted by peripheral blood monocytes and macrophages [22].

CXCL4 and CXCL4L1 are described as platelet-related agonists, whereas CXCL9, CXCL10, and CXCL11 expression is strongly inducible by interferons (IFN). All of the latter cytokines can be induced by IFN- γ , but CXCL11 is the only one being also induced by IFN- α [47]. CXCL11 show the highest binding affinity towards CXCR3 followed by CXCL10 and CXCL9. Among these three chemokines, CXCL9 shows the weakest activation upon receptor binding [22]. Interestingly, competition with CXCL11 receptor binding by CXCL9 or CXCL10 is always incomplete. Furthermore, CXCL11—in contrast to CXCL10—does also bind to CXCR3, when the receptor is uncoupled from G protein-dependent signaling [48]. Receptor activation by its three ligands also results in different effects. CXCL11 was described to be the most potent inducer of receptor internalization, whereas CXCL9 and CXCL10 mainly induce chemotaxis as well as Ca²⁺ influx [49].

Besides induction of migration and Ca²⁺ influx, ligand/receptor interaction can also lead to downstream phosphorylation of target proteins, e.g., transcription factors of the STAT family. CXCL9 and CXCL10 lead to the phosphorylation of STAT1, STAT4, and STAT5 and subsequently to an activation of T-bet and ROR γ T, two differentiation regulators, resulting in polarization of CD4-positive T cells towards the Th1 and Th17 effector lineage. Contrariwise, CXCL11 binding induces phosphorylation of STAT3 and STAT6, which leads to a regulatory phenotype of CD4-positive T cells (Th2 or Tr1) [50–52].

Thus, together with the varying susceptibility to post-translational modification, differences concerning ligand-receptor interaction and GAG binding, and the fact that CXCL9, CXCL10, and CXCL11 display not completely

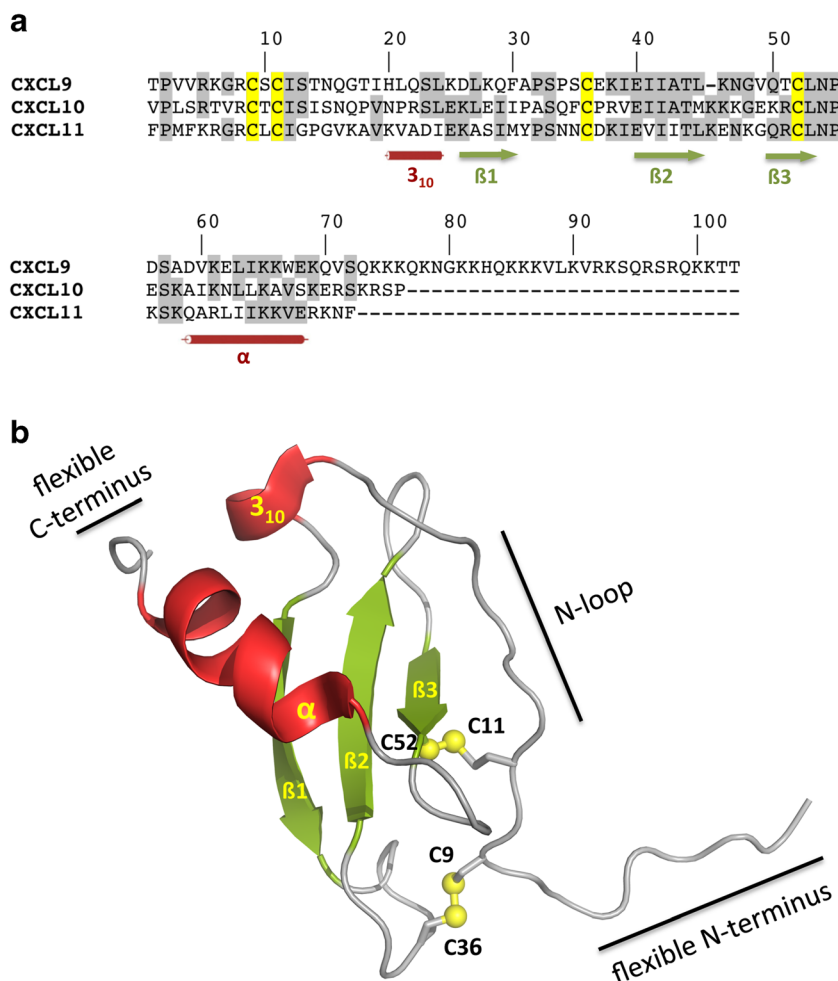


Fig. 2 Primary and tertiary structure of CXC chemokines. **A** Sequence alignment of human CXCL9, CXCL10, and CXCL11. Secondary structure elements are indicated by green arrows (β -strands) and red cylinders (α -helix and 3_{10} -helix). Cysteine residues are indicated in yellow, whereby Cys⁹ forms a disulfide bridge with Cys³⁶ and Cys¹¹ with Cys⁵², respectively. The numbering is derived from CXCL9. **B**

Ribbon plot of model 1 from the NMR structure of CXCL11 (PDB code 1RJT). A flexible unstructured N-terminus is followed by the N-loop, a 3_{10} -helix (red), and the central three-stranded anti-parallel β -sheet (green), which is stabilized by two disulfide bridges, depicted as yellow spheres. Near the C-terminus an α -helix (red) is present, which packs onto the β -sheet structure, followed by a flexible C-terminus

overlapping functions, a complex regulatory network is formed affecting diverse biological functions.

4 The CX3CR1/CX3CL1 chemokine system

Cells expressing the seven-transmembrane receptor CX3CR1 (355 aa) are monocytes, macrophages, NK cells, lymphocytes, and dendritic cells [53]. Its ligand, CX3CL1, also named fractalkine, is the only member of the CX3C chemokine family. It is expressed as a membrane-bound protein, but is also found in soluble forms [54, 55]. The 317 aa long extracellular domain encompasses a stalk formed by mucin-like domains with a chemokine domain on top. This N-terminal extracellular part is followed by a transmembrane domain and a short C-terminal cytoplasmic tail (34 aa). The soluble forms of CX3CL1 carrying the mucin-like stalk [56] and possibly

also the cytokine domain only [57] provide chemotactic recruitment of CX3CR1-positive cells. As a membrane-bound protein, CX3CL1 provides adhesive capacity and helps to stabilize the interaction between CX3CL1-expressing cells and cells expressing the corresponding receptor CX3CR1, respectively [58]. CX3CL1/CX3CR1-mediated adhesion can be further enhanced by synergistic interaction with other adhesive molecules like integrins [59]. Due to functions of CX3CL1 as chemo-attractant and adhesion molecule, it plays an important role in the process of recruiting cells from the blood stream to the site of action in an integrin-independent manner, e.g., the extravasation of CX3CR1-positive leukocytes [60]. CX3CL1 expression is mainly found in endothelial cells and neurons, but based on the involvement in inflammatory processes it is also expressed in tissue showing rather dense immune cell populations like the CNS, lung, cardiac muscles, liver, small bowel, colon, and pancreas [61].

5 The role of CXCR3 and CX3CR1 chemokines in cancer

The role of the CXCR3 chemokine system in cancer biology may be a double-edged sword. On the one hand, the CXCR3 chemokines, especially CXCL9 and CXCL10, facilitate the recruitment of CXCR3-positive Th1, NK, and NKT cells as well as cytotoxic T lymphocytes to the tumor microenvironment, which trigger the development of a tumor-suppressive immune milieu [46, 62, 63]. On the other hand, tumor cells can exploit the CXCR3 receptor to escape from the primary tumor and to metastasize to niches with high CXCR3 ligand concentrations, e.g., to the lymph nodes or to the lungs [64–69]. Moreover, all three IFN-inducible CXCR3 ligands are anti-angiogenic *in vitro* and *in vivo*, which may result in undersupplied, stagnating tumors, but also in more aggressive tumor cells with metastasizing potential [70, 71].

Raising the intratumoral concentration of intact and functional CXCR3 chemokines, e.g., by inhibition of their proteolytic inactivation, might thus kill two birds with one stone: tumor-suppressive immune cells would be attracted to the tumor site, and CXCR3-positive tumor cells would be chemotactically prevented from escaping the primary cancer. This idea is supported both by preclinical and clinical findings. Overexpression of CXCR3 ligands in murine cancer models of ovarian, breast, skin, or colon cancer caused an enhanced Th1 and NK cell infiltration and less metastatic spread [72–75]. Moreover, in human cancers, overexpression of CXCL9 and CXCL10 is associated with a higher number of tumor-infiltrating lymphocytes and improved survival, e.g., in breast, ovarian, colon, lung, and several other cancers [67, 76–83]. Although CXCR3 expression by tumor cells is associated with worse prognosis in these cancers [67, 72, 84, 85] and CXCR3 ligands may also attract tumor-promoting regulatory T cells [86], the net effect of CXCR3 chemokine overexpression seems to favor tumor suppression. These results are confirmed by large-panel gene expression analyses of breast and ovarian cancer, in which CXCR3 chemokines represented the most upregulated genes in the tumors of those patients exhibiting the best prognosis [76, 87, 88].

However, CXCR3 chemokines do not only cause a tumor-suppressive milieu *per se*, they also contribute to the effect of multiple current cancer therapeutics. Immune checkpoint inhibitors of the PD-1/PD-L1 axis rely on immune cell attraction and on the intratumoral T cell activation by CXCR3 chemokines [20, 89–91]. Moreover, an increase in CXCR3 chemokine serum concentrations under therapy with checkpoint inhibitors is predictive for therapy response [92]. CDK4/6 inhibitors, which have emerged as a new cornerstone in the treatment of

advanced estrogen receptor positive breast cancer, recruit cytotoxic T cells *via* induction of CXCR3 chemokines, which is indispensable for their therapeutic effect *in vivo* [93]. Inhibition of the poly[ADP-ribose] polymerase 1 (PARP1), which is now an established therapy in recurrent ovarian and metastatic BRCA-mutated breast cancer, also induces CXCR3 chemokines *via* the STING (stimulator of interferon genes) pathway in tumor cells, whereby the subsequent attraction of immune cells is critical for their function [94–99]. In all of these therapies, proteolytic inactivation of CXCR3 chemokines, thus, represents a new resistance mechanism, which renders inhibitors of CXCR3-chemokine cleaving proteases feasible adjuvants to all of these therapies.

CX3CL1 is also capable of recruiting tumor-suppressive immune cells that express the CX3CR1 receptor such as NK cells and cytotoxic T lymphocytes (CTLs) [21]. However, as described for the CXCR3 system, the CX3CR1 receptor is also able to facilitate tumor cell migration and, thereby, metastasis in CX3CL1-rich tissues, e.g. the bone or the brain [100, 101]. Moreover, expression of the transmembrane form of CX3CL1 in neurons, endothelial cells or peritoneal cells promotes tumor cell adhesion and site-specific metastasis of CX3CR1-expression prostate, ovarian, or pancreatic tumor cells [102–106].

Preclinical studies confirm the tumor-suppressive effect of CX3CL1 in several cancer models [107–112]. However, there are also studies in support of tumor-promoting effects of the CX3CL1-system: one study attributed a pro-metastatic function to the CX3CR1 receptor; however, the authors did not use an immuno-competent mouse model and, thereby, excluded the influence of immune modulatory effects [101]. Another study shows that CX3CL1 promotes the development of tumors, but not metastasis, in HER2 transgenic mice *via* transactivation of the EGF pathway [113]. This direct effect on tumor cells may relate to the fact that the tumor cells themselves express CX3CR1, whose activation directly triggers proliferation and migration [100, 101]. So far, it has not been satisfactorily clarified yet to what extent the two forms of CX3CL1 (membrane-bound *vs.* soluble) contribute to these different effects.

6 Posttranslational modification of chemokines *via* proteolytic cleavage

6.1 Cleavage of CXCR3 ligands

The activity of CXCR3 ligands is regulated within a complex and well-orchestrated network of different modulatory processes. Besides the regulation on both the transcriptional and translational level, modulation of chemokine activity by proteases comes more and more into focus. Moreover, a

regulatory loop has been proposed in breast cancer, in which an increased expression of CXCL9 and/or CXCL10 leads to induction of cathepsin B gene expression and, in consequence cathepsin B protein levels, which then may reduce chemokine activity [84]. Similar effects were reported for the matrix metalloproteinases MMP-2 and MMP-9 in breast cancer, gastric cancer, colon cancer, and multiple myeloma [114–118]. Finally, a linear correlation of dipeptidyl peptidase 4 (DPP4) and CXCL10 expression was observed in ovarian cancer patients, suggesting a regulatory association between chemokine substrate and the protease DPP4 [33].

6.1.1 MMPs: N- and C-terminal cleavage of CXCR3 ligands

Several members of the matrix metalloproteinase family carry out N- and C-terminal cleavages of CXCR3 ligands (Fig. 3). CXCL9 was reported to be C-terminally truncated by MMP-9 cleaving after Lys⁹⁰, Lys⁹³, and Ser⁹⁴ [119]. In another study, in which several MMPs were analyzed, cleavage by MMP-7 and MMP-12 after Lys⁹⁰ was observed as well. Interestingly, under the conditions used in the latter study, MMP-9 did not at all process CXCL9 [120].

For CXCL10, the cleavage sites for both MMP-8 and MMP-9 were mapped to Glu⁷¹↓Arg and Ser⁷³↓Lys⁷⁴. In addition, MMP-9 cleaved CXCL10 after Val⁶⁸ [119]. In another study, MMP-9 was reported to cleave only at Ser⁷³↓Lys⁷⁴ coupled with an additional N-terminal cleavage after Ser⁴. N-terminal cleavage was observed also for MMP-2, after Arg⁵, together with C-terminal cleavage at Ser⁷³↓Lys⁷⁴ [121]. Finally, Cox and co-workers [120] reported that MMP-12 cleaves CXCL10 C-terminally after Glu⁷¹ and Ser⁷³. Of note, in contrast to the study by van den Steen and co-workers [119], cleavage of CXCL10 by MMP-8 and MMP-9 was not observed, even with high enzyme-substrate ratios. MMP-7 degraded CXCL10 without generating stable intermediates.

For CXCL11, both N- and C-terminal processing by MMPs was reported. MMP-12, MMP-8, and MMP-9 cleave after Phe⁴ and after Ser⁵⁸. Only MMP-8 displays an additional cleavage site at Leu⁶³↓Ile⁶⁴. Similar to CXCL10, MMP-7 mainly degrades CXCL11. However, a transient cleavage product could be detected by mass spectrometry corresponding to CXCL11_{1–58} [120]. N-terminally truncated forms, CXCL11_{5–73} and CXCL11_{5–58}, have no detectable agonist activity in Ca²⁺ mobilization assays and only a very low activity in chemotactic migration assays [120] (Table 1).

6.1.2 Furin and carboxypeptidase: C-terminal truncation of CXCL10 does not change chemokine activity

In primary human keratinocytes, besides the full length form, a C-terminally truncated form of CXCL10, lacking

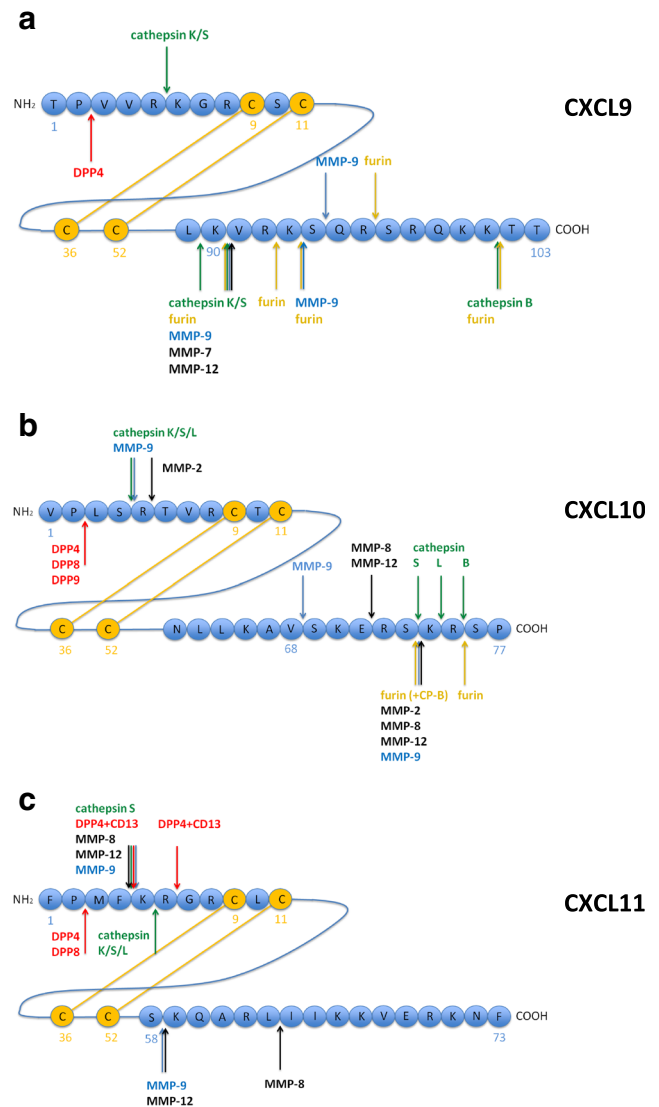


Fig. 3 Reported cleavage sites in the CXC chemokines CXCL9, CXCL10, and CXCL11. N-terminal and C-terminal amino acid sequences are depicted for CXCL9 **A**, CXCL10 **B**, and CXCL11 **C**. Cleavage sites by DPPs and CD13 are indicated in red, by cathepsins in green, by furin (in the presence or absence of CP-B) in orange, by MMP-9 in blue and by other MMPs in black

the last four amino acids, was detected [128]. Whereas a broad range MMP inhibitor had no effect on processing, an inhibitor directed against furin completely inhibited processing. Subsequently, it was shown *in vitro* that furin is able to cleave off two amino acids, which generates the dibasic sequence Lys-Arg at the newly generated C-terminus representing a high-affinity substrate for carboxypeptidase B. This truncated CXCL10_{1–73} form retains its chemotactic activity [128] (Table 1). Under conditions, where full conversion of CXCL10_{1–77} to CXCL10_{1–75} was obtained, CXCL9 was only partially cleaved at five different positions. CXCL11 was no substrate for furin (and CB) (Fig. 3).

Table 1 Characteristics of truncated chemokine forms

	Truncated form	Effects on chemokine-mediated processes	Reference
CXCL9 (1–103)	6–90	Reduced induction of Ca ²⁺ influx	[122]
	6–89	Reduced induction of Ca ²⁺ influx	[122]
	3–103	Lack of chemotactic activity, reduced induction of Ca ²⁺ influx, full angiostatic activity	[123]
	1-78 ^a	Reduced induction of Ca ²⁺ influx	[124]
CXCL10 (1–77)	5–77	Lack of chemotactic activity, no induction of Ca ²⁺ influx	[122]
	3–77	Reduced CXCR3 binding, reduced chemotactic activity, no induction of Ca ²⁺ influx, full angiostatic activity, CXCR3 antagonist ^b	[123]
CXCL11 (1–73)	1–73	Full chemotactic activity	[125]
	5–73	Reduced chemotactic activity, no induction of Ca ²⁺ influx, CXCR3 antagonist ^c	[120]
	5–58	Lack of chemotactic activity, no induction of Ca ²⁺ influx, no heparin binding	[120]
	3–73	Reduced CXCR3 binding, reduced chemotactic activity, no induction of Ca ²⁺ influx, CXCR3 antagonist ^d	[123, 126]
mCX3CL1 (1–76)	6–73	Lack of chemotactic activity	[122]
	5-78 ^e	Lack of chemotactic activity, no induction of Ca ²⁺ influx, CX3CR1 antagonist	[127]

^a CXCL9_{1–78} corresponds to a truncated version of recombinantly expressed human CXCL9_{1–103} in Chinese hamster ovary cells generated by (an) unknown protease(s)

^b CXCL10_{3–77} inhibits CXCL10_{1–77}-mediated chemotaxis

^c CXCL11_{5–73} inhibits CXCL11_{1–73}-induced Ca²⁺ influx and CXCL11_{1–73}-mediated chemotaxis

^d CXCL11_{3–73} inhibits CXCL11_{1–73}, but not CXCL10_{1–77}-mediated chemotaxis

^e The chemokine domain of CXCL11/fractalkine (aa 1–76 or its truncated form 5–76) was expressed as recombinant protein with a two amino acid extension at the C-terminus

6.1.3 Cathepsins: cleavage modulates chemokine activity

Different internal cleavage sites for chemokines of the CXCR3 ligand family were mapped for some cysteine proteases of the cathepsin protease family [122] (Fig. 3). Interestingly, GAGs seem to stabilize cleavage intermediates or even prevent (further) processing in the C-terminal region of the cytokines. CXCL9 is internally cleaved by both cathepsin K and S near the N-terminus after Arg⁵ as well as in the C-terminal part after Leu⁸⁹ and Lys⁹⁰, respectively. Cathepsin B clips off the C-terminal two amino acids, whereas cathepsin L rapidly degrades CXCL9. Cathepsin K, L, and S cleave CXCL10 between Ser⁴ and Arg⁵. Furthermore, incubation of CXCL10 with cathepsin B, L, and S leads to C-terminally truncated forms lacking two, three and four amino acids, respectively. In CXCL11, major cleavage sites were mapped after Phe⁴ and Lys⁵ for cathepsin S. The latter cleavage site was also allocated to both cathepsin K and L. Some of the cathepsin-generated forms were further analyzed in cell biological assays in comparison with the respective chemotactically active full length cytokines: in Ca²⁺ mobilization assays, cathepsin S-cleaved CXCL9 (CXCL9_{6–89}; CXCL9_{6–90}) displayed considerably reduced chemokine activity, whereas cathepsin S-cleaved CXCL10 (CXCL_{5–77}) showed no activity

at all (Table 1). In migration assays, CXCL10_{5–77} (generated by incubation with either cathepsin S or L) and CXCL11_{6–73} (generated by cathepsin L), respectively, were inactive as well [128].

6.1.4 Dipeptidyl peptidases and aminopeptidase N (CD13): N-terminal cleavage impairs chemotactic function

Other naturally occurring cleavage products result from N-terminal cleavage events. In this respect, another protease family has gained attention in recent years, namely the exopeptidase family of dipeptidyl peptidases (DPPs), which cleave dipeptides from the N-terminus. Especially, DPP4 removes N-terminal amino acids from all classical CXCR3 ligands (Fig. 3) causing impaired chemotactic activity as well as reduced Ca²⁺ influx [123, 126, 129]. Similarly, other members of the DPP family process CXCR3 chemokines, e.g., DPP8 inactivates CXCL10 and CXCL11 [130]. In addition, CXCL10 was suggested as a substrate for DPP9 [131]. Since the cleavage pattern is similar to that of DPP4 and DPP8, inactivation of the chemotactic ability of the cleavage product seems plausible as well. Aminopeptidase N (CD13), which removes single N-terminal residues, generates a truncated

version of CXCL11 devoid of any chemotactic activity or binding to the CXCR3 receptor [132] (Table 1).

6.2 Cleavage of CX3CL1

For CX3CL1, proteolytic cleavages play a major role in revealing the full potential function of the protein. Proteolytic cleavages convert the membrane-bound form to the soluble form, especially the metalloproteases ADAM17 and ADAM10 [133–135]. Activity of these ADAMs releases a soluble form of CX3CL1 encompassing the chemokine domain and large parts of the mucin-like stalk (Fig. 4). Since addition of ADAM10 and/or ADAM17 inhibitors did not completely prevent the release of soluble CX3CL1, other proteases were proposed to be involved in the shedding process as well [136]. In an unbiased mass spectrometry-based substrate screen for MMP-2, CX3CL1 was identified as a substrate for this matrix metalloproteinase. Cleavage by MMP-2 results in the release of a soluble form of CX3CL1 lacking the

mucin-like stalk [57]. The MMP-mediated release of CX3CL1 peptides was validated *in vitro* by the addition of TIMP2 and TIMP3, natural inhibitors of MMP-2 and other MMPs [137]. MMP-2 also generates an N-terminally truncated form of CX3CL1 lacking four amino acids [57]. Recombinantly expressed CX3CL1_{5–78} did neither induce Ca²⁺ influx nor displayed any chemotactic activity. Furthermore, competitive chemotaxis assays revealed CX3CL1_{5–78} as a CX3CR1 antagonist [127] (Table 1).

Another sheddase for CX3CL1 was found in the context of microglia signaling, where cathepsin S was shown to be a major regulator of generation of soluble CX3CL1 [138]. CX3CL1 may also be targeted by MMP-9: in a non-obese diabetic (NOD) mouse model, Wildenberg and co-workers [139] detected 17 kDa and 19 kDa cleavage products of CX3CL1 in the salivary gland, which was accompanied by an increased gelatinase and α -secretase activity. *In vitro* cleavage assays excluded ADAM10 and 17 as well as MMP-2 as responsible enzymes and, thus, pointed to MMP-9 as one of

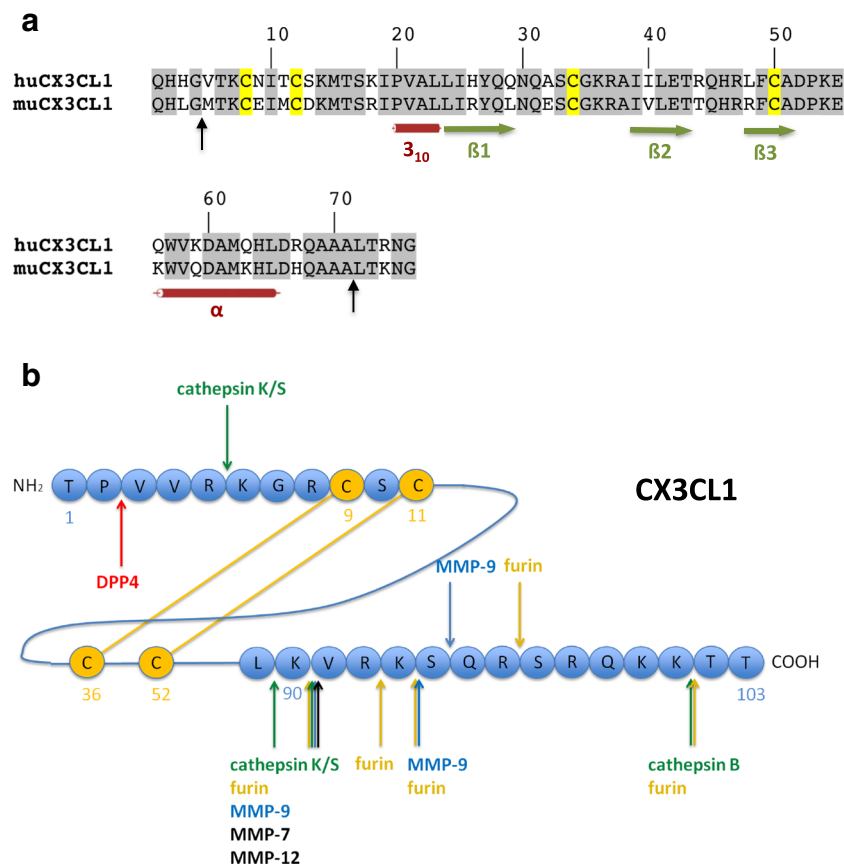


Fig. 4 Cleavage of CX3CL1/fractalkine by proteases. **A** Alignment of the cytokine domain of human and murine CX3CL1. Secondary structure elements are indicated by green arrows (β -strands) and red cylinders (α -helix and 3_{10} -helix). The cleavage sites of MMP-2 (black arrows) were determined *via* cleavage of the murine recombinant ectodomain of CX3CL1 with human MMP-2. **B** Schematic representation of CX3CL1. The N-terminally located cytokine domain (aa 1–76) is linked to the transmembrane domain (plus short cytoplasmic tail) *via* a mucin-

like stalk. N-terminal and C-terminal amino acid sequences of the cytokine domain of murine CX3CL1 are depicted. The cytokine domain can be shedded either together with the mucin-like domains by cathepsin S (green) as well as ADAM10 and ADAM17 (violet) or as isolated domain by MMP-2 (black; Ala⁷¹↓Leu⁷²). The proteolytic cleavage by MMP-2 near the N-terminus (black; Gly⁴↓Leu⁵) results in inactivation of the cytokine

the responsible proteases for this organ-specific cleavage *in vivo*. It should be noted, however, that *in vitro* MMP-9 led to the degradation of the target protein and its inactivation [139].

All these data suggest that proteases have a strong impact on CX3CL1 release suggesting involvement in the modulation of the immune response. Nevertheless, until now it is not fully understood, whether the membrane-bound or soluble form of CX3CL1 has a more regulatory impact.

6.3 *In vivo* relevance of proteolytic chemokine processing

While cleavage and its functional consequences of CXCR3 and CX3CR1 chemokines by proteases have been rather well characterized on the biochemical level, there is hardly any *in vivo* data on the impact of these cleavage processes on tumor-immune interactions.

Only indirect data exists showing that protease expression is associated with unfavorable prognosis and less lymphocytic tumor infiltrates in certain cancers. Regarding matrix metalloproteinases, a recent study showed that inhibition of MMP-9 by a monoclonal antibody in an immuno-competent model of HER2-positive breast cancer increased tumor-suppressive T cell infiltration and CXCR3 chemokine expression. Whether MMP-9-antagonism increases T cell trafficking due to CXCR3 chemokine cleavage *in vivo* was, however, not demonstrated [140].

Most detailed *in vivo* data are available for the DPP4-mediated cleavage of CXCR3 chemokines. In syngeneic models of melanoma and colorectal cancer (B16F10 and CT26 models, respectively), inhibition of DPP4 by sitagliptin or DPP4 knockout led to enhanced T cell infiltration, impaired tumor growth and less metastatic spread [19]. This effect was abrogated in *Cxcr3*^{-/-} mice and dependent on N-terminal CXCL10 truncation. Moreover, immune checkpoint inhibitor therapy was improved by DPP4 inhibition with sitagliptin [19]. These data were confirmed in both a xenograft and a fully immuno-competent model of hepatocellular carcinoma, in which DPP4 inhibition also impaired tumor growth by enhancing the CXCR3-mediated NK and T cell infiltration [141]. However, in another HCC model as well as in a triple-negative breast cancer model, the tumor-suppressive effect of sitagliptin could not be traced back to the CXCR3 chemokine cleavage, but was instead dependent on a CCL11-mediated higher eosinophilic infiltration [142]. Seemingly, the underlying mechanisms of DPP4-mediated anti-tumor immune modulation could be different across tumor types (and maybe across mouse models). In human ovarian cancer, the existence of DPP4-mediated CXCL10 cleavage products was demonstrated, suggesting

a relevant role for these mechanisms also in patients [143].

7 Conclusion

Taken together, CXCR3 and CX3CR1 chemokines are part of a complex regulatory network which orchestrates a broad variety of physiologic functions that can modulate the anti-tumoral immune response. On the one hand, chemokines help to shape the tumor-microenvironment by regulating the amount and type of infiltrating lymphocytes. On the other hand, they are enhancing cell biological processes such as proliferation, invasion, and angiogenesis and, thereby, promote tumor aggressiveness and metastatic potential. Posttranslational modifications of these chemokines by tumor cells, such as proteolytic cleavage, can exert a strong regulatory impact resulting in a shift towards a tumor-promoting environment. Proteolytic cleavage of the tumor-suppressive CXCR3 and CX3CR1 chemokines impairs their functions and, on top of this, in feedback loops, the chemokines may even lead to increased expression of the proteases targeting themselves. Thus, we suggest that cleavage of the ligands of the CXCR3 and CX3CR1 chemokine systems represents a potent immune escape mechanism in cancer. Deeper knowledge of the mechanisms behind this also provides a panel of interesting novel target structures to support existing therapies or develop new ones.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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