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How post-translational modifications influence the biological activity of chemokines

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ABSTRACT

Chemokines are important proteins involved in the regulation of directed leukocyte migration during inflammation and the homeostatic homing of immune cells. In addition, they play a role in angiogenesis, hematopoiesis, organogenesis, tumor growth and metastasis. Therefore, the chemokine/chemokine receptor network is highly complex and needs to be tightly controlled. An important mechanism of fine-tuning chemokine activity and reducing its apparent redundancy is post-translational modification (PTM) of chemokines and their receptors. Under inflammatory conditions, enzymes such as matrix metalloproteinases (MMPs), plasmin, CD13, CD26, and peptidylarginine deiminases (PADs) and protein-modifying agents, such as peroxynitrite, are upregulated and released and may provoke truncation, degradation, nitration or citrullination of chemokines. Most modified chemokines show altered biological activity. This review reports how PTMs influence the biological functions of chemokines, with special attention for the impact beyond chemotaxis.

1. Introduction

1.1. Chemokines

Chemokines are small proteins (8–14 kDa) which mediate a great variety of functions, but are mainly involved in the regulation of leukocyte trafficking [1–4]. Chemokines are locally secreted by different cell types such as resident leukocytes, endothelial cells and fibroblasts. In total, about 50 human chemokine ligands have been identified and classified based on their function in homeostasis or housekeeping or as inducible inflammatory chemokines with a role in disease [5–7]. The latter subclass is locally secreted upon infection or tissue damage and requires prior induction by endogenous or exogenous stimuli. In contrast, homeostatic or housekeeping chemokines are expressed constitutively in lymphoid or other organs and mediate homeostatic migration and homing of various immune cells. Moreover, an emerging number of chemokines fullfils both homeostatic and inflammatory roles, illustrating that the functional classification is non-absolute (*vide infra*). In addition, chemokines can be classified into four families (CC, CXC, CX_3C and C chemokines) based on the pattern of two conserved cysteine residues in the NH_2-terminal region [3].

To exert their biological functions, chemokines need to interact with two major interaction partners namely (1) glycosaminoglycans (GAGs) and (2) seven transmembrane G protein-coupled receptors (GPCRs) designated CCR, CXCR, CX_3CR and XCR according to the nomenclature of the ligands [3,8]. GAGs are linear polysaccharides consisting of repeating disaccharide subunits with a molecular weight of 10–100 kDa. In brief, once inflammatory chemokines are secreted they create, through GAG binding, a gradient along which leukocytes can migrate from the blood vessel to the site of inflammation [9–12]. Subsequently, GAG-bound chemokines interact with their leukocyte-specific chemokine receptor resulting in adhesion to and extravasation of leukocytes through the endothelium [13–15]. This GAG binding of chemokines has been proven to be indispensable for chemokine activity *in vivo* [16–18]. The interaction between chemokines and GAGs occurs between basic amino acid motifs, frequently of the form BBXB or BBBXXBBX (in which B represents a basic and X represents any non-basic amino acid), and sulfated or carboxylated domains of GAGs [19]. On some chemokines, GAG binding motifs are located in the COOH-terminal region of the chemokine, at a site distant from the specific receptor-binding site. However, on a number of chemokines these sites are at least partially overlapping. For example, it was recently shown that interaction

Abbreviations: ACKR, atypical chemokine receptor; ADAM, a disintegrin and metalloprotease; $[Ca^{2+}]_i$, intracellular calcium concentration; CTAP-III, connective tissue-activating peptide III; DPP4, dipeptidyl peptidase 4; ERK, extracellular signal-regulated kinase; GAG, glycosaminoglycan; GPCR, G protein-coupled receptor; MMP, matrix metalloproteinase; PAD, peptidylarginine deiminase; PBP, platelet basic protein; PTM, posttranslational modification

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between CXCL13 and heparan sulfate relies on residues present in the COOH-terminal region and α helix of the chemokine, whereas CXCL13/CXCR5 binding depends on its NH_2-terminal domain [20]. Contrastingly, for several chemokines including CXCL10, mutation of specific residues was found to affect both the affinity for GAGs and GPCRs [21]. In line with these observations, we previously found that natural deimination of Arg5 of CXCL10 negatively affects its GAG binding and CXCR3 signaling potencies [22].

Chemokines can affect other cell types, thereby playing a role in angiogenesis, hematopoiesis, organogenesis, tumor growth and metastasis [23–25]. For example, CXCL4 and CXCL4L1 are known to shift the angiogenic/angiostatic balance in favor of angiostasis by inhibiting endothelial cell proliferation and migration. In addition, CXCL4L1 prevents the development and metastasis of various tumors [26]. In contrast, it has been shown that CXCL8 expression in humans correlates with an increase in tumorigenesis of bronchogenic carcinomas [27]. In addition, CXCL8 is associated with various cancers such as pancreatic cancer, prostate cancer and ovarian cancer [28–30]. Besides CXCL8, other CXC chemokines are involved in angiogenesis and metastasis [24]. For example, the CXCL12-CXCR4 axis has been shown to play a critical role in tumor metastasis, since it promotes the migration of tumor cells into metastatic sites. Moreover, CXCR4 is the chemokine receptor which is most often overexpressed in human tumors [31]. Finally, homeostatic chemokines control basal cell migration [32]. However, the relevance of homeostatic chemokines may extend beyond merely homeostatic processes. For example, a significant correlation was demonstrated between CXCL13 and its receptor CXCR5 – initially designated as homeostatic chemokine – chemokine receptor pair – and prostate cancer, with CXCL13 being a more reliable predictor of prostate cancer than prostate-specific antigen (PSA) [33]. In healthy peripheral tissues, homeostatic chemokines are responsible for leukocyte migration for immune surveillance and maintenance of mucosal immunity [34–36]. Furthermore, they navigate leukocytes during hematopoiesis in bone marrow and thymus, during initiation of adaptive immune responses in the spleen and lymph nodes and during organ development. Genetic deficiency of CCL21, for example, results in impaired dendritic cell migration and T cell priming in the lymph nodes [37]. Mice deficient for CXCL13 or CXCR5 show defective lymphoid tissue development [38]. Moreover, a defect in CXCR7/ACKR3 or its ligand CXCL12 even results in perinatal lethality due to disrupted cardiac development [39–42]. In addition, targeted mutation of CXCL12 and CXCR4 results in defective myelopoiesis and B cell lymphopoiesis. Noteworthy, some chemokines can fall into both categories depending on the biological context or pathological state, demonstrating that the initially proposed harsh distinction between inflammatory and homeostatic chemokines is rather non-absolute [43].

1.2. The regulation of chemokines

The availability and activity of chemokines are regulated at multiple levels to control the inflammatory response and physiological leukocyte homing [44–46]. Upon infection or tissue damage, it is essential to have an immediate up-regulation of chemokines and their receptors for the generation of a rapid influx of leukocytes. However, this influx also requires to be terminated upon resolution of the challenge. Otherwise, the persistence of an inflammatory response may lead to tissue damage and chronic inflammation. Therefore, several mechanisms of chemokine regulation are known [44,45]. As discussed before, the expression of, especially, inflammatory chemokines is upregulated by local transcription of chemokine mRNA by inflammatory stimuli. Chemokine mRNA is often highly unstable and a target for degradation [47,48]. Moreover, for some chemokines, different isoforms are generated from a single gene. This process of alternative splicing has been shown to have significant consequences for the biological activity and the tissue distribution of chemokines, such as CXCL12 [49–52]. Second, chemokines tend to synergize directly or indirectly with other chemokines thereby providing a powerful mechanism to strengthen leukocyte recruitment [53]. In contrast, chemokines can also counteract each other, thereby increasing the selectivity of cell recruitment or reducing the inflammatory responses. A third mechanism is the fine-tuning of chemokine availability by binding to atypical chemokine receptors (ACKRs), such as Duffy antigen receptor for chemokines (DARC) (ACKR1), D6 (ACKR2), CXCR7 (ACKR3), Chemocentryx chemokine receptor (CCX-CKR) (ACKR4) and CCRL2 (ACKR5) [54–56]. Due to the presence of a modified or missing canonical DRYLAIV motif and the resulting inability to couple to G proteins these atypical receptors are unable to induce conventional G protein-coupled signaling. In addition, chemokines have been shown to bind to GAGs which influences the chemokine availability by binding of the chemokines to the endothelium and presentation to their specific chemokine receptor [8]. Finally, different types of posttranslational modifications (PTMs) of chemokines, including proteolytic cleavage, glycosylation, citrullination and nitration, have been reported [57]. These PTMs have consequences on the chemokine activity and/or receptor selectivity, thereby decreasing, inactivating or potentiating the chemokine function.

In this review, we will give a summary of PTMs of chemokines and the functional consequences for their biological activity. Since chemokines contribute to a variety of functions on different cell types, we report the influence of the alterations by biological effect, namely chemotaxis, hematopoiesis, angiogenesis, tumor growth and metastasis and antiviral activity. In addition, part of this manuscript will be dedicated to the interaction of chemokines with GAGs, the influence of PTMs on GAG binding and PTMs of chemokine receptors.

2. General aspects of PTMs of chemokines

Emerging evidence points towards a potentially central role for PTMs in the regulation of protein activity [58–64]. Chemokine isoforms generated by PTM were isolated from natural sources including cell culture supernatants and body fluids [57,65]. The currently described chemokine modifications are proteolytic processing resulting in truncation or degradation, nitration, citrullination and glycosylation. Depending on the ligand and mode of processing, different isoforms of a specific chemokine can display dramatically altered biological activities and receptor interactions.

2.1. Truncation & degradation

Proteolytic truncation may occur at the NH_2- and COOH-terminal chemokine domain, and is the best studied and probably most common way of chemokine modification [57]. Additionally, certain endopeptidases cleave chemokines internally followed by subsequent degradation of the ligand involved. Inflammatory chemokines in particular seem most susceptible to proteolysis. Moreover, the abundance of chemokine-modifying proteases is especially high in an inflammatory environment [66–69]. Proteases such as CD13 and CD26 can be present as membrane-associated molecules or exist as soluble variants in body fluids, e.g. plasma [70,71]. Members of the matrix metalloproteinase (MMP) family, among others, are stored in intracellular vesicles and are rapidly released upon appropriate stimulation [66]. Consequently, one may speculate that proteolytic processing of chemokines may become predominantly relevant during inflammation. The consequences of truncation for chemokine functioning are highly complex, including increased or decreased biological activity, inactivation, change of receptor preference or generation of receptor antagonists [57]. An interesting example in this context is the prototype CXCR1/2 agonist and most potent neutrophil-attracting chemokine in humans, i.e. CXCL8. Upon its discovery, it left no doubt that natural CXCL8 displays an exceptional degree of NH_2-terminal heterogeneity, with most of the identified CXCL8 isoforms being characterized by loss of up to eight NH_2-terminal residues [72–78]. For CXCL8, NH_2-terminal shortening

usually correlates with an increased biological activity. For example, CXCL8(6–77) was ten times more potent in *in vivo* neutrophil chemotaxis assays compared to authentic CXCL8 [79]. Minor truncation by CD13 generating CXCL8(2–77) or CXCL8(3–77) only minimally alters its biological activity, whereas the shortest identified natural isoform CXCL8(9–77) displays the strongest neutrophil-chemotactic capacity, at least *in vitro* [80–82]. Enzymes responsible for CXCL8 truncation include MMP-1, MMP-8, MMP-9, MMP-13, MMP-14, plasmin, thrombin, cathepsin L, proteinase-3 and CD13 [79,83–87]. Interestingly, truncated CXCL8 is a more potent inducer of MMP release by neutrophils, providing a positive feedback loop enhancing neutrophil migration and activation [87]. Another example is the potentiation of CXCL5 by MMP-2 and MMP-9, thereby promoting neutrophil migration *in vivo* [88]. Interestingly, the activities of MMP-2 and MMP-9 were shown to promote chemokine-induced leukocyte migration at the blood-brain barrier [89]. The potential importance of PTMs in the regulation of chemokine activity is in particular clearly illustrated by the processing of CXCL7 and CCL14, as truncation is a prerequisite for these chemokines to become chemotactically active (Appendix A and B). Specifically, the exceptional chemokine CXCL7 is a 70 amino acid derivative generated by enzymatic cleavage of the 96 residue-containing precursor platelet basic protein (PBP) or connective tissue-activating peptide III (CTAP-III) [90–92] (Appendix B). The CCR1 and CCR5-dependent chemotactic activity of the constitutively expressed CCL14 also truly depends on NH_2-terminal truncation of its inactive pro-form CCL14(1–74) [93,94] (Appendix A). Worth mentioning, NH_2-terminal cyclization of glutamine into pyroglutamic acid is mandatory for chemotactic activity of the monocyte chemotactic protein CCL2 [95]. This modification also protects CCL2, CCL7, CCL8 and CCL13 from cleavage by CD26 or dipeptidyl peptidase 4 (DPP4) [96]. The isoenzyme of glutaminyl cyclase was recently identified to be responsible for pyroglutamic acid conversion of human CCL2 and its murine equivalent [97]. In a murine model for atherosclerosis, inhibition of this enzyme resulted in reduced CCL2-induced monocyte influx, supporting the idea that *in vivo* interference with chemokine PTMs can be therapeutically beneficial [97].

Whereas limited NH_2-terminal truncation potentiates the activity of most ELR^+CXC chemokines including CXCL1, CXCL5 and CXCL8 and several CC chemokines including CCL3, CCL4, CCL14 and CCL23, other CC chemokines such as CCL2, CCL7 and CCL22 are characterized by impaired chemotactic activity upon NH_2-terminal cleavage by MMPs for example (Appendix A and B and [57]). Additionally, truncated isoforms of CC relatives including CCL5 are featured by altered receptor preference. The CCR1 and CCR3 affinity of CCL5(3–68), for example, is attenuated compared to intact CCL5, but its CCR5 binding and signaling potency are enhanced [98,99]. During the purification of natural CCL5 from *in vitro* cell cultures, our lab previously demonstrated that the ratio of CCL5(3–68) over native CCL5 depends on the cellular source [98] . Specifically, CCL5(3–68) and no intact CCL5 was identified in the supernatant of sarcoma cells. Also in whole blood and conditioned medium from diploid fibroblasts, the truncated variant CCL5(3–68) was the predominant CCL5 isoform. However, mononuclear cells produced mostly intact CCL5. Moreover, CCL5(3–68) was also found to occur *in vivo* [100]. In addition to NH_2-terminal truncation, specific enzymes including MMPs and furin may also truncate chemokines COOH-terminally. For example, the ELR^-CXC chemokines CXCL9 and CXCL10 can be truncated at the COOH-terminus by MMP-12 [101,102]. Remarkably, upon analysis of cell culture supernatant from dsRNA and IFN-γ-stimulated PBMCs, and from dsRNA-, IFN-γ-, or LPS-stimulated fibroblasts, no native CXCL9 was detected but only COOH-terminally truncated isoforms [103]. The COOH-terminal heterogeneity of CXCL9 was already noticed upon its discovery in 1995 [104]. Hence, although probably less common than NH_2-terminal cleavage, COOH-terminal processing seems biologically relevant for certain chemokines. Many question marks remain regarding enzymes responsible for COOH-terminal truncation of chemokines including CXCL9 and the implications for their biological functioning. However, in case of COOH-terminal truncation of CXCL9, the isoform CXCL9(1–78) showed an impaired activity on T cells. MMPs such as MMP-8 for CXCL9 and MMP-7 and MMP-9 for CXCL10, may rather provoke complete chemokine digestion [101,102]. MMP-9 also degrades several ELR^+CXC chemokines including CXCL1 and CXCL7. Proteases responsible for digestion of CC chemokines include cathepsin D (CCL2, CCL4, CCL15), tryptase (CCL5, CCL11) and chymase (CCL11) [105,106].

Regarding proteolytic processing of chemokines *in vitro* and *in vivo*, one of the most intensely studied enzymes is the serine protease CD26 (reviewed in [107]; [108]). Hence, the complex interactions between CD26 and chemokines will be discussed.

2.2. Truncation by CD26

It was already described that numerous chemokines including CCL3L1, CCL4, CCL5, CCL11, CCL14, CCL22, CXCL2, CXCL6, CXCL9, CXCL10, CXCL11 and CXCL12, are substrates for DPP4 or CD26 (reviewed in [107]; [108]). The related enzyme DPP8 also cleaves some of these, including CXCL10 and CXCL11 [109]. The consequences of CD26-mediated truncation are substrate-dependent and have been studied in an *in vitro* and *in vivo* context most intensely for CXCL10 and CXCL12. CD26 is a cell-bound enzyme ubiquitously expressed on blood cells, especially on activated T cells, fibroblasts, epithelial and endothelial cells [110–112]. However, a soluble and enzymatically fully active form of CD26 exists in plasma and abundantly occurs in seminal fluid. CD26 exhibits serine protease catalytic activity resulting in the cleavage of dipeptides from substrates with a (hydroxy)Pro or Ala in the penultimate NH_2-terminal position. Therefore, the three CXCR3 ligands CXCL9, CXCL10 and CXCL11 are substrates for CD26, with especially CXCL10 and CXCL11 as short half-life substrates [113,114]. Both the CD26-dependent cleavage of CXCL10(1–77) and CXCL11(1–73) into CXCL10(3–77) and CXCL11(3–73), respectively, results in a loss of chemotactic activity for $CXCR3^+$ cells and lymphocytes [113,115,116]. Moreover, this site-specific truncation turns CXCL10 and CXCL11 into CXCR3 antagonists because they retain CXCR3-binding properties. CD26 can provide a positive- or negative-feedback loop since most of the chemo-attracted leukocytes express CD26. For example, cytokine-stimulated fibroblasts produce CXCL10 and enhanced membrane-bound CD26 activity, revealing the simultaneous induction of CXCL10 and CD26 and suggesting the existence of a negative feedback machinery [116]. In addition, activated T lymphocytes simultaneously express CXCR3 and CD26. Migration of activated T lymphocytes towards CXCR3 ligands, such as CXCL11, leads to accumulation of these CD26-expressing cells at sites of inflammation. This results in the truncation and inactivation of the local CXCR3 ligands by CD26, even generating antagonistic variants preventing further infiltration of activated T cells [115]. Analogously, CD26-mediated truncation of CXCL12 results in an inactive isoform CXCL12(3–68) with reduced CXCR4 affinity, loss of its calcium-dependent signaling potency and chemotactic properties for peripheral blood lymphocytes [117–120]. Recently, it was shown that CXCL12(3–68) was no longer able to induce IP_3, Akt and extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling through CXCR4 [121]. In addition, CXCL12-induced chemotaxis of mononuclear cells and endothelial cells was abrogated by CD26. Identical to CXCL10 and CXCL11, the site-specific truncation turns CXCL12 into a CXCR4 antagonist through desensitization [118]. *In vivo*, it was shown that CXCL12 is converted to CXCL12(3–67) very rapidly [122]. Furthermore, the presence of CD26-truncated CXCL12 in murine, human and rhesus monkey plasma confirms the hypothesis that CXCL12 gets cleaved by CD26 *in vivo* [123–125]. Pretreatment of mice or rhesus monkeys with a CD26 inhibitor reduced the plasma levels of CXCL12(3–68). Analogously, plasma of wild-type mice contains significant amounts of CD26-truncated CXCL12, whereas $CD26^{-/-}$ mice contain intact CXCL12. In addition, in mice treated with the CD26 inhibitor sitagliptin, the potential of CXCL12 to induce lymphocyte migration to tissues was enhanced. The importance of CXCL12 processing

in vivo was further evidenced by the observation that CXCL12 improved wound healing [58]. However, constant CXCL12 delivery was essential since it was rapidly inactivated. Local production on the skin of CXCL12 by Lactobacilli created an acidic environment in which CD26 was inactive and unable to inactivate this chemokine.

Most ELR$^+$CXC chemokines contain no penultimate NH_2-terminal proline or alanine and are no substrates for CD26. However, CXCL2 and CXCL6 are cleaved by CD26 *in vitro* [109]. The biological activity of CXCL6(3–77) on neutrophils is not altered compared to intact forms [126,127]. An important side note is that the mouse chemokine CXCL1, in contrast to human CXCL1, contains a proline residue in the penultimate position, suggesting it to be a substrate for CD26. The monocyte chemotactic proteins CCL2, CCL7, CCL8 and CCL13 are protected against CD26-mediated truncation by their NH_2-terminal pyroglutamic acid [96]. However, several CC chemokines are substrates for CD26, including CCL3L1, CCL4, CCL5, CCL11 and CCL22 [108].

2.3. Nitration

The infiltration of immune cells able to mount an oxidative burst is a major and recurring cause of tissue injury during inflammation. Both the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been reported in pathological conditions associated with oxidative stress including diabetes, organ transplantation and cancer [128–131]. Macrophages and neutrophils produce nitric oxide (NO) and the superoxide anion (O_2^-) which can directly oxidize molecules or form the highly reactive peroxynitrite ($ONOO^-$). This peroxynitrite can spontaneously nitrate aromatic amino acids including tyrosine and tryptophan [132,133]. Although, the short half-life of peroxynitrite prevents its detection *in vivo*, the presence of nitro-tyrosine is a commonly used marker in inflammation and in several human cancers, including liver, breast and colon cancers [132,134–136]. In this way, RNS including NO and the nitration of proteins by peroxynitrite are key regulators in the immune system and can have both a beneficial or malignant role [137,138]. For example, in the tumor microenvironment, nitration of proteins is one of the key mechanisms involved in tumor-induced immune dysfunctions [139]. This PTM of proteins may result in a decrease or loss of function, an increase of protein activity or no alteration in activity [135,138,140]. Until now, *in vitro* nitration of the chemokines CCL2, CCL3, CCL5, CCL11, CXCL8 and CXCL12 by peroxynitrite has been described [130,141–144] (Appendix C and D). The presence of nitro-tyrosine and/or nitro-tryptophan were only shown for CCL2 and CXCL12 by mass spectrometry or Edman degradation, respectively [145,146]. Chemical nitration of CCL2 resulted in a significant reduction of monocyte chemotactic activity *in vitro* and *in vivo* [147]. This could be explained by a reduced affinity for CCR2b and for GAGs. Moreover, intravenous administration of nitrated CCL2 blocked the *in vivo* recruitment of leukocytes towards the non-nitrated wild-type CCL2. However, another report showed that the CD14$^+$ monocyte chemotaxis remained unaltered upon nitration of CCL2, although an impaired capacity to attract antigen-specific CD8$^+$ T cells into the tumor tissue in mice was observed [146]. In addition, blocking of the CCL2 modification with the small molecule AT38 {[3-(aminocarbonyl)furoxan-4-yl]methyl salicylate} facilitated cytotoxic T lymphocyte invasion of the tumor, suggesting that this drug may be effective in cancer immunotherapy. In addition, the neutrophil and monocyte chemotactic activity of CCL3 and the eosinophil chemotactic activity of CCL5 and CCL11 were attenuated by peroxynitrite treatment [141–143]. However, it has been reported that treatment with peroxynitrite could result in protein degradation, which would also destroy the chemotactic activity directly. In addition, the neutrophil chemotactic activity of CXCL8 was attenuated upon treatment with peroxynitrite [144]. More recently, Janssens et al. described the nitration of CXCL12 on Tyr7. This nitrated form of CXCL12, [3-NT^7]CXCL12, was produced by bone marrow stromal cells enriched with primary leukocytes under inflammatory conditions and was shown to have reduced monocyte and lymphocyte chemotactic activity *in vitro* [145]. Also *in vivo,* [3-NT^7]CXCL12 was not able to recruit lymphocytes into the joint. However, unlike CCL2, nitration of CXCL12 did not change the GAG binding affinity or the receptor binding. The difference in chemotactic activity between wild-type CXCL12 and [3-NT^7]CXCL12 is suggested to rely on a reduced ability to enhance intracellular calcium concentrations ($[Ca^{2+}]_i$), to generate inositol triphosphate and to phosphorylate ERK1/2. In addition, it was shown that a second tyrosine residue in CXCL12 may be nitrated (unpublished data).

2.4. Glycosylation

Although N- or O-glycosylation have been described for some chemokines, the *in vivo* importance of this phenomenon is still to be elucidated. For example, O-glycosylation of CCL11 at the COOH-terminus (Thr71) and of CCL5 at serine residues does not affect their eosinophil chemotactic activity [148,149] (Appendix C). In addition, an O-glycosylated form (on Ser7) of CCL14 was detected in human plasma [150] and O-glycosylation of CCL2 was described in the COOH-terminal region of the protein [151–153]. Due to the presence of sialic acid residues, the charge of the chemokine is altered. In this way, glycosylated CCL2 showed less *in vitro* chemotactic activity compared to the unglycosylated natural CCL2. However, it was shown that glycosylation of CCL2 improves its functional stability [154]. In this way, the partial loss of specific activity due to glycosylation is probably balanced by the advantage of prolonging the effectiveness of the chemokine. XCL1, which only contains one disulfide bridge, occurs in different O-glycosylated or sialylated forms [155] (Appendix D). Although glycosylation of synthetic XCL1 does not alter its calcium signaling potency, recombinant glycosylated XCL1 is more potent in chemotaxis and calcium assays and inhibits T cell proliferation more efficiently [156,157]. CX3CL1 is a protein that contains an additional glycosylated mucin-like stalk and a COOH-terminal transmembrane region with cytoplasmic loop. Deglycosylation is suggested to facilitate the interaction of the chemokine with its receptor, although no difference in adhesive potency is detected [158]. *In vivo* studies are restricted because of the difficulty to produce recombinant chemokines that contain specific sugar chains identical to the natural human sugars and the limited availability of purified natural glycoforms of human chemokines.

2.5. Citrullination

Peptidylarginine deiminase (PAD)-mediated conversion of Arg to citrulline (Cit) barely alters the protein mass (plus one mass unit for each modified arginine) but results in the loss of a positive charge, potentially altering the 3D structure of the substrate and its interactions with GPCRs, GAGs and lipids [22,79,159,160]. Although concentrations of naturally-occurring citrullinated chemokines may be rather low, experimental evidence suggests that protein citrullination potentially becomes more common during inflammation [161,162]. Thus, one may speculate that citrullinated chemokine isoforms are potential biomarkers for specific diseases. In line with this hypothesis, serum and synovial fluid from patients with rheumatoid arthritis contained enhanced levels of citrullinated CXCL5 [163] (Appendix D). Since most conventional immunoassays do not distinguish between citrullinated and native isoforms of a specific chemokine, combined with the minor mass shift associated with citrullination, the knowledge on the relevance of chemokine citrullination *in vivo* remains limited. Hence, the importance of this modification for chemokine biology may be underestimated. However, in addition to CXCL5, naturally-occurring citrullinated isoforms were also found for CXCL8 and CXCL10, whereas CCL17, CCL26, CXCL11 and CXCL12 can be site-specifically citrullinated upon PAD-incubation *in vitro* [22,79,164] (Appendix C and D). PAD-mediated citrullination significantly altered the *in vivo* activity of CXCL5 and CXCL8 [79,162,165,166]. Citrullinated CXCL8 displayed an increased capacity to induce neutrophil mobilization into the blood

upon i.v. injection in rabbits but reduced chemotactic activity upon i.p. injection in mice [79,166]. As a result of citrullination, CXCL8 is no longer susceptible to enzymatic processing by thrombin or plasmin into NH_2-terminally truncated and more potent CXCL8 isoforms [79]. Also the neutrophil chemotactic activity of CXCL5 is attenuated upon citrullination towards [Cit^9]CXCL5, but remarkably the citrullinated isoform was reported to acquire monocyte chemotactic properties [163,165]. However, it remains to be elucidated whether this is a direct effect of [Cit^9]CXCL5 on monocytes or a secondary result due to [Cit^9]CXCL5-induced release of monocyte chemoattractants by endothelial cells or leukocytes. Also the overall activity of CXCL10 and CXCL11 is attenuated upon citrullination, as shown by an impaired potency to elicit CXCR3 signaling [22]. The citrullinated CXCR3 ligands can still bind to their receptor but exhibit a reduced GAG affinity and are less potent inducers of directed T cell migration compared to the corresponding native chemokines. CXCL12 contains three potential citrullination sites (Arg8, Arg12 and Arg20) in its NH_2-terminal domain. Interestingly, PAD and CXCL12 are co-expressed in Crohn's disease [164]. Citrullination negatively affects binding of CXCL12 to its receptors CXCR4 and ACKR3, and consequently hinders the inflammatory activity of CXCL12 [164].

3. Effects of PTMs on the biological function of chemokines

As indicated above, proteolysis, nitration, citrullination and glycosylation may have important consequences for the biological functioning of chemokines. Numerous enzymes efficiently process natural chemokines generating molecules with differential biological activities and/or receptor preferences, pointing to PTMs as a fast and efficacious manner to shape the chemotactic gradient and inflammatory response. However, the fact that chemokines are central players in various (patho)physiological processes, immediately suggests that the impact of PTMs is not necessarily restricted to chemotaxis, but can be multidimensional. Here, we overview the potential consequences of PTMs on the biological activity of chemokines, with special attention for the impact beyond chemotaxis.

3.1. Effect on angiogenesis

The most potent neutrophil-attracting chemokine in humans, CXCL8, displays a high degree of natural NH_2-terminal heterogeneity and provides an excellent example showing that PTMs of ELR^+CXC chemokines may influence their impact on angiogenesis. Especially a variant missing the five most NH_2-terminal amino acids of native CXCL8 and resulting from cleavage by MMP-8, MMP-13, MMP-14, plasmin, thrombin or cathepsin L [82–86,167], namely CXCL8(6–77), is usually highly abundant in cell culture supernatant from stimulated leukocytes [75–77]. Our lab previously demonstrated that the loss of its five most NH_2-terminal amino acids, but not citrullination of Arg5, increased the angiogenic potency of CXCL8 [79]. Artificial cleavage beyond its ELR motif, which is adjacent to two disulfide bridges and as such probably is protected from proteolytic cleavage, abolished CXCL8-mediated angiogenesis [168]. However, up to now, no natural truncation in or beyond the ELR motif has been observed.

Posttranslational processing can be an intelligent way to control a chemokine's role in angiogenesis, as emphasized by the fact that isoforms of a specific chemokine with minimal amino acid variations may differentially affect angiogenesis. For example, the CD26-truncated isoforms CXCL9(3–103), CXCL10(3–77) and CXCL11(3–73) remain inhibitors of angiogenesis, but the subsequent CD13-mediated loss of additional NH_2-terminal residues of CXCL11 strongly attenuates its angiostatic effect [85,113,114]. Noteworthy, the fact that CXCL9 and CXCL10 retain their angiostatic activity in a wound healing assay after processing by CD26 suggests that the angiostatic potential of these chemokines is either not mediated through CXCR3 or implicates the existence of an alternative CXCR3-triggered signal transduction pathway.

3.2. Effect on hematopoiesis

PTMs may modulate the role of particular CC as well as CXC chemokines in hematopoiesis. For example, the CD26-truncated isoform CCL4(3–69), which is highly secreted by peripheral lymphocytes, acquires affinity for CCR1 and CCR2, thereby changing the receptor specificity (Appendix A). By achieving this affinity for CCR1 and CCR2, CCL4(3–69) is suggested to play a role in the migration of monocytes, immature dendritic cells and lymphocytes. In contrast to CCL4(1–69), the CD26-truncated form no longer enhances proliferation of hematopoietic progenitor cells *in vitro* and counteracts the proliferative effect of authentic CCL4 on single cytokine sensitive hematopoietic progenitor cells *in vitro* [169]. Additionally, murine CCL3 loses its myelosuppressive activity upon cleavage by CD26 and inhibits myelosuppression induced by native CCL3 *in vivo*. However, the receptor through which intact CCL3 exerts its myelosuppressive activity remains to be defined, since this effect was reported to occur in a CCR1 and CCR5 independent way [169]. In contrast to murine CCL3, human CCL3 has no Pro in the penultimate NH_2-terminal position and is consequently not susceptible to processing by CD26. Noteworthy, murine CCL3 is more related to human CCL3L1, which may be truncated by CD26, than to human CCL3 [170]. Also truncation of CXCL9 by CD26 limits its capacity to trigger CXCR3-mediated signal transduction *in vitro* and its myelosuppressive function *in vitro* and *in vivo* [113,169].

CD26-truncated CXCL12 has lost its capacity to recruit hematopoietic progenitor cells [171]. Moreover, the attraction of these cells was enhanced *in vivo* in $CD26^{-/-}$ mice and in wild-type mice that received a CD26 inhibitor [172–174]. Granulocyte-colony stimulating factor (G-CSF), which is clinically used for the mobilization of progenitor cells from the bone marrow, upregulates CD26 expression on $CD34^+$ progenitor cells and induces degradation of CXCL12 by neutrophil elastase. So in addition to its effects as a growth factor, it is suggested that administration of G-CSF leads to CXCL12 cleavage resulting in inactivation of CXCL12 and loss of its function as a retention signal for hematopoietic progenitor cells. Therefore, the dominant presence of truncated CXCL12 within the bone marrow plasma of patients with chronic myeloproliferative neoplasms after G-CSF-induced conversion has been described to cause the key hematopoietic stem cell mobilization [175]. In addition, it was found that CD26 inhibition by sitagliptin administration enhances engraftment and clinical cord blood transplantation in humans with hematological malignancies [176,177].

3.3. Effect on tumor growth and metastasis

As emphasized by their role in angiogenesis and potential to invite immune cells into the tumor tissue, chemokines are implicated in tumor development and metastasis. Hence, PTMs of chemokines potentially play an important role also in cancer biology. An interesting chemokine in this context is the angiogenic protein CXCL16. On the one hand, native CXCL16 is a membrane-bound adhesion molecule and scavenger receptor, but on the other hand, processing by 'disintegrin and metalloproteases' (ADAMs) releases a soluble CXCL16 isoform that acts as a chemoattractant for T cells among others [178,179]. Various types of cancer were associated with increased mRNA levels of CXCL16 and its receptor CXCR6 [180]. Specifically, in contrast to membrane-associated CXCL16, the processed CXCL16 isoform mediated migration and proliferation of CXCR6 positive cancer cells [181]. In addition, the short half-life CD26 substrates, CXCL10 and CXCL12, are involved in tumor biology, with different chemokine isoforms potentially fulfilling opposing roles. In mice, *in vivo* post-translational processing of CXCL10 by CD26 limits lymphocyte migration to tumors, thereby reducing the natural host antitumor immunity [63]. Moreover, inhibition of CD26

activity by sitagliptin enhanced tumor rejection and improved adjuvant-based immunotherapy by preserving biologically active CXCL10 and increasing the infiltration of $CXCR3^+$ lymphocytes into the tumor. Also patients with bladder carcinoma might benefit from the use CD26 inhibitors upon immunotherapy. Recently, inhibition of CD26 in humans was reported to preserve intact CXCL10, offering new therapeutic opportunities for CD26 inhibitors [182]. In patients with Sézary syndrome, however, cancer cells show increased expression of CXCR4 and CXCL12 and absence of membrane-bound CD26, preventing the inactivation of CXCL12 and accelerating CXCL12-mediated homing of neoplastic cells to the skin [64]. Depending on the substrates involved and the mechanism underlying tumor development and progression, CD26 expression in different tumor types has been associated with good as well as poor prognosis [183].

3.4. Effect on antiviral activity

Infection of human leukocytes by M-tropic (R5) and T-tropic (X4) HIV strains involves, respectively, chemokine receptors CCR5 and CXCR4 as coreceptors [37]. Thus, CCR5 and CXCR4 ligands may interfere with HIV entrance. Specifically, CXCL12, the only identified chemokine agonist for CXCR4, exerts antiviral activity by competing with the viral protein gp120 and through initiation of CXCR4 internalization. However, upon processing by CD26 the antiviral activity of CXCL12 is lost [117,118]. The rapid CD26-mediated inactivation of natural CXCL12 may explain why the chemokine fails to act as an efficient HIV-inhibitor *in vivo* [184]. Also citrullination was found to impair the antiviral activity of CXCL12, since citrullinated CXCL12 is characterized by reduced CXCR4 binding properties [164]. In addition, peroxynitrite exposure impairs the anti-HIV activity of CXCL12 (Janssens et al., submitted manuscript). However, several other chemokines become more potent HIV-inhibitors upon enzymatic truncation. CD26-truncated CCL3L1, produced by peripheral blood mononuclear cells, shows increased affinity for CCR1 with enhanced chemotactic activity for monocytes [170]. Moreover the affinity of CCL3L1(3–70) for CCR5 is also very strong and therefore, CCL3L1(3–70) is a potent HIV-1 inhibitor. Also for CCL5, CD26-mediated cleavage results in a change of receptor specificity, increasing the affinity for CCR5 but decreasing the affinity for CCR1 and CCR3 [98,99]. In this way, CCL5(3–68) is no longer able to recruit monocytes and eosinophils, and, moreover, acts as an inhibitor of CCL5(1–68)-, CCL3-, CCL4- and CCL7-induced chemotaxis. In addition, CCL5(3–68) has been shown to be a potent HIV-1 inhibitor [126,185]. Finally, CCL22 has a decreased biological effect on CCR4, implying the loss of its lymphocyte chemotactic activity [186]. However, the monocyte chemotactic property of CCL22(3–69) stays unaltered and, moreover, its anti-HIV-1 activity was shown to be increased [187,188].

The effect of PTMs seems not restricted to anti-HIV activity of certain chemokines. For example, in plasma of patients infected with hepatitis C virus, the most dominant form of circulating CXCL10 is the truncated, antagonistic isoform CXCL10(3–77), supporting that a correlation between viral infection and chemokine processing may exist *in vivo* [189,190]. Furthermore it was shown that, in patients who do not respond to therapy with PEGylated IFN-α2/ribavirin, the concentration of CXCL10(3–77) and CD26 activity are significantly higher. This suggests that CD26-mediated cleavage of CXCL10, resulting in perturbed lymphocyte migration to the liver, inhibits the development of an efficacious immune response to HCV and favors the evolution of viral persistence [191,192].

4. GAGs and chemokine PTMs

Usually the COOH-terminal chemokine domain is considered a major GAG interaction region and therefore COOH-terminal modification may have a drastic effect on chemokine - GAG binding. A remarkable example is proteolysis of CXCL11 (Appendix B). On the one hand, NH_2-terminal cleavage by MMP-8, MMP-9 or MMP-12 results in loss of the four most NH_2-terminal residues and increases the affinity of this most potent CXCR3 ligand for heparin and converts the chemokine into a receptor antagonist [102]. On the other hand, subsequent COOH-terminal truncation nullifies receptor antagonism and heparin binding. However, it has been evidenced that the GAG-binding domain of chemokines is not strictly limited to the COOH-terminal region. Hence, also NH_2-terminal modification of chemokines can affect GAG-interactions. For example, PAD-mediated citrullination on Arg5 of CXCL8 results in reduced GAG affinity in heparin and heparan sulfate binding assays [79]. Also NH_2-terminal truncation by CD26 generating CXCL12(3–68) negatively affects the interaction between CXCL12 and GAGs [121].

Emerging evidence points towards an important role for GAGs in chemokine regulation that is not limited to chemokine immobilization and presentation to their receptors (*vide supra*). PTMs may not only affect GAG binding, but also GAG binding potentially alters a chemokine's susceptibility to enzymatic processing. Indeed, GAGs protect intact CXCL12 against CD26-mediated cleavage towards CXCL12(3–68) [193]. We recently demonstrated that GAGs also protect the IFN-inducible CXCR3 ligands CXCL9, CXCL10 and CXCL11 against processing by CD26 in a dose-dependent manner [194]. Interestingly, GAGs exert this protective effect either directly or indirectly, depending on the enzyme involved. Regarding processing by CD26, no evidence was found for GAGs to directly inhibit the enzymatic activity of the serine protease [193,194]. GAGs may therefore exert their protective effect probably though direct interactions with chemokines, thereby sterically hindering CD26. Consistently, heparin was found to indirectly protect murine CCL11 against processing by plasmin [195]. However, heparin-mediated protection against digestion by cathepsin G and elastase was due to a direct inhibitory effect of GAGs on these enzymes [196–198]. A direct inhibitory effect of GAGs was also found on MMP-2 and MMP-7 [199,200].

5. PTMs of chemokine receptors

In addition to chemokines, also chemokine receptors are subject to PTMs, including phosphorylation, glycosylation and tyrosine sulfation which have implications for chemokine recognition and signaling. For many chemokine receptors, tyrosine residues in the NH_2-terminal domain are modified by tyrosylprotein sulfotransferase enzymes which are located in the trans-Golgi network [201,202]. This modification, adding a sulfate group and a negative charge to the phenolic hydroxyl of a tyrosine side chain, was shown to occur in a consensus sequence of negatively charged amino acids (Asp and Glu) in the proximity of tyrosine residues. This modification has been described for several chemokine receptors, including CCR2B, CCR3, CCR5, CCR7, CX3CR1, CXCR3 and CXCR4 [203–211]. Mostly, this modification leads to enhanced chemokine-receptor recognition [212]. For example, the sulfation of tyrosines in CCR5 contributed to the binding of CCL3, CCL4 and, interestingly, of HIV-1 gp120/CD4 complexes [204]. Inhibition of sulfation resulted clearly in a decreased chemokine and gp120/CD4 binding affinity. In contrast, the sulfation of Tyr21 in CXCR4 was less important for CXCR4-dependent HIV-1 entry [205]. For human CCR2B, Tyr26 was shown to be sulfated and mutation of this tyrosine residue resulted in a decreased binding affinity of CCL2 [203]. Moreover, the interaction with the sulfated NH_2-terminus of CCR2 destabilized the dimerization of CCL2 [213]. In contrast, the tyrosine sulfation of CXCR4 stabilized the dimeric state of the CXCR4/CXCL12 complex [214]. Noteworthy, many chemokine receptors contain two or more tyrosine residues in their NH_2-terminal region and it has been shown that the pattern of chemokine receptor tyrosine sulfation modulates the selectivity for specific chemokine ligands [215,216]. Mutation of Tyr30 in the decoy receptor ACKR1/DARC suppressed binding of only CXCL8 and mutation of Tyr41 suppressed binding of CCL2, CCL5 and CXCL1 but not of CXCL8. In addition, high affinity binding of CXCL12 required sulfation of three tyrosine residues (Tyr7, Tyr12 and Tyr21) in the

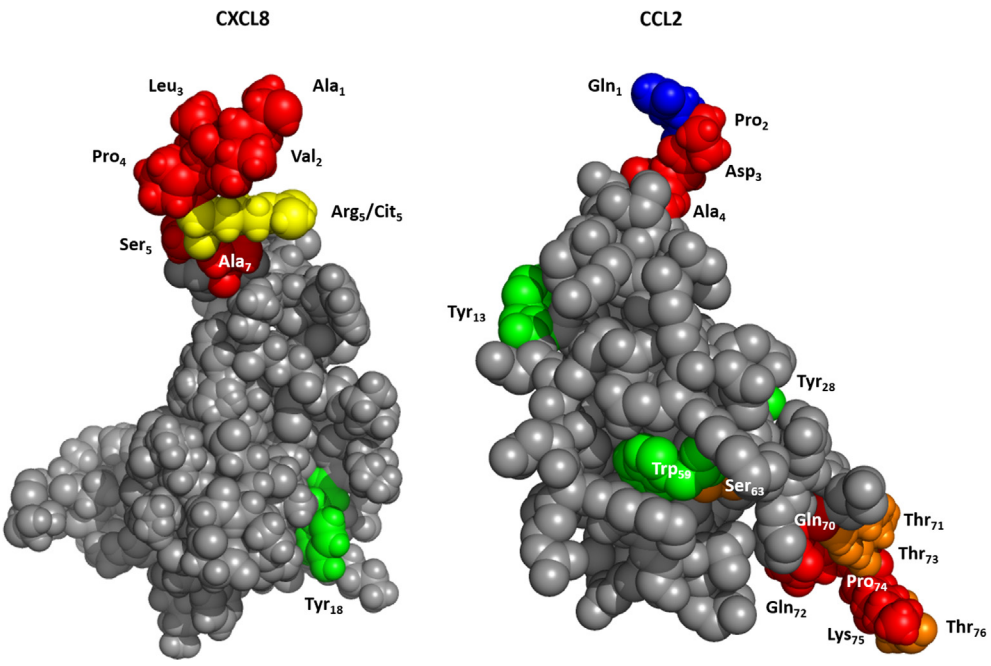

Fig. 1. The 3D structure of human CXCL8 and CCL2 and their post-translational modifications. 3D models of human CXCL8 and CCL2 were drawn from PDB accession codes 2BDN and 4XDX, respectively, to visualize the location of the potentially removed (red), citrullinated (yellow), nitrated (green) or O-glycosylated (orange) amino acids. The three-letter code of the removed or modified amino acids are indicated. The NH_2-terminal glutamine of CCL2, which is always modified in natural human CCL2 to a pyroglutamic acid, is shown in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extracellular NH_2-terminal domain of CXCR4 [205,217,218]. Interestingly, these tyrosine residues were sulfated sequentially and CXCL12 binding affinity increased with the number of sulfotyrosines present [219]. More recently, it was described that CXCL12γ interacts with sulfotyrosines of CXCR4 with high affinity, resulting in a nonproductive binding and reduced signaling and chemotactic activity. Heparan sulfate prevented the interaction between CXCL12γ and the CXCR4 sulfotyrosines, thereby functionally presenting the chemokine to its receptor such that its activity was similar to that of CXCL12α [220]. Since other human chemokine receptors including CXCR1, CXCR2 and CCR1 also contain tyrosine residues, these receptors might also be subject to post-translational sulfation [221].

Many chemokine receptors, including CXCR1, CXCR4, CCR5 and CCR7, undergo heterogeneous N- and/or O-glycosylation. CCR5 has been shown to be O-glycosylated at Ser6, with particularly sialic acid moieties, thereby contributing to the binding of chemokines [209]. Interestingly, this O-glycosylation of CCR5 had little effect on HIV-1 infection. In contrast, the N-glycosylation of CXCR4 has been proven to influence the ability of CXCR4 to function as a co-receptor for different HIV-1 strains [222]. It is suggested that the added sugars mask extracellular residues of CXCR4 which are involved in the interaction with the HIV envelope glycoprotein. For CCR7, it was even shown that different leukocyte subtypes express different sialylation patterns that differentially contribute to receptor signaling and fine-tune chemotactic responses [223]. In addition, glycosylation of CXCR2 protected this receptor from proteolysis, thereby suggesting a role for glycosylation in maintaining neutrophil responsiveness to CXC chemokines during inflammation [224]. Finally, also decoy receptors, such as ACKR1/DARC and ACKR2/D6, may be glycosylated [225,226].

6. Modifications due to recombinant expression

In addition to PTMs that may occur on mature naturally secreted proteins, a number of chemokines display serendipitous modifications upon recombinant expression in *E. coli* (Appendix E). The most frequently observed serendipitous modification is the presence of an additional NH_2-terminal Met residue. The existing knowledge regarding the implications of this type of modification for the biological activity of chemokines is rather limited. However, for a number of chemokines it turned out that the presence of an NH_2-terminal Met has significant functional consequences. Met-CCL5 for example, is a receptor antagonist with an increased affinity for heparan sulfate [227,228]. Also Met-CXCL10 shows a reduced potency to induce CXCR3-mediated $[Ca^{2+}]_i$ mobilization and chemotaxis, but is protected against proteolytic inactivation by CD26 [113].

7. Concluding remarks

At first sight, the complex chemokine/chemokine receptor network looks highly redundant. To ensure appropriate functioning of chemokines and to enhance *in vivo* specificity, they need to be tightly regulated. One of the mechanisms involved in the fine-tuning of chemokine activity is the PTM of chemokines and their receptors. The influence of these PTMs is variable in different chemokines and may lead to potentiation, inactivation, altered receptor affinity or specificity, the generation of receptor antagonists and/or altered GAG binding. In Fig. 1, all PTMs identified up to now on natural chemokines are shown exemplified on two different chemokines, namely CXCL8 and CCL2. In CXCL8, NH_2-terminal truncation results in the potentiation of CXCL8 thereby creating a positive feedback loop between upregulation of protease activity by CXCL8 and activation of CXCL8 by the protease. In contrast, site-specific citrullination of CXCL8 results in reduced neutrophil extravasation *in vivo* and reduced GAG affinity. In addition, nitration of CXCL8 on Tyr18 attenuates the neutrophil chemotactic activity and the binding to neutrophils. While NH_2-truncation of CCL2 results in the generation of a receptor antagonist, COOH-terminal truncation has no effect. Nitrated CCL2 has reduced affinity for CCR2b and GAGs, thereby recruiting less monocytes both *in vitro* and *in vivo*. Although the NH_2-terminal pyroglutamic acid is essential for the chemotactic activity of CCL2, only a minor biological effect of O-glycosylation on CCL2 is known.

In conclusion, PTM of chemokines is a fast and efficacious way to shape their biological activity. The activation and potentiation of chemokines mediated by different enzymes can be important in the early phase of inflammation thereby attracting more cells to combat the infection or repair tissue damage. Since chemokines are central players in various patho- and physiological processes, the impact of PTMs is not only restricted to chemotaxis. The consequences of PTMs on the biological activity of chemokines are broad, and even comprise an effect on antiviral activity and wound healing, for example.

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Conflict of interest

None.

Appendix A. Overview of the identified enzymatic truncations on human CC chemokines

Chemokine	Modification and enzymes involved	Amino acids (without signal peptide)	Effect of PTM on receptor interaction	Effect of PTM on chemotactic activity	Effect of PTM beyond chemotaxis	Refs.
CCL1	COOH-terminal truncation by CPM	1–70	Reduced CCR8 binding, increased Ca^{2+} signaling	N.D.	Pronounced anti-apoptotic activity	[229]
CCL2	COOH-terminal truncation	1–69	Retained Ca^{2+} signaling	Retained monocyte chemotaxis	N.D.	[153]
	NH_2-terminal truncation by MMP-1, MMP-2, MMP-3 and MMP-9	5–76	Retained CCR2 and CCR3 binding, reduced Ca^{2+} signaling	Reduced monocyte chemotaxis, antagonist	N.D.	[153,230,231]
	NH_2-terminal truncation	6–76	Reduced Ca^{2+} signaling	Reduced monocyte chemotaxis	N.D.	[153,230]
CCL3	Degraded by cathepsin D	N.A.	Inactivated	Inactivated	Inactivated	[105]
CCL3L1	NH_2-terminal truncation by CD26	3–70	Increased CCR1 and CCR5 binding, impaired CCR3 binding and Ca^{2+} signaling through CCR3; increased Ca^{2+} signaling in neutrophils; impaired Ca^{2+} signaling in eosinophils	Increased monocyte and lymphocyte chemotaxis; impaired eosinophil chemotaxis; retained neutrophil chemotaxis	Increased anti-HIV activity	[170,232]
CCL4	NH_2-terminal truncation by CD26	3–69	Increased Ca^{2+} signaling through CCR1 and CCR2, retained Ca^{2+} signaling through CCR5; retained Ca^{2+} signaling in macrophages; acquired Ca^{2+} signaling in monocytes; retained CCR5 internalization	N.D.	Retained anti-HIV activity	[233,234]
	Degradation by cathepsin D	N.A.	Inactivation	Inactivation	Inactivation	[105]
CCL5	NH_2-terminal truncation by CD26	3–68	Impaired Ca^{2+} signaling through CCR1 and CCR3, increased Ca^{2+} signaling through CCR5 ; retained Ca^{2+} signaling in macrophages; impaired Ca^{2+} signaling in monocytes	Impaired monocyte and eosinophil chemotaxis; unaltered lymphocyte chemotaxis; monocyte chemotaxis antagonist *in vitro*	Increased anti-HIV activity	[98,99,126]
	NH_2-terminal truncation by cathepsin G	4–68	Decreased CCR5 binding	Impaired lymphocyte chemotaxis	Impaired anti-HIV activity	[100,235,236]
	Degradation by tryptase	N.A.	Inactivation	Inactivation	Inactivation	[106]
CCL7	NH_2-terminal truncation by MMP-1, MMP-2, MMP-3, MMP-13, MMP-14	5–76	Retained CCR1, CCR2 and CCR3 binding, abrogated Ca^{2+} signaling	Abrogated chemotaxis, antagonist	N.D.	[237]
CCL8	NH_2-terminal truncation by MMP-3	5–76	Retained receptor binding on CCR2 transfectants		N.D.	[153,231]

				Impaired chemotaxis of THP-1 cells and CCR2 transfectants, antagonist		
	NH_2-terminal truncation	6–76	Impaired Ca^{2+} signaling in THP-1 cells	Impaired monocyte chemotaxis, antagonist	N.D.	[153,231]
CCL11	NH_2-terminal truncation by CD26	3–74	Impaired receptor binding and Ca^{2+} signaling in CCR3 transfectants	Impaired eosinophil chemotaxis, antagonist	Unaltered anti-HIV activity	[99,149,187,238]
	NH_2-terminal truncation	4–74	N.D.	N.D.	N.D.	[149]
	Degradation by tryptase or chymase	N.A.	Inactivated	Inactivated	Inactivated	[106]
CCL13	NH_2-terminal truncation by MMP-1 and MMP-3	4–75 5–75 8–75	Retained CCR2 and CCR3 binding	N.D. for 4–75 and 5–75 Impaired chemotaxis for 8–75, antagonist	N.D.	[231]
CCL14	NH_2-terminal truncation	3–74 4–74	N.D.	N.D.	N.D.	[150]
	UPA and plasmin	9–74	Mandatory for CCR1, CCR3 and CCR5 activation and signaling; increased degradation by ACKR2	Mandatory for T lymphoblast, monocyte, and eosinophil chemotaxis	Anti-HIV activity	[94,239,240]
	NH_2-terminal truncation of CCL14(9–74) by CD26	11–74	Impaired receptor activity, reduced ACKR2-mediated degradation (compared to CCL14(9–74)	Inactivated	N.D.	[240,241]
	NH_2-terminal truncation	12–74	Reduced CCR1 and CCR5 binding and signaling; antagonistic activity against CCL14(9–74) in Ca^{2+} assays	Impaired chemotaxis	Moderate anti-HIV activity	[242]
CCL15	NH_2-terminal truncation by elastase	22–92	N.D.	N.D.	N.D.	[243,244]
	NH_2-terminal truncation by cathepsin G	24–92	Increased CCR1 binding; increased Ca^{2+} signaling in monocytes	Increased monocyte chemotaxis	Increased adhesiveness to fibronectin	[243]
	Truncation at both termini	24–91	N.D.	N.D.	N.D.	[243]
	NH_2-terminal truncation by MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, or MMP-14	25–92	Increased Ca^{2+} signaling in CCR1 transfectants and THP-1 cells	Increased THP-1 chemotaxis	N.D.	[243,244]
	NH_2-terminal truncation by cathepsin G	27–92	Increased CCR1 binding; increased Ca^{2+} signaling in monocytes and CCR1 transfectants	Increased monocyte chemotaxis	Increased adhesiveness to fibronectin	[243]
	NH_2-terminal truncation by MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13	28–92	Increased Ca^{2+} signaling in CCR1 transfectants and THP-1 cells	Increased THP-1 chemotaxis	N.D.	[244]
	NH_2-terminal truncation by cathepsin G or chymase	29–92	N.D.	N.D.	N.D.	[243,245]
CCL16	Truncation at both termini by MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, or MMP-14	8–77 8–85	Retained Ca^{2+} signaling in THP-1 cells	Retained THP-1 chemotaxis	Enhanced GAG binding	[244]
	NH_2-terminal truncation by MMP-2, MMP-8 or MMP-14	5–97	Retained potency to induce Ca^{2+} signaling in THP-1 cells	Retained CCR1-transfectant and THP-1 cell chemotaxis	N.D.	[244]
CCL21	Degradation by cathepsin D	N.A.	Inactivated	Inactivated	Inactivated	[105]
CCL22	NH_2-terminal truncation by CD26	3–69	Impaired Ca^{2+} signaling in CCR4 transfectants			[188,246]

				Impaired lymphocyte chemotaxis; retained monocyte chemotaxis	Increased anti-HIV activity	
	NH_2-terminal truncation of CCL22(3–69) by CD26	5–69	Impaired receptor binding and Ca^{2+} signaling in CCR4 transfectants	Reduced chemotaxis of lymphocytes and monocyte-derived dendritic cells; no effect on monocyte chemotaxis	Retained anti-HIV activity	[186]
CCL23	NH_2-terminal truncation in the presence of synovial fluid from arthritis patients	19–99	Enhanced CCR1 activation	N.D.	N.D.	[245]
	NH_2-terminal truncation by MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, or MMP-14	26–99	Increased CCR1 signaling and Ca^{2+} signaling in THP-1 cells	Increased chemotaxis of CCR1-transfectants and THP-1 cells	N.D.	[244]
	NH_2-terminal truncation by chymase or cathepsin G	27–99	Increased CCR1 binding	Increased chemotaxis of CCR1 + cells	N.D.	[245]
	NH_2-terminal truncation by MMP-14 or elastase or in the presence of synovial fluid	30–99	Increased on CCR1	N.D.	N.D.	[244,245]

ACKR, atypical chemokine receptor; CCR, CC chemokine receptor; CPM, carboxypeptidase M; GAG, glycosaminoglycan; MMP, matrix metalloproteinase; N.A., not applicable; N.D., not determined; THP-1, Tohoku Hospital Pediatrics-1 (human monocytic cell line); UPA, urokinase-type plasminogen activator.

Appendix B. Overview of the identified enzymatic truncations on human CXC and CX3C chemokines

Chemokine	Modification and involved enzyme (s) (if determined)	Amino acids (without signal peptide)	Effect of PTM on receptor interaction	Effect of PTM on chemotactic activity	Effect of PTM beyond chemotaxis	Refs.
CXCL1	NH_2-terminal truncation	2–73 3–73	N.D.	N.D.	N.D.	[127]
	NH_2-terminal truncation	4–73 6–73	Increased potency to induce Ca^{2+} signaling in neutrophils	Increased neutrophil chemotaxis	N.D.	[127]
	NH_2-terminal truncation by cathepsins B, K, L or S	5–73	Increased Ca^{2+} signaling in neutrophils	Increased neutrophil chemotaxis	N.D.	[247,248]
	Degradation by MMP-9, proteinase-3 or trypsin	N.A.	Inactivated	Inactivated	Inactivated	[87]
CXCL2	NH_2-terminal truncation by CD26	2–73	N.D.	N.D.	N.D.	[109]
	NH_2-terminal truncation by cathepsins B, K, L or S	5–73	Increased Ca^{2+} signaling in neutrophils	Increased neutrophil chemotaxis	Increased synergistic growth stimulant for colony forming cells and in respiratory burst assays	[248,249]

CXCL3	NH_2-terminal truncation by cathepsins B, K, L or S	5–73	Increased Ca^{2+} signaling in neutrophils	Increased neutrophil chemotaxis	N.D.	[247,248]
CXCL4	NH_2-terminal truncation	17–70	N.D.	N.D.	Increased inhibition of endothelial cell growth	[250]
	Degradation by MMP-9	N.A.	Inactivated	Inactivated	Inactivated	[87]
CXCL4L1	COOH-terminal truncation	1–69 −4 to 69	N.D.	N.D.	N.D.	[251]
CXCL5	NH_2-terminal truncation by CD13	2–78	N.D.	Unaltered neutrophil chemotaxis	Impaired activation by cathepsin G	[165]
	NH_2-terminal truncation by CD13	3–78	N.D.	N.D.	N.D.	[165]
	NH_2-terminal truncation by MMP-9	6–78 7–78	N.D.	N.D.	N.D.	[82]
	NH_2-terminal truncation by MMP-8 or MMP-9	8–78	Increased Ca^{2+} and ERK1/2 signaling and CXCR2 internalization	Increased neutrophil chemotaxis	N.D.	[82,86,165,247]
	NH_2-terminal truncation by cathepsins B, G, K, L or S, or chymotrypsin	9–78	N.D.	Increased neutrophil chemotaxis	Higher neutrophil elastase release	[247,248,252]
	NH_2-terminal truncation by MMP-1	10–78	N.D.	Impaired neutrophil chemotaxis	Reduced neutrophil elastase release	[86,252]
	Degradation by MMP-9	N.A.	Inactivated	Inactivated	Inactivated	[82]
CXCL6	NH_2-terminal truncation by CD26	3–77	N.D.	Unaltered neutrophil chemotaxis	Unaltered neutrophil degranulation	[109,127]
	NH_2-terminal truncation by MMP-9	5–77	Unaltered Ca^{2+} signaling in neutrophils	N.D.	N.D.	[82]
	NH_2-terminal truncation by MMP-8 or MMP-9	6–77	Unaltered Ca^{2+} signaling in neutrophils	Unaltered neutrophil chemotaxis	Unaltered neutrophil degranulation	[82,127]
	NH_2-terminal truncation by MMP-8 or MMP-9	7–77	Unaltered potency to induce Ca^{2+} signaling in neutrophils	N.D.	N.D.	[82]
	NH_2-terminal truncation	9–77	N.D.	Unaltered neutrophil chemotaxis	Unaltered neutrophil degranulation	[127]
CXCL7	NH_2-terminal truncation	35–128 (also known as PBP)	N.D.	No neutrophil chemotaxis	No neutrophil-activating properties	[90,253]
	NH_2-terminal truncation	44–128 (also known as CTAP-III)	N.D.	No neutrophil chemotaxis	No neutrophil-activating properties; microbicidal activity	[73,253–255]
	NH_2-terminal truncation	43–128 46-128	N.D.	N.D.	No neutrophil-activating properties	[73,254,256]
	NH_2-terminal truncation	45–128 47–128	N.D.	N.D.	N.D.	[256]

	NH_2-terminal truncation by plasmin or trypsin	48–128 bTG	N.D.	N.D.	No neutrophil-activating properties	[73,90,254]
	NH_2-terminal truncation	49–128 52–128	N.D.	N.D.	N.D.	[90]
	NH_2-terminal truncation	50–128	N.D.	N.D.	No neutrophil-activating properties	[73,90,254]
	NH_2-terminal truncation	57–128 58–128	N.D.	N.D.	Acquired capacity to stimulate GAG synthesis	[257,258]
	NH_2-terminal truncation by cathepsin G, chymase, chymotrypsin or trypsin	59–128 (also known as NAP-2)	Acquired Ca^{2+} signaling in neutrophils	Acquired chemotactic activity for neutrophils	Acquired elastase and lactoferrin release by neutrophils, and increased microvascular permeability; microbicidal activity; capacity to stimulate GAG synthesis	[73,90,253–255,257–261]
	Truncation at both termini	59–126 44–126	N.D.	N.D.	Microbicidal activity	[262]
	Truncation at both termini	59–121 59–124	Increased neutrophil binding compared to NAP-2	N.D.	Increased neutrophil degranulation compared to NAP-2	[263]
	Truncation at both termini	44–124	N.D.	N.D.	N.D.	[263]
	Degradation by elastase or MMP-9 (CTAP-III); proteinase-3 or trypsin (NAP-2)	N.A.	Inactivated	Inactivated	Inactivated	[87]
CXCL8	NH_2-terminal truncation by CD13	2–77 3–77	No effect on CXCR1 and CXCR2 binding or signaling	Unaltered neutrophil chemotaxis	Increased heparin affinity	[80]
	NH_2-terminal truncation by MMP-8, MMP-13, MMP-14, cathepsins B, G, K, L or S, plasmin, thrombin	6–77	Increased CXCR1 and CXCR2 binding, increased Ca^{2+} signaling in neutrophils	Increased neutrophil chemotaxis	Increased angiogenic potency	[73,79,83–86,167,248,264]
	NH_2-terminal truncation by MMP-1 or MMP-9; NH_2-terminal truncation of CXCL8(6–77) by CD13	7–77	Increased CXCR1 and CXCR2 binding and Ca^{2+} signaling, most profoundly for CXCR1	Increased neutrophil chemotaxis	Increased neutrophil degranulation	[73,85,87]
	NH_2-terminal truncation by cathepsins B, G, K, L or S or proteinase-3 or NH_2-terminal truncation of CXCL8(6–77) by CD13	8–77	Increased receptor binding on neutrophils	Increased neutrophil chemotaxis	Increased neutrophil elastase release	[73,81,84,85,248]
	NH_2-terminal truncation	9–77	Increased receptor binding on neutrophils	Increased neutrophil chemotaxis	Increased neutrophil elastase release	[73,81]

	Degradation by (chymo)trypsin, elastase, or cathepsin G	N.A.	Inactivated	Inactivated	Inactivated	[84]
CXCL9	NH_2-terminal truncation by CD26	3–103	Retained CXCR3 binding but impaired Ca^{2+} signaling	Impaired lymphocyte chemotaxis	Retained angiostatic properties	[113,114]
	COOH-terminal truncation by furin	1–92 1–96 1–101	N.D.	N.D.	N.D.	[265]
	COOH-terminal truncation by MMP-9	1–94	N.D.	N.D.	N.D.	[101]
	COOH-terminal truncation by MMP-9 or furin	1–93	N.D.	N.D.	N.D.	[101,265]
	COOH-terminal truncation by MMP-7, MMP-9, MMP-12 or furin	1–90	N.D.	N.D.	N.D.	[101,102,104,265]
	COOH-terminal truncation	1–74 1–75 1–76 1–77 1–81 1–82 1–83 1–84 1–85	N.D.	N.D.	N.D.	[103,104]
	COOH-terminal truncation	1–78	Impaired Ca^{2+} signaling in TIL cell lines	N.D.	N.D.	[103,104]
	Degradation by MMP-8 or cathepsins B, K, L or S	N.A.	Inactivated	Inactivated	Inactivated	[101,248]
CXCL10	NH_2-terminal truncation by CD26 or DPP8	3–77	Reduced CXCR3 binding and Ca^{2+} signaling	Impaired lymphocyte chemotaxis; antagonizes chemotaxis induced by intact CXCL10	Retained angiostatic properties	[63,109,113,114,116,182,190,266]
	NH_2-terminal truncation	4–77 6–77	N.D.	N.D.	N.D.	[116,267]
	NH_2-terminal truncation by cathepsins B, K, L or S	5–77	N.D.	N.D.	N.D.	[116,248,267]
	COOH-terminal truncation by Furin + CPB, MMP-8 or MMP-12	1–73	Retained CXCR3 binding and Ca^{2+} signaling	Unaltered T cell chemotaxis	Unaltered inhibition of *E. coli* and *L. monocytogenes* growth	[101,102,265,268]
	COOH-terminal truncation by MMP-8 or MMP-12	1–71	N.D.	N.D.	N.D.	[101,102]
	Truncation at both termini, *vide supra*	3–73 4–73 5–73 6–73	N.D.	N.D.	N.D.	[116,265,267]
	Degradation by MMP-7 or MMP-9	N.A.	Inactivation	Inactivation	Inactivation	[101]

CXCL11	NH_2-terminal truncation by CD26 or DPP8	3–73	Reduced CXCR3 binding; impaired ACKR3 binding	Impaired lymphocyte chemotaxis; antagonizes chemotaxis induced by intact CXCL11	Retained potency to inhibit microvascular endothelial cell migration.	[85,109,113–115]
	NH_2-terminal truncation of CXCL11(3–73) truncation by CD13	4–73	N.D.	N.D.	N.D.	[85]
	NH_2-terminal truncation by MMP-8, MMP-9 or MMP-12 or cathepsins B, K, L or S, or NH_2-terminal truncation of CXCL11(3–73) by CD13	5–73	Reduced CXCR3 binding, Ca^{2+} signaling and receptor internalization; Impaired ACKR3 binding	Impaired lymphocyte chemotaxis, antagonizes chemotaxis induced by intact CXCL11	Impaired inhibition of microvascular endothelial cell migration; enhanced affinity for heparin	[85,102,248]
	NH_2-terminal truncation by cathepsins B, K, L or S, or NH_2-terminal truncation of CXCL11(3–73) truncation by CD13	6–73	N.D.	N.D.	N.D.	[85,248]
	NH_2-terminal truncation of CXCL11(3–73) truncation by CD13	7–73	Reduced CXCR3 and ACKR3 binding	Impaired lymphocyte chemotaxis	Impaired inhibition of microvascular endothelial cell migration.	[85]
	COOH-terminal truncation by MMP-7	1–58	N.D.	N.D.	N.D.	[102]
	Truncation at both termini by MMP-8, MMP-9 or MMP-12	5–58	Reduced Ca^{2+} signaling and CXCR3 internalization	Impaired chemotaxis, antagonizes chemotaxis induced by intact CXCL11	Reduced affinity for heparin	[102]
	Truncation at both termini by MMP-8	5–63	N.D.	N.D.	N.D.	[102]
	Degradation by MMP-7 or MMP-12	N.A.	Inactivated	Inactivated	Inactivated	[102]
CXCL12	NH_2-terminal truncation by CD26 or DPP8	3–72	N.D.	Impaired lymphocyte chemotaxis	Impaired anti-HIV activity	[117,120]
	NH_2-terminal truncation by CD26 or DPP8	3–68	Reduced CXCR4 affinity, Ca^{2+}, IP_3, Akt or ERK1/2 signaling in lymphocytes and endothelial cells, impaired recruitment of β-arrestin 2 via CXCR4 and ACKR3	Impaired chemotaxis of lymphocytes, HPC and endothelial cells	Impaired anti-HIV activity, impaired heparin affinity	[109,114,117–121,124,125,172,269–271]

	NH_2-terminal truncation by elastase	4–68	N.D.	N.D.	N.D.	[125,272]
	NH_2-terminal truncation by cathepsin G	6–68	N.D.	N.D.	N.D.	[125,273]
	NH_2-terminal truncation by MMP-1, MMP-2, MMP-3, MMP-9, MMP-13 or MMP-14	5–72	N.D.	N.D.	N.D.	[274]
	NH_2-terminal truncation by MMP-1, MMP-2, MMP-3, MMP-9, MMP-13 or MMP-14	5–67	Impaired CXCR4 binding	Impaired chemotaxis of pre-B cells and CD34+ stem cells	N.D.	[274]
	COOH-terminal truncation by CPN or CPM	1–67	Unaltered Ca^{2+} signaling; reduced CXCR4 and ACKR3 binding	Impaired chemotaxis of lymphoma cells and CD34+ cells; unaltered HCP chemotaxis	Impaired heparin binding; impaired proliferation of pre-B cells	[120,125,275,276]
	COOH-terminal truncation by cathepsin X	1–53	N.D.	Impaired chemotaxis of CD34+ cells	N.D.	[277,278]
	Truncation at both termini, *vide supra*	3–67	Impaired Ca^{2+} signaling	Impaired chemotaxis of lymphoma cells	Impaired heparin binding; impaired proliferation of pre-B cells	[120]
	Truncation at both termini	4–67	Impaired CXCR4 and ACKR3 binding, impaired Ca^{2+} signaling	Impaired HPC and T cell chemotaxis	Impaired heparin binding and anti-HIV activity	[125,272]
	Truncation at both termini	6–67	Impaired CXCR4 and ACKR3 binding and Ca^{2+} signaling	Impaired HPC and lymphocyte chemotaxis	Impaired heparin binding	[125,273,274]
	Truncation at both termini	8–67	Impaired CXCR4 and ACKR3 binding	Impaired HPC chemotaxis	Impaired heparin binding	[125]
	Degradation by cathepsins B, K, L or S	N.A.	Inactivated	Inactivated	Inactivated	[248]
CXCL16	Shedding of the membrane-bound chemokine by ADAM-10	Without TM domain	N.D.	Acquired chemotaxic activity for T cells, bone marrow plasma cells and NK cells	soluble CXCL16 induces migration and proliferation of CXCR6+ cancer cells	[178,180,279,280]
CX3CL1	Shedding of the membrane-bound chemokine by MMP-2, ADAM-10 or ADAM-17	Without TM domain	N.D.	Acquired chemotactic activity for macrophages		[281–284]

ADAM, a disintegrin and metalloprotease; ACKR, atypical chemokine receptor; CTAP-III, connective tissue-activating peptide III; CP-, carboxypeptidase; CXCR, CXC chemokine receptor; DPP, dipeptidyl peptidase; ERK, extracellular signal-regulated kinase; GAG, glycosaminoglycan; HPC, hematopoietic progenitor cell; MMP, matrix metalloproteinase; N.A., not applicable; NAP-2, neutrophil activating peptide 2; N.D., not determined; TIL, tumor-infiltrating lymphocytes; TM, transmembrane.

Appendix C. Overview of the amino acid side chain modifications on human CC chemokines

Chemokine	Modification and enzyme (s) involved	Affected amino acid(s)	Effect on receptor interaction	Effect on chemotactic activity	Effect beyond chemotaxis	Refs.
CCL2	O-glycosylation	In COOH-terminal region	Slightly reduced Ca^{2+} signaling	Reduced monocyte chemotaxis	Enhanced functional stability	[151,153,154,285]
	Conversion of NH_2-terminal Gln to pyroglutamic acid by glutaminyl cyclase	1	N.D.	Essential for chemotactic activity	N.D.	[95,97,286]
	Nitration by peroxynitrite	N.D.	N.D.	Different groups observed an unaltered or reduced monocyte chemotactic activity; reduced chemotactic activity for CD8+ cells	N.D.	[146,287]
CCL3	Nitration by peroxynitrite	N.D.	N.D.	Attenuated neutrophil and monocyte chemotaxis	N.D.	[141]
CCL5	Nitration by peroxynitrite	N.D.	N.D.	Impaired eosinophil chemotactic activity	N.D.	[287]
	O-glycosylation	N.D.	N.D.	Unaltered eosinophil chemotactic activity	N.D.	[148]
CCL7	Conversion of NH_2-terminal Gln to pyroglutamic acid	1	N.D.	Essential for chemotactic activity	N.D.	[148]
CCL8	Conversion of NH_2-terminal Gln to pyroglutamic acid	1	N.D.	Essential for chemotactic activity	N.D.	[96]
CCL11	O-glycosylation	71	N.D.	No effect on eosinophil chemotaxis	N.D.	[149]
CCL13	Conversion of NH_2-terminal Gln to pyroglutamic acid	1	N.D.	Essential for chemotactic activity	N.D.	[288]
CCL14	O-glycosylation	7	N.D.	N.D.	N.D.	[150]

N.A., not applicable; N.D., not determined.

Appendix D. Overview of the amino acid side chain modifications on human CXC, XC and CX3C chemokines

Chemokine	Modification and involved enzyme(s) (if determined)	Affected amino acid(s)	Effect of PTM on receptor interaction	Effect of PTM on chemotactic activity	Effect of PTM beyond chemotaxis	Refs.
CXCL5	Citrullination by PAD	9	Impaired Ca^{2+} signaling, phosphorylation of ERK and CXCR2 internalization	Reduced neutrophil chemotaxis; obtains monocyte chemotactic properties	More efficiently cleaved and consequently activated by cathepsin G	[163,165]
CXCL8	Citrullination by PADs	5	Impaired Ca^{2+} signaling and ERK1/2 phosphorylation; impaired erythrocyte binding	Impaired neutrophil chemotaxis to tissues in mice but increased potency to mobilize neutrophils into the blood	Impaired affinity for GAGs; resistant to thrombin- or plasmin-dependent proteolysis; increased CD62L shedding and CD11b expression	[79,166]
	Nitration by peroxynitrite	N.D.	Impaired receptor binding on neutrophils	Impaired neutrophil chemotactic activity	N.D.	[144]
CXCL10	Citrullination by PADs	5	Unaltered CXCR3 binding, impaired Ca^{2+} signaling and ERK1/2 phosphorylation	Impaired T cell chemotaxis	Impaired affinity for heparin	[22]
CXCL11	Citrullination by PADs	6	Unaltered CXCR3 and ACKR3 binding, impaired Ca^{2+} signaling	Impaired T cell chemotaxis	Impaired affinity for heparin	[22]

CXCL12	Citrullination by PADs	8	Impaired CXCR4 binding and capacity to induce ERK1/2, Ca^{2+} and protein kinase B signaling; Unaltered ACKR3 binding	Impaired monocyte and lymphocyte chemotaxis	Impaired anti-HIV activity	[164]
	Citrullination by PADs	8,12,20	Impaired CXCR4 binding and ERK1/2, Ca^{2+} and protein kinase B signaling; Impaired ACKR3 binding	Impaired monocyte and lymphocyte chemotaxis	N.D.	[164]
	Citrullination by PADs	8,12,20,41,46	Impaired CXCR4 binding and ERK1/2, Ca^{2+} and protein kinase B signaling; Impaired ACKR3 binding	Impaired monocyte and lymphocyte chemotaxis	N.D.	[164]
	Nitration by peroxynitrite	7	Unaltered CXCR4 and ACKR3 binding, impaired CXCR4 internalization, Ca^{2+}, ERK1/2 and IP_3 and Akt signaling	Impaired monocyte and lymphocyte chemotaxis	Unaltered GAG binding	[145,146]
XCL1	O-glycosylation	N.D.	Increased Ca^{2+} signaling in CD4+ T cells (recombinant glycosylated XCL1); unaltered Ca^{2+} signaling in XCR1 transfected cells (synthesized glycosylated XCL1)	Increased T cell chemotaxis (recombinant glycosylated XCL1)	Increased inhibition of CD4+ T cell proliferation (recombinant glycosylated XCL1)	[155–157]
CX3CL1	glycosylation	mucin-like stalk	N.D.	N.D.	No effect of loss of glycosylation on adhesiveness	[158]
	Conversion of NH_2-terminal Gln to pyroglutamic acid by glutaminyl cyclase	1	Increased ERK1/2, Akt and p38 signaling	N.D.	Increased induction of *CCL2*, *CX3CL1*, and *ICAM1* expression in HCASMCs and HUVECs	[289]

ACKR, atypical chemokine receptor; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; HCASMC, human coronary artery smooth muscle cell;) N.A., not applicable; N.D., not determined; PAD, peptidylarginine deiminase.

Appendix E. Overview of observed serendipitous modifications generated during recombinant production of human chemokines

Chemokine	Modification	Effect of PTM on receptor interaction	Effect of PTM on chemotactic activity	Effect of PTM beyond chemotaxis	Refs.
CCL5	NH_2-terminal Met	Retained receptor binding but reduced Ca^{2+} signaling, antagonist	Impaired chemotaxis, antagonist	Increased affinity for heparan sulfate	[227,228]
	NH_2-terminal Met and acetylation of Lys26, Lys34, Lys46, and Lys57	N.D.	N.D.	N.D.	[290]
CCL21	NH_2-terminal Met	N.D.	N.D.	N.D.	[291]
CXCL4	NH_2-terminal Met	N.D.	Unaltered chemotaxis	Unaltered heparin binding	[292]
	NH_2-terminal Ala-Glu-Phe-Gln-Ala-Ser-Met	N.D.	N.D.	Retained capacity to reverse concanavalin A-induced immunosuppression in BALB/c mice	[293]
CXCL7	NH_2-terminal Met (CTAP-III)	N.D.	N.D.	N.D.	[294]
CXCL10	NH_2-terminal Met	Reduced Ca^{2+} mobilization in CXCR3 transfectants	Reduced chemotactic activity for T cells and CXCR3 transfectants	Resistant to proteolytic inactivation by CD26	[113]

CXCL12	NH_2-terminal Met (CXCL12α)	N.D.	N.D.	N.D.	[295]
CXCL13	NH_2-terminal Met	NH_2-terminal sequence is folded over the first β-strand of the chemokine, which is not compatible with receptor activation and dimerization	N.D.	N.D.	[20]

N.A., not applicable; N.D., not determined.

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